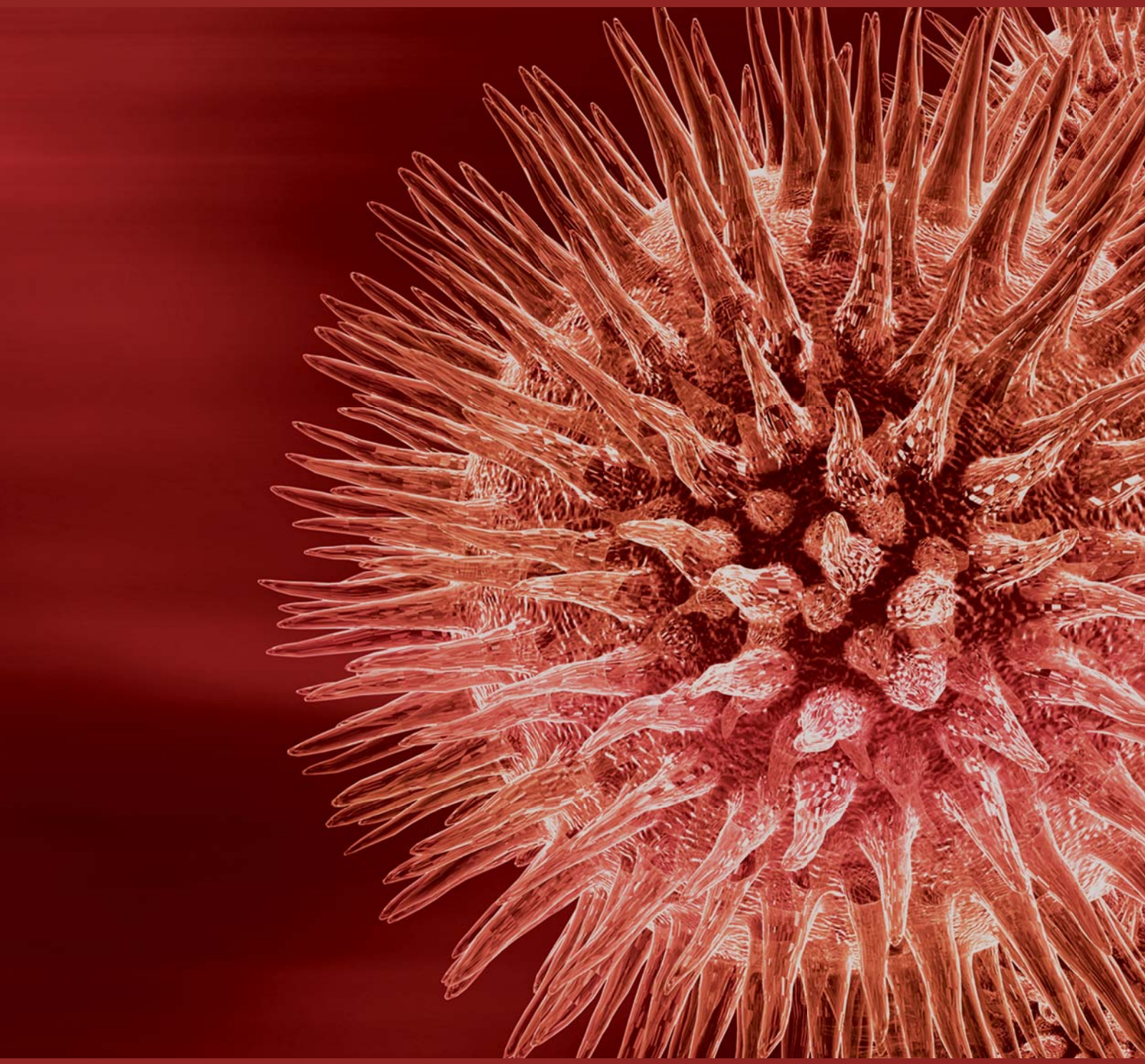
Journal of Biomedicine and Biotechnology

Plasminogen Receptors

Guest Editors: Lindsey A. Miles, Edward F. Plow, David M. Waisman, and Robert J. Parmer



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Editorial
Plasminogen Receptors

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Plasminogen is the circulating zymogen of the broad spectrum serine protease, plasmin, the enzyme responsible for thrombus dissolution. In the mid 1980's it was first recognized that plasminogen also interacts with the surfaces of cells. This interaction is functionally important because the plasminogen activators, urokinase, and tissue plasminogen activator have, by themselves, a very limited capability to activate plasminogen. However, once the plasminogen activators bind to cellular receptors their ability to activate plasminogen is increased dramatically. The plasmin that is produced remains associated with the cell surface and is protected from inactivation by the plasmin inhibitor, α_2-antiplasmin. This results in localization of the broad proteolytic activity of plasmin on cell surfaces, promoting the ability of cells to degrade extracellular matrices and activate other matrix-associated proteolytic enzymes and growth factors that facilitate cell migration. Thus, the interaction of plasminogen with cells plays a major role in macrophage recruitment during the inflammatory response, tumor cell invasion and metastasis, wound healing, tissue remodeling, neurite outgrowth, and skeletal myogenesis. Additional key functions of cell-associated plasmin are promotion of prohormone processing and induction of intracellular signaling pathways. Plasminogen-binding sites are broadly distributed on both eukaryotic and prokaryotic cell types, and the majority of cells have a very high capacity for binding plasminogen. Thus, no single molecule can account for the entire plasminogen-binding capacity of a given cell type. Notably, a subset of plasminogen-binding sites that expose a C-terminal basic residue on the cell surface are predominantly responsible for the ability of eukaryotic cells to promote plasminogen activation. Over the past two and a half decades specific plasminogen-binding proteins that promote plasminogen activation on both prokaryotic and eukaryotic cells have been identified. Recent studies using overexpression, specific knockdown, specific antibody blockade, proteomics approaches, and transgenic mice have identified new functions for plasminogen receptors. The papers selected for this issue are representative of state-of-the-art studies in plasminogen receptor biology in 2012.

This special issue contains seventeen papers, where thirteen address plasminogen receptors on eukaryotic cells and four address plasminogen receptors on prokaryotic cells.

Within the eukaryotic category, six papers address key functional consequences of the interaction of plasminogen with the entire complement of plasminogen receptors on a given cell surface and address mechanisms of these interactions.

In "*Cell surface remodeling by plasmin: a new function for an old enzyme,*" E. Deryugina and J. Quigley present the evidence linking *de novo* generated activity of plasmin and its catalytic manifestations in oncogenesis. For example, this group has shown that cell-associated plasmin catalyzes the cleavage, *in vivo,* of the CUB domain-containing protein 1 (CDCP1), a transmembrane protein overexpressed in many cancers and thought to regulate cell resistance to anoikis.

Plasmin-mediated cleavage of CDCP1 leads to outside-in signaling involving activation of Akt and suppression of PARP1-induced apoptosis *in vivo*. This signaling cascade ultimately regulates the survival potential of tumor cells in the late stages of the metastatic cascade, namely, during extravasation and early tissue colonization. The results also suggest that the original link of the uPA-plasmin system with cancer may not all be via protease-mediated invasive migration, but rather via plasmin cleavage of cell survival signaling molecules.

In "*The serine protease plasmin triggers expression of the CC-chemokine ligand 20 in dendritic cells via Akt/NF-kappaB-dependent pathways,*" X. Li et al. demonstrate that plasmin triggers release of the chemokine CCL20 via activation of Akt and MAP kinase followed by activation of NF-κB. This may facilitate accumulation of $CCR6^+$ immune cells in areas of plasmin generation such as inflamed tissues including atherosclerotic tissues.

In "*The plasminogen system in regulating stem cell mobilization,*" Y. Gong and J. Hoover-Plow present the potential mechanisms by which the plasminogen system regulates stem cell mobilization, focusing on step-wise proteolysis and signal transduction during the egress of hematopoietic progenitor and stem cells (HPSCs) from their bone marrow niche. Clear elucidation of the underlying mechanisms may lead to the development of new plasminogen-based therapeutic strategies to improve stem cell mobilization in treating hematological and cardiovascular diseases.

In "*Characterization of plasminogen binding to NB4 promyelocytic cells using monoclonal antibodies against receptor-induced binding sites in cell-bound plasminogen,*" M. Jardí et al. demonstrate that NB4 cells, which display many of the characteristics of acute promyelocytic leukemia blast cells, exhibit reduced binding of plasminogen when treated with all-*trans* retinoic acid, as detected with a monoclonal antibody that specifically reacts with cell-associated, compared with soluble plasminogen. This cell line constitutes an unique model to explore plasminogen binding and activation that can be modulated by all-*trans* retinoic acid treatment.

In "*Ocriplasmin for vitreoretinal diseases,*" I. Tsui et al. discuss a series of clinical trials to study ocriplasmin (Microplasmin, ThromboGenics, Iselin, NJ), a novel ophthalmic medication, for the treatment of vitreoretinal diseases such as vitreomacular traction, macular hole, and exudative age-related macular degeneration. The results are promising and may impact patient care.

In "*Accelerated fibrinolysis and its propagation on vascular endothelial cells by secreted and retained tPA,*" T. Urano and Y. Suzuki discuss successful visualization of the secretory dynamics of tissue-type plasminogen activator (tPA) tagged by green fluorescent protein (tPA-GFP) from cultured vascular endothelial cells (VECs) using total internal reflection fluorescence (TIRF) microscopy and demonstrate that tPA-GFP secreted from VECs is retained on cell surfaces in a heavy-chain-dependent manner. Progressive binding of Alexa568-labeled Glu-plasminogen was also observed on the surface of active tPA-GFP-expressing cells, which was not observed on cells expressing an inactive active site mutant tPA-GFP. These results suggest that retained tPA on VECs effectively activated plasminogen to plasmin, which then facilitated the binding of additional plasminogen on the cell surface by proteolytically cleaving surface-associated proteins and exposing their C-terminal lysyl residues.

Also within the eukaryotic cell category, seven papers address the functions of specific plasminogen receptors.

In "*Alpha-enolase, a multifunctional protein: its role in pathophysiological situations,*" A. Diaz-Ramos et al. review the multiple roles of α-enolase as a plasminogen receptor and its role in several pathologies. For example, this group has shown that α-enolase is expressed on the surfaces of differentiating myocytes and that inhibitors of plasminogen binding to α-enolase block myogenic fusion *in vitro* and skeletal regeneration in mice.

In "*The biochemistry and regulation of S100A10: a multifunctional plasminogen receptor involved in oncogenesis,*" P. Madureira et al. present the structure, function, and regulation of the plasminogen receptor S100A10. The S100A10-null mouse model has established the critical role that S100A10 plays as a regulator of fibrinolysis and two of its roles in oncogenesis, firstly as a regulator of cancer cell invasion and metastasis and secondly as a regulator of the recruitment of tumor-associated cells, such as macrophages, to the tumor site.

In "*The Annexin A2/S100A10 system in health and disease: emerging paradigms,*" N. Hedhli et al. review evidence that the annexin A2/S100A10 heterotetramer is dynamically regulated in settings of hemostasis and thrombosis and that this complex functions in regulating generation of plasmin. The manipulation of the annexin A2/S100A10 system may offer promising new avenues for treatment of a spectrum of human disorders.

In "*Potential role of kringle-integrin interaction in plasmin and uPA actions (a hypothesis),*" Y. Takada proposes a model in which upon plasminogen activation, an integrin-binding site in plasmin is exposed and, once activated, plasmin is able to bind to integrins on the cell surface through the kringle domains (since integrin-binding sites are exposed) and proteolytically activates PAR-1, which induces intracellular signaling.

In "*The plasminogen receptor, Plg-R_{KT}, and macrophage function,*" L. Miles et al. describe the use of proteomics to identify a plasminogen receptor with a unique structure, the novel transmembrane protein, Plg-R_{KT}, which is synthesized with and exposes a C-terminal lysine on the cell surface. Plg-R_{KT} promotes plasminogen activation, cell migration, and macrophage recruitment in the inflammatory response.

In "*The plasminogen activation system and the regulation of catecholaminergic function,*" H. Bai et al. present a mechanism by which neurotransmitter release from catecholaminergic cells is negatively regulated by cleavage products formed from plasmin-mediated proteolysis. Plg-R_{KT} is highly expressed in chromaffin cells of the adrenal medulla as well as other catecholaminergic cells and tissues, and Plg-R_{KT}-dependent plasminogen activation plays a key role in regulating catecholaminergic neurosecretory function.

In "*So many plasminogen receptors: why?*" E. Plow et al. discuss potential reasons for the expression of different plasminogen receptors. Different plasminogen receptors may be

utilized to achieve specific steps in plasminogen-dependent physiologic and pathologic functions.

The four papers addressing prokaryotic plasminogen receptors are summarized below.

In "*Bacterial plasminogen receptors utilize host plasminogen system for effective invasion and dissemination,*" S. Bhattacharya et al. review mechanisms by which pathogenic bacteria engage plasminogen, which is activated to plasmin to trigger development of a proteolytic surface on the bacteria. Bacteria, thus, exploit the host plasminogen and fibrinolytic system for the successful dissemination within the host.

In "*Plasminogen binding proteins and plasmin generation on the surface of Leptospira spp.—the contribution to the bacteria-host interactions,*" M. Vieira et al. review the ability of several species of pathogenic *Leptospira* to bind human plasminogen and to generate enzymatically active plasmin on the bacteria surface and the identification and characterization of several proteins that may act as plasminogen receptors. The presence of plasmin on the leptospiral surface may facilitate host tissue penetration and help the bacteria to evade the immune system and, as a consequence, permit *Leptospira* to reach secondary sites of infection.

In "*The role of nephritis-associated plasmin receptor (NAPlr) in glomerulonephritis associated with streptococcal infection,*" T. Oda et al. review the isolation of a nephritogenic antigen for acute poststreptococcal glomerulonephritis, highly identical to the reported plasmin(ogen) receptor of Group A Streptococcus. Deposition of this nephritogenic antigen may contribute to the pathogenesis of acute poststreptococcal glomerulonephritis by maintaining plasmin activity.

In "*Bacterial plasminogen receptors: mediators of a multifaceted relationship,*" M. Sanderson-Smith et al. provide an overview of bacterial plasminogen receptors and discuss the diverse roles bacterial plasminogen acquisition plays in the relationship between bacteria and host. Numerous bacterial plasminogen receptors have been identified, and the mechanisms by which they interact with plasminogen are diverse.

Thus, the papers in this special issue, representing a broad spectrum of experimental approaches and areas of investigation, demonstrate the wide array of cellular events and functions that are mediated by the interaction of plasminogen with cellular receptors. This unique and informative collection of papers on plasminogen receptors summarizes the research of the past 25 years and highlights the direction of future studies. This special issue, therefore, showcases the fundamentally important role that plasminogen receptors play in physiological and pathophysiological processes.

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David M. Waisman
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Review Article

Potential Role of Kringle-Integrin Interaction in Plasmin and uPA Actions (A Hypothesis)

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Academic Editor: Edward F. Plow

We previously showed that the kringle domains of plasmin and angiostatin, the N-terminal four kringles (K1–4) of plasminogen, directly bind to integrins. Angiostatin blocks tumor-mediated angiogenesis and has great therapeutic potential. Angiostatin binding to integrins may be related to the antiinflammatory action of angiostatin. We reported that plasmin induces signals through protease-activated receptor (PAR-1), and plasmin-integrin interaction may be required for enhancing plasmin concentration on the cell surface, and enhances its signaling function. Angiostatin binding to integrin does not seem to induce proliferative signals. One possible mechanism of angiostatin's inhibitory action is that angiostatin suppresses plasmin-induced PAR-1 activation by competing with plasmin for binding to integrins. Interestingly, plasminogen did not interact with $\alpha v\beta 3$, suggesting that the $\alpha v\beta 3$-binding sites in the kringle domains of plasminogen are cryptic. The kringle domain of urokinase-type plasminogen activator (uPA) also binds to integrins. The uPA-integrin interaction enhances uPA concentrations on the cell surface and enhances plasminogen activation on the cell surface. It is likely that integrins bind to the kringle domain, and uPAR binds to the growth factor-like domain (GFD) of uPA simultaneously, making the uPAR-uPA-integrin ternary complex. We present a docking model of the ternary complex.

1. The Kringle Domains of Plasmin Interact with Integrins

The integrins are a superfamily of cell adhesion receptors that bind to extracellular matrix ligands, cell-surface ligands, and soluble ligands. They are transmembrane $\alpha\beta$ heterodimers and at least 18 α and eight β subunits are known in humans, generating 24 heterodimers [1]. The α and β subunits have distinct domain structures, with extracellular domains from each subunit contributing to the ligand-binding site of the heterodimer. The sequence arginine-glycine-aspartic acid (RGD) was identified as a general integrin-binding motif, but individual integrins are also specific for particular protein ligands. Immunologically important integrin ligands are the intercellular adhesion molecules (ICAMs), immunoglobulin superfamily members present on inflamed endothelium and antigen-presenting cells. On ligand binding, integrins transduce signals into the cell interior; they can also receive intracellular signals that regulate their ligand-binding affinity.

Angiostatin, a proteolytic fragment of plasminogen, contains either the first three or four kringle domains of plasminogen and is a potent inhibitor of tumor-induced angiogenesis in animal models [2, 3]. Angiostatin has promising therapeutic potential and is now in clinical trials. Plasminogen is first converted to the two-chain serine protease plasmin by cleavage of a single Arg561-Val562 peptide bond by urokinase-type plasminogen activator (uPA), and plasmin serves as both the substrate and enzyme for the generation of angiostatin [4]. Several other mechanisms have been proposed for the generation of angiostatin from the plasminogen molecule [5]. The antiangiogenic functions of plasminogen kringles have been extensively studied using recombinant plasminogen kringles and kringle fragments produced by elastolytic processing of native plasminogen. Smaller fragments of angiostatin display differential effects on the suppression of endothelial cell growth [6].

We found that bovine arterial endothelial (BAE) cells adhere to angiostatin in an integrin-dependent manner and

that integrins $\alpha v\beta 3$, $\alpha 9\beta 1$, and to a lesser extent $\alpha 4\beta 1$, specifically bind to angiostatin. $\alpha v\beta 3$ is a predominant receptor for angiostatin on BAE cells, since a function-blocking antibody to $\alpha v\beta 3$ effectively blocks adhesion of BAE cells to angiostatin, but an antibody to $\alpha 9\beta 1$ does not. ε-Aminocaproic acid, a Lys analogue, effectively blocks angiostatin binding to BAE cells, indicating that an unoccupied Lys-binding site of the kringles may be required for integrin binding. It is known that other plasminogen fragments containing three or five kringles (K1–3 or K1–5) have an antiangiogenic effect, but plasminogen itself does not. We found that K1–3 and K1–5 bind to $\alpha v\beta 3$, but plasminogen does not. These results suggest that the anti-angiogenic action of angiostatin may be mediated via interaction with $\alpha v\beta 3$. Angiostatin binding to $\alpha v\beta 3$ does not strongly induce stress-fiber formation, suggesting that angiostatin may prevent angiogenesis by perturbing the $\alpha v\beta 3$-mediated signal transduction that may be necessary for angiogenesis [7].

Plasmin, the parent molecule of angiostatin and a major extracellular protease, induces platelet aggregation, migration of peripheral blood monocytes, and release of arachidonate and leukotriene from several cell types [8]. We found that plasmin specifically binds to $\alpha v\beta 3$ through the kringle domains and induces migration of endothelial cells. In contrast, angiostatin does not induce cell migration. Notably, angiostatin, anti-$\alpha v\beta 3$ antibodies, RGD-peptide, and a serine protease inhibitor effectively block plasmin-induced cell migration. These results suggest that plasmin-induced migration of endothelial cells requires $\alpha v\beta 3$ and the catalytic activity of plasmin and that this process is a potential target for the inhibitory activity of angiostatin [9].

We found that plasmin specifically interacts with integrin ($\alpha 9\beta 1$) and that plasmin induces migration of cells expressing recombinant $\alpha 9\beta 1$ ($\alpha 9$-Chinese hamster ovary (CHO) cells). Migration was dependent on an interaction of the kringle domains of plasmin with $\alpha 9\beta 1$ as well as the catalytic activity of plasmin. Angiostatin, representing the kringle domains of plasmin, alone did not induce the migration of $\alpha 9$-CHO cells, but simultaneous activation of the G protein-coupled protease-activated receptor (PAR)-1 with an agonist peptide induced the migration on angiostatin, whereas PAR-2 or PAR-4 agonist peptides were without effect. Furthermore, a small chemical inhibitor of PAR-1 (RWJ 58259) and a palmitoylated PAR-1-blocking peptide inhibited plasmin-induced migration of $\alpha 9$-CHO cells. These results suggest that plasmin induces migration by kringle-mediated binding to $\alpha 9\beta 1$ and simultaneous proteolytic activation of PAR-1 [10]. It is likely that other integrins that bind to plasmin may exert similar effects on plasmin signaling.

We propose a model (Figure 1) in which (1) upon plasminogen activation, integrin-binding site in plasmin is exposed. Note that plasminogen does not bind to integrins $\alpha v\beta 3$ or $\alpha 9\beta 1$. (2) Once activated, plasmin is able to bind to integrins on the cell surface through the kringle domains (since integrin-binding sites are exposed) and proteolytically activates PAR-1, which induces intracellular signaling. Plasmin is concentrated to the cell surface through integrin binding, and this process is probably critical since plasmin has much lower affinity to PAR-1 than thrombin. Angiostatin, in contrast, binds to integrins, but does not activate PAR-1. Angiostatin is expected to suppress plasmin action by competing with plasmin for binding to integrins.

It has been reported that integrins $\alpha M\beta 2$ [11], $\alpha D\beta 2$ [12], and $\alpha 5\beta 1$ [13] bind to plasminogen, while we did not detect binding of $\alpha v\beta 3$ or $\alpha 9\beta 1$ to plasminogen. One possibility is that integrins $\alpha M\beta 2$, $\alpha D\beta 2$, and $\alpha 5\beta 1$ recognize plasminogen in the ways different from those of $\alpha v\beta 3$ or $\alpha 9\beta 1$. Another possibility is that integrin-binding sites in plasminogen (perhaps kringle domains) are exposed in partially denatured plasminogen. Supporting the second possibility we observed that freshly prepared plasminogen did not significantly bind to $\alpha v\beta 3$, but plasminogen binding to $\alpha v\beta 3$ appeared to increase as plasminogen preparations aged (data not shown). This issue should be clarified in future studies.

In conclusion, the kringle domains in plasmin are involved in direct integrin binding, in addition to binding to the C-terminal Lysine residues of many proteins, and playing a role in inducing intracellular signals through proteolytic activation of PAR-1. The kringle-integrin interaction may enhance the cell surface concentration of plasmin, or directly induce intracellular signals through outside-in integrin signaling. Interestingly, plasminogen does not interact with integrins $\alpha v\beta 3$ or $\alpha 9\beta 1$ (possibly the integrin-binding sites are cryptic in plasminogen) (Figure 1) Based on our results on the plasmin kringle-integrin interaction, we hypothesized that the kringle domains of other serine proteases may interact with integrins and the interaction may play a role in their functions. Consistent with this idea, kringle domains from other proteins such as tissue-type plasminogen activator (tPA) [14] and apolipoprotein [15] have been reported to interact with integrins. This suggests that kringle-integrin interaction is a common mechanism in kringle-containing proteins.

2. uPA Kringle-Integrin Interaction

uPA is a highly restricted serine protease that converts the zymogen plasminogen to active plasmin. uPA binds with high affinity to a cell-surface uPA receptor (uPAR) that has been identified in many cell types. uPAR is a glycosylphosphatidylinositol- (GPI-) anchored 35–55 kDa glycoprotein. This system mediates pericellular proteolysis of extracellular matrix proteins including fibrin degradation (fibrinolysis) and plays an important role in cancer, inflammation, and immune responses [16–19]. The single chain form of uPA has three independently folded domains: the growth factor-like domain (GFD) (residue 1–46), kringle (residue 47–135) domain, and serine protease domain (residue 159–411). Enzymatic digestion of single chain-uPA yields an amino terminal fragment (ATF), which consists of the GFD and kringle domains, and the low molecular weight fragment (LMW-uPA), which consists of the serine protease domain. The uPAR-binding site of uPA is located in the GFD domain [20]; this binding is stabilized by the kringle [21]. It has generally been accepted that uPA signaling involves its binding to uPAR through its GFD [22].

uPA binding to uPAR on the cell surface facilitates activation of plasminogen to plasmin in vitro by increasing the rate

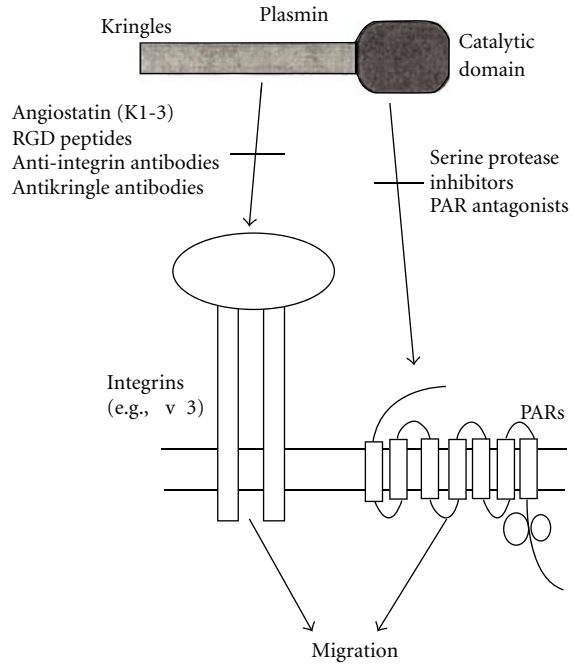

Figure 1: A model of plasmin-induced cell migration and the potential mechanism of angiostatin action. uPA activates plasminogen to plasmin pericellularly. Plasmin is accumulated on the cell surface by binding to integrins and stabilized. Free plasmin would be rapidly inactivated by circulating serine protease inhibitors (e.g., β2-antiplasmin). The catalytic activity of plasmin on the cell surface is directly involved in signal transduction, possibly through activating G-protein coupled PARs. The binding of the kringle domain may not be directly involved in signaling through integrin pathways. Angiostatin effectively blocks plasmin-induced cell migration possibly by competing with plasmin for binding to integrins. Aprotinin, a serine protease inhibitor, also effectively blocks migration. It should be noted that other antiangiogenic agents, RGD-peptide and anti-$\alpha v\beta 3$, are effective inhibitors of this process.

of pro-uPA activation by plasmin, by decreasing the apparent Km of uPA to plasmin, and by increasing the Kcat/Km of uPA to plasmin [23]. It is interesting that uPA-knockout mice do not have major thrombotic disorders [24]. This is probably because of the redundant fibrinolytic function by tPA. Indeed, combined uPA and tPA knockout mice show extensive thrombotic disorders very similar to those observed in plasminogen-knockout mice, but these are rarely detected in animals lacking uPA or tPA alone [25]. In contrast to uPA, studies performed in uPAR-knockout mice do not really support a major role of uPAR in fibrinolysis. Fibrin deposits are found within the livers of mice with a combined deficiency in uPAR and tPA, but not in uPAR-knockout mice, indicating a minor role for uPAR in plasminogen activation [25]. The extraordinarily mild consequences of combined uPAR and tPA deficiency raised the question of whether there are other receptors for uPA that might facilitate plasminogen activation [19, 25].

Besides plasminogen activation, uPA has been shown to induce the adhesion and chemotactic movement of myeloid cells [26, 27], to induce cell migration in human epithelial cells [28] and bovine endothelial cells [29], and to promote cell growth [30–32]. Notably these signaling functions of uPA do not require its proteolytic activity. Several studies suggest that uPA has additional, unidentified cell-surface receptor(s) other than uPAR that are involved in signaling events. For example, blocking of uPA binding to uPAR using a monoclonal antibody or by depletion of cell surface uPAR with phosphatidylinositol-specific phospholipase C (PIPLC) did not inhibit uPA-induced mitogenic effects in smooth muscle cells [33]. uPA-induced mitogenic effects in melanoma cells are independent of high-affinity binding to uPAR, and this suggests the existence of a low-affinity binding site on this cell type based on the kinetic data [34]. The chemotactic action of uPA on smooth muscle cells depends on its kringle domain, and kinetic evidence indicates that these cells express a lower-affinity kringle receptor distinct from uPAR [35]. The isolated uPA kringle augments vascular smooth muscle cell constriction in vitro [36] and in vivo [37]. Taken together these observations all suggest that cells express uPA-binding proteins (other than uPAR) that mediate signaling from uPA.

We found that uPA binds specifically to integrin $\alpha v\beta 3$ on CHO cells depleted of uPAR (Figure 2). The binding of uPA to $\alpha v\beta 3$ required the uPA kringle domain (Figure 3). The isolated uPA kringle domain binds specifically to purified, recombinant soluble, and cell surface $\alpha v\beta 3$, and other integrins ($\alpha 4\beta 1$ and $\alpha 9\beta 1$), and induces migration of CHO cells in an $\alpha v\beta 3$-dependent manner. The binding of the uPA kringle to $\alpha v\beta 3$ and uPA kringle-induced $\alpha v\beta 3$-dependent cell migration is blocked by angiostatin. We studied whether the binding of uPA to integrin $\alpha v\beta 3$ through the kringle

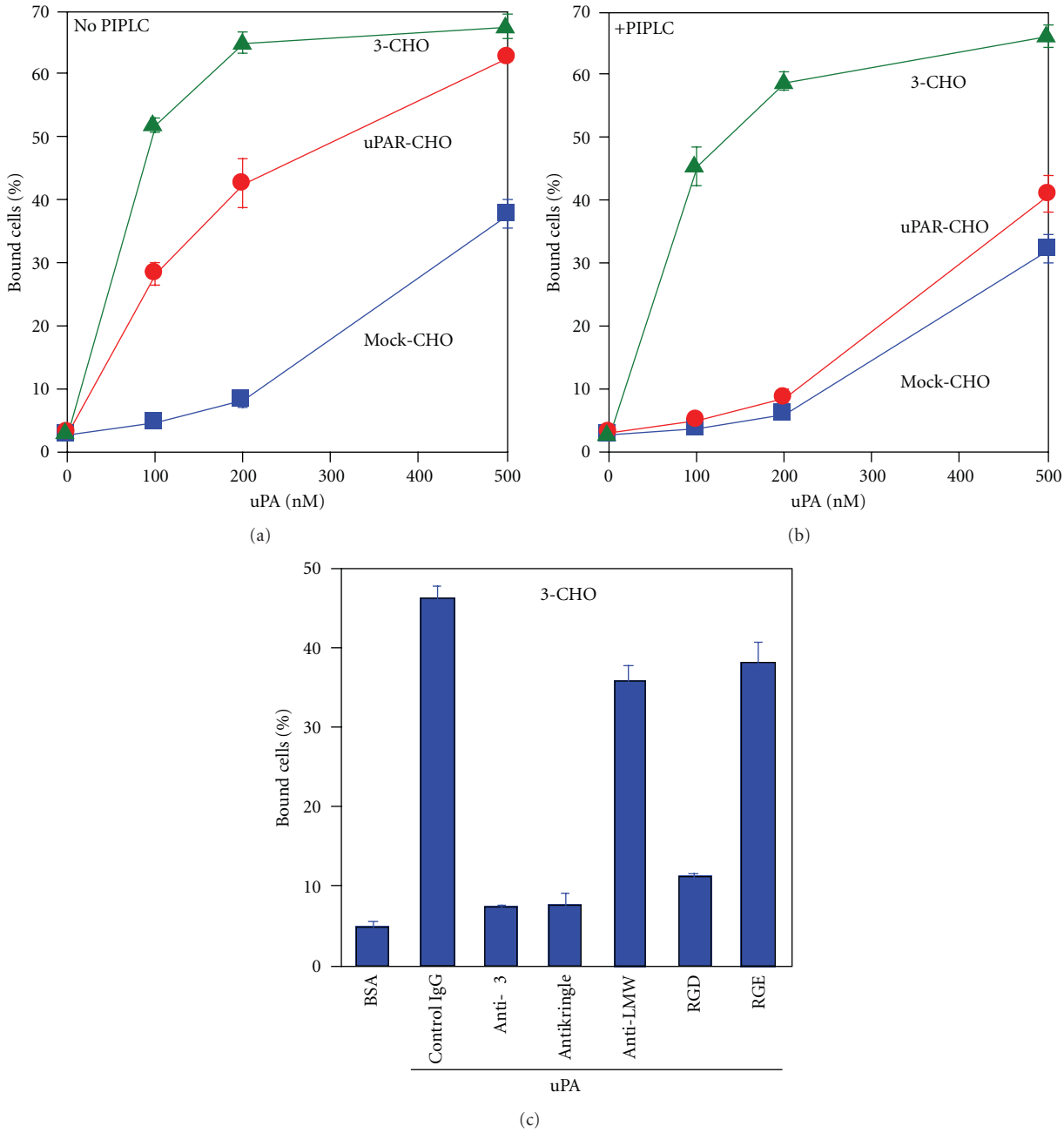

Figure 2: uPA binding to the cell surface in an integrin $\alpha v\beta 3$-dependent and uPAR-independent manner [38]. (a) and (b) Depletion of uPAR from the cell surface blocked uPA binding to uPAR-CHO cells, but did not affect uPA binding to $\beta 3$-CHO cells. To deplete GPI-linked uPAR on the cell surface, $\beta 3$-CHO, uPAR-CHO, or control mock-transfected CHO cells were treated with PIPLC. The treatment removed more than 95% of human uPAR from uPAR-CHO cells as determined by flow cytometry with anti-uPAR mAb 3B10 (data not shown). uPA was immobilized to wells of 96-well microtiter plates at the indicated coating concentrations, and incubated with cells without (a) or with (b) pretreatment with PI-PLC. Bound cells were quantified. (c) uPA binding to $\beta 3$-CHO cells is specific to $\alpha v\beta 3$ and the kringle domain. uPA (200 nM coating concentration) was immobilized to wells of 96-well microtiter plates and incubated with $\beta 3$-CHO cells in the presence of mAb 16N7C2 (anti-$\beta 3$), Ab 963 (anti-kringle), mAb UNG-5 (anti-LMW-uPA), or RGD or RGE peptides (100 μM).

domain plays a role in plasminogen activation. On CHO cell depleted of uPAR, uPA enhances plasminogen activation in a kringle and $\alpha v\beta 3$-dependent manner (Figure 4). Endothelial cells bind to and migrate on uPA and uPA kringle in an $\alpha v\beta 3$-dependent manner. These results suggest that uPA binding to integrins through the kringle domain plays an important role in both plasminogen activation and uPA-induced intracellular signaling. The uPA kringle-integrin interaction may represent a novel therapeutic target for cancer, inflammation, and vascular remodeling [38].

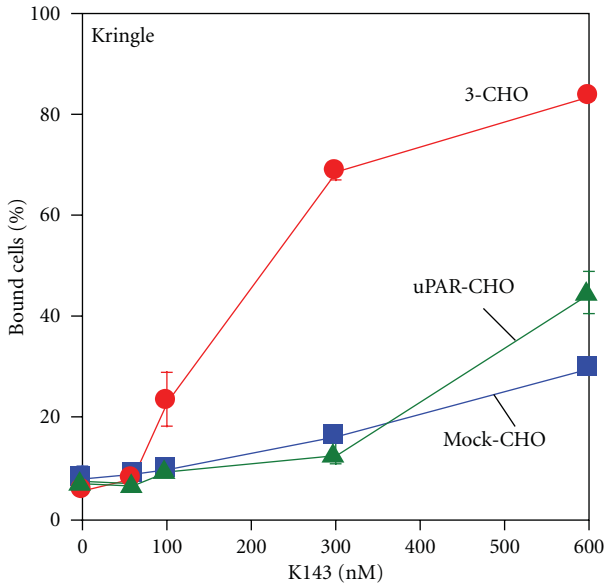

Figure 3: The kringle domain of uPA mediates binding to $\alpha v\beta 3$. The uPA kringle domain was immobilized onto wells of 96-well microtiter plates at the indicated coating concentrations and incubated with $\beta 3$-CHO, uPAR-CHO, or mock-CHO cells. The ability of the uPA fragments to support adhesion of these cells was determined [38].

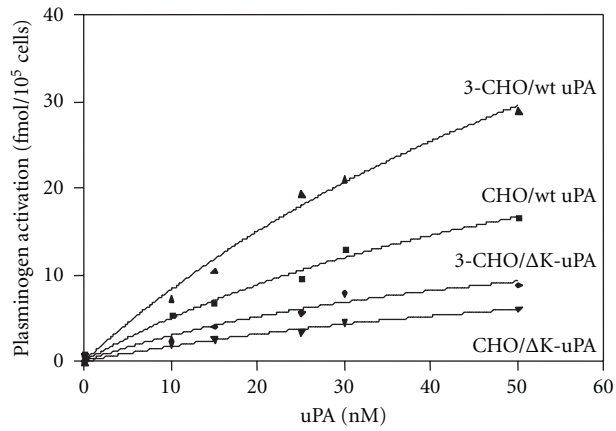

Figure 4: Integrin-dependent plasminogen activation on the cell surface. Parental CHO cells and $\beta 3$-CHO cells in wells of 96-well plates were treated with PIPLC to deplete uPAR, and incubated with wt or delta kringle (ΔK) uPA in the cold binding buffer for 1 h at 4°C. The cells were washed with the binding buffer, and plasminogen activation was determined using Glu-plasminogen and SpectrozymePL chromogenic substrate at 37°C. We found that $\beta 3$-CHO cells showed much higher ability to activate plasminogen in a manner dependent on the uPA added. Deletion of the kringle domain (with ΔK-uPA) markedly reduced the plasminogen activation on $\beta 3$-CHO, indicating that $\alpha v\beta 3$ and uPA-dependent plasminogen activation required the kringle domain of uPA. These results suggest that the binding of uPA kringle to integrin $\alpha v\beta 3$ induces plasminogen activation [38].

We propose a model, in which the N-terminal GFD of uPA binds to uPAR and the kringle domain of uPA binds to integrins, leading to the uPAR-uPA-integrin ternary complex on the cell surface. It is likely that the ternary complex formation may be involved in uPA signaling and plasminogen activation. The isolated kringle or the isolated GFD domain may suppress uPA signaling or plasminogen activation by suppressing the process. Indeed isolated kringle domain or GFD have been shown to suppress tumorigenesis [39].

3. Another Example of the Role of $\alpha v\beta 3$ in uPA Signaling: uPA Kringle and Integrin $\alpha v\beta 3$ in Neutrophil Activation

It has been reported that antibody to integrin $\alpha v\beta 3$ and RGD peptide suppress the signaling action of uPA in neutrophils, although it is unclear if this include direct uPA-$\alpha v\beta 3$ interaction [40]. The study examined the ability of specific uPA domains to increase cytokine expression in murine and human neutrophils stimulated with lipopolysaccharides (LPS). Whereas the addition of intact uPA to neutrophils cultured with LPS increased mRNA and protein levels of interleukin-1β, macrophage-inflammatory protein-2, and tumor necrosis factor α, deletion of the kringle domain from uPA resulted in loss of these potentiating effects. Addition of purified uPA kringle domain to LPS-stimulated neutrophils increased cytokine expression to a degree comparable with that produced by single-chain uPA. Inclusion of the RGD but not the RGE peptide to neutrophil cultures blocked uPA kringle-induced potentiation of proinflammatory responses, demonstrating that interactions between the kringle domain and integrins are involved. Antibodies to the αv or $\beta 3$ subunit or to $\alpha v\beta 3$ heterodimer prevented uPA kringle-induced enhancement of expression of proinflammatory cytokines and also of adhesion of neutrophils to the uPA kringle domain. These results demonstrate that the kringle domain of uPA, through interaction with $\alpha v\beta 3$ integrins, potentiates neutrophil activation.

4. A Docking Model of uPAR-uPA Kringle-Integrin Interaction

How does integrin $\alpha v\beta 3$ interact with uPA kringle? This has recently been predicted by docking simulation [41]. They modeled the interaction of uPA on two integrins, αIIb$\beta 3$ in the open configuration and $\alpha v\beta 3$ in the closed configuration. They found that multiple lowest energy solutions point to an interaction of the kringle domain of uPA at the boundary between α and β chains on the surface of the integrins. This region is not far away from peptides that have been previously shown to have a biological role in uPAR/integrins dependent signaling. They demonstrated that in silico docking experiments can be successfully carried out to identify the binding mode of the kringle domain of uPA on the scaffold of integrins in the open and closed conformation. Importantly they found that the binding mode is the same on different integrins and in both configurations. To get a molecular view of the system is a prerequisite to unravel the complex

Table 1: Amino acid residues involved in $\alpha v\beta 3$-uPA kringle interaction in the docking model. Amino acid residues at the binding interface (within the 6 Angstrom) were selected using Swiss pdb viewer (v. 4.02).

αv	β3	uPA kringle
Ala149, Asp150, Tyr178, Gln214, Ala215, Ile216, Asp218, Asp219, Arg248	Tyr122, Ser123, Met124, Lys125, Asp126, Asp127, Asp179, Met180, Lys181, Thr182, Arg214, Arg216, Asp217, Ala218, Asp251, Ala252, Lys253, Thr311, Glu312, Asn313, Val314, Asn316, Val332, Leu333, Ser334, Met335, Asp336, Ser337	Ser47, Lys48, Thr49, Tyr51, Glu52, Gly53, Asn54, Gly55, His56, Phe57, Tyr58 Arg59, Tyr84, Asp90, Leu92, Gln93, Leu94, Asn104, Pro105, Asp106, Asn107, Arg108, Arg109, Arg110, Glu125

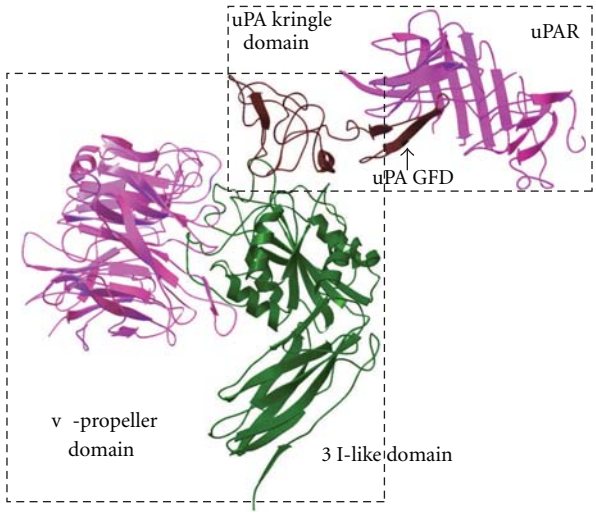

Figure 5: A model of integrin, uPA kringle, and uPAR complex. We performed docking simulation of the interaction between uPA kringle (PDB code 2URK) and integrin $\alpha v\beta 3$ (PDB code 1L5G) using Autodock3. The simulation predicted the poses in which uPA kringle interacts with $\alpha v\beta 3$. The uPA kringle-integrin complex was superposed with the ATF-uPAR complex (PDB code 2I9B).

protein-protein interactions underlying uPA/uPAR/integrin mediated cell motility, adhesion, and proliferation, and to design rational in vitro experiments.

However, in their paper which amino acid residues in uPA kringle are involved in integrin interaction is unclear. Thus, we presented our model here (Figure 5). We performed docking simulation of the interaction between uPA kringle (PDB code 2URK) and integrin $\alpha v\beta 3$ (PDB code 1L5G) using Autodock3. The simulation predicted the poses in which uPA kringle interacts with $\alpha v\beta 3$ (docking energy -22.3 kcal/mol). The amino acid residues involved in the interaction are shown in Table 1. The uPA kringle-binding site in $\alpha v\beta 3$ appears to be common to other known $\alpha v\beta 3$ ligands. The uPA kringle-integrin complex was superposed with the ATF-uPAR complex (PDB code 2I9B). Our model predicts that integrin $\alpha v\beta 3$ and uPAR can bind to ATF (GFD and kringle) simultaneously without steric hindrance. Obviously, it would be important to identify amino acid residues in uPA kringle that are critical for integrin binding by site-directed mutagenesis. In future studies, using uPA kringles that cannot bind to integrins or uPAR, it would be important to study the role of uPA kringle-integrin interaction in the proinflammatory action of uPA and to establish the role of uPAR in this process.

5. uPAR-Integrin Interaction

Previous studies suggest that uPAR directly binds to integrins [42–44]. How can our hypothesis explain this interaction? Our preliminary docking simulation studies of interaction between uPAR and integrin $\alpha v\beta 3$ did not detect high-affinity $\alpha v\beta 3$ binding sites in uPAR (not shown). In contrast the docking simulation of interaction between uPA kringle and $\alpha v\beta 3$ predicted high affinity binding of $\alpha v\beta 3$ to uPA kringle (as shown above). Since uPA binds to uPAR at high-affinity through GFD of uPA, one possibility is that previous studies detected interactions between the uPA-uPAR complex and integrins, in which integrins bind indirectly to uPAR through uPA kringle, but not those between uPAR and integrins. uPA is widely expressed in different cell types and tissues. This hypothesis should be rigorously tested in future studies.

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Review Article

Plasminogen Binding Proteins and Plasmin Generation on the Surface of *Leptospira* spp.: The Contribution to the Bacteria-Host Interactions

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Leptospirosis is considered a neglected infectious disease of human and veterinary concern. Although extensive investigations on host-pathogen interactions have been pursued by several research groups, mechanisms of infection, invasion and persistence of pathogenic *Leptospira* spp. remain to be elucidated. We have reported the ability of leptospires to bind human plasminogen (PLG) and to generate enzimatically active plasmin (PLA) on the bacteria surface. PLA-coated *Leptospira* can degrade immobilized ECM molecules, an activity with implications in host tissue penetration. Moreover, we have identified and characterized several proteins that may act as PLG-binding receptors, each of them competent to generate active plasmin. The PLA activity associated to the outer surface of *Leptospira* could hamper the host immune attack by conferring the bacteria some benefit during infection. The PLA-coated leptospires obstruct complement C3b and IgG depositions on the bacterial surface, most probably through degradation. The decrease of leptospiral opsonization might be an important aspect of the immune evasion strategy. We believe that the presence of PLA on the leptospiral surface may (i) facilitate host tissue penetration, (ii) help the bacteria to evade the immune system and, as a consequence, (iii) permit *Leptospira* to reach secondary sites of infection.

1. Introduction

The spirochete *Leptospira interrogans* is a highly invasive pathogen and the causal agent of leptospirosis, one of the most widespread zoonosis of human and veterinary concern. The disease represents a great economic burden because it has an effect on the public health system and the livestock [1–4]. The disease occurs mainly in urban regions lacking adequate sanitary conditions, associated with activities that involve direct contact with contaminated water, soil, or animals [3, 5, 6]. The leptospires chronically infect mammal species, which harbor the bacteria in their renal tubules, shedding them through the urine into the environment, thus constituting a source of reinfection to other animals. Humans are accidental and terminal hosts in the transmission process of leptospirosis [1, 7]. The leptospires enter the body via abrasions on skin or actively through mucosa, spreading to any tissue, but specially colonizing kidneys and liver [2].

The understanding of molecular aspects of the pathogenesis, the virulence, and invasion processes by which the leptospires infect the hosts and initiate tissue colonization remains to be elucidated, despite the availability of genomic sequencing of five strains of *Leptospira* that have identified more than 200 putative membrane proteins that might be involved in pathogenesis [8–12]. Yet, only few virulence

factors related to the pathogenesis of the disease have been reported [13–16].

The ability of the leptospires to adhere to extracellular matrix (ECM) proteins is assumed to be crucial during the first steps of pathogenesis [17]. Indeed, this ability has been recently and firstly described by our group [18] and a number of adhesins, ECM-binding proteins, have been identified [15, 18–26]. After leptospiral adherence, the next fundamental step must be to overcome the barriers imposed by epithelial tissues and extracellular matrixes, to reach the bloodstream and secondary sites of infection within the hosts. Proteolytic activity has been demonstrated to be important during the penetration of several pathogenic microorganisms [27]. Because the presence of ECM degrading enzymes is limited to few bacterial pathogens, one of the strategies employed to surmount this deficiency is the interaction with protease-mediated systems of the hosts [27].

Plasmin is a broad-spectrum serine protease component of the fibrinolytic system, which has PLG as a main component. PLG is a circulating single-chain zymogen that is converted to plasmin by cleavage of the peptide bond between Arg-560 and Val-561, mediated by PLG activators-like uPA (urokinase-type PLG activator) and tPA (tissue-type PLG activator). Once activated to plasmin the bacteria are endowed with membrane-associated proteolytic activity, a characteristic that have been demonstrated to contribute to the degradation of ECM components, tissue penetration, and invasion. It has been reported that several pathogens, including the spirochetes *Borrelia* spp. and *Treponema* spp., bind PLG on the surface and converts it to plasmin by host activators [28–30]. The binding of PLG and plasmin generation promotes ECM molecules degradation and it is essential for dissemination of the bacteria through the host tissues, thus suggesting its importance in invasiveness process [27, 31–35].

In this paper, we highlight and compile the recent studies performed by our group describing and characterizing the leptospiral binding to PLG [36], the identification of possible PLG receptors [37–40], and the aspect of the leptospiral immune escape strategy associated to plasmin proteolytic activity [41]. The disclosure of this proteolytic system on the surface of *Leptospira* spp. and the implications associated to bacterial infectivity are discussed.

2. Materials and Methods

2.1. Bacteria Isolates and Culture Conditions. Virulent *L. interrogans* serovar Copenhageni strain Fiocruz L1-130 is routinely cultured at Faculdade de Medicina Veterinária da Universidade de Sao Paulo by iterative passages in Golden Syrian hamsters for maintenance of virulence [1]. The organs-derived leptospires were cultured at 28°C in semisolid modified Elinghausen-McCullough-Johnson-Harris (EMJH) medium supplemented with 10% rabbit sera. Nonvirulent culture-attenuated *L. interrogans* serovar Copenhageni strain M20 are equally routinely cultured by maintenance in liquid-modified EMJH medium supplemented with 10% rabbit sera [42]. For the experiments of PLG binding, to exclude the PLG interference from the rabbit serum supplementing the culture medium, serum-free leptospires were obtained by three passages in liquid modified EMJH medium supplemented with 10% *Leptospira* enrichment EMJH (BD, Difco), cultured at 28°C.

2.2. Labeling of Leptospires with Plasmin. The leptospires were treated with PLG and uPA based in the protocol described by Coleman et al. [43], with modifications. A total of 7.0×10^9 leptospires were centrifuged at $6,000 \times g$ for 10 min at 25°C, resuspended in 1.4 mL of EMJH culture medium supplemented with 10% *Leptospira* enrichment EMJH, divided into 7 aliquots of 0.2 mL each (1.0×10^9 leptospires) in 2 mL microcentrifuge tubes, and recentrifuged. The 7 tubes containing the leptospires received different treatments: (a) 30% plasma and 3 U uPA (urokinase, Sigma) in 100 μL low-salt PBS (lsPBS—with 50 mM NaCl), (b) 5 μg PLG (native PLG purified from human plasma was from Calbiochem) and 3 U uPA in 100 μL lsPBS, (c) 2 μg PLG and 3 U uPA in 100 μL lsPBS, (d) 0.5 μg PLG and 3 U uPA in 100 μL lsPBS, (e) 5 μg PLG in 100 μL lsPBS, (f) 3 U uPA in 100 μL lsPBS, and (g) 100 μL lsPBS. All the preparations were incubated for 1 h at 37°C, under gentle shaking, prior to the addition of the uPA, and followed by 1 h incubation at 37°C. Leptospires were then centrifuged and washed three times with 0.7 mL lsPBS.

2.3. Measurement of Enzymatic Activity of Plasmin-Coated Leptospires. The treated leptospires (1.0×10^9 per sample) were resuspended in 300 μL lsPBS and divided into 3 aliquots. Each aliquot received 100 μL of 0.5 mg/mL of the chromogenic substrate D-Val-Leu-Lys 4-nitroanilide dihydrochloride (Sigma) in lsPBS, to a final substrate concentration of 0.25 mg/mL. The suspensions were incubated for 1.5 h at 37°C, under gentle shaking, and then centrifuged at $6,000 \times g$ for 10 min at 25°C. The supernatants (150 μL) were transferred to 96-well microplates and the cleavage of the specific plasmin substrate was quantified with a microplate reader set at a wavelength of 405 nm.

2.4. 6-Aminocaproic Acid (ACA) Binding-Inhibition Assay. Low-passage virulent *L. interrogans* serovar Copenhageni strain Fiocruz L1-130 (1.0×10^9 leptospires/sample) was treated with PLG and uPA, as described above, except for the addition of increase concentrations of ACA (Sigma) ranging from 0 to 1,000 mM, added together with the PLG. PLG quantity was set at 5 μg and uPA at 3 U. Aliquots treated with only PLG, uPA, or ACA, and just lsPBS treated, were used as controls. The enzymatic plasmin activity was measured as described before.

2.5. Liquid-Phase Immunofluorescence Assay (L-IFA). Live bacteria suspensions (2.5×10^9) were harvested at $12,800 \times g$ for 15 min, washed twice with lsPBS, resuspended in 200 μL lsPBS containing 8 μg of human PLG, and incubated for 45 min at 37°C. After the incubation, 6 μg/mL of propidium iodide (Sigma) were added to stain the nuclei, and the suspensions were incubated for more 45 min at 37°C. After

this time, the leptospires were gently washed three times with lsPBS and incubated for 45 min at 37°C with mouse-produced antiserum against human PLG (Sigma) at a 1 : 50 dilution. The leptospires were washed three times and incubated with goat anti-mouse IgG antibodies conjugated to fluorescein isothiocyanate (FITC; Sigma) at a dilution of 1 : 50 for 45 min at 37°C. After this incubation, the leptospires were washed twice and resuspended in lsPBS-antifading solution (ProLong Gold; Molecular Probes). The immunofluorescence-labeled leptospires were examined by the use of a confocal LSM 510 META immunofluorescence microscope (Zeiss, Germany). As control for cell integrity, we used antibodies against recombinant *Leptospira* proteins LipL32 or GroEL, produced in mice, following all the mentioned procedures for PLG.

2.6. SDS-PAGE and Affinity Blotting. Total leptospiral protein extracts for SDS-PAGE were prepared from 10 mL of $\sim 10^9$ bacteria in EMJH serum-free cultures. The cells were harvested by centrifugation, washed three times with 5 mM $MgCl_2$ in lsPBS, and resuspended in 100 μL PBS. The proteins were loaded into 10% SDS-PAGE and transferred to nitrocellulose membranes (Hybond-ECL, GE Healthcare) in semidry equipment. The membranes were blocked for 2 h at 37°C with 5% BSA, washed three times (10 min for each wash) with PBS-0.5% Tween-20 solution (PBS-T), and incubated overnight with 3 μg/mL PLG or 3 μg/mL PLG + 100 mM ACA at 4°C, followed by a 2 h incubation at room temperature. Then, the membranes were washed three times and incubated with mouse anti-human PLG (1 : 750, Sigma) for 3 h at room temperature, following by more than three washings and 1 h incubation at room temperature with anti-mouse IgG-peroxidase conjugated (1 : 5,000, Sigma). The membranes were washed and the protein's reactivity was revealed by ECL reagent (GE Healthcare) with subsequent exposition to X-Ray films (Kodak).

2.7. Assay for the Degradation of Immobilized ECM Macromolecules. 96-well plates were coated overnight at 37°C with 0.5 μg/well of cellular fibronectin (Sigma) or laminin (Sigma), washed four times with 200 μL per washing with PBS-T, and blocked with 2% BSA in PBS-T for 2 h at 37°C, followed by two washings. The spirochetes (1.0×10^8 leptospires/per sample) were treated as described above with 10 μg PLG and 3 U uPA or lsPBS (untreated). Bacteria were washed, resuspended in 100 μL lsPBS, and transferred to the plate previously coated wells. The plates were centrifuged at 180 ×g for 15 min to ensure the contact of the leptospires with the immobilized ECM components. The plates were incubated at 37°C for 20 h and washed 5 times to remove the bacteria. The degradation of the ECM components were detected by reduction in absorbance followed by incubation with antifibronectin or antilaminin IgG antibodies (1 : 5,000 dilution in 100 μL PBS-T for 45 min at 37°C, Sigma), anti-IgG peroxidase conjugated antibodies (1 : 5,000 dilution in 100 μL PBS-T for 45 min at 37°C, Sigma), and 100 μL/well of 1 mg/mL *o*-phenylenediamine—OPD—in citrate phosphate buffer, pH 5.0 plus 1 μL/mL H_2O_2. The reaction was stopped with 50 μL/well 4N H_2SO_4, and the absorbance was measured at 492 nm. Percentage degradation was calculated by de formula $(A - B)/A(100)$, where A = the mean PBS samples absorbance (positive control group) and B = the mean experimental group absorbance (untreated or plasmin).

2.8. Bioinformatics Characterization of the Proteins. Predicted coding sequences (CDSs) were analyzed as their cellular localization predictions by PSORT program, http://psort.hgc.jp/ [44, 45]. The SMART http://smart.embl-heidelberg.de/ [46, 47], PFAM http://www.sanger.ac.uk/resources/software/ [48], and LipoP, http://www.cbs.dtu.dk/services/LipoP/ [49] web servers were used to search for predicted functional and structural domains within the amino acid sequences of the CDSs.

2.9. Cloning, Expression, and Purification of Recombinant Proteins. Amplification of the CDSs was performed by PCR from *L. interrogans* serovar Copenhageni genomic DNA using complementary primer pairs. The gene sequences were amplified without the signal peptide tag and predicted by SignalP (http://www.cbs.dtu.dk/services/SignalP/). The final constructs were verified by DNA sequencing on an ABI Prism 3730_L sequencer (Seq-Wright, Houston, TX, USA) with appropriate vector-specific T7 (F: TAATACGACTCACTATAGGG) and pAE (R: CAGCAGCCAACTCAGTTCCT) primers. Cloning, expression, and purification of the recombinant proteins have been previously described as summarized in Table 1.

2.10. Plasminogen Binding to Recombinant Proteins. The binding of the recombinant proteins to PLG was evaluated by a modified ELISA, based in the protocol described by Brissette et al. [50], as follows: 96-well plates (Costar High Binding, Corning) were coated overnight in PBS at 4°C with 100 μL of 10 μg/mL of the recombinant proteins or bovine serum albumin (BSA, Sigma) as negative control. Plates were washed once with PBS-T and blocked for 2 h at 37°C with PBS with 10% (wt/vol) nonfat dry milk. The blocking solution was discarded and 100 μL of 10 μg/mL human PLG (Calbiochem) in PBS was incubated for 2 h at 37°C. Wells were washed four times with PBS-T and incubated for 1 h at 37°C with mouse anti-human PLG (1 : 4,000 in PBS, Sigma). Plates were washed again and incubated with horseradish peroxidase-conjugated anti-mouse immunoglobulin G (IgG, Sigma), diluted 1 : 5,000 in PBS. After three washings, 100 μL/well of 1 mg/mL OPD plus 1 μL/mL H_2O_2 in citrate phosphate buffer (pH 5.0) were added. The reactions were carried out for 5 min and stopped by the addition of 50 μL/well of H_2SO_4 (4 N). Readings were taken at 492 nm.

2.11. Enzymatic Activity Assay of Plasmin Bound to Recombinant Proteins. ELISA plates were coated overnight with 10 μg/mL recombinant proteins or BSA in PBS at 4°C. Plates were then washed once with PBS-T and blocked with PBS with 10% (wt/vol) nonfat dry milk for 2 h at 37°C. The blocking solution was discarded and 100 μL/well of 10 μg/mL human PLG (Calbiochem) was added, followed by

Table 1: Leptospiral selected proteins for characterization of the binding to PLG. Gene locus, protein name, NCBI reference sequence, features, gene conservation, sequence of the primers employed for DNA amplification, and molecular mass of expressed recombinant proteins.

Gene locu[1]	Recombinant protein given name	NCBI reference sequence number[2]	Description/Function	Conservation (identity)[3]	Sequence of primers for PCR amplification	Recombinant protein molecular mass (kDa)
LIC11352	LipL32[a,h]	YP_001316	Major outer membrane protein (MOMP), LipL32 lipoprotein	Lai (100%); LBH (98%)	F: 5′CACCGGTGCTTTCGGTGGTCTG 3′ R: 5′ATTACTTAGTCGCGTCAGAAGC 3′	30.2
LIC10314	Lsa63[b,h]	YP_000304	Conserved hypothetical protein with Borrelia_P83 domain	Lai (98%); LBH (87%); LBP(39%)	F: 5′GGATCCTTATTTTCTCAGGAAAG 3′ (BamH I) R: 5′GGTACCCTAAGGTTTAATTTTTTT 3′ (Kpn I)	63.0
LIC10509	rLIC10509[c,h]	YP_000493	Putative lipoprotein	Lai (98%)	F: 5′CCGGGATCCAAAAAGAGCAAAGAAG 3′ (BamH I) R: 5′GGTACCCTACTCGAGACAGCCAGGACCTTC 3′ (Kpn I)	22.0
LIC12892	Lp29[d,h]	YP_002808	Putative lipoprotein		F: 5′CTCGAGGCAGTACATTACAATCTTGCT 3′ (Xho I) R: 5′CCATGGCTCTTAGGAGCCTGGAAA 3′ (Nco I)	29.6
LIC10793	Lp49[d,h]	YP_000772	Putative lipoprotein, Surface antigen	Lai (99%); LBH (86%)	F: 5′CTCGAGAGCGGAGACTTTTCTTTACTT 3′ (Xho I) R: 5′CCATGGTTAAAAACCATCTCTACGATAAAC 3′ (Nco I)	49.1
LIC12895	Lsa27[e,h]	YP_002811	Putative lipoprotein	Lai (79%)	F: 5′GGATCCCTGAAATATACGAA 3′ (EcoRI) R: 5′GAATTCTTACTGTTCTCCTTC 3′ (BamHI)	27.0
LIC13131	MPL21[a,f,h]	YP_003039	Hypothetical protein with Ycel domain	Lai (98%); LBP (45%)	F: 5′CACCACGTCTCAAAGTTACGCTTCAG 3′ R: 5′ TTCTCACCATCCAGCTCGG 3′	21.9
LIC10765	MPL17[a,f,h]	YP_000745	Conserved hypothetical protein	Lai (100%); LBH (80%); LBP (41%)	F: 5′ CACCGAAAGTCCCGTAAGGTTCAAA 3′ R: 5′TGCAGGAGTTCCCACATTTTA 3′	15.4
LIC10091	LipL40[a,h]	YP_000088	Putative lipoprotein	Lai (100%); LBH (84%)	F: 5′CCATGGGACTCGAGACGCCTCCTCCTAAAGATCC 3′ R: 5′CTCCATGGTCATTTCAAAACTTCTACGGGGC 3′	39.0
LIC10054	MPL36[a,h]	YP_000054	Putative lipoprotein with Rare lipoprotein A (RplA) like domain	Lai (100%); LBH (88%); LBP (50%)	F: 5′CACCACGTCTTGTGCGTCGGTAGAG 3′ R: 5′CCAAGTATTCTATTTATACGTCCGAG 3′	35.1
LIC10494	rLIC10494[g,h]	YP_000478	Putative lipoprotein	Lai (99%)	F: 5′CACCACTGCTAGGGCTGCAGAAA 3′ R: 5′ACTTTGAGAGCTTCGTCTCGT 3′	25.8
LIC12730	rLIC12730[g,h]	YP_002650	Hypothetical protein with TPR domain and 4 NHL repetition	Lai (100%); LBH (90%); LBP(37%)	F: 5′CACCAGTTCTGACGGACTTCCCAA 3′ R: 5′TCTTGCGAATGAGTTGATCC 3′	77.4

Table 1: Continued.

Gene locu[1]	Recombinant protein given name	NCBI reference sequence number[2]	Description/Function	Conservation (identity)[3]	Sequence of primers for PCR amplification	Recombinant protein molecular mass (kDa)
LIC12922	rLIC12922[h]	YP_002837	Conserved hypothetical protein	Lai (100%); LBH (89%); LBP (48%)	F: 5′CACCGAATCACTCAACAGAGTCATTGC 3′ R: 5′ATCAATCTAAATGAAACGTCTCTTC 3′	40.0
LIC12238	rLIC12238[h,i]	YP_002173	Hypothetical protein	Lai (99%); LBH (77%); LBP (39%)	F: 5′CTCGAGTGTTTTAAACCTACCGGAG 3′ (Xho I) R: 5′AAGCTTCTACTTCATCGCTTTTTCTATATC 3′ (Hind III)	17.6
LIC10258	Lsa66[i]	YP_000249	Hypothetical protein with ompA domain	Lai (99%); LBH (79%)	F: 5′GGATCCGAAGCCTTCTCACCCAATTG 3′ (BamH I) R: 5′CCATGGTTAAAGTGAAAGATAAAAATCGATTC 3′ (Nco I)	65.7
LIC12880	Lp30[i]	YP_002796	Putative lipoprotein	Lai (99%); LBH (76%)	F: 5′CTCGAGGAAGTTGTCCGAGTCTAT 3′ (Xho I) R: 5′CCATGGTTATTGATTGTTTAATTCAG 3′ (Nco I)	30.7
LIC11469	Lsa20[j]	YP_001430	Hypothetical protein	Lai (100%); LBH (82%); LBP (29%)	F: 5′CTCGAGCCAATTTCTTTCGATCCAAATC 3′ (Xho I) R: 5′AAGCTTTCAATCCTCTACTGCAGCCC 3′ (Hind III)	20.0
LIC11030	rLIC11030[g,j]	YP_001000	Putative lipoprotein with unknown domain (DUF1565)	Lai (100%)	F: 5′CTCGAGTGTACAAACGAAAAAGAAGGT 3′ (Xho I) R: 5′AAGCTTTTAGTTGCAAGGATTTGGA 3′ (Hind III)	37.0
LIC11834	rLIC11834[k] (Lsa33)	YP_001783	Putative lipoprotein	Lai (99%); LBH (87%); LBP (31%)	F: 5′CTCGAGGATCTACAAGGTGGGGTTTTTAC3′ (Xho I) R:5′CCATGGTTACTGAGGTTTTACTTGGTCC3′ (Nco I)	33.1
LIC12253	rLIC12253[k] (Lsa25)	YP_002188	Conserved hypothetical protein	Lai (100%); LBH (77%); LBP (39%)	F: 5′CTCGAGGAGGAGAAACCGGACGATAC 3′ (Xho I) R: 5′CCATGGTTAGGGAAGACTTCTAACACATC 3′ (Nco I)	24.0

[1] http://aeg.lbi.ic.unicamp.br/world/lic/.
[2] http://www.ncbi.nlm.nih.gov/protein/.
[3] Protein BLAST—http://www.ncbi.nlm.nih.gov/blast/Blast.cgi.
[a] Previously published by Gamberini et al. [78];
[b] Previously published by Vieira et al. [37];
[c] Previously published by Gómez et al. [79];
[d] Previously published by Neves et al. [80];
[e] Previously published by Longhi et al. [19];
[f] Previously published by Oliveira et al. [81];
[g] Previously published by Barbosa et al. [18];
[h] Previously published by Vieira et al. [37];
[i] Previously published by Oliveira et al. [38];
[j] Previously published by Mendes et al. [39];
[k] Previously published by Domingos et al. [40];
Lai: *L. interrogans* serovar Lai [8]; LBH: *L. borgpetersenii* serovar Hardjo-bovis [11]; LBP: *L. biflexa* serovar Patoc [12].

incubation for 2 h at 37°C. Wells were washed three times with PBS-T, and then 4 ng/well of human uPA (Sigma) were added. Subsequently, 100 μL/well of the plasmin-specific substrate *D*-valyl-leucyl-lysine 4-*p*-nitroanilide dihydrochloride (Sigma) was added at a final concentration of 0.4 mM in PBS. Plates were incubated overnight at 37°C and substrate was measured by taking readings at 405 nm.

2.12. Dose-Response Curves and Dissociation Constant of the Proteins-PLG Interactions. ELISA plates were coated overnight in PBS at 4°C with 100 μL of 10 μg/mL PLG. Plates were then blocked and increasing concentrations of the purified recombinant proteins (0-1 μM) were added (100 μL/well in PBS). The assessment of bound proteins was performed by incubation for 1 h at 37°C with the antiserum raised against each protein at appropriate dilutions, followed by horseradish peroxidase-conjugated anti-mouse IgG (1 : 10,000 in PBS, Sigma). The binding was evaluated by the peroxidase substrate OPD and readings were taken at 492 nm. The ELISA data were used to calculate the dissociation constant (K_D) according to the method described by Pathirana et al. [51] and Lin et al. [52], based on the equation: $A = A_{max}$ [protein]/(K_D + [protein]), where A is the absorbance at a given protein concentration, A_{max} is the maximum absorbance for the ELISA plate reader (equilibrium), [protein] is the protein concentration, and K_D is the dissociation equilibrium constant for a given absorbance at a given protein concentration (ELISA data point).

2.13. Two-Dimensional Gel Electrophoresis (2-DE Gels). Virulent low passage leptospires culture was harvested by centrifugation at 12,800 ×g at 4°C for 10 min. Pellet was washed (×5) by resuspension in PBS containing 5 mM $MgCl_2$. The pellet was resuspended in DeStreak rehydration solution(GE Healthcare, USA) and lysed by vigorous vortexing. The cellular debris was separated by centrifugation at 20,800 ×g for 10 min, and the supernatant was collected. Total protein content was determined according to the Bradford method (Pierce Biotechnology, USA). Samples containing 200 μg of protein were adjusted to 125 μL with DeStreak rehydration solution(GE Healthcare, USA), along with 0.8% (v/v) IPG buffer, with a pH range of 3–10 (GE Healthcare).

First-dimension isoelectric focusing was performed using the IPGphor-System (GE Healthcare, USA), and the second dimension was conducted on the Ettan DALT*six* system (GE Healthcare). The IPG gel strips (7 cm) with a linear separation of immobilized pH ranging from 3 to 10 were rehydrated directly with the solubilized samples. The focusing protocol was 30 V for 180 Vh, 150 V for 300 Vh, 350 V for 350 Vh, 500 V for 500 Vh, 1000 V for 1,000 Vh, 3,000 V for 3,000 Vh, and 5,000 V for 40,000 Vh, with a 50 μA/strip maximum-setting at 20°C. The strips were equilibrated twice (reduced and alkylated) for 15 min in 15 mL equilibration solution (0.05 M Tris-HCl, pH 8.8, 6.0 M urea, 30% [v/v] glycerol, and 2% [w/v] SDS), first with the addition of 1% DTT, and finally with 2.5% iodoacetamide. After equilibration, the strips were attached to the 12% SDS-PAGE. The gels were stained by Coomassie Blue R350 (PhastGel BlueR-GE Healthcare). The gels were analyzed with Image Master-2D Platinum version 6.0 software (GE Healthcare, USA).

2.14. Mass Spectrometry and Protein Identification. The samples were analyzed by MALDI-TOF (Matrix Assisted Laser Desorption Ionization-Time of Flight) mass spectrometry, using α-cyano-4-hydroxycinnamic acid as the matrix on an Ettan MALDI-TOF/Pro instrument (Amersham Biosciences, USA). The spots preparation, mass spectrometry, and database search for protein identification were performed according to the protocol already described in Vieira et al. [53]. Prediction for protein localization was performed by LipoP program [49].

2.15. Human Sera and Microscopic Agglutination Test. Confirmed-leptospirosis serum samples were obtained from Instituto Adolfo Lutz collection, Sao Paulo, Brazil, as previously described [41]. In brief, a laboratory-confirmed case of leptospirosis was defined by the demonstration of a four-fold microagglutination titer rise between paired serum samples. The probable predominant serovar was considered the titer that was the highest sample dilution with 50% of agglutination. MAT (microscopic agglutination test) was considered negative when the titer was below 100. In addition to the MAT-negative and MAT-positive paired samples of the same individuals with laboratory and clinic leptospirosis confirmations, we also employed sera from normal healthy donors (normal human sera—NHS), without a known history of leptospirosis, with confirmed negative MAT.

2.16. Human IgG and C3b Deposition on Leptospires. L. interrogans serovar Pomona strain LPF (2.5×10^7 cells/well) were diluted in 50 μL lsPBS, coated onto microplates, and allowed to stand at 37°C for 1 h, followed by overnight at 4°C. The plates were washed twice with 200 μL lsPBS and blocked with 5% nonfat dry milk and 2.5% BSA for 2 h at 37°C. After addition of 50 μL of pooled sera/well (NHS, MAT-negative or MAT-positive) as a source of IgG, the plates were allowed to incubate for 30 min on ice, and then washed three times. The coated leptospires were treated with 50 μL/well of 5 μg/mL uPA (urokinase, Sigma) and 40 μg/mL PLG (Calbiochem) for 2 h at 37°C, or only with uPA, only with PLG, or no additions, as negative controls. After three washings, the plates were incubated with anti-human IgG Fc region specific antibodies (Calbiochem) at a dilution of 1 : 5,000 for 1 h at 37°C, and then with secondary antibodies conjugated with peroxidase at a dilution of 1 : 10,000 for 1 h at 37°C. The IgG depositions on leptospires were evaluated by the peroxidase substrate OPD and readings were taken at 492 nm. When dilutions of NHS were used, they were performed with lsPBS.

The protocol of C3b deposition on leptospires was similar to the IgG deposition described before, with exception of the incubation with NHS, placed at 37°C for 50 min, and use of anti-human C3b primary antibodies (1 : 5,000)

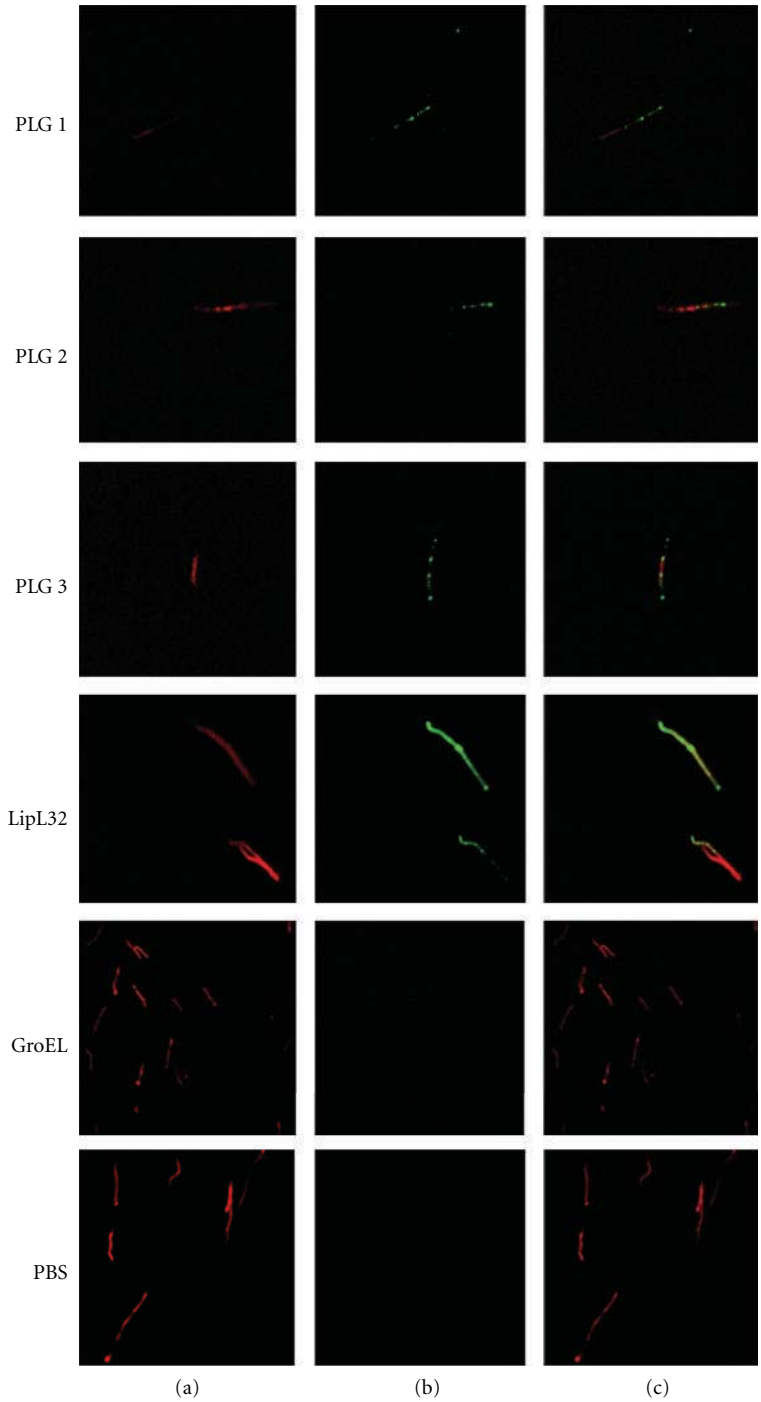


Figure 1: Recognition of PLG binding to *Leptospira* by L-IFA. Live virulent *L. interrogans* serovar Copenhageni isolates were treated with PLG, and the recognition was assessed through polyclonal anti-PLG antibodies under a confocal immunofluorescence microscope (PLG 1 to 3). PLG-treated leptospires stained for the proteins LipL32 (outer surface protein marker) or GroEL (a protoplasmic-cylinder marker) and untreated leptospires (PBS), are shown as controls (a) DNA propidium iodide-stained, (b) FITC-stained and (c) A+B composite images.

(Calbiochem), followed by secondary antibodies conjugated with peroxidase (1 : 5,000).

2.17. Human C3b Degradation by Leptospires. *L. interrogans* serovar Pomona strain LPF (10^8 leptospires/sample) were treated in 200 μL lsPBS with the addition of 10 μg PLG and 3 U uPA (plasmin), 10 μg PLG, or no additions (untreated). The cells were incubated for 1 h at 37°C with the PLG, and for one more hour after the addition of uPA. The cells were washed three times with lsPBS, and then resuspended in 100 μL lsPBS containing 15 μg/mL human purified C3b (Calbiochem), being incubated for 20 h at 37°C. The leptospires were removed by centrifugation, 20 μL of the supernatants

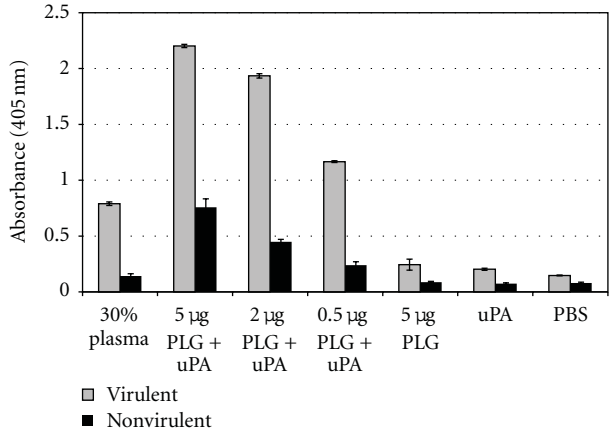


Figure 2: Cleavage of the plasmin-specific chromogenic substrate by plasmin-bound leptospires. Live low-passage virulent and high-passage nonvirulent *L. interrogans* serovar Copenhageni cells received the following treatments: PBS only (PBS), uPA alone (uPA), 5 μg PLG alone (PLG), PLG in crescent quantities (0.5, 2 and 5 μg) together with uPA, and 30% human plasma together with uPA (30% plasma). Bars represent mean absorbance as a measure of relative substrate degradation ± the standard deviation of three replicates for each experimental group and are representative of three independent experiments. *Virulent leptospires experiments: statistically significant ($P < 0.0001$) in comparison to the PBS control; **nonvirulent leptospires experiments: statistically significant ($P < 0.01$) in comparison to the PBS control. The 30% plasma, as PLG source, and all the PLG + uPA samples of the virulent leptospires were statistically significant ($P < 0.001$) in comparison to the same nonvirulent bacteria tested samples.

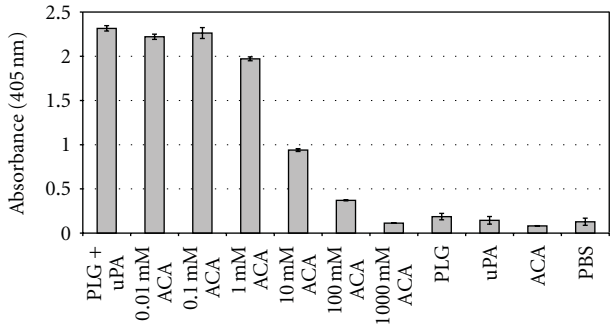


Figure 3: Inhibition of PLG binding to *L. interrogans* by ACA. Low-passage virulent *L. interrogans* serovar Copenhageni cells were treated with the following: PLG together with uPA (PLG + uPA), PLG together with uPA with the addition of crescent concentrations of ACA (0.01 to 1,000 mM ACA), PLG alone (PLG), uPA alone (uPA), ACA alone (ACA), and no additions (PBS). The cleavage of the plasmin-specific substrate D-Val-Leu-Lys 4-nitroanilide dihydrochloridein by the treated spirochetes was measured by absorbance readings at 405 nm. Bars represent mean absorbance ± the standard deviation of three replicates for each experimental group and are representative of three independent experiments. Statistically significant substrate degradation inhibition results in comparison to the positive control (PLG + uPA) are shown: $P < 0.01$ (*) and $P < 0.0001$ (**).

were separated by 10% SDS-PAGE and then transferred to nitrocellulose membranes in semi-dry equipment. The membranes were blocked by incubating overnight at 4°C with 5% nonfat dry milk and 1% BSA. The C3b detection was made by incubations with anti-human C3b antibodies and secondary antibodies conjugated with peroxidase, followed by ECL (GE Healthcare) development.

2.18. Serum Susceptibility Testing for Treated Leptospires. Complement-mediated killing of leptospires was evaluated after incubation with nonimmune NHS, as described by Meri et al. [54], with some modifications. Approximately 5×10^8 *L. interrogans* serovar Pomona strain LPF per sample were treated with 40 μg PLG and 4 U uPA in 200 μL lsPBS for 3 h at 37°C. As a control, one aliquot of *L. interrogans* serovar Pomona strain LPF was incubated only with PBS. Then, the samples were divided into aliquots containing 1.0×10^8 cells, which were added by 80 μL NHS and 80 μL EMJH medium supplemented with 10% *Leptospira* enrichment. The bacteria suspensions were incubated at 37°C for one hour, followed by a incubation of 5 min on ice for stopping complement activation. After, 20 μL of each sample (four replicates) were transferred to microplates filled with 180 μL of EMJH medium/well. The plates were sealed with a sterile adhesive film and incubated at 30°C for 4 days. Bacterial growth was determined by counting leptospires in a Petroff-Hausser chamber under dark-field microscopy.

2.19. Statistics. For the data that were tested for statistical significance, the student's two-tailed test was applied, considering the minimum of $P < 0.05$.

3. Results and Discussion

3.1. Binding of Human Plasminogen by L. interrogans Cells. Live-immunofluorescence microscopy (L-IFA) was employed in order to evaluate the ability of *L. interrogans* cells to bind human PLG. L-IFA revealed that leptospires bind PLG in their outer surface along the entire cell, as seen when the bacteria were probed with antibodies anti-PLG (Figure 1). PLG bound to leptospires does not seem to damage the bacterial membrane because no fluorescence of GroEL, a cytoplasmatic heat-shock protein [55], was achieved when serum anti-GroEL was employed (Figure 1). The leptospiral recombinant proteins LipL32, an abundant outer membrane protein of *Leptospira* [56], used as positive control, showed an intense surface fluorescence. We also observed that the spirochete regular movement was conserved, suggesting that the flagella remained undamaged. Taken together, these data suggest that the PLG-binding does not interfere with the membrane structure or dynamics [36].

3.2. Activation and Enzymatic Activity of Plasminogen-Bound Leptospira. The binding of PLG to leptospiral surface was further analyzed by plasmin enzymatic activity. Leptospires were incubated with purified PLG, followed by plasmin activation after addition of uPA, conferring the bacteria a surface-associated plasmin activity (Figure 2), as measured

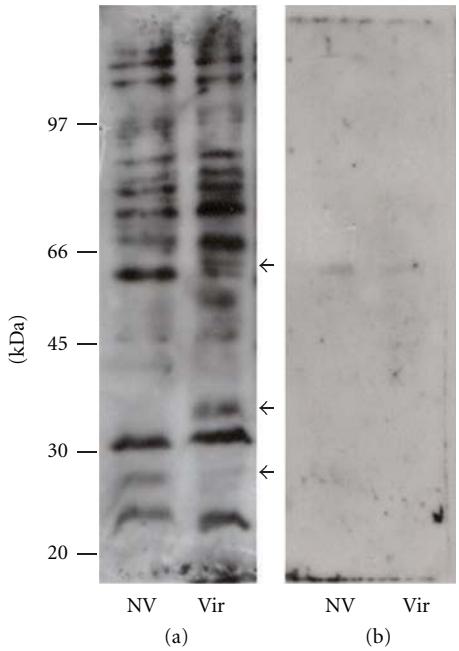


Figure 4: Binding of human PLG to *L. interrogans* proteins by affinity blotting. Total protein extracts were resolved by 10% SDS-PAGE and electroblotted into nitrocellulose membranes. The membranes were incubated with PLG (a) or PLG + ACA (b), and the binding was detected by anti-PLG antibodies and peroxidase-conjugated antibodies. The reactivity was revealed by ECL (GE Healthcare) and exposure to X-Ray films. NV: high passage, nonvirulent *L. interrogans* serovar Copenhageni strain M 20; Vir: low-passage, virulent *L. interrogans* serovar Copenhageni strain Fiocruz L1-130. The arrows indicate reactivity protein regions seen in the virulent but absent in the nonvirulent bacterial extract. Positions of protein molecular mass markers are shown on the left.

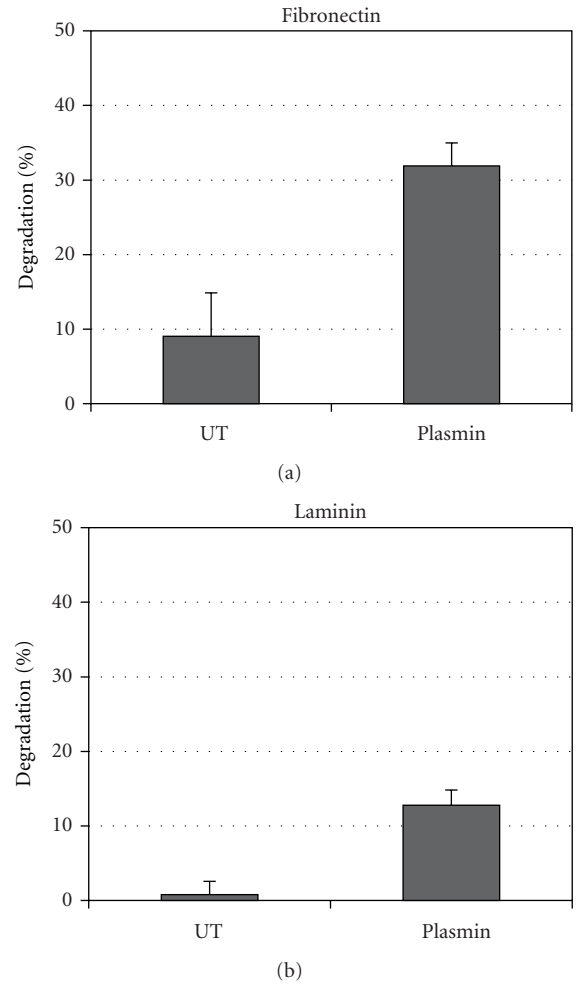


Figure 5: Fibronectin and laminin degradation by plasmin-coated leptospires. Spirochetes were incubated in lsPBS with no additions (UT) and with addition of PLG and uPA (plasmin). ELISA plate wells coated with 0.5 μg/well fibronectin (a) or laminin (b) were incubated for 20 h with the spirochetes from each experimental group to allow substrate digestion. Plates were then incubated with antibodies antilaminin or antifibronectin. A reduction in absorbance was interpreted as substrate digestion, relative to controls lacking bacteria (0.5 μg/well purified laminin or cellular fibronectin, corresponding to 0% degradation). Percent degradation (a reduction in absorbance value relative to control lacking bacteria) was calculated as described in Section 2. Bars represent mean percent substrate degradation relative to the positive control (0% degradation) ± the standard deviation of three replicates. The experiment was independently performed three times with similar results. $P < 0.001$ (*) and $P < 0.01$ (**).

by the degradation of plasmin's chromogenic specific substrate [36].

The labeling with PLG alone did not trigger proteolytic activity suggesting that *L. interrogans* serovar Copenhageni is dependent on the host PLG activation system, probably not possessing an endogenous mechanism, as already reported for other pathogens [57–64]. In contrast, the ability of PLG activation is shown for a number of pathogenic microorganisms [57–64].

Both the virulent and nonvirulent strains of *L. interrogans* serovar Copenhageni tested were capable of capturing PLG, although the virulent strain seems to be more efficient in the PLG-binding than the nonvirulent bacteria (~2.5 fold) (Figure 2) [36]. PLG is present in plasma at a concentration of approximately 20.8 ± 1.9 mg/100 mL [65]. Our data shows that virulent *L. interrogans*, but not the attenuated strain, have the ability to sequester PLG from human plasma in the conditions assayed. At any rate, once in the host, the infectious leptospires can acquire PLG when reach the blood circulation.

3.3. Inhibition by 6-Aminocaproic Acid (ACA) of Proteolytic Activity Acquired by L. Interrogans. It is well known that the lysine-binding sites of PLG kringle domains frequently mediate interactions with lysine residues of the cellular receptors [27]. ACA, a lysine analogue, decreased the proteolytic activity of virulent *L. interrogans* serovar Copenhageni incubated with PLG, in a dose-dependent manner (Figure 3). The total inhibition (100%) of plasmin activity achieved with the higher ACA concentrations employed shows that the PLG interaction with these spirochetes occurs trough the lysine-binding sites of the kringle domains [36].

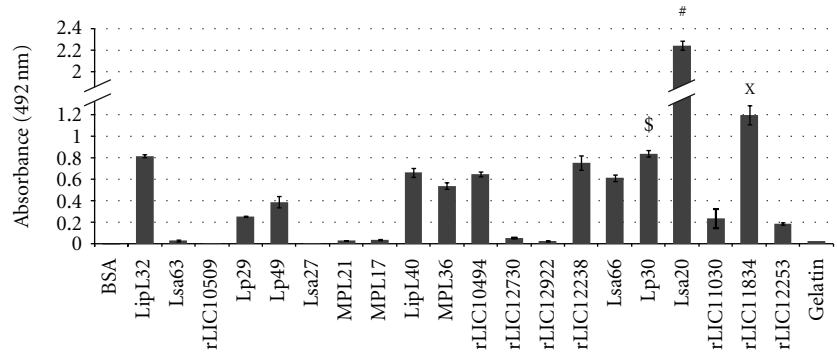


Figure 6: Binding of leptospiral recombinant proteins to human plasminogen. The recombinant proteins LipL32, Lsa63, rLIC10509, Lp29, Lp49, Lsa27, MPL21, MPL17, LipL40, MPL36, rLIC10494, rLIC12730, rLIC12922, rLIC12238, Lsa66, Lp30, Lsa20, rLIC11030, rLIC11834, and rLIC12253 were coated onto 96-well ELISA plates (10 μg/mL) and allowed to interact with purified human PLG (10 μg/mL). BSA and gelatin were used as a negative control for nonspecific binding. Binding was detected and quantified by specific antibodies. Bars represent the mean absorbance values at 492 nm ± the standard deviation of four replicates for each protein and are representative of three independent experiments. Statistically significant binding in comparison to negative control BSA are shown by $^{*}(P < 0.001)$; $^{**}(P < 0.0001)$; $^{\$}(P < 0.02)$; $^{\#}(P < 0.0005)$. The binding of rLIC11834 and rLIC12253 was compared to its binding to gelatin $^{X}(P < 0.005)$.

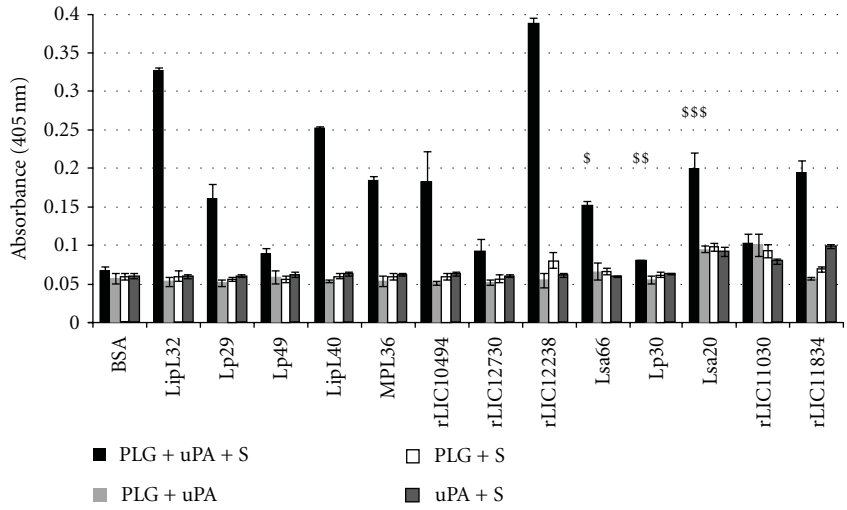
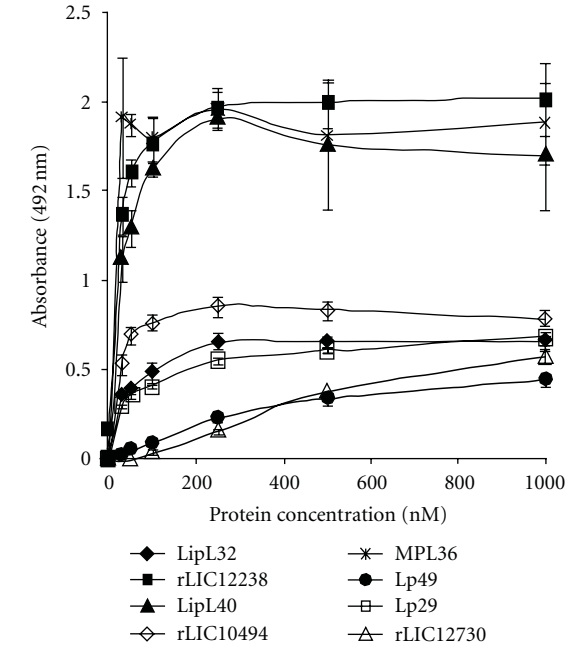


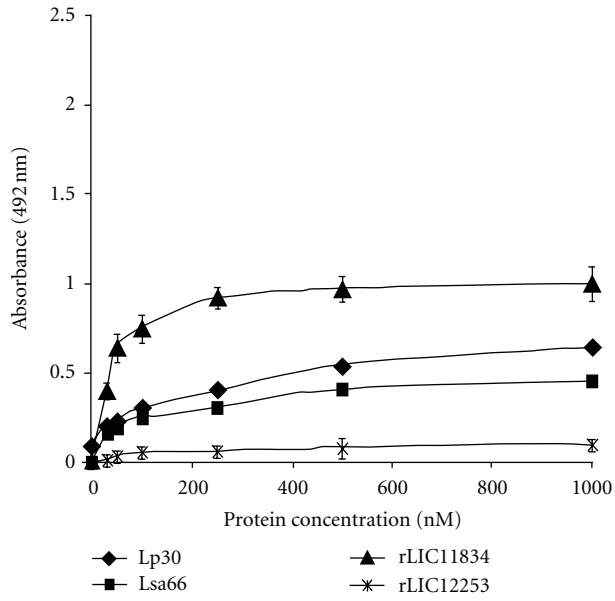
Figure 7: Activation of recombinant protein-PLG to enzymatically active plasmin. Cleavage of specific plasmin substrate by PLG bound to recombinant proteins was assayed by modified ELISA as immobilized proteins received the following treatment: PLG + uPA + specific plasmin substrate (PLG + uPA + S) or controls lacking one of the three components (PLG + uPA; PLG + S; uPA + S). BSA was employed as a negative control. Bars represent the mean absorbance values at 405 nm, as a measure of relative substrate cleavage, ± the standard deviation of four replicates for each experimental group and are representative of two independent experiments. Statistically significant differences are shown by $^{*}(P < 0.00001)$, $^{**}(P < 0.005)$, $^{***}(P < 0.05)$, $^{\$}(P < 0.001)$, $^{\$\$}(P < 0.02)$, and $^{\$\$\$}(P < 0.003)$.

3.4. Analysis of Plasminogen-Binding L. Interrogans Proteins on Cell Lysates. Assessment of leptospiral proteins binding to PLG was first visualized by affinity blotting using total whole-cell lysates (Figure 4(a)). Our results are indicative that multiple leptospiral proteins are capable of binding PLG, although these affinity blotting experiments do not simulate the protein-PLG interaction *in vivo*. As expected, the data also demonstrate that there are similarities between the PLG-binding proteins of the virulent and nonvirulent strains, but also revealed the presence of some reactive bands on the low-passage virulent strain that are absent in the nonvirulent one, indicating that virulent strains have more PLG binding proteins. Thus, based on the data from enzymatic activity and affinity blotting presented here we cannot discriminate whether the increased bound activity of the virulent strain is due to only differential expression of a different set of membrane receptors, an increased expression of the same receptors, or both. Comparable to the results obtained with the intact leptospires, the affinity blotting was inhibited by the presence of the lysine analog, ACA, supporting the central role of lysine residues of the proteins in the PLG interactions (Figure 4(b)) [36].

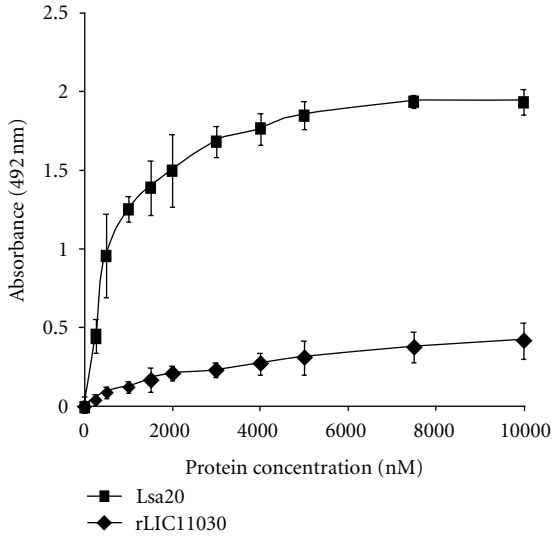
3.5. Plasmin-Coated Leptospira Degrades Immobilized ECM Molecules. Plasmin is a broad-spectrum serine protease that



(a)



(b)



(c)

Recombinant proteins	Dissociation constant (K_D)
LipL32	25 87 ± 5 09 nM
rLIC 12238	11 97 ± 106 nM
LipL40	18 24 ± 2 45 nM
rLIC 10494	10 98 ± 2 33 nM
MPL36	3 52 ± 3 95 nM
Lp49	ND
Lp29	ND
rLIC 12730	ND
Lsa66	68 82 ± 25 54 nM
Lp30	167 39 ± 60 14 nM
rLIC 11834	23 53 ± 4 66 nM
rLIC 12253	ND
Lsa20	509 13 ± 77 47 nM
rLIC 11030	ND

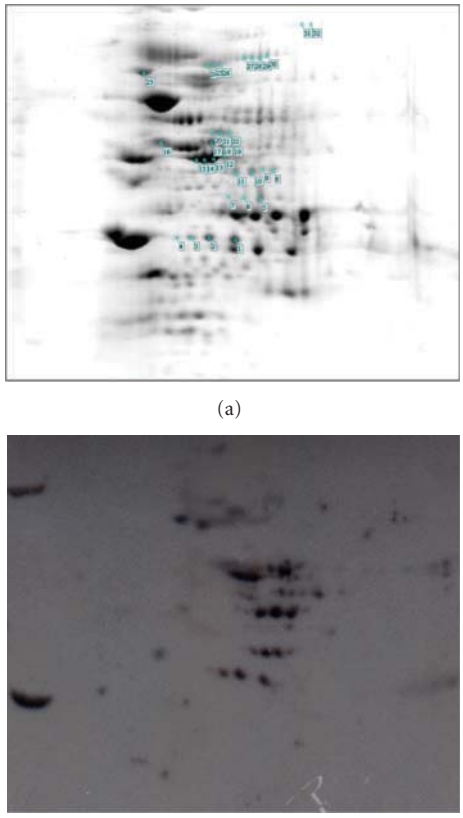
ND not determined.

(d)

Figure 8: Characterization of recombinant proteins binding to PLG. In (a), (b), and (c) PLG (10 μg/mL) was immobilized in 96-well ELISA plates, and each recombinant protein at 0 to 1,000 nM was added for interaction. The binding was detected using antiserum raised in mice against each protein at appropriate dilutions (1 : 4,000 for LipL32; 1 : 5,000 for rLIC12238, LipL40, and MPL36; 1 : 1,000 for Lp29, Lp49, Lsa20, and rLIC11030; 1 : 500 for rLIC12730, Lsa66, and Lp30; 1 : 750 for rLIC11834 and rLIC12253), followed by horseradish peroxidase-conjugated anti-mouse IgG. Data represent the mean absorbance values ± the standard deviation of six replicates for each experimental group. The results are representative of two independent experiments. In (d) The dissociation constant (K_D) was calculated based on ELISA data for the recombinant proteins that reached equilibrium up to a concentration of 1,000 nM.

is capable to degrade directly laminin and fibronectin. To evaluate whether the plasmin associated to the leptospires surface can directly break these ECM components, purified soluble human fibronectin and laminin were adhered into 96-well plates, and the reaction was evaluated by exposing the ECM proteins with low-passage virulent *L. interrogans*, pretreated with PLG/uPA. As shown in Figure 5, the plasmin-coated leptospires were able to significantly cleave down the immobilized ECM macromolecules, when compared to the ones exposed to untreated bacteria [36].

Given that leptospires are highly invasive pathogens that are thought to penetrate the skin or break in the skin to initiate infection, the ability of digesting ECM macromolecules via plasmin activity might be an important step for leptospiral pathogenesis. To date, this is the first proteolytic activity detected in *Leptospira* that can promote



(a)

(b)

Figure 9: Two-dimensional gel analysis of *L. interrogans* PLG binding proteins. Leptospiral total cell lysates were separated by isoelectric point (pH 3–10) and molecular mass on 12% SDS-PAGE. Replicate gels were Coomassie Blue stained (a) or transferred to membranes and submitted to PLG affinity immunoblotting (b). The reactive spots are enumerated on (a). The results are representative of three independent experiments performed in duplicates.

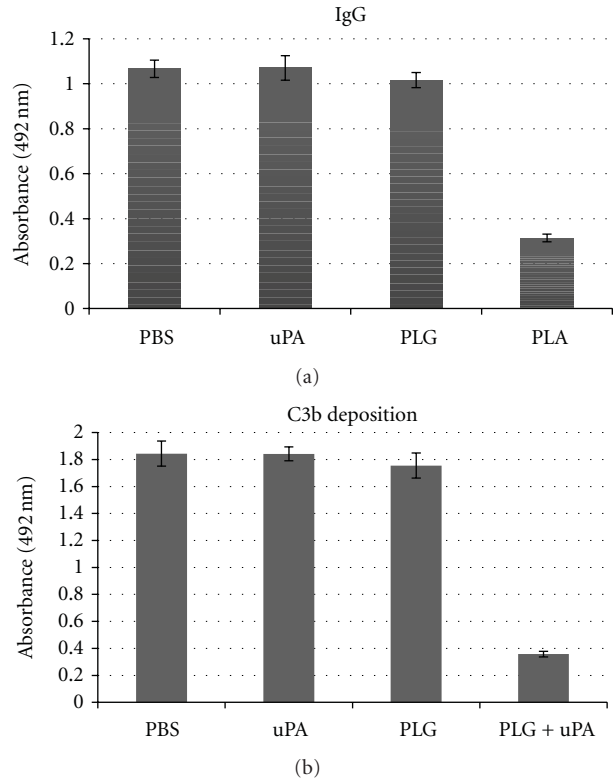


Figure 10: Human IgG and C3b binding to *L. interrogans*. Bacteria were coated onto microtiter plates (25×10^6 leptospires/well) and IgG (a) or C3b (b)were bound by incubating bacteria with 100% NHS. Bacteria were treated with PBS, 40 μg/mL PLG (PLG), 5 μg/mL uPA (uPA), or PLG + uPA (Pla). Presence of human IgG or C3b deposited on leptospires was determined by ELISA. Bars represent the mean absorbance values at 492 nm ± the standard deviation of three replicates for each experimental group and are representative of two independent experiments.

ECM degradation and it is a plausible mechanism that can contribute to leptospiral invasiveness [1, 66].

3.6. Recombinant Leptospiral Proteins Bind Human Plasminogen. In order to investigate PLG binding proteins in *Leptospira* we benefitted from the availability of several recombinant proteins of *L. interrogans* available in our laboratory. We evaluated the ability of these proteins to interact with PLG *in vitro*. Table 1 summarizes the features of these proteins and gene conservation within the sequenced genomes [8–12]. The binding of these proteins to PLG was quantified by ELISA and the results are shown in Figure 6. Proteins LipL32, Lp29, Lp49, LipL40, MLP36, rLIC10494, rLIC12730, rLIC12238, Lsa66, Lp30, Lsa20, and Lsa33 (rLIC11834) presented significant binding to PLG while proteins Lsa63, Lsa27, rLIC10509, MPL21, MPL17, rLIC12922, rLIC11030, Lsa25 (rLIC12253), BSA, and gelatin did not show significant binding activity (Figure 6) [37–40].

3.7. Activation of Plasminogen-Bound Proteins. To evaluate whether purified PLG-binding proteins were independently capable to generate active plasmin in the presence of uPA PLG activator, we measured the degradation of plasmin specific chromogenic substrate in a modified ELISA. The PLG captured by the proteins could be converted into plasmin, as demonstrated indirectly by specific proteolytic activity (Figure 7). The negative control BSA, that showed no PLG binding, produced no significant proteolytic activity, as well as the controls lacking PLG, uPA, or the chromogenic substrate [37–40].

3.8. Characterization of the Binding of Recombinant Proteins to PLG. The binding between each recombinant protein and PLG was assessed on a quantitative basis as illustrated in Figures 8(a), 8(b), and 8(c). Dose-dependent and saturable binding was observed when increasing concentrations (0 to 1,000 nM) of the recombinant proteins MPL36, LipL40, LipL32, rLIC10494, rLIC12238, Lsa66, Lp30, Lsa33 (rLIC11834), and Lsa20 was allowed to individually adhere to a fixed amount of PLG (1 μg), indicating the specificity of the binding. For the proteins Lp29, Lp49, rLIC12730, Lsa25 (rLIC12253), and rLIC11030, the saturation level was not

Table 2: *L. interrogans* PLG-binding proteins identified by MALDI-TOF mass spectrometry. The proteins are identified as the genomic nomenclature (LIC: *Leptospira interrogans* serovar Copenhageni), and are listed and respective NCBI reference sequence, predicted name/function and predicted cellular localization, in theoric molecular mass and isoelectric point (pI).

Gene locus[1]	NCBI reference sequence number[2]	Gene/protein function	Localization[3]	Theoric molecular mass (kDa)	pI
LIC12816	YP_002733	Hypothetical protein	Membrane	14.7	9.5
LIC10788	YP_000767	flaA-1 (Flagellar filament sheath protein)	Membrane	35.0	7.6
LIC11194	YP_001164	Putative citrate lyase	Cytoplasm	37.2	6.1
LIC12875	YP_002791	tuf (Elongation factor Tu)	Cytoplasm	43.6	5.7
LIC12795	YP_002712	Acetyl-CoA acetyltransferase	Cytoplasm	47.4	6.0
LIC12407	YP_002339	Glutamine synthetase protein	Cytoplasm	55.4	5.9
LIC10524	YP_000508	dnaK (Heat shock protein)	Cytoplasm	69.1	5.1

[1] http://aeg.lbi.ic.unicamp.br/world/lic/.
[2] http://www.ncbi.nlm.nih.gov/protein/.
[3] Lipo P: Juncker et al., [49].

reached, even at the highest concentration tested (1,000 nM). Based on the ELISA data, the calculated dissociation equilibrium constants (K_D) for the recombinant proteins with PLG are depicted in Figure 8(d); for the ones that reached equilibrium, the highest and the lowest K_D values were for Lsa20 (509.13 ± 77.47 nM) and MPL36 (3.52 ± 3.95 nM), respectively, [37–40].

3.9. Proteomic Approach to Leptospiral PLG Binding Protein Identification. In order expand our knowledge on PLG binding proteins, we decided to employ two-dimensional gel electrophoresis followed by affinity immunoblotting, as an alternative strategy to identify novel leptospiral PLG-binding proteins. Total protein extracts of low passage *L. interrogans* were separated by isoelectric point and molecular mass (Figure 9(a)), transferred to membranes, and incubated with PLG and anti-PLG antibodies for detection of reactive spots (Figure 9(b)). The reactive spots were excised and prepared for MALDI-TOF mass spectrometry and protein identification. Under the conditions assayed, we could identify seven proteins capable of binding PLG, as depicted in Table 2. Two were annotated as membrane and five as cytoplasmatic proteins (Table 2). Along with the membrane proteins, one is hypothetical and the other is a putative flagellar filament sheath protein (FlaA-1); the cytoplasmic proteins are citrate lyase, Tuf (elongation factor), acetyl-CoA acetyltransferase, glutamine synthetase, and the heat shock protein DnaK. While the role of membrane proteins on the PLG sequestering within the host is expected, the meaning of cytoplasmatic proteins remains to be investigated. Intriguingly, none of the PLG-binding recombinant proteins was detected in these experiments. One possible explanation is that the identified proteins, except LIC12816, are expressed in high amounts per cell, while the proteins assayed in our laboratory were below the detection limit, as reported by quantitative proteomics [67]. However, LipL32 that was shown to have the highest expression level per leptospiral cell, was not detected in our proteomics studies. Although the reason for this is unknown, we might speculate that because LipL32 is always presented as isoforms in 2D gels [53, 68, 69], it is possible that these isoforms do not bind PLG. Several cytoplasmatic proteins of other pathogenic microrganisms have been identified as PLG binding proteins, such as *Bifidobacterium animalis* DnaK [70], *Paracoccidioides brasiliensis*, *Schistosoma bovis* and *Streptcoccus pneumoniae* enolases [71] and *Streptococcus pneumoniae* GAPDH [72]. For some of these proteins, there is the possibility that at some moment they could be exposed in the bacterial outer membrane. Indeed, it has been recently reported that *B. burgdorferi* enolase, which is both a cytoplasmic and membrane-associated protein, is also a PLG-binding protein [73]. It is also possible that the PLG binding to these cytoplasmic proteins are not relevant for the bacteria.

3.10. Human IgG and C3b Deposition on Leptospires. Plasmin-generation on surface of *L. interrogans* may facilitate the bacteria to degrade opsonizing IgG and C3b molecules, and therefore contribute to immune evasion. To test this hypothesis, microtiter plates coated with virulent *L. interrogans* serovar Pomona were incubated with NHS, as a source of C3b and nonspecific IgG molecules. Bacteria were then treated with PLG and uPA, and the resulting surface-associated opsonins were quantified by specific antibodies. The results show that the incubation of bacteria with PLG and uPA resulted in a decrease in IgG Fc region (Figure 10(a)) and C3b depositions (Figure 10(b)) at the leptospiral surface. No effect on IgG or C3b binding to *L. interrogans* was observed with only PLG or uPA [41].

3.11. IgG Binding from Human Leptospirosis Immune Serum to L. Interrogans. To evaluate whether PLA activity observed against nonspecific human IgG bound to leptospires could also be functional to immune sera from patients diagnosed with leptospirosis, we performed the same experiment using MAT negative (preimmune phase) and MAT positive (convalescent immune phase) pools of human sera. As expected, MAT positive sera resulted in significant higher IgG depositions on bacterial surface when compared to NHS and MAT negative sera (Figure 11). When PLA was generated on leptospires, a noticeable decrease in the amount of IgG

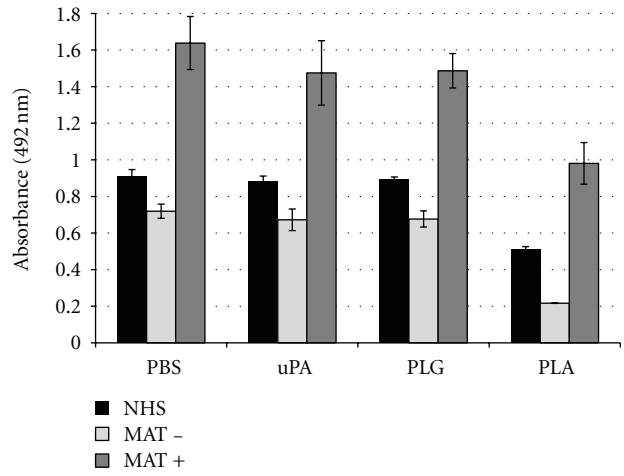


Figure 11: IgG binding from human leptospirosis immune serum to *L. interrogans*. Bacteria were coated onto microtiter plates (25×10^6 leptospires/well) and IgG was bound by incubating bacteria 50% nonimmune human sera (NHS) or confirmed paired leptospirosis patients sera: MAT negative (MAT−) or MAT positive (MAT+). Bacteria were treated with PBS, 40 μg/mL PLG (PLG), 5 μg/mL uPA (uPA), or PLG + uPA (Pla). Presence of human IgG deposited on leptospires was determined by ELISA using Fc-specific anti-human IgG antibodies. Bars represent the mean absorbance values at 492 nm ± the standard deviation of three replicates for each experimental group and are representative of two independent experiments.

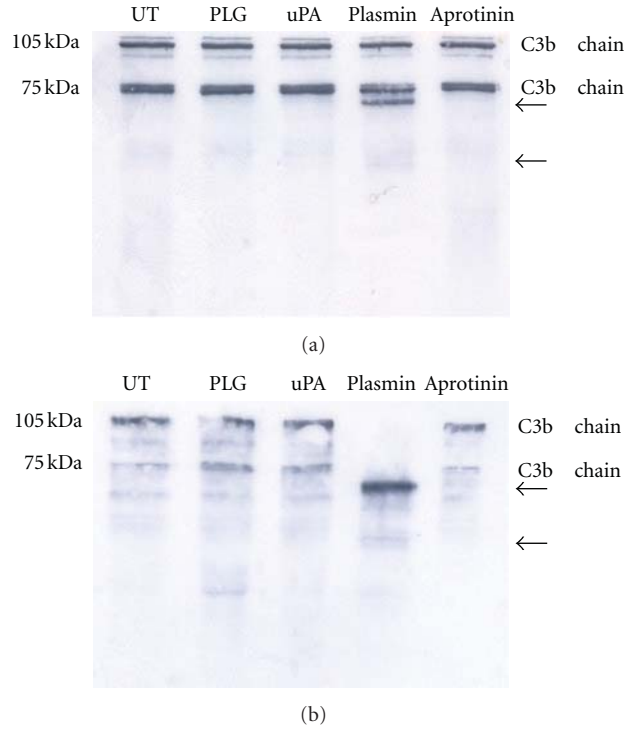


Figure 12: Detection of human C3b. Leptospires (10^8/sample) were treated with 10 μg PLG and 3 U uPA (Plasmin), only with PLG (PLG), or left untreated (UT). The cells were incubated with 15 μg/mL human purified C3b for 20 h at 37°C, and the supernatants were resolved by SDS-PAGE and transferred to membranes. The C3b was detected by specific antibodies followed by ECL reagent development and exposition to X-Ray films. There are indicated the native human C3b alpha chain (105 KDa) and beta-chain (75 KDa), as well as the degradation products (arrows).

deposition from both negative and positive MAT sera was detected [41].

Opsonization obstruction by antibodies or complement system appears to be an interesting approach for the bacteria to evade the first line of human immune defense upon infection. In fact, the expression of proteins on the bacterial surface that sequester host complement regulators FH, FHL-1, or C4BP and therefore the inhibition of the complement cascade by the alternative or classical pathways has been employed by several pathogens [74–77]. Moreover, the proteolytic cleavage of antibodies that prevents the activation of complement classical pathway or Fc receptor-mediated phagocytosis, is another well-known strategy employed by pathogenic bacteria [74–77].

3.12. Human C3b Degradation by Leptospires. Meri and colleagues [54] reported the capability of *Leptospira*-bound factor H (FH) to act as a cofactor for the serum protease factor I in cleaving C3b. Thus, we decided to assess whether the decrease in C3b deposition by *Leptospira*-generated PLA was due to C3b cleavage. *L. interrogans* serovar Pomona were coated with PLA by treatment with PLG and uPA, and then incubated with human purified C3b in fluid-phase. The resulting supernatant together with anti-C3b-specific antibodies was analyzed by Western blotting (Figure 12). The data revealed the appearance of two-lower molecular mass bands of estimated 60 and 20 kDa, concurrently with the decrease in the masses of the alpha (105 kDa) and beta (75 kDa) C3b chains that took place only with PLA-coated leptospires. Untreated or leptospires only treated with PLG showed no gradation. The results unmistakably show that the human C3b is cleaved by leptospires-coated PLA, indicating that the reduction in deposition is most probably due to degradation [41].

3.13. Serum Susceptibility Testing for Treated Leptospires. It has been described that *L. interrogans* serovar Pomona is partially susceptible to complement killing upon incubation with normal human sera [54]. To evaluate whether the PLG/PLA binding to leptospires confers protection on exposure to NHS, leptospires were treated with PLG+uPA (Pla) or with PBS, as negative control, and incubated for one hour with pooled NHS. The bacteria were harvested and counted in a dark-field microscopy using Petroff-Hausser chamber, at the fourth day of incubation. The number of untreated leptospires was almost four times less when compared to the plasmin-coated leptospires ($0.35 \times 10^8 \pm 0.1 \times 10^8$ versus $1.28 \times 10^8 \pm 0.3 \times 10^8$ leptospires/mL), suggesting that the leptospires endowed with active plasmin are less vulnerable to components present in NHS [41].

4. Conclusions

The interaction of the human PLG system has been suggested to be a feature that significantly contributes to the virulence of many bacterial pathogens by facilitating the initial anchoring to endothelium and penetration [27]. Our group has been studying the interaction of *Leptospira* with PLG/plasmin generation system and the possible implications for pathogenesis. We demonstrated that leptospires interact with PLG and can acquire plasmin activity associated to the surface with no apparent damage to the surface, what occurs through multiple proteins. Although nonvirulent leptospires can interact with PLG, there seems to be a correlation between the efficiency of PLG capturing and virulence, suggesting a role in virulence and infection. Moreover, the generation of enzymatically active PLA on the leptospiral surface decreased C3b and IgG depositions that may constitute a novel mechanism by which leptospires could evade the immune system until reach immunologically safe environments. This latter assertion will also have implication to the bacterial persistence within the host. We believe that the disclosure of this enzymatically system will shed light on the molecular mechanism of leptospiral pathogenesis.

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Research Article

Characterization of Plasminogen Binding to NB4 Promyelocytic Cells Using Monoclonal Antibodies against Receptor-Induced Binding Sites in Cell-Bound Plasminogen

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The NB4 promyelocytic cell line exhibits many of the characteristics of acute promyelocytic leukemia blast cells, including the translocation (15 : 17) that fuses the PML gene on chromosome 15 to the RARα gene on chromosome 17. These cells have a very high fibrinolytic capacity. In addition to a high secretion of urokinase, NB4 cells exhibit a 10-fold higher plasminogen binding capacity compared with other leukemic cell lines. When tissue-type plasminogen activator was added to acid-treated cells, plasmin generation was 20–26-fold higher than that generated by U937 cells or peripheral blood neutrophils, respectively. We found that plasminogen bound to these cells can be detected by fluorescence-activated cell sorting using an antiplasminogen monoclonal antibody that specifically reacts with this antigen when it is bound to cell surfaces. All-*trans* retinoid acid treatment of NB4 cells markedly decreased the binding of this monoclonal antibody. This cell line constitutes a unique model to explore plasminogen binding and activation on cell surfaces that can be modulated by all-*trans* retinoid acid treatment.

1. Introduction

Interaction of components of the plasminogen system with fibrin or extracellular matrix promotes plasminogen activation [1]. In a similar way, when components of the plasminogen system are bound to cell surfaces, plasmin generation is increased [2–4]. Binding of plasminogen to cell surfaces is the most important event in the enhancement of plasmin-mediated pericellular proteolysis. Plasminogen binding has two major consequences: (1) plasminogen activation by either tissue-type plasminogen activator (tPA) or urokinase (uPA) is enhanced when plasminogen is bound to cells [5, 6] and (2) plasmin generated on the cell surface is protected from α_2-antiplasmin [4, 7], arming the cell with a more efficient proteolytic activity [5]. The promotion of plasminogen activation by cells can be explained by kinetic interactions with and on the cell surface, conformational effects, and/or receptor occupancy [5].

The amount of plasminogen bound to cell surfaces can be quantified. By using radiolabeled plasminogen, it is possible to detect specific binding of plasminogen to cells and to calculate binding affinities and the number of plasminogen binding sites. With this approach, a wide number of cell types have been analyzed (reviewed in [8]). In humans, platelets and all nucleated cells bind plasminogen with a Kd in the 0.3 to 2.8 μM range in agreement with the plasminogen concentration in plasma (1-2 μM). Cells exhibit a high capacity for plasminogen ranging from 10^4–10^5 binding sites for most nontransformed cells to 10^5–10^7 for malignant cells and human umbilical vein endothelial cells. In general, plasminogen binding capacity is paralleled by the capacity of the cells to promote plasminogen activation. For

example, in studies of plasminogen receptor modulation, it has been demonstrated that upmodulation of plasminogen binding sites, a process that can be induced by several effectors, induces an increase in the promotion of plasmin generation by cells [9]. Among malignant cells, the study of fibrinolysis on acute promyelocytic leukemia (APL) cells offered new insights in the understanding of this disease and its hemorrhagic complications.

APL is due to a clonal proliferation of promyelocytic blast cells carrying the $t(15:17)$ that fuses the PML gene on chromosome 15 to the RARα gene on chromosome 17 [10, 11]. In contrast to other leukemic processes, APL onset is frequently associated with life-threatening bleeding complications due to disseminated intravascular coagulation, abnormal fibrinolysis, or both [10–12]. Immature promyelocytes secrete high amounts of uPA [13, 14] that can promote plasmin formation *in vivo* and cause abnormal bleeding. In addition, a mechanism of promotion of plasminogen activation by cell surfaces has been explored on APL blast cells and on the promyelocytic cell line NB4. This promyelocytic cell line carries the typical translocation found in APL blast cells and has been used in a wide variety of biological studies on APL. The introduction of all-*trans* retinoid acid (ATRA) in the treatment of this disease in the nineties has dramatically changed the outcome of APL. In most APL patients, ATRA treatment induces differentiation of immature promyelocytic cells and corrects bleeding disorders. ATRA has several dramatic effects on the hemostatic system on both APL blast cells and on NB4 cells [15].

In this study, we sought to characterize plasminogen binding to NB4 cells using three different approaches. First, using radiolabeled plasminogen, we analyzed the plasminogen binding capacity of NB4 cells compared with other leukemic cells lines of different lineages. Second, we explored the functional consequences of plasminogen activation on NB4 cell surfaces analyzing plasmin generation by these cells. Finally, we measured plasminogen bound to this cell line by fluorescence-activated cell sorting analysis using an antiplasminogen monoclonal antibody that specifically interacts with plasminogen bound to cell surfaces [16] and explored the effect of ATRA treatment of NB4 cells on plasminogen binding.

2. Material and Methods

2.1. Proteins, Protein Iodination, and Antibodies. Glu-plasminogen was obtained from Chromogenix (Mölndal, Sweden). tPA (Actilyse) and high-molecular-weight uPA were obtained from Boehringer Ingelheim and Roger Laboratories (Molins de Rei-Barcelona, Spain), respectively. Glu-plasminogen was radiolabeled using a modified chloramine T method [17]. The labeled and unlabeled preparations of plasminogen used in this study had the characteristics of previously described preparations from our laboratory [17–20]. Antiplasminogen monoclonal antibody 49 (mAb49) was raised and characterized as previously described [16]. Fluorescein isothiocyanate (FITC) conjugated goat anti-mouse monoclonal antibodies were from Sera-Lab, Ltd.

2.2. Cells. Neutrophils, monocytes, and lymphocytes were isolated from blood collected into heparin (5 U/mL) as described [21]. NB4 cells were provided by Dr. M. Lanotte (Hôpital St. Louis, Paris, France). The human cell line, Nalm6, was provided by Dr. J. Inglés-Esteve (IDIBELL, Barcelona). Other cell lines were from the American Type Tissue Culture Collection (ATCC) and cultured in RPMI-1640 (Bio-Whitakker/MA Bioproducts) containing 1 mM Na pyruvate and 5–10% fetal bovine serum. Blast cells from peripheral blood were analyzed from a patient with acute nonlymphoblastic leukemia (ANLL), categorized according to the FAB classification [22].

2.3. Ligand Binding Analyses. Ligand binding analyses were performed as previously described by separating bound from free ligand by centrifugation over 20% sucrose [17–20]. Molecules of ligand bound per cell were calculated based on the specific activities of the radiolabeled ligands.

2.4. Cell-Dependent Promotion of Plasminogen Activation. Plasminogen activation studies were carried out in microtitre plates in reaction volumes of 100 μL as previously described [5, 6]. Briefly, 20 μL of plasminogen activators (tPA or uPA) (final concentration 70 and 37 pmol/L, resp.) were mixed with 40 μL of cells (final concentration 1.5×10^6 cells/mL) and 40 μL of substrate mix containing Glu-plasminogen (final concentration 100 nmol/L) and chromogenic substrate S-2251 (Val-Leu-Lys-p-nitroanilide; Chromogenix) (final concentration 0.15 mmol/L). Reactions were performed in assay buffer consisting of Tris-HCl, pH 7.4, at 37°C, and a final ionic strength of 0.12, containing 1 mg/mL human serum albumin. Absorbance was monitored at 405 nm, using a Thermomax thermostatted plate reader (Molecular Devices Corporation, Stanford, CA). Rates of plasmin generation were calculated as previously described [5, 6].

2.5. Fluorescence-Activated Cell Sorting (FACS) Analysis. Cells were washed with PBS containing 1% BSA and 0.1% sodium azide (PBA), incubated with PBA containing 10% heat-inactivated normal rabbit serum, washed again, and incubated with mAb49 (130 nM) or isotype control, washed, and then stained with FITC-goat anti-mouse IgG, which was detected in a flow cytometry analyzer (Coulter's EPICS XL-MCL). Plasminogen binding to cells in whole peripheral blood collected into EDTA was determined as above with the following exceptions. Cells were incubated in 10% heat-inactivated human AB serum in PBS, washed with PBA and incubated with anti-mouse IgG conjugated to PE, washed and incubated with FITC-conjugated antibodies to specific leukocyte antigens. Cells were incubated in Ortho-mune Lysing Reagent (Ortho Diagnostic Systems Inc.), centrifuged, and resuspended in PBA containing 7-aminoactynomycion D (Molecular Probes) at 1 mg/mL.

2.6. Reagents. Heparin, Tween 80, Tween 20, ε-ACA, and bovine serum albumin were from Sigma (St. Louis, MO). All-*trans* retinoic acid was from Hoffmanm-La Roche.

Table 1: Plasminogen binding to cell surfaces of several leukemic cell lines and normal peripheral blood cells.

Cell type	Cell lineage	Molecules of plasminogen bound/cell ($\times 10^6$)
NB4	Promyelocytic	38.0 ± 1.8
KG1a	Myeloblastoid	1.5 ± 0.2
K562	Erythromyeloid	3.1 ± 0.2
HL-60	Promyeloid	1.7 ± 0.3
U937	Monocytoid	2.0 ± 0.7
THP-1	Monocytoid	1.6 ± 0.2
Nalm6	Pre-B-Cell	1.2 ± 0.2
Molt4	Undifferentiated T cell	1.1 ± 0.4
	Neutrophils	0.35 ± 0.07
	Monocytes	0.58 ± 0.08
	Lymphocytes	0.59 ± 0.06

Binding analyses were carried out by incubation of washed cells ($2–5 \times 10^6$/mL) with radiolabeled plasminogen (100 nM) in a total volume of 200 μL for 2 hours a 4°C. Cells were then separate from the whole reaction mixture by centrifugation of aliquots in 20% sucrose solution. The specific binding of radiolabeled plasminogen was determined by subtracting counts bound in the presence of 0.15 M ε-ACA. Results are the mean ± SD of 2–4 separate experiments.

Neutrophils, monocytes, lymphocytes, and RBC were isolated from blood collected into heparin (5 U/mL), theophylline (10 mM), and prostaglandin $E_{1\alpha}$ (10 U/mL) (Sigma) as described [6].

3. Results

3.1. Plasminogen Binding Capacity of NB4 Promyelocytic Cells. To explore the plasminogen binding capacity of this cell line with respect to other leukemic cells lines and peripheral blood cells, radiolabeled plasminogen (100 nmol/L) was added to washed cells ($2–5 \times 10^6$/mL) and incubated for 1 hr at 37°C. After incubation, free ligand was separated by centrifugation on 20% sucrose. Specific binding was calculated by subtracting counts bound in the presence of 0.15 mol/L ε-ACA. Under these conditions, NB4 cells specifically bound $38.0 \pm 0.7 \times 10^6$ molecules of plasminogen per cell. For comparison, plasminogen binding was also explored on several leukemic cell lines of distinct lineages and in several types of normal peripheral blood cells. As shown in Table 1, NB4 cells bound plasminogen with a capacity at least one order of magnitude higher than the other cells analyzed.

3.2. Functional Consequences of Plasminogen Binding to NB4 Cells. Plasminogen binding to leukocytoid cells promotes plasmin formation in the presence of either tPA or uPA plasminogen activators [4–6, 22–26]. In previous kinetic studies, we have demonstrated that plasmin generation is promoted by leukocytoid cells by 60- to 30-fold when tPA or uPA, respectively, was used as plasminogen activators [5–9]. With a similar approach, we explored the promotion of plasminogen activation by NB4 cells.

NB4 cells secrete high amounts of uPA [10, 13]. uPA has a high affinity for uPA receptors, and an autocrine mechanism of saturation of uPA receptors has been previously described in several cell lines [27]. To assess whether NB4 cells could promote plasmin formation in the absence of extrinsic plasminogen activators, washed cells were incubated with plasminogen and the rate of plasminogen activation was measured as previously described [5, 6]. Under this conditions, NB4 cells generated 0.3 ± 0.01 pmol/L of plasmin $\cdot$ sec^{-1}, whereas 0.07 ± 0.02 and 0.06 ± 0.01 pmol/L of plasmin $\cdot$ sec^{-1} were generated by U937 cells or peripheral blood neutrophils, respectively. These data suggested that a significant fraction of uPA secreted by NB4 cells was bound to their cell surface and could activate plasminogen.

To better define the role of plasminogen binding sites in the promotion of plasmin formation, NB4 cells were acid treated to remove uPA from uPA receptors. Following acid treatment, <0.01 pmol/L $\cdot$ sec^{-1} of plasmin was generated when cells were incubated with plasminogen, indicating that the uPA was efficiently removed from cells by this treatment. Acid-treated NB4 cells were incubated with plasminogen and tPA and the rate of plasminogen activation determined. Under these conditions, plasmin generation on acid treated NB4 cells was 0.8 ± 0.04 pmol/L $\cdot$ sec^{-1} while U937 and peripheral blood neutrophils generated 0.3 ± 0.08 and 0.016 ± 0.04 pmol/L $\cdot$ sec^{-1}. of plasmin, respectively. Taken together, these data suggested that the high number of plasminogen binding sites detected on NB4 cells was paralleled by a high capacity to promote plasminogen activation on their cell surface.

3.3. Detection of Plasminogen Bound to the Cell Surface of NB4 Cells. To explore whether plasminogen could be detected on the cell surface of NB4 cells, we used an antiplasminogen monoclonal antibody (mAb49) that recognizes receptor-induced binding sites (RIBSs) in plasminogen and, therefore, preferentially react with cell-associated plasminogen in the presence of soluble plasminogen [16]. Cells were washed and incubated with plasminogen (10 μmol/L) for 1 hr at 37°C. Then, mAb49 was added to the system and processed for FACS analyses as described in Section 2. As depicted in Figure 1, mAb49 detected plasminogen bound to these cells. The mean fluorescence intensity was 2.2-fold higher in the system containing added plasminogen (5.5 units) than in the negative control without plasminogen (2.5 units).

To assess whether plasminogen binding changes induced by ATRA treatment of NB4 cells [12, 29] could also be detected by mAb49, these cells were treated for 48 hour with 5 μM ATRA. The positive FACS signal observed with untreated NB4 cells, preincubated with plasminogen, was markedly decreased after treatment of NB4 cells with ATRA for 48 hr (Figure 2(a)). For comparison, we explored with a similar approach blast cells from a patient with APL with a large proportion of blast cells (80%), both prior to and after ATRA treatment *in vivo*. A strong FACS signal with mAb49 was detected prior to ATRA treatment compared with the isotype control (Figure 2(b)). After both a 4-day and a 5-day of treatment with ATRA, blast cells exhibited a

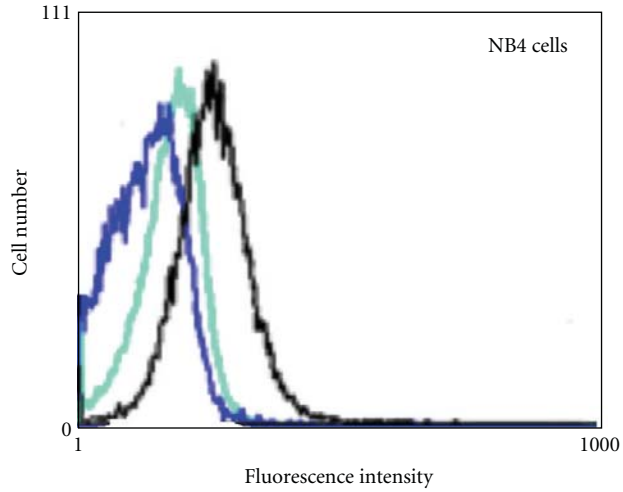

Figure 1: Detection of plasminogen bound to the surface of NB4 cells. Cells (5×10^5) were washed with PBS containing 1% BSA and 0.1% sodium azide (PBA) and incubated with 10 μM plasminogen (black tracing) or buffer (blue tracing) for 1 hour at 37°C, washed again and incubated with PBA containing 10% heat-inactivated normal rabbit serum for 10 minutes a room temperature. As an additional control, NB4 cells were treated with carboxypeptidase B (200 U/mL) before adding plasminogen (teal tracing). After incubation, supernatants were removed by centrifugation, incubated with mAb49 (130 nM) for 30 minutes a 4°C, washed and then stained with FITC-goat anti-mouse IgG, which was detected by FACS analyses. This research was originally published in [28].

markedly decreased FACS signal, compared with untreated cells (Figure 2(b)). As an additional control, FACS analysis of M1 blast cells (that have not been reported to bind plasminogen) with mAb49 did not show a positive signal compared with isotype control (Figure 2(c)).

Taken together, these results demonstrate that mAb49 can be used to monitor modulation of plasminogen to NB4 cells following ATRA treatment. Changes induced by ATRA are similar to the ones observed in blast cells of patients with APL.

4. Discussion

In this study, we have characterized plasminogen binding to the NB4 promyelocytic leukemia cell line by analyzing plasminogen binding to these cells in comparison to other blood cell lines or peripheral blood cells. The functional consequences of the high capacity of plasminogen binding by NB4 cells were explored in kinetic studies measuring plasmin generation. In addition, plasminogen binding to NB4 cells was also explored using an antiplasminogen monoclonal antibody that specifically recognizes plasminogen bound to cells. This antibody allows detection of downmodulation of plasminogen binding induced by ATRA treatment of these cells.

NB4 cells exhibited a high capacity for binding of plasminogen. This capacity is one order of magnitude higher than that displayed by other leukemic cells lines of distinct lineages and two order of magnitude higher than normal peripheral nucleated blood cells. Thus, the NB4 cell line has the highest capacity for plasminogen among the cell lines tested. This characteristic is also complemented with a high capacity to produce uPA and therefore NB4 cells have been used as models to study the bleeding complications of APL patients [10–15, 29–31]. The high plasminogen binding capacity of NB4 cells has been related to the overexpression of plasminogen binding molecules. Several proteins have been identified as cell surface binding molecules for plasminogen, including α-enolase, annexin II, tissue factor, and the complex S100A10-Annexin II [7–9, 12, 29–39]. Although annexin II was highly expressed by NB4 and APL blast cells antiannexin II antibodies reduce plasminogen activation mediated by these cells by 35%, ε-aminocaproic acid (EACA) gave a 71% reduction [12]. Because EACA inhibits the interaction of plasminogen with cells, this result implies that other plasminogen receptors in addition to annexin II could be important for stimulation of plasminogen activation. An antibody to S100A10 molecule fully blocks endothelial cell plasmin production [40], but no data have been generated using this antibody on NB4 or APL cells. A monoclonal antibody to α-enolase (11G1) blocks the cell surface promotion of plasminogen activation in a wide variety of leukemic cell lines and abrogates NB4 mediated plasminogen activation by 70–80% [41]. Thus, in addition to the Annexin II-S100A10 complex, α-enolase also mediates plasminogen binding to NB4 cells. Tissue factor is also a plasminogen-binding molecule [39] but very high concentrations of soluble TF are required to reduce plasminogen binding or cell-dependent promotion of plasminogen activation. Thus, the physiological role of TF in APL blast cells as a plasminogen binding molecule should be further studied.

NB4 cells secrete high amounts of uPA, and therefore we explore the capacity of these cells to promote plasmin generation in the absence of added plasminogen activators. Under these conditions, NB4 cells generated plasmin with a 4-5-fold higher efficiency that U937 cells or peripheral blood neutrophils. When tPA was added to acid-treated cells, promotion of plasmin generation by NB4 cells was 20–26-fold greater than by U937 cells or neutrophils, respectively. These data suggest a parallelism between plasminogen binding capacity and promotion of plasmin formation on NB4 cell surfaces.

In flow cytometric and radioimmunometric studies, we have previously demonstrated that a fraction of blood plasminogen is bound to surfaces of peripheral nucleated blood cells and platelets. In these studies, plasminogen bound to cells was detected using a monoclonal antibody to plasminogen which preferentially reacts with plasminogen bound to cell surfaces, suggesting that plasminogen binding to cells induces a conformational change in plasminogen and that latent epitopes in soluble plasminogen become available when plasminogen is bound to cells. These antibodies detect receptor-induced binding sites (RIBS) in plasminogen induced by its interaction with cells. Thus, they have been named RIBS antibodies (see details in [16]). A practical

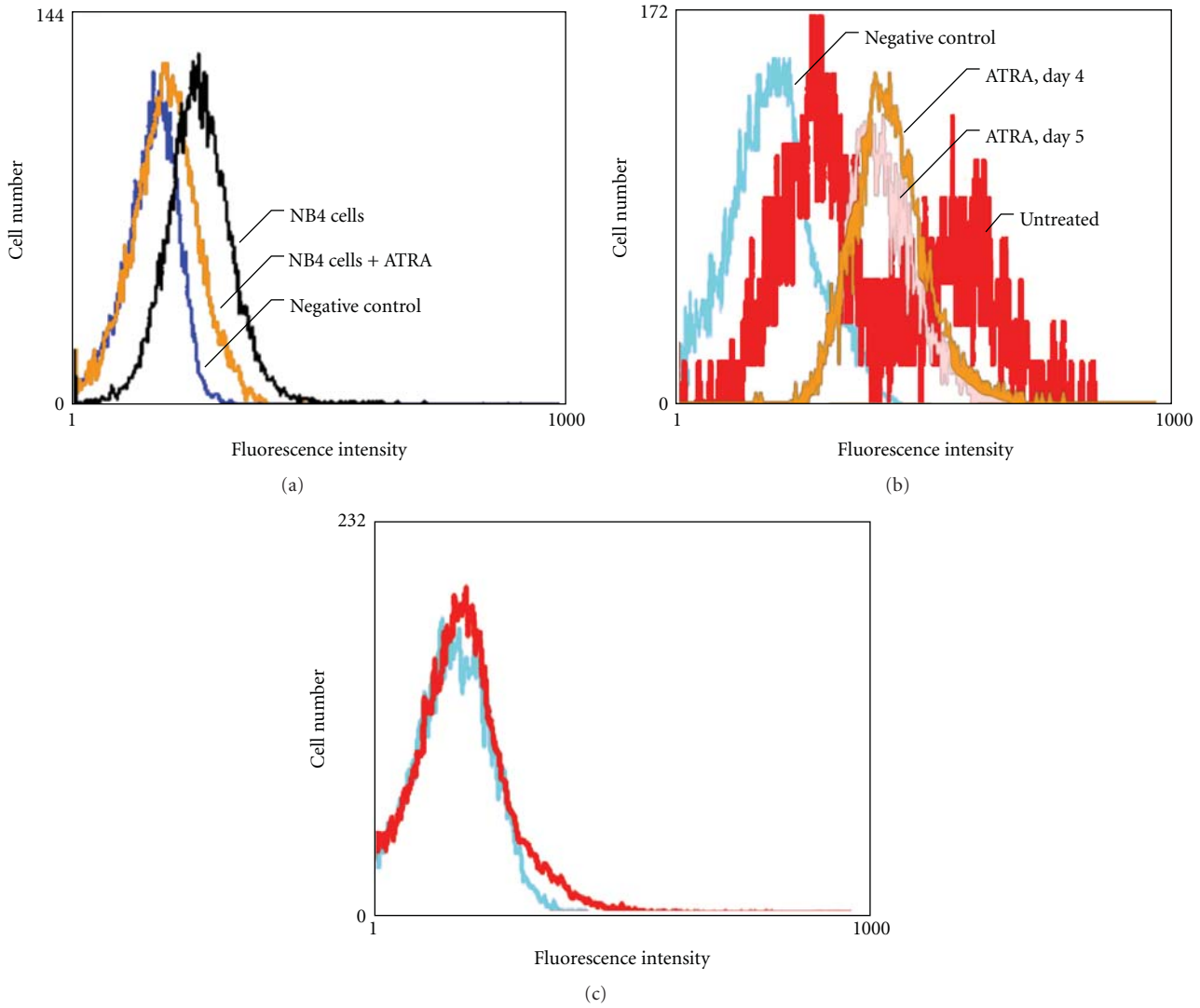

Figure 2: *In vitro* and *in vivo* ATRA modulation of plasminogen binding to NB4 cells and APL blast cells. (a) NB4 cells were incubated with 1 μM all-*trans* retinoic acid (ATRA) for 48 hours, washed and preincubated with plasminogen (10 μM) followed by FACS analyses with mAb49 (orange tracing). FACS analyses with mAb49 of untreated NB4 cells preincubated with either plasminogen (black tracing) or buffer (blue tracing) detected by antiplasminogen mAb49. (b) Plasminogen bound to blast cells from a patient with APL (CD33+; HLDR Negative) was monitorized in whole blood using mAb49. Analyses were performed at day 0 of ATRA treatment (red tracing) and after ATRA treatment for either 4 days (orange tracing) or 5 days (pink tracing). As a negative control FACS analysis with an isotype control antibody (turquoise tracing). (c) FACS analysis using mAb49 (red tracing) or isotype control (turquoise tracing) of blood from a patient with an M1 leukemia. This research was originally published in [28].

application of the use of antiplasminogen RIBS antibody has been explored on acute promyelocytic leukemia (APL) blast cells. This antibody gives a clear positive signal in FACS analyses and can be used to explore changes in the amount of plasminogen bound to blast cells in whole blood during all-*trans* retinoid acid (ATRA) treatment of APL patients [41]. Similar results were also obtained here with NB4 cells. Plasminogen was detected on NB4 cells incubated with plasminogen, whereas culture of NB4 cells with ATRA for 48 hours markedly reduced the antiplasminogen RIBS signal [41]. Again, these data reinforce the similarity between APL blast cells and the NB4 cell line. In recent studies, ATRA treatment of NB4 cells induces a downregulation of the plasminogen-binding molecule S100A10 that is paralleled by reduction in fibrinolytic activity [29]. In addition, depletion of S100A10 by RNA interference abrogates cell-dependent fibrinolytic activity in NB4 cells. Thus, these cells constitute an excellent model to explore the modulation of plasminogen binding to cells using mAb49.

5. Conclusions

Taken together, these data suggest that the NB4 cell line constitutes a unique cell model for plasmin generation on cell surfaces. The individual contribution of molecules that bind plasminogen on these cells should be explored in future

studies. In addition, the downregulation of plasminogen receptors induced by ATRA treatment of NB4 cells offers an exciting model to study the modulation of these receptors and their functional consequences.

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Review Article

Bacterial Plasminogen Receptors: Mediators of a Multifaceted Relationship

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Multiple species of bacteria are able to sequester the host zymogen plasminogen to the cell surface. Once localised to the bacterial surface, plasminogen can act as a cofactor in adhesion, or, following activation to plasmin, provide a source of potent proteolytic activity. Numerous bacterial plasminogen receptors have been identified, and the mechanisms by which they interact with plasminogen are diverse. Here we provide an overview of bacterial plasminogen receptors and discuss the diverse role bacterial plasminogen acquisition plays in the relationship between bacteria and the host.

1. Introduction

Recruitment of plasminogen to the bacterial cell surface is emerging as a central theme in host/pathogen interactions. The glycoprotein plasminogen is found in plasma and extracellular fluids at concentrations of approximately 2 μM. Upon activation, plasminogen is converted to the serine protease plasmin [1]. Plasmin is able to degrade fibrin clots, connective tissue, extracellular matrix (ECM), and adhesion proteins. Plasmin itself contributes to a number of amplification loops which leads to increased plasminogen activation. Plasmin-mediated proteolysis of cell membrane proteins exposes cryptic plasminogen-binding sites within receptors, subsequently enhancing the recruitment of plasminogen to cell surfaces [2]. Similarly, cleavage of the inactive form of the urokinase plasminogen activator pro-uPA by cell bound plasmin generates the active two-chain uPA. This feedback activation results in a significant increase in plasmin activation within biological systems [3]. Additionally, activation of prometalloproteases by plasmin results in degradation of the collagen structural components of the ECM, leading to widespread tissue destruction. Recruitment of plasminogen to the surface of bacteria by specific plasminogen receptors was first reported over 20 years ago [4]. Since then, the importance of this interaction in bacterial virulence has become the focus of a large body of research. It is now clear that recruitment of plasminogen to bacterial cell surfaces is a feature common to both pathogenic and commensal bacteria. This paper provides an overview of known bacterial plasminogen receptors and examines the diverse roles they play in the host-bacteria interaction.

2. Plasminogen

Plasminogen is the inactive zymogen form of the enzyme plasmin [5, 6]. Posttranslational processing results in several different forms of plasminogen (Figure 1). The circulating mature form of plasminogen is known as Glu-plasminogen as a consequence of the glutamic acid residue at the N-terminus. Glu-plasminogen consists of the preactivation peptide followed by five characteristic kringle domains and then the serine protease active site in the C-terminal region [6] (Figure 1). The amino acid residues His^{603}, Asp^{646} and Ser^{741}, make up the catalytic triad of the serine protease domain. This domain catalyses the hydrolysis of peptide bonds, resulting in peptides with C-terminal arginine and lysine residues [6]. The kringle domains of plasmin(ogen) mediate interactions with multiple ligands, including fibrin(ogen) and mammalian cellular plasmin(ogen) receptors

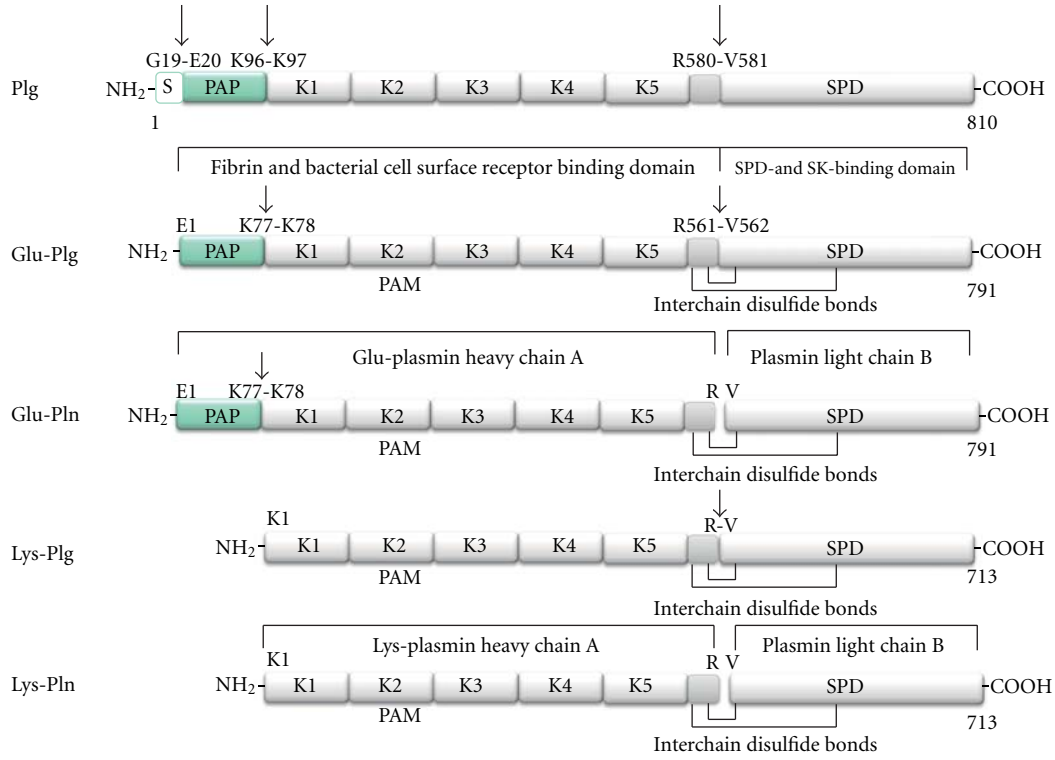

Figure 1: Structural domains of human plasmin(ogen) forms. Human plasminogen is synthesised as an 810 amino acid protein. The 19 amino acid residue signal sequence is removed resulting in the circulating mature form (791 amino acids, ~90,000 kDa) known as Glu-plasminogen (Glu-plg) as it contains an N-terminal glutamic acid. Glu-plg contains a hairpin-loop structure called the PAN domain encompassing the preactivation peptide (PAP), followed by 5 homologous kringle domains (K1–K5) containing three intradomain disulfide bridges, followed by a peptidase S1 domain (SPD). The preactivation peptide is generated by plasmin cleavage giving rise to Lys-plg (713 amino acids, ~80,000 kDa). The conversion of Glu-plg or Lys-plg to their respective plasmin forms occurs by hydrolysis of the Arg-Val peptide bond shown by either uPA or tPA, yielding chain A and the smaller chain B, which remain covalently associated by interchain disulfide bonds. Kringles 1, 2, 4, and 5 contain lysine-binding sites (LBS) with affinity for free lysine and lysine-like compounds such as ω-aminocarboxylic ligands in the following order of binding affinity K1 > K4 > K5 > K2 [8]. Kringle 3 shows no detectable binding to Lys or Lys-like compounds [9], related to a sequence variation in its LBS. Glu-plg thus binds to various lysine-containing proteins via Kringles 1, 2, 4, and 5. Streptokinase (SK) and staphylokinase (not shown) bind in a 1 : 1 complex with the SPD to generate an activator complex. Not shown: Mini plasminogen (K5 plus the SPD) can also be generated by stromelysin-19 cleavage of the Pro466-Val467 bond of Plg. Sequence data are derived from UniProt (swiss-prot entry P00747). Plasminogen (EC = 3.4.21.7) (http://www.uniprot.org/uniprot/P00747).

[7]. In particular Kringles 1, 2, 4, and 5 (K1-K5) contain lysine-binding sites (LBS) comprised of a hydrophobic cleft formed by aromatic residues that most commonly bind C-terminal lysine residues and internal lysine residues of receptors. As described in Figure 1, the kringles show differing affinities for free lysine and lysine-like compounds such as ω-aminocarboxylic ligands, in the following order of binding affinity K1 > K4 > K5 > K2 [8]. Kringle 3 shows no detectable binding to Lys or Lys-like compounds [9]. Intramolecular binding between lysine residues and the LBS of these Kringles maintains Glu-plasminogen in a closed conformation which is less susceptible to activation [6, 10]. Competitive binding interactions with fibrin(ogen) or plasminogen receptors allows Glu-plasminogen to adopt an open conformation, exposing the activation loop (Arg^{561}-Val^{562}) to cleavage by specific mammalian plasminogen activators thus forming Glu-plasmin [6, 7] (Figure 1). Alternatively, cleavage of the Lys^{77}-Lys^{78} peptide bond may also occur leaving the plasminogen molecule with a Lys residue at the-N terminus (Lys-plasminogen) [6] (Figure 1). Lys-plasminogen has a more open, U-shaped conformation than Glu-plasminogen making it more readily activated to Lys-plasmin by the plasminogen activators [11, 12]. The resulting two-chain Glu- or Lys-plasmin molecule consists of the plasmin heavy chain A in the N-terminal region and the plasmin light chain B in the C-terminal region held together by interchain disulfide bonds (Figure 1).

Two differentially glycosylated variants of human Glu-plasminogen exist. Both variant 1 and 2 contain O-linked glycosylation site, whereas variant 1 contains an additional N-linked glycosylation site (located at Asn^{289} within Kringle 3) [13–15]. These glycosylation patterns appear to affect both the stability and affinity of the protein to interact with lysine moieties as well as its subsequent activation rate [16]. Differences in glycosylation were recently shown to affect the positioning of Kringle 3 (a non-LBS containing Kringle) in the X-ray crystal structure of plasminogen, which has consequences for efficient Glu-plasminogen activation [10].

3. Plasminogen-Binding Bacteria

Commandeering the host plasminogen activation system is a common mechanism employed by a variety of bacteria [17–20]. The ability to acquire cell surface plasminogen is not host species restricted or limited to specific sites of infection. Rather, the ability to recruit plasminogen is emerging as a central theme in the interaction between host and bacteria. Early studies by Ullberg et al. showed that 5 out of 11 species of gram-negative bacteria tested and 9 out of 17 species of gram-positive bacteria tested displayed a specific and high affinity interaction with Glu-plasminogen [21, 22], although plasminogen acquisition by different strains within each species varied significantly. Many studies have since focused on the ability of highly pathogenic bacteria to interact with plasminogen, including *Streptococcus pyogenes*, *S. pneumoniae*, *Staphylococcus aureus*, *Helicobacter pylori*, *Mycobacterium tuberculosis*, *Neisseria meningitides,* and *N. gonorrhoeae,* [23–25]. There is also a growing body of evidence to indicate that animal pathogens sequester plasminogen. Examples of this include *Mycoplasma hyopneumoniae* and *M. gallisepticum* which bind porcine and chicken plasminogen, respectively, [26, 27] and the canine pathogen *S. suis* [28].

Interactions with plasminogen are not solely the domain of pathogenic bacteria, with a number of commensal species also reported to bind plasminogen with both high affinity and specificity, including several species of oral *streptococci* [29], *bifidobacteria* [30], and *lactobacillus* [31]. The role of bacterial-plasminogen recruitment in pathogenesis will be discussed in more detail later; however, given the above findings it appears that plasminogen recruitment by bacteria may have a multifaceted role in the interaction with the host. This may underlie the diversity of plasminogen receptors expressed by bacteria and the different mechanisms of interaction which have been described to date.

4. Bacterial Plasminogen Receptors

Recruitment of plasminogen to the bacterial cell surface is mediated directly by either specialised cell surface receptors or cytoplasmic and glycolytic pathway proteins localised to the bacterial cell surface or indirectly via interactions with host plasma proteins such as fibrinogen. Table 1 gives an overview of the most well-characterised bacterial plasminogen receptors.

4.1. Specialised Cell Surface Receptors. Cell surface expressed receptors can be defined as those proteins which have a recognisable N-terminal signal sequence and membrane anchor motif. Several cell surface expressed plasminogen receptors have been well characterised, and it is interesting to note that many of these appear to have internal plasminogen-binding sites. Among the best characterised of these is the group A streptococcal plasminogen binding M protein. This coiled-coil alpha helical protein extends from the streptococcal cell surface and binds Glu-plasminogen with an affinity of K_d 1-2 nM [32, 33]. A combination of bacterial mutants, synthetic peptides and amino-acid substitution in recombinant proteins has been utilised to demonstrate that plasminogen binding to group A streptococcal M proteins is dependent on the presence of an internal plasminogen-binding repeat domain, consisting of positively charged arginine and histidine residues [34–36]. X-ray crystallography studies of the interaction between a 30-amino acid peptide comprising the plasminogen binding domain of streptococcal M protein (VEK-30) and a modified version of K2 of plasminogen indicate that Arg^{17} and His^{18} of VEK-30 form a pseudolysine structure that interacts with the LBS of this kringle [36]. This work supports earlier studies which showed that group A streptococcal plasminogen-binding M proteins interact with K2 of plasminogen, which contains a low affinity lysine-binding site [37]. Despite the fact that plasminogen binding by M proteins is readily inhibited by the lysine analogue EACA [32], mutation of the lysine residues within the bacterial interaction motif is not sufficient to fully abrogate plasminogen binding [34]. This highlights the important point that EACA competition alone is insufficient to demonstrate the role of lysine residues in interactions with plasminogen and its many receptors. Rather, the ability of lysine analogues to compete out plasminogen binding can be interpreted as demonstrating a role for the LBS within the kringle domains of plasminogen.

Plasminogen-binding M proteins are expressed by approximately 15% of group A streptococcal isolates, and similar proteins have been identified in a variety of group C and G streptococcal strains [38, 39]. Recently, a plasminogen-binding M protein expressed by the group G streptococci *S. canis* was reported to bind to miniplasminogen, a plasminogen variant consisting of only K5 and the serine protease domain [28]. Similarly, the M-like protein of group C streptococcus GCS3 likely interacts with K4 or K5 of plasminogen [40]. K4 and K5 show a high affinity for lysine-based ligands when compared with K2 [8], so, whilst specific plasminogen-binding sites within the M proteins of group C streptococcus and *S. canis* are yet to be defined, it is likely that they display markedly different properties to the internal motif described for the group A streptococcal plasminogen-binding M proteins. It is possible that these receptors mediate plasminogen binding at different sites or stages of infection; however, this hypothesis has yet to be fully explored. Based on the crystal structure of plasminogen, it has been suggested that the interaction of K5 with lysine residues is key to the structural change of plasminogen from its closed to open form [41]. It is tempting to hypothesise that bacteria which do not express their own plasminogen activators, such as *S. canis* may have evolved plasminogen interaction mechanisms that allow more efficient activation by host activators.

Internal plasminogen-binding sites have also been proposed for several bacterial lipoproteins identified as plasminogen receptors. *B. burgdorferi* binds plasminogen via an array of lipoproteins, including ErpP, ErpC, Erp, and OspA [46], while *B. recurrentis* and *B. hermsii* mediate plasminogen binding by the lipoproteins HcPA and BhCRASP1 [87, 88]. Similarly, several as yet uncharacterised lipoproteins of *Francisella tularensis* have been found to interact with plasminogen in human plasma via ligand blot analysis [89]. Whilst specific plasminogen binding sites within all

Table 1: Bacterial plasminogen receptors and their interactions with plasminogen.

Plasminogen receptor	Bacterial species	Cell surface attachment	Binding affinity (K_D)	Binding interactions and characteristics	References
Bfp60	*Bacteroides fragilis*	Anchored	ND	ND	[42]
Choline-binding protein E (CBPE)	*Streptococcus pneumoniae*	Anchored	ND	Binds plg via internal lysine residues K^{259}, K^{267}, and K^{319} present in the phosphorylcholine esterase domain.	[43, 44]
CRASP-1, 3, 4, and 5	*Borrelia burgdorferi*	Anchored	ND	ND	[45]
ErpP, ErpC, and ErpA	*Borrelia burgdorferi*	Anchored	Glu-plg: K_D = 25 nM	Plg binding is associated with C-terminal lysine residues. Bound plg can be activated by uPA.	[46]
Erp63	*Borrelia spielmanii*	Anchored	ND	ND	[47]
Flagella	*Escherichia coli*	Anchored	ND	ND	[48]
GlnA1	*Mycobacterium tuberculosis*	Anchored	ND	Interact with LBS within plg	[49]
LenA	*Leptospira interogans*	Anchored	ND	Interacts with the K1–K3 plg fragment	[46, 50]
Leptospiral surface adhesin Lsa66	*Leptospira interrogans*	Anchored	Plg: K_D = 68.8 nM	ND. Bound plg can be activated by uPA	[51]
Lp30	*Leptospira interrogans*	Anchored	Plg: K_D = 167.39 nM	ND. Bound plg can be activated by uPA	[51]
LIC12238	*Leptospira interrogans*	Anchored	Plg: K_D = 11.97 nM	ND. Bound plg can be activated by uPA	[52]
LIC10494			Plg: K_D = 10.98 nM		
LIC12730			ND		
LipL32, LipL40	*Leptospira interrogans*	Anchored		ND	[52]
Lp29, Lp49	*Leptospira interrogans*	Anchored		ND	[52]
Lsa20	*Leptospira interrogans*	Anchored	ND	ND	[53]
Lsa66			Plg: K_D = 68.8 nM		[51]
M and M-like protein	*Streptococcus pyogenes*	Anchored	Glu-plg: K_D = 1.6 nM–7.6 nM	K2	[34, 35]
	Streptococcus canis		Mini-plg: K_D = 2.7 nM	High affinity for plg K5	[28]
	Streptococcus equi		Plg: K_D = 18 nM	Plg binding not competed out by excess K1–3, but inhibited by EACA, suggesting a role for K4 or K5	[54]
Mhp 107	*Mycoplasma hyopneumoniae*	Anchored	Plg (porcine): K_D = ND	ND	[26]
MPL36	*Leptospira interrogans*	Anchored		ND	[52]
Outer surface protein A (OspA)	*Borrelia burgdorferi*	Anchored	Glu-plg: K_D = 260 μM	Interacts with LBS within plg and pln. Bound plg can be activated by both uPA and tPA	[55]
70 kDa surface protein (OppA)	*Borrelia burgdoferi*	Anchored	ND	ND	[55, 56]
PavB	*Streptococcus pneumonia*	Anchored	ND	ND	[57]
PfbB	*Streptococcus pneumonia*	Anchored	ND	ND	[58]
PfbA	*Streptococcus pneumonia*	Anchored	ND	ND	[59]
Plasminogen-binding protein (Pbp)	*Bacteroides fragilis*	Anchored	ND	ND	[60]
PbbA and pgbB	*Helicobacter pylori*	Anchored	ND	Interacts with LBS of plg	[61]

Table 1: Continued.

Plasminogen receptor	Bacterial species	Cell surface attachment	Binding affinity (K_D)	Binding interactions and characteristics	References
P116	*Mycoplasma hyopneumoniae*	Anchored	Asp-plg (porcine): K_D = 44 nM	ND	[26]
Protein E	*Haemophilus influenzae*	Anchored	ND	Interacts with LBS of plg	[62]
Type 1fimbriae	*Escherichia coli*	Anchored	Glu-plg: K_D = 200 nM	ND	[63, 64]
	Streptococcus pneumoniae		Plg: K_{D1} = 0.55 nM; K_{D2} = 86.2 nM	Residues 248–256; C-terminal lysyl residues LL^{433} and LL^{434}. Interacts with LBS within Plg	[65–67]
	Streptococcus pyogenes		Glu-Plg: K_D = 1.6 nM; Lys-plg: K_D = 127 nM	C-terminal lysine residues K^{434} and K^{434}; Residues 252–255. Interacts with LBS within Plg	[68, 69]
	Streptococcus suis		Plg: K_D = 14 nM	Contains internal nonapeptide motif	[70]
	Bifidobacterium lactis		Plg: K_D = 42 nM	Lysine and glutamic acid residues K^{251}, K^{251}, and E^{252}	[30]
	Bacillus anthracosis		ND	Plg binding partially mediated by C-terminal lysine. Interacts with LBS within Plg	[71, 72]
α-enolase	*Neisseria meningitidis*	Nonanchored	ND	Undefined internal plg-binding motif	[73]
	Streptococcus mutans		ND	Binds plg via C-terminal lysine	[74]
	Streptococcus agalactiae		Glu-Plg: ND; Lys-Plg: ND	ND	[75]
	Mycoplasma gallisepticum		ND	ND	[27]
	Mycoplasma fermentas		ND	ND	[76]
	Borrelia burgdorferi		Glu-plg: K_D = 125 nM	Interacts with LBS within Plg	[77]
Ag85B	*Mycobacterium tuberculosis*	Nonanchored	ND	Interacts with LBS within plg	[49]
Aspartase	*Haemophilus influenzae*	Nonanchored	ND	K4. Potent stimulator of tPA but not uPA	[78]
	Bifidobacterium animalis		Plg: K_D = 11.97 nM	Interacts with LBS within plg	[79]
DNaK	*Neisseria meningitidis*	Nonanchored	ND	Undefined internal plg-binding motif	[73]
	Mycobacterium tuberculosis		ND	Interacts with LBS within plg	[49]
Elongation factor-tu (EF-tu)	*Bacillus anthracosis*	Nonanchored	ND	Interacts with LBS within plg	[71]
Fructose-1,6-bisphosphate aldolase	*Mycoplasma tuberculosis*	Nonanchored	Plg: K_D = 6.73 nM	ND	[80]
	Streptococcus pneumoniae		Pln: K_{D1} = 28 nM; K_{D2} = 52 nM Plg: K_{D1} = 0.43 μM; K_{D2} = 0.16 nM	Binds plg via two C-terminal lysine residues separated by isoleucine and alanine	[67, 81]
Glyceraldehyde 3-phphate dehydrogenase (GAPDH; GAPC; SDH; Plr)	*Streptococcus pyogenes*	Nonanchored	ND	ND	[82]
	Bacillus anthracis		Plg: K_D = 78.5 nM; 572 nM	ND	[83]
	Streptococcus equisimilis		Plg: K_D = 220 nM; Pln: K_D = 25 nM	ND	[84]
Peroxiredoxin	*Neisseria meningitidis*	Nonanchored	ND	Undefined internal plg-binding motif	[73]
Phosphoglycerate kinase	*Streptococcus equisimilis*	Nonanchored	ND	Shown to bind both plg and pln	[85]
SkzL	*Streptococcus agalactiae*	Nonanchored	Glu-plg: K_D = 3−16 nM; Lys-plg: K_D = 80 nM; Pln K_D = 50 nM	ND	[86]

ND: not determined, plg: plasminogen, pln: plasmin, LBS: lysine binding site, K1–5: kringle 1–5.

these proteins have not been fully defined, the role of C-terminal lysines appears limited for those that have been characterised. Truncated Erp proteins lacking three native C-terminal lysine residues show only a partial reduction in plasminogen binding, supporting a role for both C-terminal lysine residues and an unidentified internal binding site in the interaction with plasminogen [46]. Similarly, mutation of residues Lys^{259}, Lys^{267}, and Lys^{319} within the choline-binding protein E (CBPE) of *S. pneumoniae* results in a 70% reduction in plasminogen when compared to the wild-type protein [43]. A number of other receptors with less well-defined plasminogen-binding sites are listed in Table 1.

4.2. Cytoplasmic and Glycolytic Pathway Proteins. In addition to specialised cell surface expressed plasminogen receptors, a number of proteins, usually considered to be restricted to the cytoplasm, have been found on the bacterial cell surface and are involved in interactions with plasminogen. Examples include the glycolytic pathway enzymes α-enolase and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), DNaK, and elongation factor Tu (efTu) [30, 71, 79]. The mechanisms underlying the cell surface localisation of these proteins are not defined; however, their cell surface location has been confirmed in multiple species of bacteria [73, 82, 83, 85]. Unlike traditional cell wall anchored proteins, the contribution of glycolytic pathway enzymes to whole cell binding can typically only be shown indirectly, using blocking antibodies or competing concentrations of soluble recombinant proteins. This stems from the fact that these proteins are often metabolically essential for bacterial survival which prevents the construction of isogenic knockout mutant strains.

Interactions between the glycolytic pathway enzyme enolase and plasminogen have been characterised for several species of bacteria (Table 1). The most extensively studied of the bacterial enolases include those expressed by *S. pneumoniae* and *S. pyogenes.* Both have been shown to have a higher affinity for Lys-plasminogen than the circulating Glu-plasminogen or plasmin [90, 91].

Reports on the mechanism of plasminogen binding by bacterial enolases have been conflicting. *Pneumococcal* enolase contains an internal nonapeptide motif (FYDKERKVY), with the C-terminal lysine residues playing only a minor role in the interaction with plasminogen [65]. Like *S. pneumoniae,* several other bacterial enolases also appear to mediate plasminogen binding via internal lysine residues, including the enolase from *Bifidobacterium lactis,* for which internal residues Lys^{251} and Lys^{255}, as well as the negatively charged Glu^{252} are responsible for plasminogen binding [30]. However a recent study of the plasminogen-binding of oral *Streptococcal* enolase variants showed that plasminogen binding activity is conserved despite the loss of lysine residues within the internal nonapeptide, with the authors suggesting that the role of the first lysine in the internal nonapeptide in plasminogen binding may not be as critical as first thought [29]. For the *S. pyogenes* enolase (SEN), internal lysines Lys^{252} and Lys^{255} contribute significantly to plasminogen binding. However, the high affinity of SEN for plasminogen is also mediated in part by two lysine residues at the C-terminus (Lys^{434} and Lys^{435}) which are thought to stabilise the conformation of SEN's plasminogen binding site [68]. Site-directed mutagenesis of either the C-terminal or internal lysine motifs abrogate binding of plasminogen by SEN [69]. In contrast, the enolase of *S. mutans* does not have a functional internal plasminogen-binding site and may mediate plasminogen binding by C-terminal lysine residues only [74]. In all reported cases, bacterial plasminogen binding by enolase is inhibited by the lysine analogue EACA, indicating a role for the lysine binding sites within plasminogen in this interaction. However, it has yet to be established which LBS within plasminogen mediate interactions with enolase. It is possible that the different motifs responsible for plasminogen binding within diverse enolases interact with distinct LBS within plasminogen. One could hypothesise that C-terminal lysines interact with K1 of plasminogen, whilst internal lysines bind to K5, akin to the model proposed by Law et al. 2012 for the interaction of plasminogen and fibrin(ogen). Plasminogen binding is not conserved in all enolases, as evidenced by the finding that enolase from *Bacteroides fragilis* does not interact with plasminogen [92].

Similar to enolase, glyceraldehyde 3 phosphate dehydrogenase is a glycolytic pathway enzyme which has been shown to be located on the bacterial cell surface and to interact with plasminogen. GAPDH of *S. pyogenes* and *S. pneumoniae* binds preferentially to Lys-plasminogen and plasmin, and this interaction is mediated by the C-terminal lysine residue in GAPDH [93]. Interestingly, it has been shown that GAPDH of group B streptococcus interacts with both Glu-and Lys-plasminogen but not plasmin [94].

4.3. Indirect Plasminogen Binding. A number of bacterial pathogens possess the ability to interact with additional plasma proteins including IgG, α_2-macroglobulin, albumin, numerous complement factors, and fibrinogen [95]. These interactions are involved in pathogenic processes such as cell adherence and colonisation, evasion of the immune system and dissemination [95–97]. For *S. pyogenes*, the interaction of bacterial cell surface receptors with fibrinogen has been shown to play a role in the acquisition of cell surface plasmin activity.

Fibrinogen is a large, 340 kDa protein made up of two identical subunits connected by numerous disulphide linkages. Each subunit consists of three nonidentical polypeptide chains denoted Aα, Bβ, and γ [98]. These polypeptide chains are folded into a number of structural domains. The central E domain consists of the N-termini of all six polypeptide chains, the two D domains (one in each subunit) consist of C-terminal regions of Bβ and γ chains and a portion of the Aα chain, while the remaining portions of the two Aα chains form 2 αC domains [99]. Cleavage of fibrinogen by thrombin is the last step in the coagulation pathway and leads to the formation of fibrin. After thrombin cleavage, previously unexposed (cryptic) sites are revealed in fibrin molecules which initiate fibrin polymerisation and clot formation [100]. Polymerisation results in the exposure of additional cryptic-binding sites for a range of cell types, growth factors, and proteins including those involved in

fibrinolysis, such as tissue plasminogen activator, plasminogen, plasminogen activator inhibitor, and α_2-antiplasmin [101–103]. This diverse range of ligand interactions allows fibrin to participate in a variety of processes involved in tissue regeneration and also facilitates the tight regulation of haemostasis.

S. pyogenes secretes streptokinase, a plasminogen activating protein. Streptokinase binds to plasminogen SPD (Figure 1) and induces conformational changes in the latent active site of plasminogen producing an enzymatically active complex which, in addition to plasmin activity, also displays plasminogen activation activity [104]. While the main physiological role of plasmin is the degradation of fibrin, plasmin can also cleave a variety of other substrates including fibrinogen. Cleavage of soluble fibrinogen exposes cryptic sites within the molecule which allow it to interact with ligands that were previously nonreactive with the intact protein. Plasminogen-binding sites have been identified in D domain fibrinogen fragments [105] and the binding of plasminogen to this fragment enhances streptokinase-mediated plasminogen activation [106]. Additionally, fragment D is sufficient for interaction with fibrinogen receptors on the GAS cell surface [107, 108]. Therefore, at the site of infection, Plg-SK activator complexes can cleave fibrinogen, producing D domain fragments. These D domain fragments are then able to interact with both plasmin(ogen) (present in the activator complex and/or as free plasmin) and bacterial cell surface fibrinogen receptors thereby mediating the acquisition of unregulated plasmin activity onto the bacterial cell surface. This mechanism of plasmin acquisition appears to be important for those GAS strains that do not possess high-affinity plasminogen-binding proteins but do express fibrinogen-binding proteins such as PrtF1 and PrtF2 variants [109], M protein variants [110] and the lipoprotein Spy_0591 [111]. It is currently not known if a similar mechanism of plasmin acquisition involving fibrinogen fragments and bacterial fibrinogen receptors is functioning in other bacterial species.

4.4. Physiological Significance of Plasminogen Acquisition by Bacteria. The broad proteolytic activity of plasmin necessitates tight *in vivo* regulation. Within the host, this is achieved by specific mechanisms that control the generation of plasmin from plasminogen and by mechanisms that restrict plasmin activity to specific locations as required. The major circulating inhibitor of plasmin is α_2-antiplasmin. Lysine residues within α_2-antiplasmin stabilise binding to the kringles of plasmin(ogen), resulting in rapid inhibition of plasmin in solution. However, once bound to surfaces such as fibrin, or cell surface receptors, plasmin is partially protected from inactivation by α_2-antiplasmin [112–114]. Bacteria circumvent host regulatory mechanisms as cell surface bound plasminogen are more readily activated to plasmin, and, as in the host, this plasmin activity is not readily inhibited by host inhibitors [18, 19, 115]. Protection of plasmin from inhibition by binding to cell surface receptors appears to be central to the pathogenesis of several bacterial species and is utilised in a variety of pathogenic processes (summarised in Figure 2) [66, 73, 80, 116, 117].

Several bacterial species associated with highly invasive infections express receptors for plasminogen and plasmin, including *S. pyogenes*, *S. pneumoniae*, *S. aureus, P. aeruginosa, Y. pestis,* and *S. enteritidis*. Local thrombosis and microvascular occlusion during the early inflammatory response to bacterial infection can capture bacteria and prevent bacterial dissemination into deeper tissues. Surface-associated plasmin activity can facilitate fibrinolysis, preventing clot formation or promote the release of bacteria from a formed clot (Figure 2) [17, 107]. Furthermore, plasmin degradation of fibrinogen can initiate the release of products that affect blood vessel permeability and the accumulation of inflammatory cells [1, 118]. A major pathogenic consequence of bacterial plasminogen recruitment thus appears to be severe tissue destruction and overstimulation of the inflammatory response.

The direct degradation of ECM and basement membrane proteins and the activation of matrix metalloproteases by plasmin may enable bacteria to break down host tissue barriers (Figure 2). This is evidenced by the repeated demonstration that plasmin-coated bacteria are capable of penetrating ECM or basement membranes *in vitro* [119–121]. Plasminogen immobilised to the surface of *E. coli*, *H. pylori,* and *N. meningitidis* shows enhanced tPA-mediated plasminogen activation; whilst tPA- and uPA-activated plasmin at the surface of *S. typhimurium, B. burgdorferi*, *S. pneumoniae*, *S. agalactiae,* and *M. fermentans* facilitates the degradation of various ECM components, migration through endothelial and epithelial cell layers, or invasion of epithelial cells [18, 75, 122, 123]. The role of plasminogen acquisition in highly invasive infections is supported by a number of studies using animal models of infection, as well as several epidemiological studies. The ability to accumulate cell surface plasmin has been shown to be a prerequisite for systemic *S. pyogenes* infection in a humanised plasminogen mouse model [124–126]. Moreover, *B. burgdorferi* with active plasmin bound to their surface causes a more severe form of bacteraemia than their counterparts without active plasmin in a mouse model of spirochetemia [116]. Additionally, the abrogation of enolase-mediated plasminogen binding by *S. pneumoniae* significantly reduces the virulence of this pathogen in mice [65]. There are also epidemiological data to support the role of plasmin acquisition in bacterial pathogenesis. *E. coli* strains isolated from patients with colonic disease have been shown to bind significantly more plasminogen than *E. coli* isolates from healthy patients [127]. Similarly a study of *S. pyogenes* isolates from Northern Australia showed that isolates associated with invasive disease acquired significantly more cell surface plasminogen than noninvasive isolates [38]. However, oral streptococci display specific, high affinity plasminogen binding irrespective of their association with either benign dental plaque or severe inflammatory disease [128], and many commensal bacteria have been shown to recruit plasminogen to the bacterial cell surface (Table 1). This suggests that sequestration of plasminogen by bacteria may be important for bacterial survival in the host environment, with reports indicating a role for this interaction in both immune evasion and host colonisation [68, 129–131].

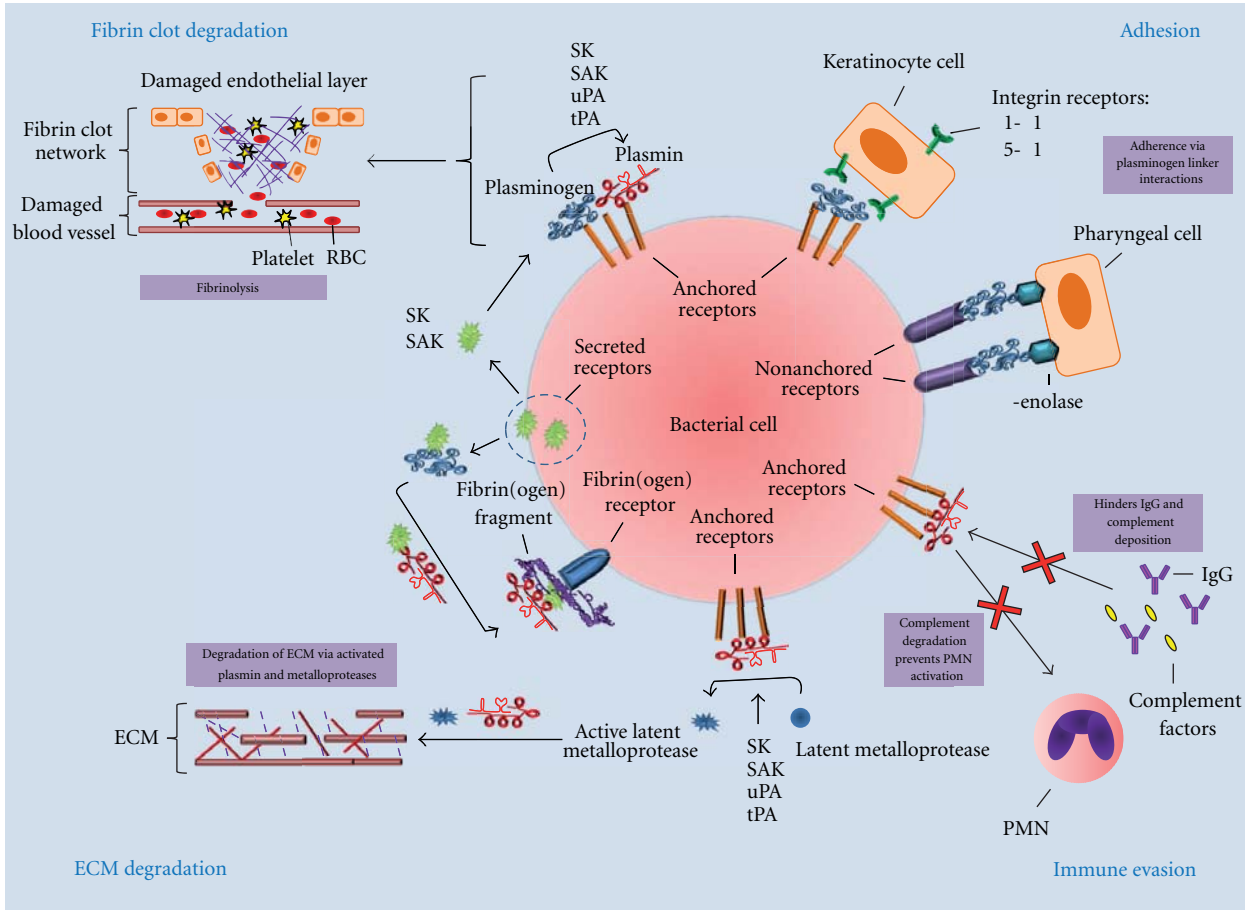

Figure 2: Mechanisms of bacterial cell surface plasmin(ogen) acquisition and its role in bacterial-host interactions. Plasmin(ogen) can be bound directly to the bacterial cell surface via cell-membrane-anchored receptors, nonanchored-cell-surface-associated receptors or indirectly through interactions with fibrinogen and cell surface fibrinogen receptors. Plasmin(ogen) localised on the bacterial cell surface is involved in four main processes; (1) ECM degradation via activated metalloproteases and plasmin; (2) fibrinolysis via plasmin; (3) immune evasion through plasmin-mediated degradation of immune effectors, including complement components and immunoglobulins; (4) adherence to host cells via plasminogen-linker interactions with host cell surface receptors. ECM: extracellular matrix; IgG: immunoglobulin G; RBC: red blood cell; SAK: staphylokinase; SEN: streptococcal α-enolase; Ska: streptokinase; tPA: tissue plasminogen activator; uPA; urokinase plasminogen activator.

Plasmin plays an integral role in the recruitment of host immune cells to sites of bacterial infection and is able to degrade essential components of the innate immune response such as the complement factors C3b, C4b, and C5 (Figure 2) [132–134]. Plasmin at the bacterial cell surface therefore provides organisms with the capacity to degrade immunoglobulins and complement proteins, thereby inhibiting the host immune response. Specifically, plasminogen activation by the bacterial activators staphylokinase (of *S. aureus*) and PgtE (of *S. typhimurium*) results in degradation of C3b, thereby preventing complement-driven phagocytosis. PgtE-generated plasmin has also been shown to degrade complement factors C4b and C5 [129, 133]. Similarly, uPA activated plasminogen at the surface of *L. interogans* and *B. anthracis* prevents deposition of IgG and C3b on the bacterial surface [135] and leads to a subsequent decrease in macrophage phagocytosis [71]. Furthermore, the ability of certain bacteria to activate plasminogen has been shown to alter the response of inflammatory cells to infection. The expression of the plasminogen activator Pla by *Yersinia pestis* appears to decrease the level of neutrophil infiltration in a mouse model of infection [136]. Clearly, there is a role for bacterial plasminogen acquisition in protecting bacteria from the host immune response. Whilst this has obvious significance for the initiation of systemic bacterial disease, it also has implications in host colonisation by commensal and pathogenic organisms alike.

A further role for plasminogen recruitment in bacterial colonisation has been demonstrated by several studies of plasminogen recruitment by streptococci. Plasminogen has been shown *in vitro* to act as a linker molecule between enolase at the surface of pharyngeal cells, and SEN at the surface of *S. pyogenes*, thus facilitating the adhesion process (Figure 2) [137]. When this bridging plasminogen molecule is activated by tPA to plasmin, it can digest intercellular junctions and disrupt cell monolayers in ECM models [137]. Similarly, the streptococcal M protein GSC3 has been shown to mediate plasminogen-dependant adherence

of streptococci to pharyngeal cells [40], implying a role for plasminogen binding in colonisation of the throat and oral cavity by bacteria. A role for plasminogen binding in colonisation has been further demonstrated for *S. pyogenes* interaction with keratinocytes. Plasminogen on the bacterial cell surface promoted the internalisation of streptococci by keratinocytes through the interaction with $\alpha 1\beta 1$- and $\alpha 5\beta 1$-integrins (Figure 2) [130]. In all the cases reported so far, the role of bacterially bound plasminogen in adherence/internalisation appears to function independently of the serine protease activity of plasmin.

5. Conclusions

The expression of receptors which enable localisation of plasminogen to the cell surface is a phenotype common to a multitude of bacteria. Since the initial identification of bacterial plasminogen receptors over 20 years ago, a myriad of receptor types have been identified, associated with both pathogenic and commensal bacterial species. The vast array of mechanisms via which different receptors interact with Glu-plasminogen, Lys-plasminogen, plasmin, and mini-plasmin suggests that these receptors may have evolved to mediate interactions with this abundant human protein under diverse physiological conditions. Indeed, recent studies show bacterial plasmin(ogen) acquisition is central to the onset of invasive pathogenesis via fibrin and ECM degradation; immune evasion via degradation of various immune effectors; and colonisation of the host (Figure 2). Much remains to be learned about how diverse plasminogen receptors interact with plasminogen. For many receptors, there is limited information on specificities of interaction with different forms of plasminogen and plasmin and on the location of binding within the plasminogen molecule. Recent structural studies suggest that the mechanism through which receptors interact with plasminogen can have different effects on the structure and activation of this protein which may ultimately influence the pathogenic process for many bacterial species [10]. Plasminogen receptors clearly play a central role in the relationship between bacteria and the host, and further elucidation of the nuances of how microbes interact with plasminogen will contribute significantly to our understanding of both the plasminogen molecule and bacterial pathogenesis in the future.

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Review Article

The Biochemistry and Regulation of S100A10: A Multifunctional Plasminogen Receptor Involved in Oncogenesis

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The plasminogen receptors mediate the production and localization to the cell surface of the broad spectrum proteinase, plasmin. S100A10 is a key regulator of cellular plasmin production and may account for as much as 50% of cellular plasmin generation. In parallel to plasminogen, the plasminogen-binding site on S100A10 is highly conserved from mammals to fish. S100A10 is constitutively expressed in many cells and is also induced by many diverse factors and physiological stimuli including dexamethasone, epidermal growth factor, transforming growth factor-α, interferon-γ, nerve growth factor, keratinocyte growth factor, retinoic acid, and thrombin. Therefore, S100A10 is utilized by cells to regulate plasmin proteolytic activity in response to a wide diversity of physiological stimuli. The expression of the oncogenes, PML-RARα and KRas, also stimulates the levels of S100A10, suggesting a role for S100A10 in pathophysiological processes such as in the oncogenic-mediated increases in plasmin production. The S100A10-null mouse model system has established the critical role that S100A10 plays as a regulator of fibrinolysis and oncogenesis. S100A10 plays two major roles in oncogenesis, first as a regulator of cancer cell invasion and metastasis and secondly as a regulator of the recruitment of tumor-associated cells, such as macrophages, to the tumor site.

1. Introduction

Several fundamental studies have shown that cellular receptors for plasminogen play a major role in the regulation of important physiological processes such as fibrinolysis and in the development of disease, such as cancer [1–4]. The binding of the blood protein and zymogen, plasminogen, to specific cell surface receptors, called plasminogen receptors, significantly increases the rate of its proteolytic conversion to plasmin, due to the colocalization of plasminogen with its activators, tissue plasminogen activator (tPA) and the urokinase-type plasminogen activator (uPA) [5, 6]. Although tPA shares the same cellular binding sites as plasminogen [7–9], uPA is localized to the cell surface by its binding to its cell surface receptor, the urokinase-type plasminogen receptor (uPAR) [10]. Many intracellular and extracellular functions have been proposed for S100A10, of which probably the most striking in terms of physiological significance and implications for disease is the extracellular function of this protein as a plasminogen receptor (reviewed in [11–14]). S100A10 binds to tPA and plasminogen and also colocalizes with the uPA/uPAR complex, which stimulates the conversion of plasminogen to the broad specificity protease, plasmin. Plasmin also binds to S100A10 which protects the newly generated plasmin from inactivation by its inhibitor, α_2-antiplasmin, and also serves to focus the proteolytic activity of plasmin to the cell surface [15]. S100A10 also stimulates plasmin autoproteolysis, resulting in the destruction of plasmin and the generation of bioactive plasmin fragments, the angiostatins (reviewed in [16]). A

main characteristic of the plasminogen receptors is the presence of a carboxyl-terminal lysine residue that binds to the kringle domains of plasminogen [9, 13, 17, 18]. S100A10 possesses two carboxyl-terminal lysine residues that have been shown to bind both tPA and plasminogen and to play a critical role in the conversion of plasminogen to plasmin by the plasminogen activators [19, 20]. Several plasminogen receptors have been reported that do not contain a carboxyl-terminal lysine residue, but it is unclear as to what extent these proteins contribute to cellular plasmin generation.

In this paper we will review, in detail, the structure and function of S100A10. Although both intracellular [21, 22] and extracellular roles [12] have been identified for S100A10, the main focus of this paper will be on the extracellular role of S100A10 as a plasminogen receptor. We will develop two themes, highlighted by studies of the S100A10-null mouse. The first is that S100A10 is a highly inducible plasminogen receptor. S100A10 is not only regulated by physiologically important signaling molecules such as thrombin [23], epidermal growth factor [24, 25], transforming growth factor-α [26], and interferon-γ [27, 28], but also by pathophysiological events such as the expression of oncogenes that occurs during the process of tumor development and progression (oncogenesis) [29, 30]. Second, we will summarize our observations that document that S100A10 is responsible for a significant amount of total cellular plasmin generation and develop the second theme that S100A10 plays a key role in physiological processes, such as fibrinolysis and inflammation. Collectively, we will showcase the concept that in response to both physiological and pathophysiological cues, cells utilize S100A10 to regulate their levels of plasmin proteolytic activity. Our working model of cellular plasmin regulation by S100A10 is presented in Figure 1.

2. Historical Perspective

S100A10 was first identified in 1984 during the purification of a 34 kDa protein substrate of Rous sarcoma virus-transforming protein tyrosine kinase ($pp60^{v\text{-}src}$) from chicken embryo fibroblasts [31]. These investigators observed the presence of a small, 6 kDa protein that was present at the dye front of Coomassie blue-stained SDS-polyacrylamide gels. Using preparations from porcine or bovine epithelial cells, this protein was estimated to have a molecular mass of 11 kDa and found to share homology with the glia-specific protein, S-100, and to share about 50% amino acid homology with S100α [32, 33].

Since its discovery, many proposed intracellular functions have been suggested for S100A10. In the late 1980s and early 1990s the binding of S100A10 to the protein annexin A2 was shown to diminish the phosphorylation of annexin A2, and thereby regulate the association of annexin A2 with phospholipid membranes [32, 34, 35]. S100A10 was also shown to stimulate annexin A2 translocation to the cortical cytoskeleton [36] and stimulate the F-actin bundling activity of annexin A2 during exocytosis [37–39]. Again, in complex with annexin A2, S100A10 was reported to enhance the annexin A2 stimulation of glial fibrillary acidic protein (GFAP) polymerization [40] and to play a role in cytomegalovirus infection [41, 42]. S100A10 was also shown to have a role in inhibiting inflammation, by targeting phospholipase A2 [43]. The interaction of S100A10 with the Bcl-2-associated death promoter (BAD) was shown to inhibit the proapoptotic activity of the protein [44]. The interaction of S100A10 with PCTAIRE-1 was reported to stimulate its protein kinase activity [45, 46]. It was also noted that S1000A10 was a transglutaminase substrate although the functional significance of this modification is unclear [47].

The early part of 2000 saw a flurry of publication on S100A10. In complex with bluetongue virus protein, NS3, S100A10 was shown to mediate virus release [48]. S100A10 in association with HBV Pol was also reported to inhibit DNA polymerase activity [49]. The binding of S100A10 to AHNAK was also reported [50]. During this time, a role for S100A10 in the regulation of plasma membrane ion channels was reported. S100A10 was shown to complex with the two-pore domain acid-sensitive potassium *channel (*TASK-1) at the plasma membrane [51], and interact with the transient receptor potential cation channel subfamily V member 5/6 (TRPV5/TRPV6) [52], the acid-sensing ion channel 1 (ASIC-1) [53], and with the voltage-gated sodium channel NaV1.8 [54, 55]. The year 2006 saw reports of the interaction of the S100A10 protein with the serotonin receptor, 5-HT1B [56]. Perhaps the most exciting development in the S100A10 field came with the development of the S100A10 gene knockout (S100A10-null) mouse. Using a mouse knockout that entailed a specific S100A10 deletion in nociceptive sensory neurons, it was reported that S100A10 played a role in nociception, resulting from decreased sodium current [57]. This study confirmed the role of S100A10 in the regulation of the expression of Na(V)1.8. This study also reported that knockout of S100A10 did not affect the protein levels of its annexin A2 binding partner. A general S100A10 mouse knockout model also indicated the physiological relevance of the S100A10–5-HT1B receptor interaction. They found that these S100A10-null mice are viable but exhibited a depression-like phenotype with reduced responses to 5-HT1B agonists, suggesting that S100A10 is not required for normal mouse development, but that lack of S100A10 causes a depressive disorder due to its regulation of the 5-HT1B channels [56].

Since S100A10 can only be purified from tissues as a complex with annexin A2, our initial studies compared the structure and function of annexin A2 monomer and the annexin A2/S100A10 complex isolated from bovine lung. We called the annexin A2/S100A10 complex, the annexin A2 heterotetramer (abbreviated as AIIt). We reported, in 1998, that annexin A2/S100A10 complex purified from bovine lung, regulated plasmin activity by stimulating plasmin autoproteolysis [58, 59]. We also observed that other purified annexins, including annexin A2, did not appreciably stimulate plasmin autoproteolysis. Later studies would reveal that the plasmin fragments produced by AIIt-dependent plasmin autoproteolysis were biologically active antiangiogenic molecules [60, 61]. An extracellular function for S100A10 as a plasminogen receptor was initially suggested in the late 90′s

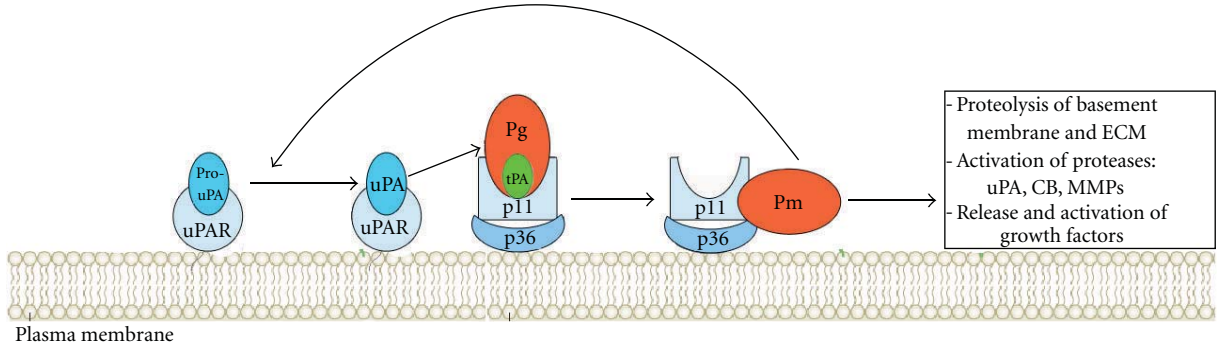

Figure 1: Regulation of cellular plasmin generation by S100A10. Normal cells and cancer cells utilize the plasminogen receptor, S100A10 (p11), for cell surface plasmin generation. The predominant form of S100A10 at the cell surface is as the heterotetramer, AIIt which consists of two copies of the annexin A2 (p36) and a S100A10 homodimer. The model shows one-half of this heterotetrameric complex. Annexin A2 acts as a regulatory subunit which utilizes its phospholipid-binding sites to anchor S100A10 to the cell surface. S100A10 binds tPA and plasminogen (Pg) at its carboxyl-terminal lysine residue. It is unclear if one molecule of AIIt can bind both tPA and plasminogen at each of the binding sites on the antiparallel S100A10 monomers or if each molecule of AIIt can only bind two molecules of tPA or two molecules of plasminogen at the S100A10 monomers. The localization of tPA and plasminogen in close proximity promotes the rapid conversion of plasminogen to plasmin (Pm) by tPA. Plasmin binds to both annexin A2 and S100A10 at sites distinct from the plasminogen-binding site. The protease, urokinase-type plasminogen activator (uPA), is secreted from cells in its inactive, zymogen form called pro-uPA. Pro-uPA is converted to uPA by several proteases including plasmin. The uPA/uPAR complex colocalizes with the AIIt where uPA cleaves the S100A10-bound plasminogen, generating plasmin. Plasmin cleaves and activates promatrix metalloproteases (MMPs), procathepsin B (pro-CB), and pro-uPA. Plasmin, MMPs, and cathepsin B degrade many extracellular matrix (ECM) proteins and both release and activate growth factors from the ECM via proteolysis. Increased cell surface concentration of uPA or pro-uPA (by binding to its receptor, uPAR) and plasmin or plasminogen (by binding to S100A10) accelerates their reciprocal activation and focuses plasmin proteolytic activity to the cell surface.

by our laboratory. We observed that S100A10 was present on the surface of cells, predominantly in a complex with annexin A2, and that this complex dramatically stimulated tPA-dependent plasmin formation [15]. Using an *in vitro* assay with purified components, we showed that the the annexin A2/S100A10 complex stimulated the rate of activation of [Glu] plasminogen about 341-fold compared with an approximate 6-fold stimulation by monomeric annexin A2. These studies suggested that S100A10 and not annexin A2 was an important regulator of plasmin generation and plasmin activity. The cloning and purification of full-length human recombinant annexin A2 and S100A10 in 1997 [62] allowed the detailed characterization of the interaction of tPA and plasminogen with S100A10 complexed with annexin A2 as well as, for the first time, with the free S100A10 homodimer. We reported that the human recombinant annexin A2/S100A10 complex stimulated tPA-dependent plasminogen activation by about 77-fold compared with about 2- and 46-fold for human recombinant annexin A2 monomer and homodimeric S100A10, respectively [19]. We also showed that the loss of the carboxyl-terminal lysine residues of homodimeric S100A10 or S100A10 complexed to annexin A2 blocked tPA-dependent plasminogen activation [19, 20]. Finally, we showed that the addition of a peptide to the S100A10 binding site of annexin A2 (the amino-terminal 15 amino acids), to the recombinant S100A10 homodimer, increased the rate of tPA-dependent plasminogen activation by two-fold compared with the stimulation by homodimeric S100A10 alone [19]. The activity of this truncated recombinant complex, formed by the binding of this peptide to the recombinant S100A10 homodimer (annexin $A2_{(1-15)}$/S100A10), was comparable to the activity of the intact annexin $A2_{(1-338)}$/S100A10 complex. This indicated that within the annexin A2/S100A10 complex, S100A10 was the subunit directly responsible for plasmin generation and that the interaction of annexin A2 with S100A10 functioned to stimulate the activity of S100A10. These studies were extended in 2003 when we used surface plasmon resonance to examine the interaction of tPA and plasminogen with homodimeric S100A10 and S100A10 complexed with annexin A2 [63]. We reported that homodimeric S100A10 bound tPA and plasminogen and that S100A10 complexed with annexin A2 bound plasminogen with higher affinity than homodimeric S100A10. Since we also observed that annexin A2 did not bind tPA or plasminogen, we concluded that the binding of annexin A2 to S100A10 increased the affinity of S100A10 for plasminogen.

In 2003-2004 we used antisense RNA or small interfering RNA (shRNA) to selectively deplete S100A10 from cells. These studies established that S100A10 contributed significantly to the total cellular plasmin generation and that S100A10-dependent plasmin generation was utilized by cancer cells to promote invasion and metastasis [64, 65]. In 2010-2011 studies with the S100A10-null mouse contributed greatly to our understanding of the role of S100A10 as a plasminogen receptor. We found that mice deficient in S100A10 had increased fibrin deposition in various tissues including the lungs, liver, spleen, and kidney. These mice exhibited impaired fibrinolysis, illustrated by an inability to clear microclots formed by the snake venom, batroxobin. These mice also showed decreased angiogenesis, evidenced by a decreased infiltration of endothelial cell into Matrigel

plugs [66]. We also demonstrated that cell surface S100A10 played an important role in the migration of macrophages to the site of inflammation [67]. Our lab group recently explored the role of S100A10 on the surface of macrophages and the ability of these cells to enhance tumor growth [68]. Mice deficient in S100A10 grew smaller tumors than their wild-type counterparts, but injection of macrophages that express S100A10 into the tumors of the S100A10-null mice resulted in an increase in tumor growth rate similar to that of wild-type mice. These studies established that S100A10 was essential and sufficient for macrophage migration to tumor sites, and that the S100A10-dependent migration of macrophages to the tumor site was a novel rate-limiting step in tumor progression.

Most recently, two studies have contributed greatly to our understanding of the regulation of S100A10. In 2011, Lin's group demonstrated that the transport of S100A10 to the extracellular surface requires annexin A2 and is mediated by the exosomal transport pathway [28]. During this time, it was also shown that the expression of the protein, DLC1, resulted in the binding of DLC1 to S100A10 which decreased the S100A10 levels because DLC1 displaced annexin A2 from S100A10 which resulted in the ubiquitin-dependent degradation of S100A10 [69]. This result established that a primary function of annexin A2 was to protect S100A10 from degradation, a property not shared by all S100A10-binding proteins.

3. S100A10 Structure

The S100 family of proteins are small acidic calcium-binding proteins of 9–13 kDa that, excluding pseudo genes and fused S100 proteins, consist of about 20 genes (reviewed in [70–72]). Sixteen S100 genes are tightly clustered in a region of the human chromosome 1q21 and are designated as S100A followed by Arabic numbers (S100A1-S100A16). Another four S100 genes outside the 1q21 locus carry a single-letter stem symbol (S100B, S100P, S100Z, and S100G). The S100 proteins belong to a large family of Ca^{2+}-binding proteins called the EF hand superfamily that includes proteins such as calmodulin, parvalbumin, troponins, and CaBPs [73]. On the basis of the parvalbumin structure, Kretsinger identified the structure responsible for Ca^{2+} binding and coined the term for this Ca^{2+}-binding structure as the EF hand motif [74].

The S100 proteins typically contain two EF hand motifs (two α-helices linked by a Ca^{2+}-binding loop): an S100-specific EF hand at the N-terminus and a canonical EF hand at the C-terminus. In all of the S100 proteins except S100A10, the EF hands are responsible for Ca^{2+}-binding. These EF hands are not equivalent as the carboxyl-terminal EF hand Ca^{2+}-binding loop contains the classical 12-amino-acid Ca^{2+} binding motif that is common to all EF hand Ca^{2+}-binding proteins such as parvalbumin and calmodulin. In this motif, Ca^{2+}-binding occurs via acidic side chains that comprise the sequence DXDGDGTIXXXE. In contrast, the N-terminal EF hand motif is a 14-amino-acid Ca^{2+} binding loop which is characteristic of all S100 proteins and is referred to as the S100 specific or pseudo EF domain. This motif binds Ca^{2+} via backbone carbonyl groups and only by one carboxylate side group of glutamic acid [75]. Some of the S100 proteins also bind zinc and copper cations at sites distinct from the EF hand motifs [76]. In addition to the EF domains, the S100 proteins also possess a carboxyl-terminal extension. Some S100 proteins have long and flexible carboxyl-terminal extensions and this region has been suggested to be required for a ligand interaction independent of the EF hand. In general, the carboxyl-terminal extension exhibits the highest sequence variation and has been suggested to be a major contributor to the specificity of S100 proteins [77].

Calmodulin was the first multifunctional EF hand containing Ca^{2+}-binding protein that was shown to be highly conserved and present in all eukaryotes [78]. In contrast, the S100 protein family has been proposed to have originated during the Ordovician period, about 460 million years ago, with the evolution of vertebrates. S100 proteins most likely originated from a calmodulin-type precursor protein by gene duplication or exon recombination with subsequent loss of two of the four EF hands [73, 75, 79]. S100 proteins have been only isolated from vertebrates, and the analysis of the available genomes of nonvertebrate eukaryotes such as nematodes, insects, and protozoa has suggested the absence of S100-like sequences from these organisms [80]. This suggests that S100 proteins form a phylogenetically young group among the EF hand proteins. Despite their phylogenetic short history, the diversification in the S100 family is so extensive, that the S100 proteins actually form the largest subgroup among the EF hand proteins. The members of the S100 protein family are multifunctional signaling proteins that are involved in the regulation of diverse cellular processes such as transcription, secretion, contraction, motility, cell growth, differentiation, and cell cycle progression. Diseases associated with altered expression levels of S100 proteins include diseases of the heart, diseases of the central nervous system, inflammatory disorders, and cancer progression (reviewed in [77]). The diversity of binding partners for the S100 proteins is illustrated by the reports that S100 proteins are involved in at least 68 interactions with non-S100 targets (IntAct database of binary interactions (http://www.ebi.ac.uk/intact/). In fact, one S100 family member, S100A8, has been reported to interact with as many as 27 partners [81]. One of the possible explanations for the functional versatility of S100 proteins is the recent suggestion that most of the S100 proteins are intrinsic disordered proteins and as a result of structural plasticity, these proteins can interact with different targets and in some cases adopt different conformations, depending upon the specific ligand bound [82].

The majority of S100 proteins form symmetric noncovalent homodimers, a feature that is unique to the EF hand family. The dimer is formed by the interaction of helices I and IV of each monomer thus forming an antiparallel structure. Although infrequent, heterodimers have been shown to form between certain S100 proteins, such as that formed between S100A8 and S100A9 [83]. The only monomeric S100 family member is S100G (calbindin-D9k). Upon Ca^{2+} binding, all S100 proteins except S100A13 [84] undergo a

conformational change that results in a large reorientation of helix III whereas helix IV and the N-terminal EF hand show only minor structural changes. Specifically, the Ca^{2+}-binding event results in the N-terminus of helix III shifting by about 40 degrees relative to helix IV which results in helix III adopting a position that is nearly perpendicular to helix IV. As a consequence five hydrophobic residues on helix IV, as well as on two hydrophobic residues in helix I and three hydrophobic residues in the hinge region which was previously buried in the Ca^{2+}-free state, are exposed and form the site utilized for ligand interactions (reviewed in [72]).

S100A10 is unique among S100 family members in that its EF hands cannot bind Ca^{2+}. Each S100A10 monomer is composed of four α-helical domains of variable length referred to as H-I (Q3-A19), H-II (K27-K36), H-III (A50-L58), and H-IV (F68-H89) (Figure 2). Separating H-I and H-II helical regions is a loop which along with contributions from H-I and H-II forms a Ca^{2+}-binding loop (L1; A19-L30) and the H-I-L1-H-II domain forms the S100-specific, EF-1 domain. The H-III and H-IV domains are separated by a second loop (L2; D59-S70) and the H-III-L2-H-IV domain forms the canonical EF hand motif, EF-2. The EF-1 and EF-2 domains are connected by a flexible linker or hinge region (HR1: P39-N44). S100A10 has three deletions in L1 thus rendering this domain incapable of binding Ca^{2+}. Two substitutions involving a glutamic and asparagine residue in EF-2 of S100A10 also render this domain incapable of binding Ca^{2+}. Although the EF hands of S100A10 are unable to bind Ca^{2+}, the conformation of S100A10 resembles the Ca^{2+} "on" state of other S100 proteins, therefore the mutations in S100A10 result in a constitutively active conformation [85]. Thus the interaction of S100A10 with its best characterized ligand, annexin A2, is Ca^{2+} independent [62].

X-ray crystallographic analysis and site-directed mutagenesis studies have revealed that a hydrophobic cleft formed by the hinge HR1 and helix H-IV of one monomer and helix H-I of the other monomer of S100A10 form the binding site with the amino-terminal region of annexin A2 [85–88]. These points of interaction between annexin A2 and S100A10 are quite extensive. Four hydrophobic amino acids of the amino terminus of annexin A2 (V3, I6, L7, and L10) form seven points of contact with helix H-I of one monomer, two points of contact with the hinge region, and nine points of contact with helix H-IV of the other monomer, for a total of nineteen points of contact between annexin A2 and S100A10 (reviewed in [71]). In addition, T2 of S100A10 interacts with E9 and F13 of H-I (A. Rety, personal communication). Site-directed mutagenesis experiments have identified important interactions between annexin A2 and Y85 and F86 of helix H-IV of one S100A10 monomer and E5 and E9 of helix H-I of the other monomer [88]. The interaction of S100A10 with annexin A2 produces a heterotetrameric complex that is referred to as AIIt. This interaction affects the structure of both molecules. Annexin A2 aggregates chromaffin granules with a $K_d(Ca^{2+})$ in the millimolar range. Partial proteolysis results in the removal of the first 27 or 43 residues of the amino terminus and reduces the $K_d(Ca^{2+})$ for chromaffin granule aggregation about 10- or 50-fold, respectively. The binding of the S100A10 to the annexin A2 also reduces the $K_d(Ca^{2+})$ of chromaffin granule about aggregation to about 2 μM. This suggests that the amino-terminus exerts an inhibitory constraint on the interaction of annexin A2 with its biological targets whereas the binding of the S100A10 reverses this inhibitory restraint (reviewed in [39]).

As discussed, S100 proteins interact with a large variety of target molecules and S100A10 is certainly no exception (reviewed in [12, 21, 22]). S100A10 (in association with annexin A2 as the heterotetrameric complex, AIIt) participates in the recruitment and/or function of Na^+, K^+, Ca^{2+}, and Cl^-channels and serotonin receptors ([51, 52, 54, 56, 89]. Other proteins have been reported to bind S100A10, including phospholipase A2 [43], cathepsin B [90, 91], BAD [44], PCTAIRE1 [46], DLC1 [69], and AHNAK [50]. The site of interaction of S100A10 with the bluetongue virus protein, NS3 (M-L-S-G-L-I-Q-R-F-E-E-E) [48], TASK-1 (G-F-R-N-V-Y-A-E-V-L-H-F) [51], AHNAK (G-K-V-T-F-P-K-M-K-I-P-K-F-T-F-S-G-R-E-L) [92], and DLC1 (S-T-F-N-N-V-V-E-Q-N-F-K) [69] has been reported. The binding site between S100A10 and annexin A2 (T-V-H-E-I-L-C-K-L-C) forms the general interaction motif (X-O-O-X-X-O-O-X-O) where X refers to hydrophobic residues and O is any other residue [93]. The binding region of AHNAK which binds to both annexin A2 and S100A10 within the AIIt complex, only partially possesses this motif. However, both NS3 and TASK-1 show similarities to this motif and both of these proteins appear to compete with annexin A2 for S100A10. Importantly, the amino-terminal region of annexin A1, (AMVSEFLKQAWFI) does not interact with S100A10 but possesses a similar motif.

The competition of ligands for the annexin A2 binding site on S100A10 has important consequences for the stability of S100A10. The detailed study of the interaction of S100A10 with DLC1 established that annexin A2 serves to protect S100A10 from ubiquitin-mediated degradation and ligands that displace annexin A2 from S100A10 will cause the rapid degradation of S100A10. It has been suggested that many of the binding partners of S100A10 may utilize the protein as a mechanism of cotransport to the plasma membrane; however if the binding of these ligands results in the disassociation of annexin A2 then rapid degradation of the S100A10-ligand complex would be expected. Therefore only ligands that bind to both subunits and/or do not dissociate annexin A2 from S100A10 would benefit from binding to S100A10. It was also interesting that overexpression of DLC1 which resulted in the depletion of S100A10 levels resulted in the inhibition of plasmin generation and invasion of aggressive lung cancer cells [69]. This study therefore confirmed the importance of S100A10 as a plasminogen receptor and regulator of cellular plasmin generation [29, 64–68]. This study also provided important structural information on the ligand binding motif between S100A10 and DLC1 and annexin A2. A comparison of the binding regions of annexin A2 (S-T-V-H-E-I-L-C-K-L-S) with DLC1 (S-T-F-N-N-V-V-E-Q-N-F-K) suggests that a minimal binding motif may be T-X-O-O-X-X-O) in which L10 of annexin A2 does not play an important role in binding to S100A10. Consistent with this suggestion is that the interaction of L10 forms only weak

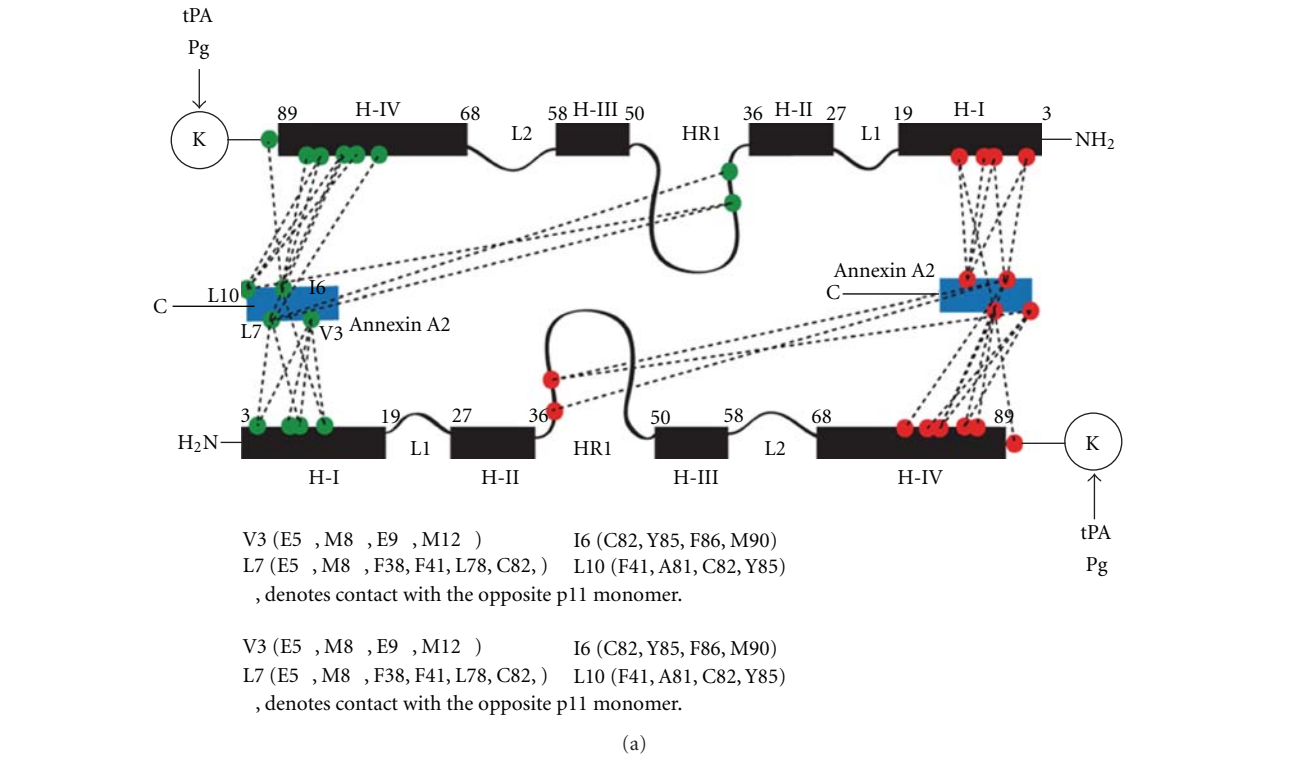

Figure 2: Structure of S100A10. A cartoon of the association of S100A10 with its two primary ligands, annexin A2 and plasminogen, is presented in *A*. The figure illustrates the structure of S100A10 and the association of S100A10 with the amino-terminus of annexin A2 and with plasminogen. Each S100A10 monomer is composed of four α-helical domains H-I, H-II, H-III, and H-IV. Separating H-I and H-II helical regions is a loop, L1. The H-III and H-IV are separated by a second loop (L2). The H-II and H-III are connected by a flexible linker or hinge region (HR1). The points of interaction between the amino-terminus of annexin A2 and S100A10 are quite extensive and four hydrophobic amino acids of the amino terminus of annexin A2 (V3, I6, L7, and L10) form seven points of contact with helix H-I of one monomer, two points of contact with the hinge region, and nine points of contact with helix H-IV of the other monomer for a total of nineteen points of contact with S100A10. Shown also are the helical wheel projections for the S100A10-binding site for B, annexin A2; C, NS3; D, DLC1; E, TASK-1. The S100A10-binding region of these ligands consists of an amphipathic α-helix in which hydrophobic residues form a binding site on one side of the helix. The program for helical wheel projections was obtained from http://www.kael.net/helical.htm.

van der Waals interactions with the residues of helix IV (S. Rety, personal communication).

Previous studies have shown that S100A10 binds both tPA and plasminogen through its carboxyl-terminal lysine [19]. Thus a binding site for tPA and plasminogen is present on each S100A10 monomer which contrasts with the annexin A2 binding site for S100A10 that requires the participation of both S100A10 monomers. Although the carboxyl-terminal extension of S100A10 plays an important role in the function of the protein, it is unclear if other amino acid residues other than the carboxyl-terminal lysine also participate in tPA or plasminogen binding. However, since removal of the carboxyl-terminal lysines of either S100A10 alone or S100A10 complexed with annexin A2 completely inhibited tPA and plasminogen binding, it is likely that the carboxyl-terminal lysine of S100A10 plays a key role in tPA and plasminogen binding [15, 20, 63]. As had been shown for many plasminogen-binding proteins, the carboxyl-terminal lysine

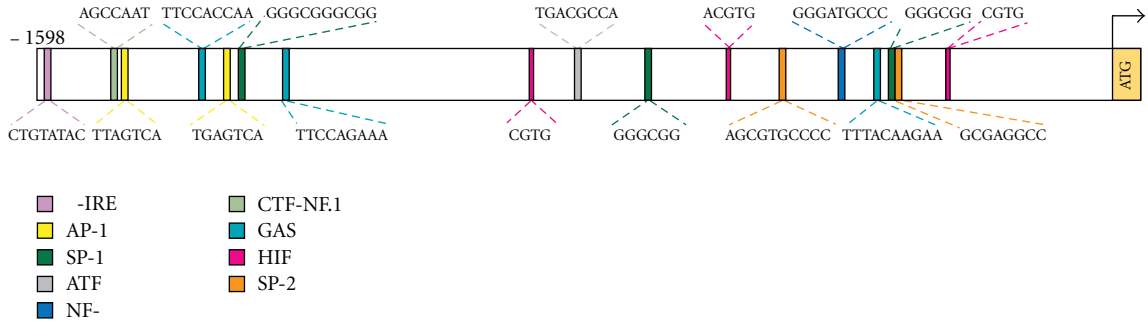

Figure 3: Schematic representation of the human *S100A10* promoter. Transcription factor-binding sites are marked with color boxes.

forms a binding site for the lysine binding domains (kringles) of both tissue plasminogen activator (tPA) and plasminogen (reviewed in [12, 13, 18, 94]). Figure 8 presents the amino acid sequence of several well-established plasminogen receptors. Although these receptors all possess carboxyl-terminal lysines it was interesting to note that several of the receptors also displayed a basic amino acid five residues distant from the carboxyl-terminal lysine, at a position corresponding to K91 in S100A10, suggesting that this residue may also play an important role in plasminogen receptors. Analysis of the phylogenetic distribution of S100A10 reveals that the carboxyl-terminal lysine is conserved across all species from human to fish (Figure 9). Only Xenopus S100A10 does not possess a carboxyl-terminal lysine. Interestingly, K91 (in the human S100A10 sequence) is absolutely conserved in all species examined. Whether this residue plays a role in maintaining the orientation of the carboxyl-terminal residue or directly participates in ligand binding is unclear. It is not unreasonable to suspect that K91 may influence the affinity of interaction between plasminogen and S100A10. The binding of annexin A2 to S100A10 increases the affinity of plasminogen binding to S100A10 by about ten fold, suggesting that the plasminogen binding to the carboxyl-terminal lysine of S100A10 is influenced by the conformation of S100A10 [63].

The other S100 family members that possess carboxyl-terminal lysines include S100A4, S100A5, S100A13, S100P, and S100Z. Interestingly, of these S100 proteins, plasminogen binding has only been reported for S100A4 [95]. As discussed, S100A10 is present in mammals, birds, reptiles, amphibians, and fish, but not in insects, nematodes, protozoa, fungi, or plants (Figure 9). Similarly, plasminogen, one of the established ligands of S100A10, is present in vertebrates including fish [96]. In contrast, S100A2, S100A7, S100A12, S100Z, and S100P are present in humans but not in mouse and rat [73]. The expression of S100A2, S100A3, S100A4, S100A5, and S100A6 is restricted to mammals and is absent in birds and fish [80]. Therefore, the presence of a carboxyl-terminal lysine has been highly conserved in the structure of S100A10 and appears to parallel the phylogenetic distribution of its ligand, plasminogen. Similarly the presence of the S100A10-annexin A2 complex in the swamp eel is consistent with the phylogenetic conservation of the annexin A2 binding site on S100A10 [97].

4. Regulation

S100A10 is expressed fairly ubiquitously in most cells and tissues yet its promoter region lacks a TATA box. Several binding motifs for various transcription factors have been identified within the promoter region of *S100A10*, such as binding sites for the transcription factor Sp1 [98]. Interferon (IFN)-γ has been shown to induce *S100A10* expression in epithelial cell lines through the transcription factor STAT1 [27] (Figure 3). STAT1-induced *S100A10* expression was dependent on the presence of GAS sites in the *S100A10* promoter region. The promoter region for *S100A10* also contains glucocorticoid response elements (GREs) and glucocorticoid stimulation has been demonstrated to induce *S100A10* expression [99–101]. *S100A10* expression may additionally be induced by transforming growth factor-β [26], gonadotrophin, epidermal growth factor, basic fibroblast growth factor, and interleukin 1β [24, 25, 102]. Decreases in *S100A10* expression have been associated with increased risk of depression. Recently, *S100A10* promoter hypermethylation has been demonstrated in studies using a rodent model of depression and hypermethylation of the *S100A10* promoter region was reduced following administration of antidepressants, suggesting potential epigenetic regulation of *S100A10* expression [103].

It was initially observed that depletion of annexin A2 resulted in a concomitant loss of cellular S100A10 and from this study it was inferred that the S100A10 homodimer was unstable in the absence of annexin A2 [104]. Subsequently, it was suggested that S100A10 was rapidly degraded by a proteosomal degradation mechanism [105]. Finally, it was observed that DLC1 decreased the cellular levels of S100A10 by binding to S100A10 which resulted in the displacement of annexin A2 from S100A10. Although annexin A2 and DLC1 bound to the similar site on S100A10, the binding of DLC1 to S100A10 did not protect S100A10 from ubiquitin-mediated degradation. Thus, the displacement of annexin A2 by DLC1 resulted in the ubiquitin-mediated degradation of S100A10 [69]. Conceptually, these experiments established the important regulatory role of annexin A2 in protecting S100A10 from ubiquitin-mediated degradation and identified a new mechanism of regulation of S100A10 by the expression of proteins such as DLC1 that displace annexin A2 from S100A10 and in doing so target S100A10 for destruction.

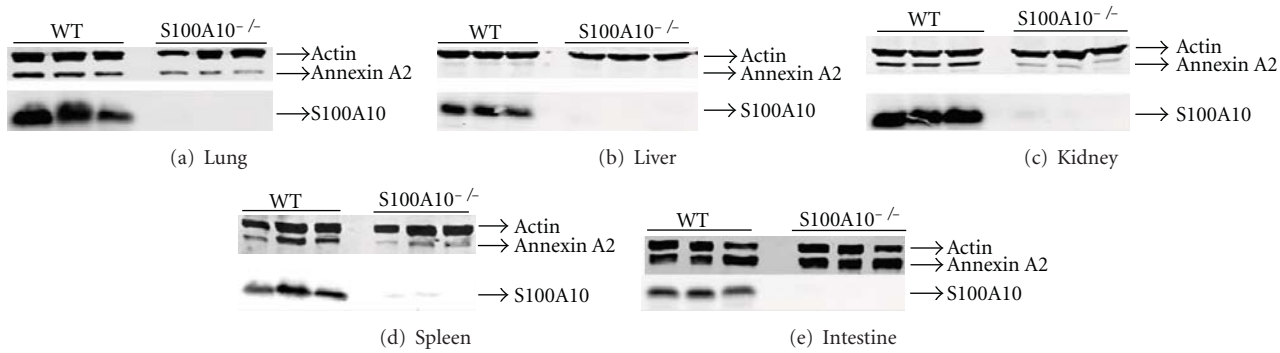

Figure 4: S100A10 and annexin A2 protein levels in tissues from S100A10$^{-/-}$ mice. Annexin A2 and S100A10 protein levels were analyzed by Western blot in tissues isolated from wild-type (WT) and S100A10$^{-/-}$ mice. Annexin A2 protein levels decreased in lung (a), kidney (c), and spleen (d), were not detectable in liver (b) and were unaltered in the small intestine (e). As expected, S100A10 protein levels were not detected in tissues isolated from S100A10$^{-/-}$ mice.

Other groups have demonstrated that annexin A2 may also stabilize S100A10 mRNA levels as loss of annexin A2 resulted in decreased S100A10 mRNA levels in MDA-MB-435 cells [106], melanocytes, and L5178Y murine lymphoma cells [107]. We have also detected S100A10 protein in murine liver in the absence of measurable annexin A2 (Figure 4). The mechanisms by which S100A10 is expressed and regulated posttranslationally by annexin A2 therefore appear to be cell-type dependent.

As discussed, S100A10 is present on the extracellular cell surface as a complex with annexin A2, called AIIt [15]. The annexin A2 subunits of AIIt serve to anchor AIIt to the plasma membrane in a Ca^{2+}-dependent fashion [108–110]. Several groups have reported that annexin A2 is required to transport S100A10 to the cell surface [111–113]. Annexin A2, however, lacks a signal peptide which suggests that the transport to the cell surface occurs by an unconventional secretion pathway [114]. Others have shown that the transport of S100A10 to the cell surface requires the phosphorylation of annexin A2 [111–113]. More recently, Fang et al. described a mechanism by which IFN-γ stimulated expression of S100A10 results in increased S100A10 levels on the cell surface. They demonstrated that S100A10 transport to the cell surface is dependent on annexin A2 and utilizes the exosomal secretion pathway [28]. A model for the transport of S100A10 to the cell surface is presented in Figure 5.

5. Cellular and Tissue Distribution

S100A10 is present in the cell primarily with annexin A2 as part of the annexin A2/S100A10 heterotetrameric complex. This complex, called AIIt, is formed by the binding of two molecules of annexin A2 to the S100A10 homodimer. Annexin A2, which binds cellular membranes, serves as the membrane anchor for S100A10. Intracellular S100A10 participates in the trafficking of several plasma membrane proteins, including the 5-HT1B receptor [56], TRPV5 and TRPV6 [52, 89], TASK-1 [51, 115], and Na(V)1.8 [54]. S100A10 present on the cell surface membrane as a complex with annexin A2 is a prominent plasminogen receptor and participates in tPA- and uPA-dependent plasminogen activation at the cell surface [15, 29, 64–67].

Expression of S100A10 has been reported in a wide range of cell types and tissues. The expression of S100A10 protein is highest in lung, kidney, and intestine. S100A10 has also been observed in various cell types, including endothelial cells [66, 111], macrophages [67, 68, 116, 117], fibroblasts [37], epithelial cells [27, 89], and various cancer cell lines [29, 64, 65, 69, 106, 107, 118–121]. S100A10 is not detected in erythrocytes [37] and relatively low levels of S100A10 are found in the liver [122]. Our laboratory has shown the expression of S100A10 protein in murine lung, liver, kidney, spleen, and intestine (Figure 4). Interestingly, S100A10 appears to regulate annexin A2 protein levels in a tissue-specific fashion. We investigated annexin A2 protein and mRNA levels in tissues isolated from the S100A10-null mouse (Figure 6) and observed that the loss of S100A10 affected annexin A2 levels in a tissue specific fashion. While loss of S100A10 did not affect annexin A2 levels in the intestine, it resulted in decreased annexin A2 levels in the lung, liver, spleen and kidney. On the other hand, annexin A2 mRNA levels were not altered in any of the S100A10-null tissues (Figure 6). S100A10 therefore appears to contribute to annexin A2 protein stability in a tissue specific fashion.

6. Function(s)

6.1. Role As a Plasminogen Receptor. In order for a protein to be considered a plasminogen regulatory protein, several critical must be met. First, the putative plasminogen regulatory protein must bind plasminogen. Surface plasmon resonance studies have demonstrated that S100A10 binds plasminogen, (K_d of 1.81 μM), plasmin (K_d of 0.36 μM), and tPA (K_d of 0.45 μM). Furthermore, S100A10 possesses the requisite carboxy-terminal lysine that has been shown to be essential for plasmin activation at the cell surface. Removal of the carboxy-terminal lysines (carboxy-terminus of S100A10- [85]-Y-F-V-V-H-M-K-Q-K-G-K-K [96]) attenuates plasminogen and tPA binding [63]. Second, binding

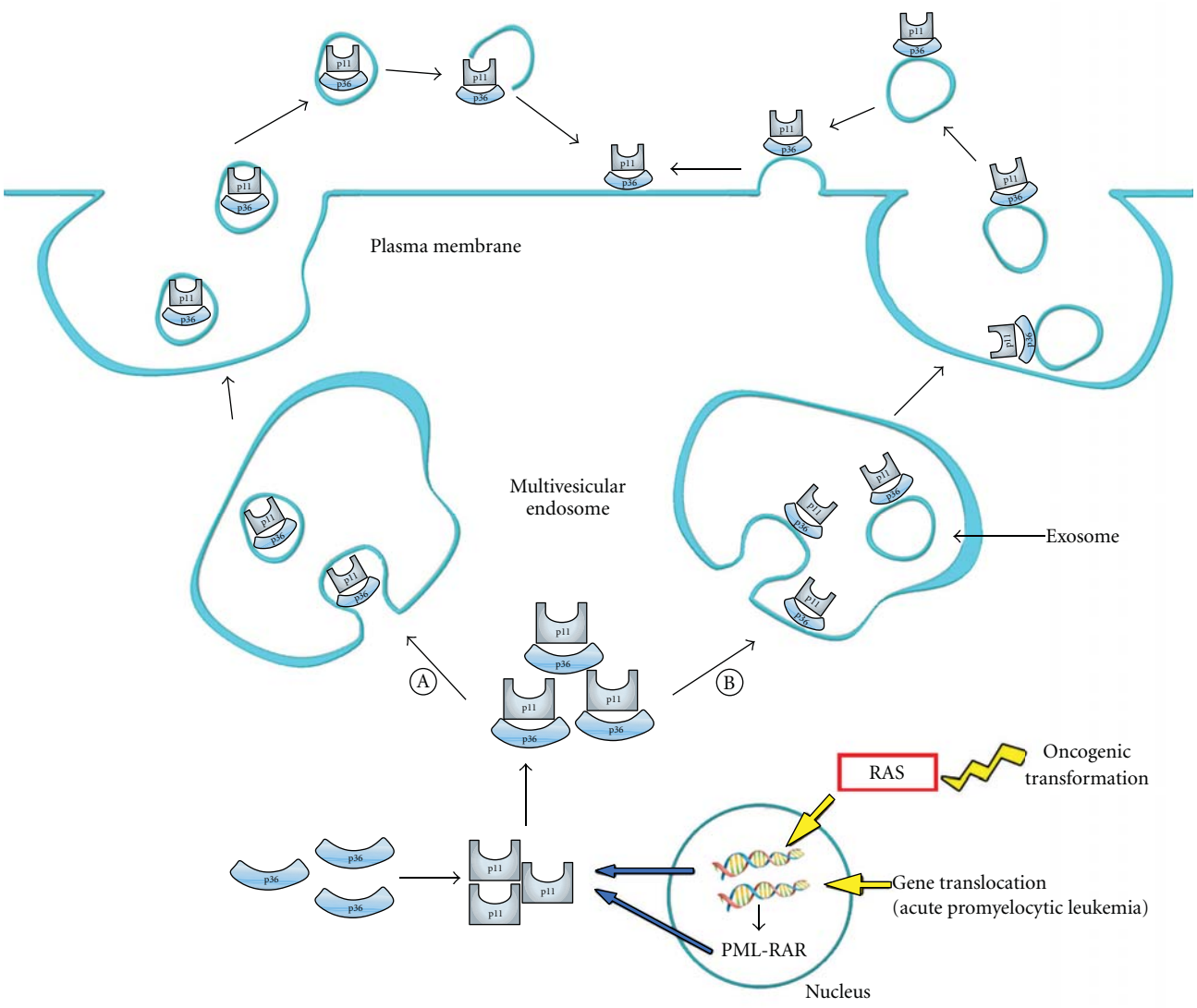

Figure 5: Regulation of the expression of extracellular S100A10. Many physiological agents (EGF, interferon-γ, transforming growth factor-α) and pathophysiological agents (PML-RARα and KRas oncogenes) stimulate the upregulation of S100A10 protein levels. Annexin A2 is thought to play two important roles in the export of S100A10. First, the association of annexin A2 with S100A10 protects S100A10 from ubiquitin-mediated proteasomal degradation as S100A10 is rapidly degraded in the absence of annexin A2. Secondly, the binding of annexin A2 with S100A10 is thought to promote the capture of the complex by the exosomes. Exosomes are small vesicles, approximately 30–100 nM in diameter, that are formed by the inward budding of large intracellular compartments called multivesicular endosomes (MVE). Proteins and RNA that are present in the cytoplasm are trapped within the lumen of the exosomes during this inward budding process and proteins that associate with the MVE lumenal membrane during MVE budding are localized to the outer surface of the exosomes. The exosomes, sequestered as intact vesicles within the MVE, are released from cells when the membrane of the MVE fuses with the plasma membrane. The association of annexin A2 with exosomes has been reported but it is unclear at this time if the S100A10/annexin A2 complex is present on the surface or in the lumen of the exosomes. In our speculative model, we show that S100A10 protein that is synthesized in response to oncogenic agents such as oncogenic Ras or the PML-RAR oncogene forms a complex with annexin A2. The S100A10/annexin A2 complex is then sequestered with the lumen of the exosomes during the inward budding of the MVE (A). These MVEs fuse with the plasma membrane resulting in the release of the exosomes into the extracellular space. These exosomes rupture and release the S100A10/annexin A2 complex from their lumen which then allows the association of the complex with the plasma membrane. Alternately, the S100A10/annexin A2 complex may be present on the lumenal surface of the MVE (B). The inward budding of the MVE results in the association of the S100A10/annexin A2 complex with the outer surface of the newly formed exosomes. The exosomes released by the fusion of the MVE with the plasma membrane fuse with the plasma membrane resulting in the incorporation of the S100A10/annexin A2 complex at the plasma membrane.

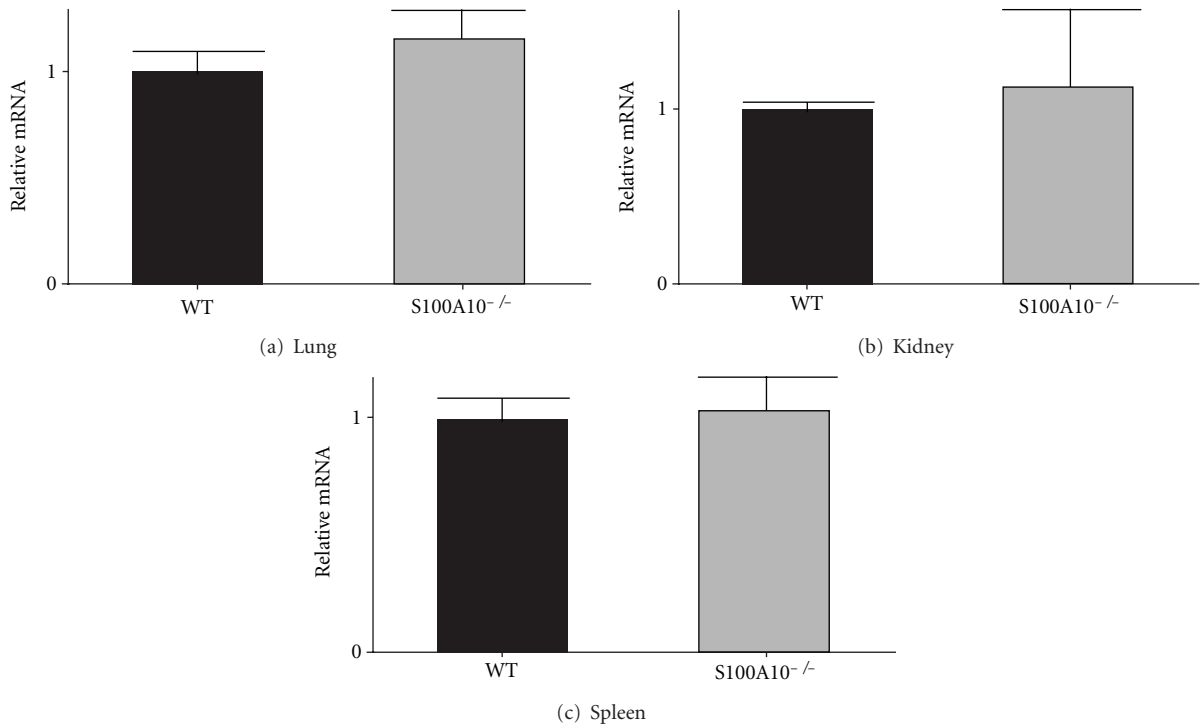

Figure 6: Annexin A2 mRNA levels in tissues from S100A10$^{-/-}$ mice. Annexin A2 mRNA levels were analyzed in tissues isolated from WT and S100A10$^{-/-}$ mice. Loss of S100A10 did not affect annexin A2 mRNA levels in lung (a), kidney (b), and spleen (c). Statistical analysis was performed using Student's t-test and the data are expressed as the mean (±) SEM of 6 independent experiments.

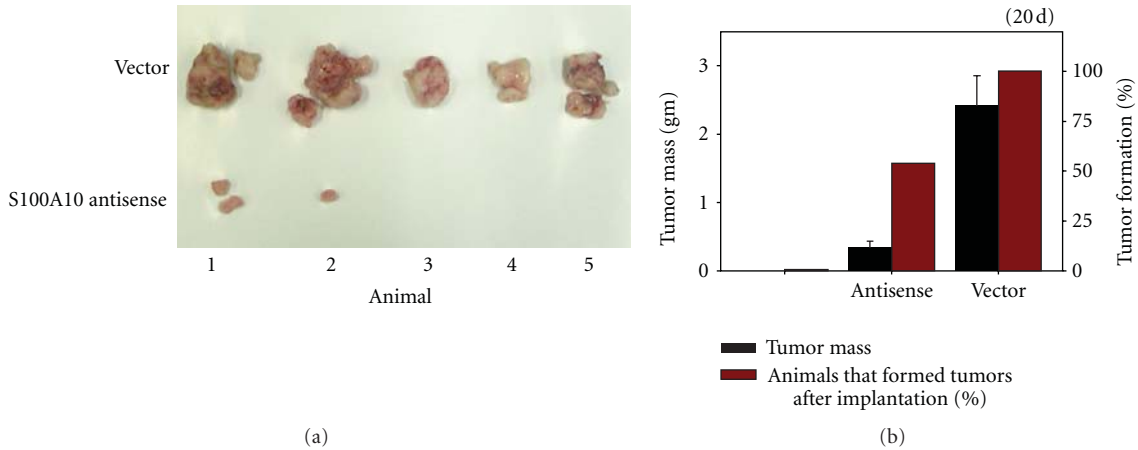

Figure 7: Role of S100A10 in Tumor Formation. HT1080 fibrosarcoma cells (106 cells) were injected intradermally into SCID mice and dissected and weighed after 20 days. Thirteen mice were used in each group. The dissected-20 day tumors were photographed. 7/13 of mice injected with S100A10 antisense HT1080 cells developed tumors compared to 100% tumor development for the WT HT1080 cells. Furthermore, the tumors grown by the S100A10-depleted HT1080 cells were much smaller. The data shows that the loss of S100A10 from the cancer cell surface and the resultant loss of protease activity inhibited tumor growth.

of plasminogen to the candidate regulatory protein must convert plasminogen into the open, activation-susceptible conformation. Studies using plasminogen that was FITC labeled at its active site showed that addition of either AIIt or S100A10 alone, but not annexin A2 alone, resulted in quenching of the fluorescence of plasminogen, meaning that S100A10 promoted an open, activatable conformation of plasminogen [19]. Third, the plasminogen receptor should be inactivated by removal of its carboxyl-terminal lysine. S100A10 is a high affinity substrate for several carboxypeptidases, which have been shown to block cellular plasminogen activation by cleaving carboxy-terminal lysines [7]. Our laboratory has demonstrated that carboxypeptidase B (CpB) ablates enhancement of plasminogen activation

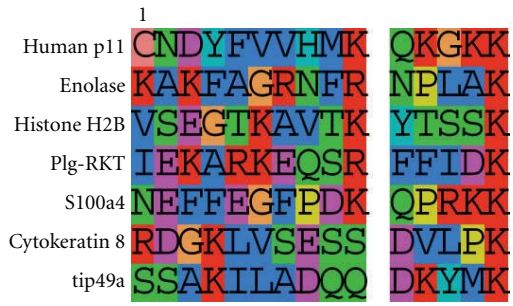

Figure 8: Plasminogen receptors.

by homodimeric S100A10 or by the S100A10/annexin A2 complex through removal of carboxy-terminal lysines of S100A10 [20]. Fourth, binding of plasminogen regulatory proteins to plasmin or plasminogen activators must protect these enzymes from inactivation by their inhibitors. S100A10 has been shown to protect tPA and plasmin from PAI-1 and alpha2-antiplasmin, respectively [15]. Fifth, although not a criterion for a plasminogen receptor *per se*, it is expected that in order for a plasminogen receptor to efficiently convert plasminogen to plasmin it should bind to or colocalize with plasminogen activators. S100A10 binds directly to tPA and colocalizes with uPAR on the cell surface. Studies from our laboratory have demonstrated the presence of uPAR in S100A10 precipitates and colocalization has been observed by immunofluorescence microscopy [15, 64, 65]. Sixth, depletion of the plasminogen receptor from the cell surface should result in a loss in cellular plasmin generation. The loss of S100A10 from the cell surface results in a loss of cellular plasmin generation. *In vitro* studies have demonstrated that when HT1080 fibrosarcoma cells were transfected with antisense S100A10, resulting in depletion of S100A10 but not annexin A2, plasmin production was reduced by 95% and extracellular matrix hydrolysis was decreased by almost 70% in comparison to the vector controls. Furthermore, tumor formation in SCID mice was dramatically reduced in S100A10-antisense-transfected cells compared to the vector control (Figure 7). Taken together, this evidence supports a key role for S100A10 in plasmin regulation and in the process of oncogenesis.

6.2. S100A10 in Fibrinolysis. Increasing evidence has shown that the plasminogen receptor, S100A10, is a main regulator of plasmin activity at the surface of endothelial cells playing a main role in vascular fibrinolysis. Our laboratory recently used a human microvascular endothelial cell line, called telomerase immortalized microvascular endothelial (TIME) cells [123], to investigate the role of S100A10 in fibrinolysis [66]. We used an shRNA system to deplete these cells of S100A10 and observed a significant impairment in both plasminogen binding (50%) and plasmin generation (60%) even though the cell surface levels of its binding partner annexin A2 were identical in the S100A10-depleted cells compared to the control cells expressing a scramble shRNA [66]. On the other hand, annexin A2-depleted TIME cells showed similar losses in plasminogen binding and plasmin generation as the S100A10-depleted TIME cells [66]. Taking into consideration that the annexin A2-depleted TIME cells were also depleted of S100A10, we concluded that the loss of cell surface annexin A2 did not affect plasminogen binding or plasmin generation in these endothelial cells. In view of these results we have proposed that annexin A2 functions to stabilize S100A10 protein levels and to localize S100A10 to the cell surface of endothelial cells, while S100A10 is directly responsible for plasminogen binding and plasmin generation by endothelial cells [12].

The role of S100A10 in fibrinolysis *in vivo* has been recently established using the S100A10-null mouse model. These studies showed an enhanced accumulation of fibrin in the S100A10-null mice tissues compared to wild-type mice litter mates. These studies initially demonstrated that the enhanced fibrin deposition observed in the S100A10-null mice tissues was not due to increased coagulation, since PT (prothrombin time) and aPTT (activated partial thromboplastin time) assays, which directly measure coagulation, were identical for both the WT and the S100A10-null mice [66]. In order to directly investigate fibrinolysis in the S100A10-null mice, we used a novel approach which involved the injection of the snake poison, batroxobin, in S100A10-null and WT mice. Batroxobin is a thrombin-like enzyme that rapidly cleaves fibrinogen resulting in the production of fibrin microclots which are removed from the vasculature through the process of fibrinolysis. We then compared the ability of these mice to dissolve the batroxobin-induced blood clots. This experiment showed that the S100A10-null mice have significantly lower rates of fibrinolysis of the batroxobin-induced blood clots *in vivo* compared to the WT mice [66]. Finally, a tail clip experiment showed that mice lacking S100A10 have an approximately 4-fold reduction in the time required for the cessation of bleeding compared to the WT mice. This is most likely due to decreased fibrinolysis of the tail clip-induced blood clot by the S100-null mice. Therefore, a central defect in the S100A10-null mice involves plasmin generation by the endothelium. These studies taken together establish that S100A10 plays a major role in fibrinolysis *in vivo.*

To investigate if S100A10 plays a significant role in angiogenesis *in vivo*, we used a well-established method where Matrigel (which mimics the extracellular matrix) is injected in mice allowing for the measurement of the angiogenic response towards a growth factor stimulus *in vivo* [66]. This study showed a defective vascularization of the Matrigel plugs in the S100A10-null mice compared to the WT mice, establishing that S100A10 plays an important role in angiogenesis *in vivo*. These results were further supported by another experiment that showed that S100A10-depleted endothelial cells have a significantly decreased ability to migrate through Matrigel barriers. In conclusion, the studies performed using the S100A10-null mouse model clearly established an important role for S100A10 as a positive regulator of fibrinolysis and angiogenesis.

6.3. S100A10 in Signalling. S100A10, as part of AIIt, has also been shown to function as a receptor in plasmin-induced

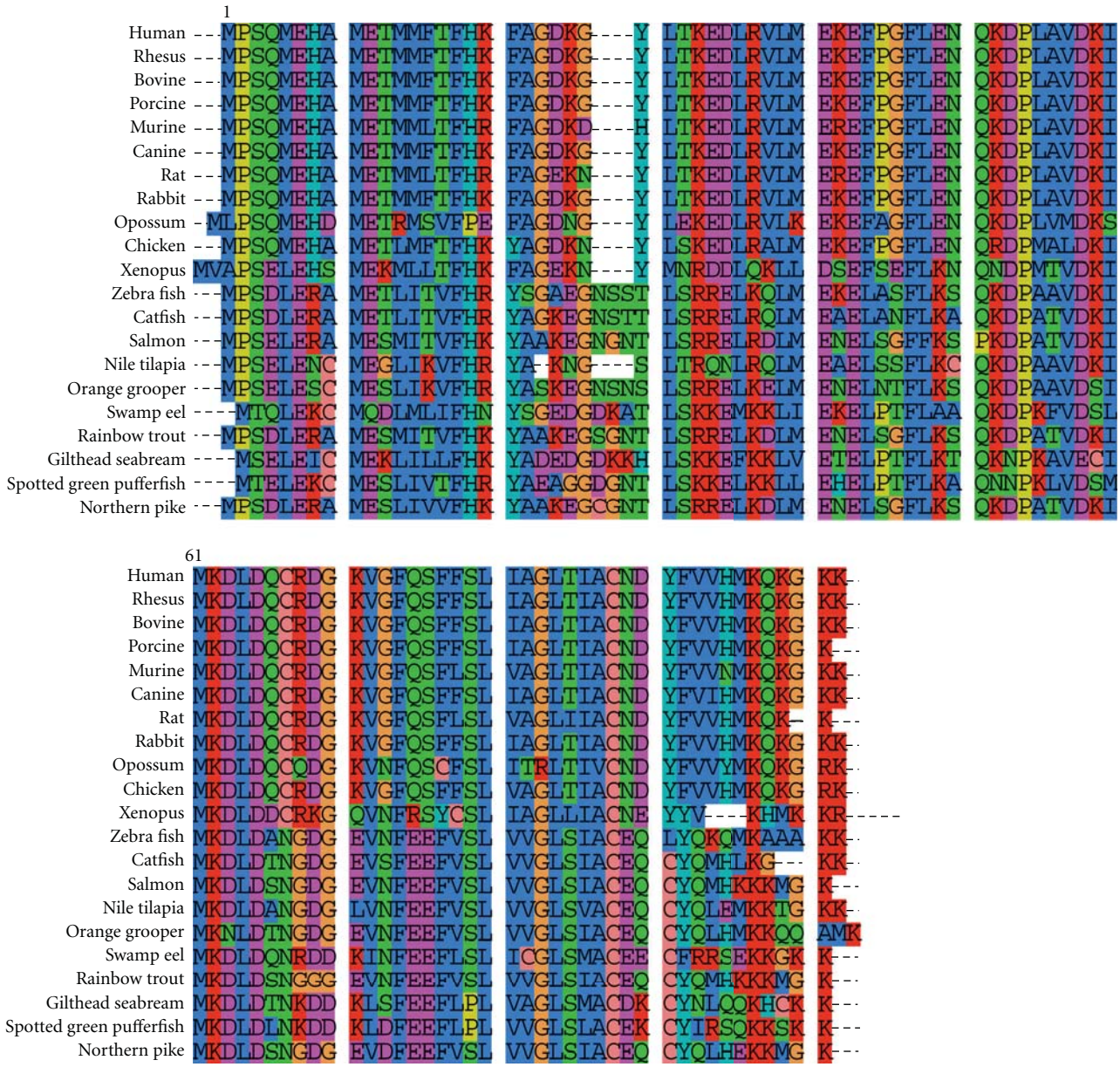

Figure 9: S100A10 phylogeny.

signaling in monocytes [124] and macrophages [125]. After having demonstrated that monocytes show a chemotactic response to plasmin that is dependent on S100A10 and annexin A2 [124], the Simmet group showed that plasmin, through S100A10 and annexin A2, activates macrophages by a mechanism involving stimulation of the Janus kinase JAK1/TYK2 signaling pathway. JAK1/TYK2 leads to STAT3 activation, Akt dependent NF-κB activation and phosphorylation of extracellular signal-regulated kinase 1/2 (ERK1/2) and mitogen-activated kinase, p38. Plasmin also triggered nuclear translocation of STAT3 and p65 transcription factors and the induction of the proinflammatory cytokines tumor necrosis factor-α and interleukin-6. The Simmet group has proposed a mechanism by which plasmin cleaves annexin A2 at lysine 27 (K27), resulting in disruption of AIIt at the cell surface and transduction of a signaling cascade inside the cell. Further work needs to be performed to identify a more complete mechanism by which AIIt serves as a signaling receptor and transduces this signal into the cell.

7. Role of S100A10 in Disease

7.1. S100A10 in Depression. S100A10 is expressed in several regions of the brain and through its interactions with 5-HT1B receptors and NaV1.8/ASIC-1 channels, the involvement of S100A10 in the regulation of depression-like states and nociception has been thoroughly examined [53, 54, 56]. 5-HT1B receptors modulate serotonin neurotransmission by

acting as both autoreceptors on serotonin-containing neurons originating from the raphe nuclei and heteroreceptors on several neurons that do not contain serotonin [126, 127]. 5-HT1B receptors have been demonstrated to play a role in the pathophysiology of multiple mental disorders, including depression [128, 129].

S100A10 levels are decreased in a mouse model of depression and in brain tissue from unipolar depressed patients. The S100A10-null mice demonstrate significantly reduced responsiveness to stimulation of 5-HT1B receptors in biochemical, electrophysiological, and behavioural tests thus providing strong evidence to suggest that the interaction between S100A10 and 5-HT1B receptors plays a role in the pathophysiology of depression-like states. Further evidence suggests that decreased levels of S100A10 correlate with greater susceptibility to depression [56]. The S100A10-null mouse demonstrate a reduced number of 5-HT1B receptor ligands which are likely mediated by multiple mechanisms, such as altered recruitment of the receptors to the cell membrane or disturbed endosomal recycling and/or degradation of the receptor. In contrast, overexpression of S100A10 leads to an increase in the number of 5-HT1B receptors at the cell surface and recapitulates certain behaviors seen after antidepressant treatment in mice [56]. Similarly, administration of antidepressants increases S100A10 expression in the brain [130].

In addition to results seen at the cellular level, the S100A10-null mouse exhibits a depressive-like phenotype in which they display a decreased thigmotaxis and increased immobility in tail suspension tests in response to imipramine [56]. Taken together, these results show that S100A10 plays an important role in the dynamic modulation of 5-HT1B receptor function and that decreased expression of S100A10 leads to a depressive-like state.

7.2. S100A10 in Inflammation

7.2.1. S100A10 Regulates Plasminogen-Dependent Macrophage Invasion. It has been shown that the cell surface generation of plasmin is required for macrophage recruitment to a site of inflammation and that macrophage recruitment is mediated in part through the plasmin-dependent activation of MMP-9 [131]. Therefore, macrophage-generated plasmin plays two roles in invasion; it directly hydrolyzes extracellular matrix (ECM) proteins and it activates MMP-9, thus promoting further ECM degradation. Analysis of the plasminogen receptors on the surface of macrophages has identified α-enolase, histone H2B, and Plg-RKT as plasminogen receptors that participate in macrophage invasion [7, 132–134]. Recent studies performed by our laboratory investigated the mechanism of plasminogen-dependent inflammatory cell recruitment *in vivo* using two different methodologies: the thioglycollate-induced peritonitis and the Matrigel plug assay [67]. We observed that in response to thioglycollate-induced peritonitis, macrophage migration through the peritoneal membrane into the peritoneal cavity of the S100A10-null mouse was reduced by about 53%. Our results with the Matrigel plug assay demonstrated that S100A10-null macrophages had a limited capability to infiltrate into the Matrigel plug *in vivo*. Analysis of the mechanism of macrophage migration through the Matrigel suggested that S100A10-null macrophages have a reduced capacity to generate plasmin and activate MMP-9. Therefore, the simplest explanation to our results is that S100A10 and other carboxyl-terminal plasminogen receptors contribute to macrophage plasmin generation that is utilized for ECM hydrolysis and MMP-9 activation. Of note was our observation that neutrophil recruitment to the peritoneal cavity in response to a thioglycollate-dependent inflammatory stimulus was also regulated by S100A10. It has been reported that neutrophil recruitment in plasminogen-null mice is identical to that of wild-type mice [135] suggesting that plasmin does not play a role in neutrophil recruitment in the thioglycollate model. We are currently investigating the possibility that S100A10 may regulate proteases other than plasmin on the surface of the neutrophil. For example, it has been reported that S100A10 regulates the activation of cathepsin B on the surface of certain cancer cells [90].

Previous studies have established the presence of S100A10 and its binding partner, annexin A2, on the surface of murine macrophages [136]. These studies showed that knockdown of annexin A2 resulted in decreased plasmin generation, matrix remodeling, and a dramatic loss in directed migration [137]. However, since annexin A2 knockdown results in concomitant loss of S100A10, it is difficult to attribute these effects to either annexin A2 or S100A10. Annexin A2 levels were reduced in macrophages isolated from S100A10-null mice, making it unclear if the reduced macrophage migration in response to thioglycollate-induced peritonitis was due to the loss of S100A10 or annexin A2 or both. Elucidating the role that annexin A2 plays in invasion is complicated by the reports from three laboratories that intact annexin A2 does not bind plasminogen [63, 133, 138]. Since annexin A2-dependent plasmin generation is blocked by the lysine analog ε-amino caproic acid or by treatment of annexin A2 by carboxypeptidase-B [138], it has been proposed that annexin A2 binds plasminogen upon proteolytic cleavage of the protein and exposure of a new carboxyl-terminal lysine [138]. Further proof that plasminogen binding to annexin A2 requires "activation" by a carboxyl-terminal cleavage event was provided by the report that the K307T mutant annexin A2 transfected into HEK 293 cells failed to bind plasminogen while a change of a lysine proximal to this site (K328I) bound plasminogen with similar affinity to the wild type [139]. The proteinase that has been proposed to cleave annexin A2 has not been identified but plasmin has been ruled out [139]. We addressed the issue of whether annexin A2 processing occurred in migrating macrophages *in vivo* by examining the molecular mass of cell surface annexin A2 by SDS-PAGE [67]. The macrophages used in this study were isolated from the peritoneal cavity of thioglycollate-stimulated mice. We have recently shown that the loss of the carboxyl-terminal 29 amino acid of annexin A2 (Ser1-Asp338) generates a truncated form of annexin A2 (Ser1-Lys307), that is easily detected on SDS-PAGE [14]. However, macrophage cell surface annexin A2 is similar in molecular mass to

unproteolyzed annexin A2. Since the macrophages isolated for these studies were macrophages that had migrated through the extracellular matrix into the peritoneal cavity and were therefore proteolytically active, this result suggests that macrophage cell surface annexin A2 is not cleaved and therefore does not participate in plasminogen binding, plasmin generation, or macrophage invasion. Similarly, it has been reported that annexin A2 is not proteolyzed during active plasmin generation by HT1080 cells [64]. It was also interesting that S100A10 and annexin A2 protein levels were higher in thioglycollate-stimulated peritoneal macrophages compared to resident peritoneal macrophages, suggesting that macrophages upregulate S100A10 after activation by inflammatory mediators.

Our observation that annexin A2 levels were lower in macrophages isolated from S100A10-null mice compared to WT mice was unexpected. RT-PCR analysis suggested that the annexin A2 mRNA levels were similar in these macrophages [67]. S100A10 has been shown to be ubiquitinated [105, 140] and rapidly degraded by the proteasome. The binding of S100A10 to annexin A2 protects S100A10 from ubiquitination and proteasomal degradation. In contrast, although annexin A2 is ubiquitinated, ubiquitination does not activate the proteasomal degradation of the protein [141]. We have also observed that the proteasomal inhibitor, MG-132, does not affect the annexin A2 levels thereby eliminating proteasomal degradation as a possible regulatory mechanism for annexin A2 [29]. However, it is known that annexin A2 is mainly cytosolic whereas the annexin A2-S100A10 complex is associated with the cytoskeleton [39, 142]. Since the turnover of cytoskeleton-bound annexin A2 ($t1/2 = 40 - 50$ h) is three to four times slower than for the cytoplasmic annexin A2 ($t1/2 = 15$ h) [37], it is possible that changes in the subcellular distribution of annexin A2 in the S100A10-null macrophages could account for the decreased annexin A2 levels.

7.3. S100A10 in Oncogenesis. Oncogenesis, the process of tumor development and progression, requires an intimate association between the cancer cells and the tumor-associated cells. The cancer cells utilize plasmin and other proteases such as cathepsin B and the matrix metalloproteases (MMPs) to proteolyse the extracellular matrix and basement membrane in order to gain access to the circulation. Once in the circulation, the cancer cells utilize proteolytic activity to enter and form stable metastatic foci in other tissues. The tumor-associated cells such as macrophages also require proteolytic activity in order to migrate from the circulation into the tumor stroma. Therefore, proteolytic activity is used by both cancer cells and tumor-associated cells during oncogenesis. Several studies have established that plasmin is an important protease involved in cancer cell migration and invasion, but the plasminogen receptor(s) that is involved in the regulation of plasmin activation during oncogenesis remains elusive.

The uPA receptor, uPAR, is an important regulator of plasmin-dependent extracellular matrix proteolysis in cancer cells. uPAR binds uPA and its zymogen form, pro-uPA, and therefore localizes these proteins to the cell surface [143]. Activated uPA that is bound to the uPAR at the cell surface cleaves plasminogen and the resultant plasmin reciprocally cleaves and activates pro-uPA (Figure 1). This positive-feedback loop is further amplified by the increased concentration of the active and zymogen forms of uPA and plasmin at the cell surface through uPA or pro-uPA binding to the uPAR and plasminogen or plasmin binding to the plasminogen receptor(s) (reviewed in [144–146]). Previous work from our laboratory has shown that S100A10 co-localizes with uPAR and positively regulates the conversion of pro-uPA to active uPA [65]. The activation of proteases at the cell surface of cancer cells plays an important role in the migration of primary tumor cells as well as in the invasiveness of these cells and establishment of metastatic foci at other sites.

Several studies have shown that S100A10 plays an important role in tumor invasion and metastasis. Choi and colleagues have shown that mice injected with S100A10-depleted HT-1080 fibrosarcoma cells have a 3-fold decrease in the number of metastatic foci in the lungs compared to mice injected with control HT1080 cells, while overexpression of S100A10 in the HT-1080 cells leads to a 16-fold increase in the number of lung metastasis in these mice [64]. Furthermore, tumor formation in SCID mice was dramatically reduced in S100A10-antisense-transfected HT-1080 cells compared to the vector control (Figure 7). These results show that the ability of HT-1080 tumor cells to extravasate and metastasize is directly related to the extracellular expression of S100A10. Another study showed that siRNA-mediated downregulation of S100A10 gene expression in CCL-222 colorectal cancer cells resulted in a significant decrease in extracellular S100A10 protein, which correlated with a 45% loss in plasminogen binding and a 65% loss in cellular plasmin generation in these cells compared to the control cells. S100A10 depletion in CCL-222 cells also abolished the plasminogen-dependent invasiveness of these cells through a matrigel barrier. A remarkable observation made by this study was that the CCL-222 cells do not express annexin A2 on their extracellular surface and for this reason the plasminogen binding, plasmin activation and invasiveness of these cells, although dependent on the presence of S100A10 at the cell surface, were independent of annexin A2. Collectively, these studies establish a role for S100A10 as an oncogenic plasminogen receptor, involved in invasiveness and metastasis of cancer cells.

7.4. The Role of S100A10 in the Migration of Macrophages to the Tumor Site. Inflammatory cell recruitment to the tumor microenvironment is indispensable to cancer progression. Tumor-associated macrophages (TAMs) represent a prominent component of the inflammatory cell population with solid tumors and density of TAMs within a tumor correlates with poor prognosis [147]. The recruitment of TAMs to the tumor microenvironment involves the migration of monocytic precursor cells from the circulation in response to various chemotactic signals originating from the tumor [148]. TAMs promote tumor growth by mediating inflammation, stimulating angiogenesis, suppressing antitumor immunity, and by matrix remodeling [149]. However, little

is known about the proteolytic mechanism by which monocytes/macrophages migrate from the circulation to the tumor site. Several studies have hypothesized that macrophages mobilize a number of cell surface plasminogen receptors to generate plasmin thereby facilitating proteolysis of basement membrane and extracellular matrices to allow migration to the tumor site. Recently, O'Connell et al. [67] demonstrated that S100A10 plays a significant role in mediating plasmin generation at the surface of macrophages.

Phipps et al. [68] recently demonstrated that S100A10 plays a significant role in oncogenesis. Wild-type and S100A10-null mice were injected with Lewis Lung carcinoma cells and the kinetics of tumor growth was measured. Interestingly, tumors grown in the S100A10-null mice reached maximum size after seven days whereas tumors in their wild-type counterparts continued to grow exponentially. Upon termination of the experiment, LLC tumors from wild-type mice were 10-fold larger than those in S100A10-null mice. Immunohistochemical analysis of the tumors revealed that macrophage recruitment was impaired in the S100A10-null mice. Macrophages were visible throughout wild-type tumors but were only found at the tumor edge in S100A10-null mice. Cytokine levels were similar in wild-type and S100A10 tumors thus eliminating the possibility that less macrophages were recruited due to impaired chemokine/cytokine production. Peritoneal injection of wild-type macrophages into S100A10-null mice prior to injection of LLC cells rescued tumor growth to levels comparable to those seen in wild-type mice, indicating that S100A10-null plays an important role in both angiogenesis and tumor growth in terms of macrophage function. Taken together, these studies demonstrated that expression of S100A10 on the surface of murine macrophages plays significant role in their ability to associate with the tumor microenvironment and, in turn, promotes tumor growth and progression, that is, oncogenesis.

7.5. Regulation of S100A10 by the Oncogene PML-RARα. Leukemia is a group of hematological malignancies characterized by clonal expansion of hematopoietic cells with uncontrolled proliferation, blocked differentiation, and decreased apoptosis. Acute myeloid leukemia (AML) is the most common leukemia affecting adults. Acute promyelocytic leukemia (APL) is a subtype of acute myeloid leukemia that is characterized by fusion of the retinoic acid receptor alpha (RARα) gene with the promyelocytic leukemia (PML) gene via the t(15; 17) translocation, resulting in the expression of a PML-RARα fusion protein [150]. Intracellular accumulation of the PML-RARα fusion protein causes the inhibition of cellular differentiation of these cells into granulocytes, resulting in the accumulation of the abnormal promyelocytes in the bone marrow. Pathogenesis of the disease includes disseminated intravascular coagulation (DIC), fibrinolysis, and proteolysis. The excessive fibrinolysis and proteolysis is thought to be a result of increased production of the fibrinolytic enzyme plasmin, and clinical evidence supports this idea. It is common for patients with new diagnoses of APL to present with elevated D-dimer level, fibrin split products, elevated prothrombin time and partial thromboplastin time, and hypofibrinogenemia [151–153]. Because activation of plasmin at the cell surface is necessary for plasmin activity, attention has turned to elucidating the plasminogen receptor on APL cells.

Previous work has shown that annexin A2 levels are elevated in APL and annexin A2 protein levels have been implicated as the cause for the excessive fibrinolysis that is associated with the disease. It was previously observed that all-trans retinoic acid (ATRA) treatment of APL primary cells and cell lines resulted in loss of cell surface annexin A2. *In vitro* treatment of t(15; 17)-positive APL cells with all-trans-retinoic acid significantly reduced both the cellular expression of annexin A2 and plasmin generation over a similar period [154, 155]. However, since annexin A2 knockdown results in concomitant loss of S100A10, it is difficult to attribute these effects to annexin A2 or S100A10.

Of the reputed plasminogen receptors, annexin A2 was reported to be present at abnormally high levels on APL cells and it was proposed that the elevated levels of annexin A2 were responsible for increased production of plasmin and the hemorrhagic complications of APL. A recent publication from our laboratory examined the role of S100A10 in regulating the generation of plasmin at the surface of APL cells in NB4 cells [29]. We used the human APL cell line, NB4, for these studies. NB4 cells possess the t(15; 17)-translocation and constitutively express the PML-RARα oncoprotein and are induced to terminally differentiate to neutrophils with ATRA. Depletion of S100A10 by RNA interference resulted in a 70% loss in plasminogen binding and a 64% loss in plasmin generation by the NB4 cells. Furthermore, depletion of S100A10 resulted in 60% fewer NB4 cells migrating through a fibrin barrier. These results established the importance of S100A10 in plasmin generation in promyelocytic leukemia cells. Treatment of the NB4 cells with ATRA resulted in a rapid reduction in the PML-RARα oncoprotein concomitant with a loss of S100A10. ATRA treatment also resulted in a 60% loss in plasminogen binding, a 40% loss in plasmin activity, and a 60% loss in migration of the ATRA-treated NB4 cell through the fibrin barrier. The complete loss of cellular levels of S100A10 after ATRA treatment presents the possibility that the remission of hemorrhagic complications observed during treatment of APL patients with ATRA could be due to the ATRA-mediated loss of S100A10 from the surface of the leukemic promyelocytes.

Since the treatment of NB4 cells with ATRA is known to result in the upregulation of about 119 genes as well as the downregulation of 17 genes [156], it was unclear if the regulation of S100A10 by ATRA was directly regulated by the PML-RARα oncoprotein or by other ATRA-regulated genes. This issue was directly addressed by examining S100A10 levels in the U937/PR9 cells, which express PML-RARα oncoprotein under the control of a zinc-inducible promoter. Expression of PML-RARα oncoprotein resulted in a rapid and dramatic upregulation of S100A10 protein and a subsequent increase in plasminogen binding and fibrinolytic activity. We also observed that annexin A2 was upregulated by induced expression of the PML-RARα oncoprotein. However, when the PML-RARα oncoprotein-expressing PR9 cells were depleted of S100A10 by RNA

interference, we observed a dramatic loss of plasminogen binding and plasmin generation [29]. These data suggest that PML-RARα oncoprotein increases the S100A10 protein levels and also implicate S100A10 as a major component of the fibrinolytic system upon development of APL. Therefore, the upregulation of S100A10 at the cell surface of promyelocytic leukemic cells is likely the cause of the bleeding complications that are associated with APL.

These results demonstrated, for the first time, the regulation of S100A10 protein levels by an oncogene. A study performed by Sloane's laboratory showed that disruption of the gene that encodes oncogenic K-Ras, a well established oncogene, results in a dramatic decrease in S100A10 protein levels [157]. This result suggests that S100A10 protein levels might also be stimulated by oncogenic Ras and that S100A10 might play a role in Ras-dependent plasmin activation and, consequently, cancer cell invasiveness. Nevertheless, further studies are necessary in order to address this hypothesis.

8. Summary

The evidence that has accumulated over the past 15 years supports the concept that one of the most important physiological functions of S100A10 is as a plasminogen receptor. As such, S100A10 plays an important role in endothelial cell function by regulating plasmin production. In the absence of S100A10, endothelial cells fail to produce sufficient plasmin to maintain vascular patency and as a consequence fibrin clots accumulate in the tissues. S100A10 also regulates the movement of macrophages to the site of inflammation. S100A10 also plays a key role in oncogenesis by regulating the plasmin proteolytic activity of cancer cells and by regulating the migration of macrophages to the tumor site. The regulation of S100A10 levels by oncogenes such as PML-RARα and oncogenic KRas presents the possibility that activation of the S100A10 gene may be of fundamental importance during the transformation process. Future studies are necessary to define exactly when during the cellular transformation process the S100A10 gene is activated and what are the functional consequences of that activation.

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Research Article

The Serine Protease Plasmin Triggers Expression of the CC-Chemokine Ligand 20 in Dendritic Cells *via* Akt/NF-κB-Dependent Pathways

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The number of dendritic cells is increased in advanced atherosclerotic lesions. In addition, plasmin, which might stimulate dendritic cells, is generated in atherosclerotic lesions. Here, we investigated cytokine and chemokine induction by plasmin in human dendritic cells. In human atherosclerotic vessel sections, plasmin colocalized with dendritic cells and the CC-chemokine ligand 20 (CCL20, MIP-3α), which is important for homing of lymphocytes and dendritic cells to sites of inflammation. Stimulation of human dendritic cells with plasmin, but not with catalytically inactivated plasmin, induced transcriptional regulation of CCL20. By contrast, proinflammatory cytokines such as TNF-α, IL-1α, and IL-1β were not induced. The plasmin-mediated CCL20 expression was preceded by activation of Akt and MAP kinases followed by activation of the transcription factor NF-κB as shown by phosphorylation of its inhibitor IκBα, by nuclear localization of p65, its phosphorylation, and binding to NF-κB consensus sequences. The plasmin-induced CCL20 expression was dependent on Akt- and ERK1/2-mediated phosphorylation of IκBα on Ser32/36 and of p65 on Ser276, whereas p38 MAPK appeared to be dispensable. Thus, plasmin triggers release of the chemokine CCL20 from dendritic cells, which might facilitate accumulation of CCR6$^+$ immune cells in areas of plasmin generation such as inflamed tissues including atherosclerotic lesions.

1. Introduction

The serine protease plasmin is mainly recognized for its central role in fibrinolysis. In addition, however, plasmin may also be generated at inflammatory sites from ubiquitously distributed plasminogen [1]. Indeed, generation of plasmin has been shown in a number of chronic inflammatory conditions including arthritis and atherosclerosis [1]. Specifically in unstable atherosclerotic lesions, plasminogen and plasmin appear to be associated with clinical complications [2–5]. Local plasmin generation at sites of inflammation might aggravate inflammatory processes by triggering proinflammatory effects. *In vitro*, plasmin is capable of stimulating lipid mediator release and of eliciting chemotaxis of human monocytes [6, 7]. In addition, plasmin is a potent inducer of proinflammatory cytokines in human macrophages [8] and monocytes, where it also causes expression of procoagulant tissue factor [9].

Dendritic cells play a crucial role in innate and adaptive immune responses [10]. Dendritic cells are crucial for immune diseases including rheumatoid arthritis, where they accumulate in synovial tissue and activate T cells [11]. Likewise, the number of dendritic cells is strongly increased in advanced atherosclerotic lesions, where they colocalize with T cells [12–14]. Dendritic cells induce differentiation of T cells into different T-cell subsets through direct interaction with T-cell receptors and the release of cytokines. Dendritic cells are heterogeneous in their origin and their ability to activate either tolerogenic or immunogenic T-cell responses [15]. A distinct dendritic cell type is monocyte-derived dendritic cells, which arise in the course of inflammation [15, 16]. We have recently shown that plasmin is a potent chemoattractant for immature dendritic cells, and that it activates dendritic cells to produce interleukin-12 (IL-12) and to promote polarization of CD4$^+$ T cells towards the interferon-γ (IFN-γ-) producing, proinflammatory Th1 phenotype [17].

Chemokines orchestrate the homing of lymphocytes and dendritic cells to lymphoid tissues as well as the recruitment

of leukocytes to sites of infection or tissue damage [18]. As a result, chemokines play crucial roles in the pathogenesis of diseases that are characterized by inflammatory cell accumulation, such as atherosclerosis [12, 14, 18, 19].

CCL20 (also known as liver- and activation-regulated chemokine, LARC, or macrophage inflammatory protein-3α, MIP-3α) is a CC-type chemokine, which activates chemokine receptor CCR6 and therefore plays an important role in homing $CCR6^+$ lymphocytes and dendritic cells into secondary lymphoid organs and to sites of inflammation [20]. Memory T lymphocytes, naïve and memory B cells, Langerhans cells, and subsets of immature dendritic cells all express CCR6 [20] and migrate to sites of CCL20 expression, for example, in atherosclerosis [20], inflammatory bowel disease [21], arthritis [19], chronic obstructive pulmonary disease [22], psoriasis [23], and tumor tissues [24]. Accordingly, an important role for CCL20 has been postulated in atherosclerosis, skin, and mucosal immunity, in rheumatoid arthritis, and in cancer [20].

Immunohistochemical colocalization of immature dendritic cells and CCL20 indicates a link between the accumulation of immature dendritic cells and the local production of CCL20 by epithelial and tumor cells [20, 22]. Indeed, subsets of immature dendritic cells express CCR6 and are able to migrate towards CCL20 [25]. However, dendritic cells might also be a source of CCL20 on their own. Thus, CCL20 secretion can be induced in dendritic cells either by stimulation with LPS [26] or extracellular nucleotides [27].

Here, we investigated whether plasmin might affect the expression of cytokines by human monocyte-derived dendritic cells and whether this might occur in human atherosclerotic lesions.

2. Materials and Methods

2.1. Materials. Antibodies used are phospho-IκBα, phospho-ERK1/2, phospho-p38, phospho-Akt (Ser473), phospho-p65 Ser536, and phospho-p65 Ser276-Cell Signaling Technology (Danvers, MA); CCL20-R&D Systems (Minneapolis, MN); p65-Santa Cruz Biotechnology (Santa Cruz, CA); actin-Chemicon International (Chemicon, Temecula, CA); HLA-DR, CD80, CD86, and CD1a-BD Biosciences (Heidelberg, Germany); S100 [28]-AbD SeroTec (Oxford, UK). Antibodies against Phycoerythrin- (PE-) conjugated donkey anti-mouse, anti-rabbit, and anti-goat $F(ab')_2$ were from Dianova (Hamburg, Germany). The catalytic inhibitor of plasmin, D-Val-Phe-Lys chloromethyl ketone (VPLCK), the kinase inhibitors SB203580, U0126, Akt inhibitor VIII, and controls to the inhibitors SB202474 and U0124 were from Calbiochem (San Diego, CA). GM-CSF was from Berlex (Bayer HealthCare). Human recombinant IL-4, proteome profiler array, and CCL20 ELISA were from R&D Systems. Endotoxic lipopolysaccharide (LPS; *Escherichia coli* serotype 055 : B5) and Histopaque 1077 were from Sigma (St. Louis, MO). Purified human plasmin (lot no. 2008-01L) was from Athens Research & Technology (Athens, GA). The plasmin lot used in this study contained no detectable LPS contamination as measured by the Limulus amoebocyte lysate assay (Sigma, sensitivity 0.05–0.1 EU/mL). The plasmin substrate S-2251 (H-D-valyl-leucyl-L-lysine-*P*-nitroanilide dihydrochloride) was supplied by Diapharma Group Inc. (Columbus, OH). Catalytically inactivated plasmin (VPLCK plasmin) was prepared by incubation of 4 mg/mL human plasmin with 200 μM VPLCK for 30 min at 37°C. Aliquots of the mixture were used to assure the complete loss of any residual proteolytic activity of plasmin using the chromogenic substrate S-2251. VPLCK was separated from VPLCK plasmin by NAP-5 Sephadex G25 chromatography (GE Healthcare), and the concentration of VPLCK plasmin was determined by the BCA protein assay (Pierce). The NF-κB/p65 transcription factor ELISA was from Active Motif (Carlsbad, CA).

2.2. Methods

2.2.1. Immunohistochemical Staining. Sections of surgical specimens from human abdominal aorta from 3 patients were stained with antibodies recognizing plasmin, CCL20, or the DC marker S100, which is exclusively expressed by dendritic cells in the arterial wall [28, 29]. For immunohistochemical double staining, HRP- and AP-conjugated secondary antibodies were visualized by DAB, Fast Red, or AEC substrates (PicTure kit, Invitrogen). The images were digitally recorded with an Axiophot microscope and a Sony MC-3249 CCD camera using Visupac 22.1 software (Carl Zeiss, Göttingen, Germany) [17, 30]. The study was approved by the University Ethics Review Board (approval reference number 114/10) and complied with the principles of the Declaration of Helsinki.

2.2.2. Cell Preparation and Differentiation. Immature dendritic cells were differentiated from human monocytes obtained from buffy coats with 1000 U/mL GM-CSF and 25 ng/mL IL-4 for 6 days in RPMI 1640 containing 10% FCS [30]. The differentiation was confirmed by flow cytometric analysis of HLA-DR, CD80, CD86, and CD1a using a FACScan (BD Biosciences, Franklin Lakes, NJ). Dendritic cells were used on day 6 of differentiation. The cells (1×10^6 cells/mL) were kept for 12 h in AIM-V medium (Invitrogen, Carlsbad, CA) without cytokines and FCS before treatment with plasmin. In some experiments, the dendritic cells were treated with catalytically inactivated plasmin, equivalent to 0.143 CTA U/mL of native plasmin. Catalytically inactivated plasmin (VPLCK plasmin) was prepared as described [31].

2.2.3. Analysis of mRNA Expression. mRNA was isolated from dendritic cells stimulated with plasmin or equivalent amounts of active site-blocked plasmin (VPLCK plasmin) [9, 30] and analyzed by RT-PCR and quantitative real-time PCR. Primer pairs for CCL20 were sense 5′-GACATAGCCCAAGAACAGAAA-3′, antisense 5′-TAATTGGACAAGTCCAGTGAGG-3′ [32]; GAPDH served as control [33]. The identity of the PCR products was confirmed by direct sequencing (Abi Prism 310, Applied

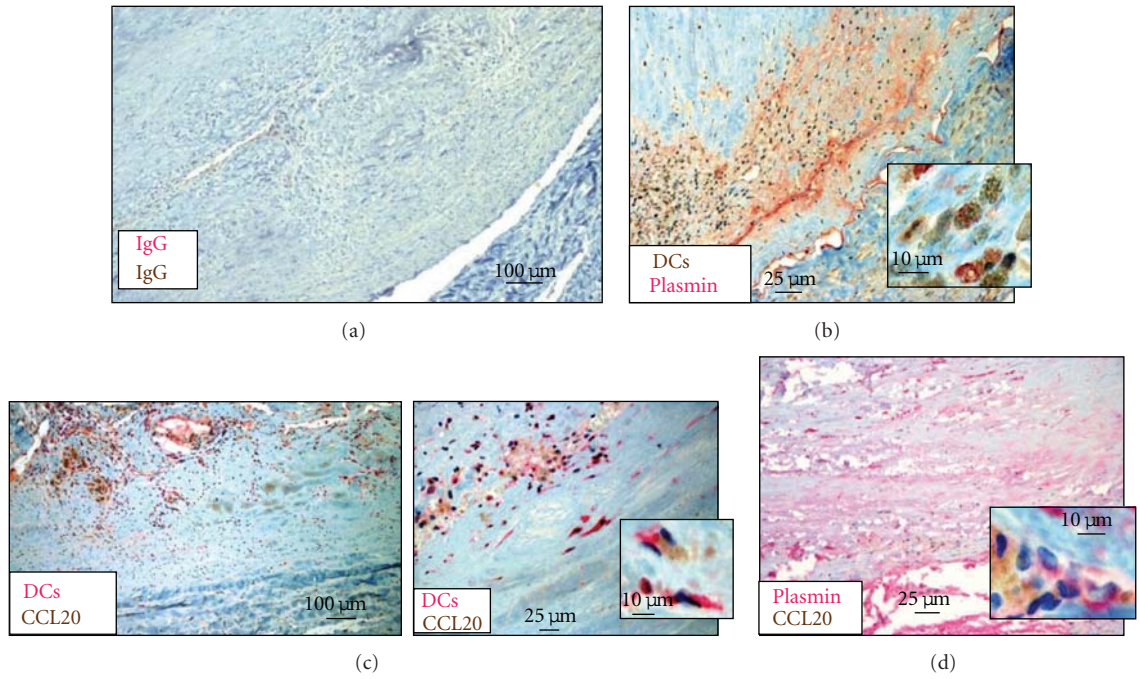

Figure 1: CCL20 is present in human atherosclerotic lesions, where it colocalizes with plasmin and dendritic cells. (a) Negative control. Sections of human atherosclerotic abdominal aorta specimens were stained with control antibodies and visualized with double immunostaining using AP- and HRP-conjugated secondary antibodies and FastRed (pink) and DAB (brown) substrates. Original magnification is ×100. (b) Plasmin colocalizes with dendritic cells. Sections of human atherosclerotic abdominal aorta specimens were stained with antibodies against plasmin (AEC, red) and dendritic cell marker S100 (DAB, brown). Original magnifications ×200 and ×400. (c) CCL20 colocalizes with dendritic cells. Sections of human atherosclerotic abdominal aorta specimens were stained with antibodies against CCL20 (DAB, brown) and dendritic cell marker S100 (FastRed, pink). Original magnifications are ×100, ×200, and ×400. (d) Plasmin colocalizes with CCL20. Sections of human atherosclerotic abdominal aorta specimens were stained with antibodies against plasmin (FastRed, pink) and CCL20 (DAB, brown). Original magnifications are ×200 and ×400.

Biosystems, Foster City, CA). Quantitative PCR was performed using real-time PCR system (7300 Real-Time PCR, Applied Biosystems), and the relative gene expression was determined by normalizing to GAPDH using the $\Delta\Delta C_T$ method.

2.2.4. Analysis of Protein Expression. Protein expression was analyzed by western immunoblotting, proteome profiler array, ELISA, and flow cytometry [30, 34]. Dendritic cells were kept in AIM-V medium for 12 h prior to stimulation. For the analysis of phosphorylated IκBα and p65, whole cell lysates were analyzed by western immunoblotting [9]. CCL20 secretion was measured by ELISA (R&D Systems) in supernatants of dendritic cells stimulated for 24 h with plasmin or the positive control LPS (0.5 μg/mL). For flow cytometric analysis, dendritic cells were pretreated with 1 μg/mL brefeldin A (Sigma) for 4 h prior to analysis to prevent release of CCL20 from the cells. Dendritic cells were fixed with paraformaldehyde, permeabilized with 0.5% saponin, stained with antibodies against CCL20 or control IgG and analyzed by FACScan (BD Biosciences). TNF-α, IL-1α, and IL-1β were analyzed by proteome profiler array (R&D Systems) in the supernatants of dendritic cells stimulated with plasmin (0.143 CTA U/mL) for 24 h.

2.2.5. NF-κB ELISA. Activation of transcription factor NF-κB p65/RelA was quantified in nuclear extracts (5 μg) using TransAM ELISA (Active Motif, Carlsbad, CA) [35]. Nuclear extracts were prepared from dendritic cells treated with plasmin (0.143 CTA U/mL) or LPS (0.5 μg/mL) for 60 min [9]. Results are expressed as fold activation compared to the control samples.

2.2.6. Statistical Analysis. Data shown represent mean ± SEM where applicable. Statistical significances were calculated with the Newman-Keuls test. Differences were considered significant for $P < 0.05$.

3. Results

3.1. Plasmin and Dendritic Cells Colocalize with CCL20 in the Human Atherosclerotic Vessel Wall. Immunohistochemical analysis of sections from atherosclerotic tissue specimens obtained from human abdominal aorta confirmed that plasmin is abundant in the atherosclerotic vessel wall, where it colocalizes with clusters of dendritic cells (Figures 1(a) and 1(b)). In addition, these immunohistochemical studies revealed that plasmin and dendritic cells are in close proximity to CCL20 (Figures 1(a), 1(c), and 1(d)) suggesting that dendritic cells might be activated by locally generated

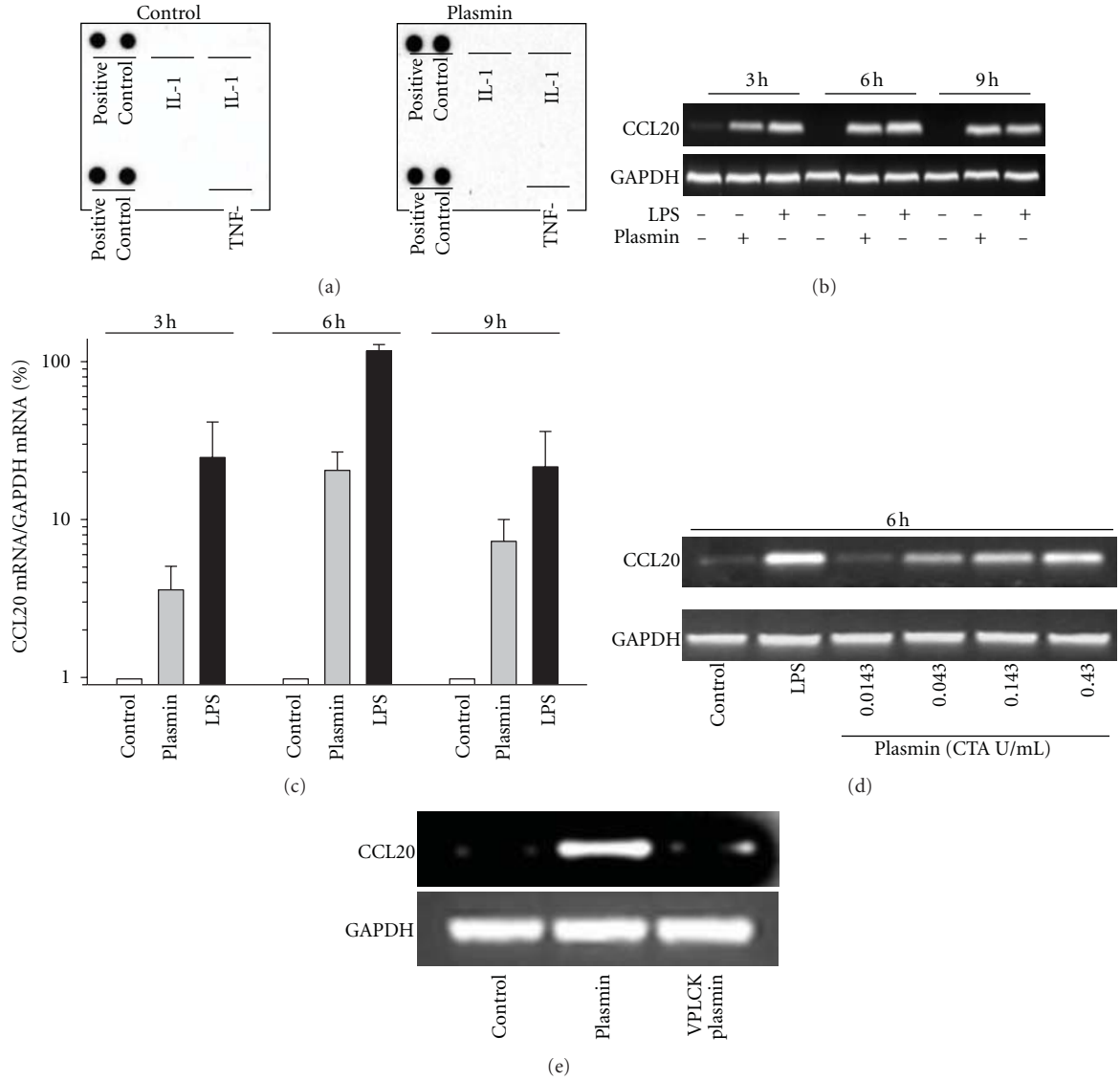

Figure 2: Plasmin induces time- and concentration-dependent expression of CCL20 mRNA in dendritic cells. (a) Plasmin does not induce release of the proinflammatory cytokines TNF-α, IL-1α, and IL-1β. At day 6, dendritic cells were left untreated or stimulated with plasmin (0.143 CTA U/mL) for 24 h. Release of cytokines in the culture media was analyzed with the proteome profiler array. (b) Plasmin induces time-dependent expression of CCL20 mRNA in dendritic cells. Dendritic cells were treated with plasmin (0.143 CTA U/mL) or the positive control LPS (0.5 μg/mL) for the indicated time. mRNA was isolated and subjected to RT-PCR (b) and real time qPCR (c) analysis using CCL20-specific primers; GAPDH served as control. Results are mean ± SEM of 3 experiments. (d) Dendritic cells were treated for 6 h with the indicated concentrations of plasmin, and CCL20 mRNA expression was analyzed by RT-PCR. (e) Proteolytic activity of plasmin is required for the induction of CCL20 mRNA expression. Dendritic cells were stimulated either with plasmin (0.143 CTA U/mL) or the equivalent amount of catalytically inactivated plasmin (VPLCK plasmin) for 6 h. CCL20 mRNA expression was analyzed by RT-PCR. All data are representative of at least 3 independent experiments.

plasmin, and that dendritic cells could serve as a source of CCL20.

3.2. Plasmin Induces CCL20 mRNA Expression in Dendritic Cells. To address the possible generation of cytokines and chemokines by plasmin-activated dendritic cells, we stimulated monocyte-derived dendritic cells with plasmin *in vitro*. Analysis of the supernatants of such cells revealed that in contrast to human monocytes [9] and macrophages [8], dendritic cells do not release proinflammatory cytokines, such as TNF-α, IL-1α and β (Figure 2(a)), or IL-16 (LCF), nor did they release chemokines such as CXCL10 (IP-10), CXCL11 (I-TAC), CXCL12 (SDF-1), CCL1 (I-309), CCL2 (MCP-1), or CCL5 (RANTES). Control dendritic cells produced CXCL8 (IL-8) and small amounts of CXCL1 (GRO), but the release of these chemotactic cytokines remained unaffected by plasmin treatment (data not shown). However, stimulation of dendritic cells with human plasmin (0.143 CTA U/mL) elicited a time-dependent increase of CCL20 mRNA expression as analyzed by RT-PCR

(Figure 2(b)) and real-time qPCR (Figure 2(c)). The maximum of the CCL20 mRNA expression was observed 6 h after stimulation with either plasmin (0.143 CTA U/mL) or the positive control LPS (0.5 μg/mL). The stimulatory effect of plasmin was concentration dependent with a maximum at 0.143–0.43 CTA U/mL (Figure 2(d)).

Previous studies had suggested that the proteolytic activity of plasmin might be required for cell activation [7, 8, 31, 36]. To test whether this is also true for the plasmin-induced activation of human dendritic cells, we generated catalytically inactivated plasmin (VPLCK plasmin) [9]. In contrast to active plasmin, catalytically inactivated plasmin did not trigger any CCL20 induction in dendritic cells (Figure 2(e)) indicating that the plasmin-mediated dendritic cell activation depends on a proteolytic signaling mechanism.

3.3. Plasmin Induces Release of CCL20 in Dendritic Cells. The transcription of CCL20 mRNA by plasmin was followed by a concentration-dependent release of CCL20 with a maximum at 0.143 CTA U/mL (Figure 3(a)). Similar to the mRNA expression levels, higher plasmin concentrations did not further increase the amount of secreted CCL20. The positive control LPS (0.5 μg/mL) induced release of higher amounts of CCL20 (792.9 ± 129.9 pg/mL, $n = 5$) compared to 0.143 CTA U/mL plasmin (132.6 ± 26.1 pg/mL, $P < 0.01$, $n = 8$); control cells released 35.8 ± 10.4 pg/mL CCL20.

Consistently, flow cytometric analysis of the CCL20-expressing cells revealed that about 41% of the dendritic cells treated with plasmin expressed CCL20 within 24 h after treatment (Figure 3(b)). Thus, plasmin triggers production of chemotactic CCL20 by human dendritic cells.

3.4. Plasmin Elicits Activation of Akt, ERK1/2 and p38 MAP Kinases, and NF-κB Signaling. Expression of cytokines and chemokines is regulated primarily at the level of transcription. The promoter region of CCL20 is known to contain an NF-κB consensus sequence indicating that the expression of CCL20 might be regulated by NF-κB [37]. In addition, it has been previously shown that NF-κB can be activated by Akt-dependent IκBα kinase phosphorylation [38, 39], and Akt mediates an IL-17A-induced expression of CCL20 in human airway epithelial cells [40]. Moreover, ERK1/2 and p38 MAP kinases have been implicated in the regulation of the NF-κB activation via MSK1/2 activation and the phosphorylation of p65 [39, 41].

Taking into account that NF-κB is involved in the expression of various proinflammatory genes including chemokines [42], and that plasmin in turn activates NF-κB in monocytes and macrophages [8, 9, 43], we investigated whether plasmin might activate Akt, MAP kinases, and NF-κB in dendritic cells.

Western immunoblot analysis of plasmin-stimulated dendritic cells indicated that plasmin triggers a rapid phosphorylation of Akt, ERK1/2, and p38 MAP kinases (Figure 4(a)). In addition, the phosphorylation of IκBα was increased with a maximum response at 15–30 min after stimulation (Figure 4(b)) indicating activation of NF-κB.

Phosphorylation of IκBα by IκB kinases is a prerequisite for IκB ubiquitination and degradation required for the release of p65 and other NF-κB subunits, and their subsequent nuclear translocation and NF-κB-dependent gene induction [42]. Among different NF-κB subunits, the p50/p65 heterodimer is the most abundant. Only the p65 subunit of the p50/p65 heterodimer contains a domain initiating transcriptional activation essential for the expression of the NF-κB-dependent genes [41]. In addition, p65 overexpression significantly increased the CCL20 mRNA expression in HeLa cells stimulated with TNF-α [44]. Therefore, we analyzed activation of p65 in the nuclear extracts of dendritic cells that had been stimulated for 1 h with either plasmin or the positive control LPS (0.5 μg/mL). Plasmin induced a significant increase in the p65 NF-κB activity (2.00 ± 0.27-fold compared to control, $P < 0.05$) (Figure 4(c)); LPS induced a higher NF-κB activation (8.20 ± 0.59-fold, $P < 0.01$), which is consistent with the higher amounts of CCL20 released by the LPS-stimulated dendritic cells (Figure 3(a)).

3.5. Plasmin Induces CCL20 Expression in Dendritic Cells through Akt- and ERK1/2 MAPK-Dependent NF-κB Activation. To analyze the role of Akt and MAPK in the plasmin-induced CCL20 expression, dendritic cells were pretreated with pharmacological inhibitors of Akt (Akt inhibitor VIII) [45], MEK/ERK1/2 (U0126), p38 (SB203580) [46], and NF-κB (AKβBA) [35, 47–49] before addition of plasmin. AKβBA is an NF-κB inhibitor targeting IκB kinases (IKK) thereby inhibiting NF-κB-dependent signaling in monocytes [48] and tumor cells [35]. In preliminary tests, we ensured that the used concentrations induced specific inhibition of the respective pathways, yet did not impair cell viability. The Akt inhibitor VIII, the MEK/ERK1/2 inhibitor U0126, and the IκB kinase inhibitor AKβBA, but not the p38 MAPK inhibitor SB203580, abolished the plasmin-induced expression of CCL20 mRNA and CCL20 protein release (Figures 5(a) and 5(b)) indicating that plasmin-induced activation of Akt, ERK1/2, and NF-κB is indispensable for the CCL20 expression.

To address whether the plasmin-induced activation of Akt and ERK1/2 would be located upstream of the NF-κB activation, we analyzed protein phosphorylation in the presence of the inhibitors. Inhibition of either Akt or ERK1/2 impaired the plasmin-induced IκBα phosphorylation and the phosphorylation of p65 at Ser276, whereas the phosphorylation of p65 at Ser536 remained unaffected (Figure 6). These data indicate that Akt and ERK1/2 activation is indispensable for the plasmin-induced NF-κB activation and the subsequent expression of CCL20.

4. Discussion

The serine protease plasmin is activated under physiological and pathological conditions. Plasmin is locally generated during tissue damage or thrombus formation, but also in the context of contact activation during inflammatory processes [1, 50–53]. It has been shown that the plasminogen activator uPA and its receptor are present on the surface of

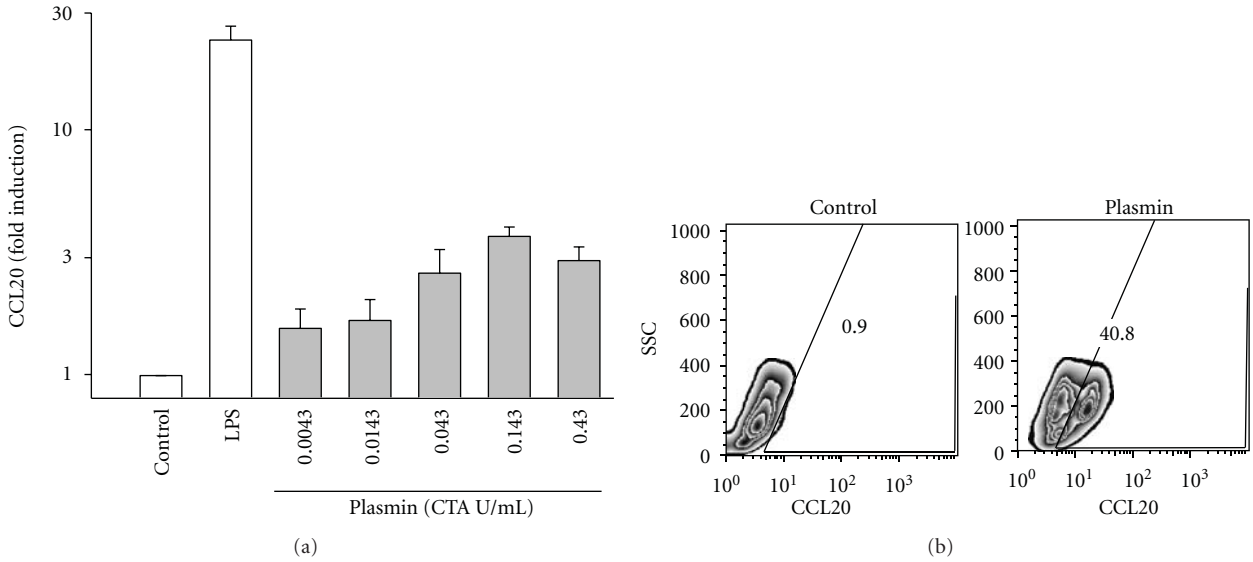

Figure 3: Plasmin elicits CCL20 protein expression in dendritic cells. (a) Plasmin induces a concentration-dependent release of CCL20. Dendritic cells were stimulated with various concentrations of plasmin or LPS (0.5 μg/mL) for 24 h before being analyzed by ELISA. The results are mean ± SEM of 8 experiments, $^*P < 0.05$, $^{**}P < 0.01$ versus control. (b) Flow cytometric analysis of CCL20 expression by dendritic cells. Dendritic cells were either unstimulated or stimulated with plasmin (0.143 CTA U/mL) for 24 h. Brefeldin A was added to the cell culture 4 h before the end of the incubation, and the cells were fixed, permeabilized, stained, and analyzed by flow cytometry. Representative data of 3 independent experiments are shown.

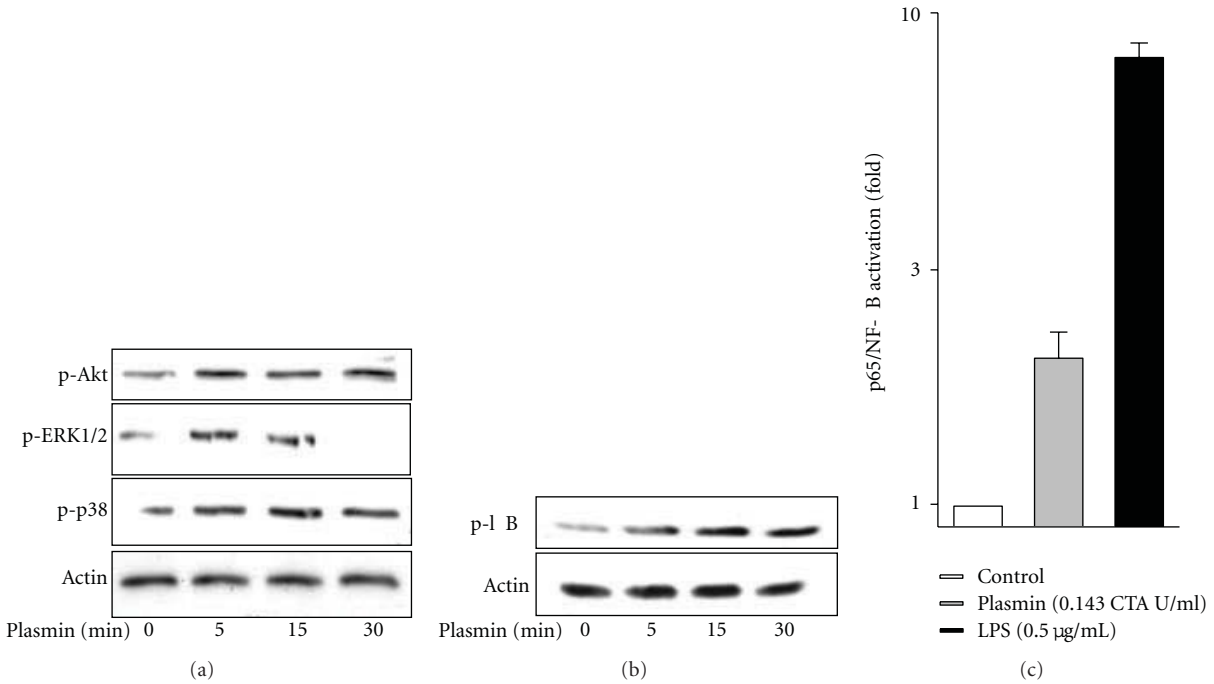

Figure 4: Plasmin activates Akt, MAPK, and NF-κB signaling in dendritic cells. (a) Time-dependent phosphorylation of Akt (Ser473) and MAP kinases in dendritic cells after stimulation with plasmin (0.143 CTA U/mL). Dendritic cells were stimulated with plasmin, and whole cell lysates were analyzed by western immunoblotting; actin served as loading control. Representative data of three experiments are shown. (b) Time-dependent phosphorylation of IκBα (Ser32/Ser36) in dendritic cells after stimulation with plasmin (0.143 CTA U/mL). Representative data of three experiments are shown. (c) Activation of p65 NF-κB as analyzed with the NF-κB TransAM ELISA. Nuclear extracts were obtained from dendritic cells stimulated with plasmin (0.143 CTA U/mL) or LPS (0.5 μg/mL) for 1 h. The results are mean ± SEM of 3 independent experiments, $^*P < 0.05$, $^{**}P < 0.01$ versus control.

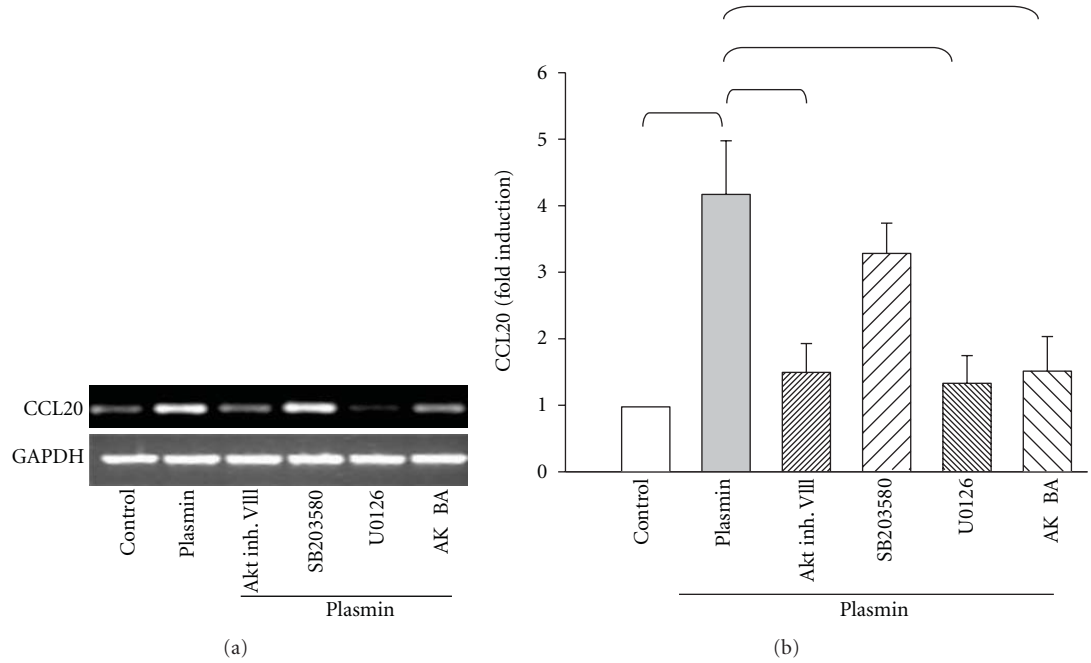

Figure 5: Activation of Akt and ERK1/2 is indispensable for the plasmin-induced CCL20 expression. (a) RT-PCR analysis of CCL20 mRNA expression. Dendritic cells were pretreated with the Akt inhibitor VIII, the MEK inhibitor U0126, the p38 inhibitor SB203580 (each at 1 μM), or the IκB kinase inhibitor AKβBA (10 μM) for 15 min and then stimulated with plasmin (0.143 CTA U/mL) for 6 h. mRNA was isolated and subjected to RT-PCR using CCL20-specific primers; GAPDH served as control. Representative data of 3 independent experiments are shown. (b) Release of CCL20 by plasmin-stimulated dendritic cells. Dendritic cells were treated as in A, but for 24 h, and CCL20 release into the supernatants was analyzed by ELISA. Results are mean ± SEM of 5 experiments, $^{**}P < 0.01$.

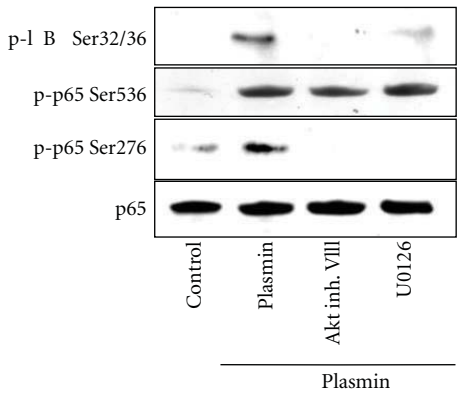

Figure 6: Akt and ERK1/2 activation is indispensable for the plasmin-induced activation of NF-κB. Analysis of IκBα and p65 phosphorylation. Dendritic cells were pretreated with the Akt inhibitor VIII or the MEK/ERK1/2 inhibitor U0126 (each at 1 μM) for 15 min and then stimulated with plasmin (0.143 CTA U/mL) for 40 min. Dendritic cells were collected, lysed, and subjected to western blot analysis with antibodies against the phosphorylated forms of IκBα (Ser32/Ser36) and p65 (Ser276 and Ser536). Staining with p65 antibody-loading control. Results are representative of 3 independent experiments.

immature dendritic cells derived from myeloid progenitors [54]. Plasmin generated at the cell surface is protected from inactivation by its physiological inhibitor α_2-antiplasmin and can, therefore, trigger cell activation [1].

CCL20 is constitutively expressed by lymphoid and nonlymphoid tissue, where it contributes to homeostatic functions and immunity [20]. Thus, mucosa-associated lymphoid tissues and different tumors constitutively express CCL20 [20]. Under inflammatory conditions, CCL20 can be rapidly induced by proinflammatory cytokines, bacterial and viral infections of epithelial cell, keratinocytes, fibroblasts, or endothelial cells [20, 27, 40, 55]. Recent studies have shown that neutrophils produce CCL20 in response to treatment with LPS or TNF-α [56]. Human monocytes express CCL20 when activated with LPS, extracellular nucleotides [20, 27], or under hypoxic conditions [37]. Similarly, dendritic cells can produce CCL20 when stimulated with LPS, CD40L [26], or extracellular nucleotides [27], but not TNF-α [26].

Here, we show for the first time that plasmin elicits CCL20 expression in dendritic cells. The plasmin-induced expression of CCL20 is very rapid and is not dependent on the release of proinflammatory TNF-α. Moreover, we show that plasmin does not induce expression of TNF-α by dendritic cells, and TNF-α does not induce expression of CCL20 in dendritic cells [26]. Similar to the plasmin-induced activation of monocytes and macrophages [8, 9, 31], the proteolytic activity of plasmin is essential for the induction of the CCL20 expression in dendritic cells.

Chemokines are regulated primarily at the level of gene transcription. The CCL20 promoter region contains binding sites for different transcription factors such as activator protein-1 (AP-1) and AP-2, CAAT/enhancer-binding protein

(C-EBP), stimulating protein 1 (SP1), and the epithelium-specific Ets nuclear factor ESE-1 [20]. However, activation of the NF-κB transcription factor family is indispensable for the CCL20 gene expression in several tissues and in response to various agonists [20, 37, 44, 55]. Plasmin induces phosphorylation of IκBα, nuclear translocation, and phosphorylation of p65 at Ser276 and Ser536, as well as binding of activated p65 to the NF-κB consensus sequence. All those events concur with NF-κB activation induced in dendritic cells by plasmin. Consistently, using an NF-κB inhibitor, we demonstrated that the NF-κB pathway is indispensable for plasmin-induced CCL20 expression in dendritic cells.

Akt and MAPK pathways have been shown to be involved in the plasmin-induced gene expression in monocytes and macrophages [8, 9]. In this study, we found that inhibitors of Akt and ERK1/2, but not of p38/MAPK, inhibited the plasmin-induced CCL20 mRNA and protein expression. Others also reported that the CCL20 expression might depend on the activation of Akt, ERK1/2, and p38 MAPK. However, the involvement of different pathways in the CCL20 gene expression strongly depends on the cell type and stimulus. Thus, stimulation of intestinal epithelial cells with IL-21 resulted in enhanced phosphorylation of ERK1/2 and p38 and increased synthesis of CCL20, but only inhibition of ERK1/2, but not of p38 MAPK, suppressed the IL-21-induced CCL20 production [57]. On the other hand, when human monocyte-derived dendritic cells were stimulated with nucleotides, the CCL20 expression was NF-κB, ERK1/2, and p38 MAPK dependent. By contrast, the release of CCL20 by LPS-stimulated dendritic cells was NF-κB and p38 dependent, yet ERK1/2 was independent [27]. These data indicate that the expression of CCL20 is differentially regulated in distinct cell types and in response to different activators.

Similar to human airway epithelial cells stimulated with IL-17A [40, 55], in plasmin-stimulated dendritic cells, the CCL20 expression was dependent on NF-κB, Akt, and ERK1/2, but not on p38 MAPK activation. Plasmin-induced ERK1/2 signaling might contribute to NF-κB activation via several independent mechanisms. In melanoma cells, constitutive ERK1/2 activation has been shown to increase the IκBα phosphorylation and the NF-κB activity [58]. On the other hand, ERK1/2 could facilitate the engagement of transcriptional cofactors CBP/p300, which may increase the transcriptional activity of NF-κB. Thus, ERK1/2 has been shown to activate nuclear kinases MSK1/2 [39, 41], which are potent activators of CREB, whose activity, in turn, is essential for the recruitment of CBP/p300. Interestingly, the CREB site phosphorylated by MSK1/2 is very similar to the site surrounding Ser276 in the sequence of p65. This led to the finding that MSK1/2 can effectively increase the transcriptional activity of p65 via phosphorylation at Ser276 [59]. ERK1/2-mediated MSK activation might also contribute to enhanced gene expression via histone 3 phosphorylation creating a more accessible chromatin structure [59]. We have observed that the inhibition of ERK1/2 activity inhibited the plasmin-induced phosphorylation of IκBα and the phosphorylation of p65 at Ser276 indicating that plasmin-induced ERK1/2 activation might contribute to the CCL20 induction through increased phosphorylation of both IκBα and p65/Ser276, which would result in increased activation of NF-κB and enhanced recruitment of transcriptional cofactors.

The role of Akt in the plasmin-induced NF-κB activation is more complex. The ability of Akt to regulate NF-κB activity might occur through the phosphorylation of IκB kinase, which in turn phosphorylates IκB and allows the release of NF-κB [38], and/or by stimulating transactivation of the p65 subunit by IκB kinase-dependent phosphorylation of p65 on Ser536 [60, 61]. However, the later process is p38 dependent. Consistent with the fact that plasmin-activated dendritic cells did not utilize the p38 MAPK pathway to induce CCL20, we did not observe any effects of p38 inhibition on p65 phosphorylation. However, the Akt inhibition impaired the plasmin-induced IκBα and p65 Ser276 phosphorylation, indicating the Akt-dependent activation of IKK. We have previously shown that plasmin-induced ERK1/2 activation in dendritic cells is Akt dependent [17]. Therefore, Akt might induce the p65 Ser276 phosphorylation via ERK1/2. The activation pathway triggered in dendritic cells by plasmin is different to the IL-17A-induced CCL20 expression in human airway epithelial cells, which is Akt and NF-κB dependent, although both pathways act independently [40]. The plasmin-induced expression of CCL20 in dendritic cells also differed from that in a transformed T-cell line, where Akt inhibition resulted in reduced phosphorylation of p65 on Ser536, whereas the IκBα phosphorylation remained unaffected [62]. Akt might also positively regulate the NF-κB activity through GSK3β inhibition. GSK3β regulates the phosphorylation and function of certain transcriptional coactivators, such as C/EBP and β-catenin, and some transcriptional repressors [40]. Therefore, it is possible that plasmin-induced PI3K/Akt/GSK3β pathway is involved in the modulation of transcriptional activators and/or repressors, which might contribute to the plasmin-induced expression of CCL20.

In summary, the present study demonstrates that plasmin and dendritic cells colocalize with CCL20 in human atherosclerotic vessels. We also show that plasmin is a potent activator of dendritic cells triggering CCL20 expression by the coordinated activation of Akt, ERK1/2, and NF-κB signaling pathways. Hence, by activating dendritic cells to produce CCL20, locally generated plasmin might control the composition of the cellular infiltrate and modulate inflammatory and immune reactions in atherosclerotic lesions. By contrast, such effects might be rather unlikely during conditions of fibrinolysis, where plasmin in the plasma phase would be spatially separated from inflammatory dendritic cells and rapidly bound to fibrin or quickly inactivated by plasmin inhibitors such as α_2-antiplasmin and α_2-macroglobulin [1].

Acknowledgments

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Review Article

Cell Surface Remodeling by Plasmin: A New Function for an Old Enzyme

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Plasmin, one of the most potent and reactive serine proteases, is involved in various physiological processes, including embryo development, thrombolysis, wound healing and cancer progression. The proteolytic activity of plasmin is tightly regulated through activation of its precursor, plasminogen, only at specific times and in defined locales as well as through inhibition of active plasmin by its abundant natural inhibitors. By exploiting the plasminogen activating system and overexpressing distinct components of the plasminogen activation cascade, such as pro-uPA, uPAR and plasminogen receptors, malignant cells can enhance the generation of plasmin which in turn, modifies the tumor microenvironment to sustain cancer progression. While plasmin-mediated degradation and modification of extracellular matrix proteins, release of growth factors and cytokines from the stroma as well as activation of several matrix metalloproteinase zymogens, all have been a focus of cancer research studies for decades, the ability of plasmin to cleave transmembrane molecules and thereby to generate functionally important cleaved products which induce outside-in signal transduction, has just begun to receive sufficient attention. Herein, we highlight this relatively understudied, but important function of the plasmin enzyme as it is generated *de novo* at the interface between cross-talking cancer and host cells.

1. Introduction

The plasminogen activation (PA) system plays an important role in various pathophysiological processes, including vascular and tissue remodeling, tumor development, and cancer progression [1–7]. In the cancer arena, which will be in the spotlight of this paper, some of the distinct molecular components of the PA system received much attention because of their rate-limiting role in plasmin generation or because their overall expression was linked to progression of certain types of cancers and patient outcome (see some original publications [8–15] and summarizing reviews [16–18]). Hence, the main focus of studies within the PA/plasmin system during the last two decades was placed on the plasminogen activators (tPA and uPA), their inhibitors (PAI-1 and PAI-2), the uPA receptor (uPAR), and various plasminogen receptors, whereas the investigation of specific effects of the actual, cleavage-executing enzyme, plasmin, appeared to be somewhat abandoned by mainstream cancer research. Moreover, a tendency has been established to ascribe direct plasmin-mediated functions, such as cleavage of certain matrix proteins or activation of specific latent growth factors, to the activity of uPA [19, 20]. Conversely, plasmin involvement *in vivo* is generally implied in the outcomes of uPA functionality without conclusive evidence of *de novo* generated plasmin or measuring or inhibiting its enzymatic activity. In addition, being a potent proteolytic enzyme, plasmin was shown early to cleave and degrade *in vitro* a broad range of proteins, frequently in a test tube or assay plate. These early findings may have hindered the later investigations of precise plasmin targets and functions in a live animal, especially within the context of the tumor microenvironment, for which the list of nonfibrin proteins proven to be actual *in vivo* plasmin substrates is rather limited. In this paper

we will concentrate on the evidence directly linking *de novo* generated activity of plasmin and its catalytic manifestations in cancer-related *in vivo* settings.

2. Soluble and Cell Surface Systems for Active uPA and Plasmin

Plasminogen is converted to plasmin *via* cleavage of the Arg561-Val562 peptide bond [21] by either tissue-type plasminogen activator (tPA) or urokinase-type plasminogen activator (uPA). The cleavage results in generation of the N-terminal α chain, containing five kringle domains with lysine-binding sites, and the β chain, containing the catalytic triad of His603, Asp646, and Ser741 [22]. Produced by the liver, plasminogen circulates at relatively high concentrations (approximately 2 μM), making it an abundant zymogen in plasma and also in interstitial fluids, where its concentration can be increased upon inflammation or injury through exudation from the vascular system [23]. Therefore, plasminogen activation by its specific activators is one of the ways to efficiently regulate plasmin functions. In the blood stream, plasminogen is activated mainly by tPA, generating the enzyme responsible for lysis of fibrin clots. In wounded tissues undergoing repair or in the tumor microenvironment undergoing constant remodeling, plasmin is generated through uPA-mediated activation [1]. The enhanced expression of single-chain pro-uPA by cancer cells and the ability of malignant tumor cells to activate pro-uPA into the two-chain, catalytically active uPA [24, 25], as well as regulation of uPA activity by the specific inhibitors, PAI-1 and PAI-2 [26], are all critical factors in plasmin generation and consequently, regulation of plasmin-mediated cell functions. A unique mechanism for plasminogen activation involves microparticles which are produced by endothelial cells under stress conditions and provide abundant surface for uPAR/uPA-mediated plasmin generation [27].

Since the major components of the uPA/plasmin systems are secreted proteins, activation of plasminogen by uPA can occur extracellularly, albeit in close proximity to the uPA-expressing cancer or endothelial cell. Pro-uPA activation by trace plasmin, constituting a distinct feedback mechanism leading to further amplified plasmin generation, also can be achieved directly on tumor cell surfaces through the binding of pro-uPA to its specific receptor, uPAR. Our findings with the highly disseminating variant of human prostate cancer cells, PC-hi/diss, indicate that most of the *in vitro* generated two-chain uPA quickly dissociates from the cell surface and accumulates in the conditioned medium, but is undetectable in the cell lysates (Casar, unpublished observations). Therefore, only a fraction of plasmin activity could be demonstrated being directly generated by the cell surface-bound uPA [28]. It is possible that uPAR and some plasminogen receptors initiate generation of uPA and plasmin at the cell surface, but do not retain the respective activated enzymes for a long time. Supporting this notion, it has been demonstrated that plasmin and plasminogen bind to distinct sites on one of the plasminogen receptors, S100A10 [29]. This finding implies that proteolytic conversion of plasminogen would result in plasmin translocation *in cis* within the same plasminogen receptor molecule or, more likely, *in trans* between two receptor molecules. Although binding of plasmin to plasminogen receptors and uPA to uPAR is viewed as a mechanism for sequestration or escape of active enzymes from their natural inhibitors, respectively α2-antiplasmin and PAI-1/PAI-2 [30], the release of active uPA and plasmin from the cell surface may actually represent a mechanism allowing for efficient re-usage of the respective zymogen-binding molecules during recurring cycles of enzyme generation.

3. Role of Cell Surface Plasminogen-Binding Receptors in Pericellular Localization of uPA-Generated Plasmin

Whether activated uPA remains bound to cell membrane *via* uPAR or is released but maintained in a pericellular pool, uPA-generated plasmin will be localized to the close vicinity of the tumor cell surface. This scenario is further supported by existence of a number of cell surface plasminogen-binding molecules [31]. These receptors present the bound plasminogen to uPAR-bound uPA or pericellular soluble uPA, and therefore localize any generated plasmin to the cell surface or to immediate proximity of the cell. Besides functioning as plasminogen receptors on tumor cells, plasminogen-binding molecules are also found on other cell types that might reside within or influx into the tumor microenvironment. The interplay between tumor cells and host cells, such as endothelial cells and activated myofibroblasts, can induce various signaling pathways as a result of uPA-mediated conversion of plasminogen bound to its receptors. This tumor-host crosstalk resulting in plasmin generation might play an important role in cancer progression through regulation of cell proliferation, apoptosis, angiogenesis, adhesion, migration, and invasion. In addition, expression of plasminogen receptors by host blood leukocytes might facilitate activation of juxtapositioned inflammatory cells presenting surface-bound plasminogen to the active uPA bound to uPAR on either tumor or endothelial cell. The latter scenario becomes even more relevant when one considers that angiogenic tumor tissues are invariably infiltrated with various types of inflammatory leukocytes [32].

Numerous cell surface molecules have been shown to be capable of binding plasminogen, thereby facilitating its conversion into plasmin. Many of these plasminogen-binding receptors are intracellular molecules having well-known specific roles in the cytoplasm or nucleus. However, being translocated to the plasma membrane, these "unusually" displayed molecules apparently serve for plasminogen trapping. Most of these cell membrane molecules possess C-terminal lysines, which allow them to function as plasminogen receptors *via* direct binding of plasminogen through the lysine-binding sites located in its kringle domains. A glycolytic enzyme enolase-1 (α-enolase) was among the first C-terminal lysine-containing plasminogen receptors identified on the surface of peripheral blood cells [33]. Recently, enolase-1 was shown to regulate, *via* plasminogen-binding

mechanisms, monocyte recruitment to injured lungs and to play a central role in inflammatory lung disease [34].

Another "unusually" localized plasma membrane molecule that possesses a C-terminal lysine is histone H2B, one of the main 5 histone proteins involved in the maintenance of chromatin structure in eukaryotic cells. It originally was shown to serve as a plasminogen receptor on the cell surface of neutrophils and monocytic cells [35], and later its plasminogen-binding capability was implicated in the regulation of macrophage recruitment [36]. Cell surface forms of actin were also identified as plasminogen receptors responsible for stimulation of plasminogen activation and plasmin-mediated processing of prohormones produced by neurosecretory cells [37].

A number of plasminogen-binding receptors belong to the families of calcium-binding proteins localized to the cell membrane as homodimers [38]. Annexin 2 (annexin II) is a member of annexin family, whereas S100A4 and S100A10 belong to the S100 family. In addition to plasminogen, annexin 2 can independently bind tPA, accelerating cell surface conversion of plasminogen to plasmin [39, 40]. Furthermore, two molecules of annexin 2 and two molecules of S100A4 or S100A10 (p11) can generate heterotetramers [29, 41, 42], thereby even further enhancing the catalytic efficiency of plasmin production. The specific functions of these molecules as plasminogen receptors are described in the designated sections of this issue. In relation to cancer biology, the role of annexin2 has been recently reviewed in [38]. Specifically, attenuation or loss of S100A10 expression in tumor cells was shown to result in a significant reduction of plasmin generation, concomitant with a dramatic inhibition of extracellular matrix degradation, invasiveness and metastasis [29, 43]. Annexin-mediated assembly of plasminogen and tPA on monocytes/macrophages contributed to the generation of plasmin activity *in vitro* and thus, facilitated matrix remodeling, invasion of monocytes and their differentiation into macrophages [44]. Plasminogen binding to the annexin 2A and S100A10 complex on human monocytes was also implicated in uPA-mediated generation of plasmin and plasmin activity, which in turn increased MMP-1 synthesis *via* multiple signaling pathways, including ERK1/2, p38 MAKK, cyclooxygenase-2, and PGE_2 [45]. PKC-dependent phosphorylation of annexin 2 was reported to be induced by plasmin generated form plasminogen bound to the annexin A2-S100A10 heterotetramer on the surface of endothelial cells [46]. Recently, the expression of S100A10 in macrophages was shown to be essential for their recruitment to the sites of primary tumor formation in murine Lewis lung carcinoma and T241 fibrosarcoma models [47].

All the aforementioned plasminogen receptors lack signal sequences and transmembrane domains and therefore their cell surface location is supposed to be regulated by yet undetermined pathways [48]. In contrast, the most recently discovered plasminogen receptor, Plg-R_{KT}, not only possesses a C-terminal lysine exposed on the cell surface, but has been predicted to have two transmembrane helix domains and four-amino acid cytoplasmic-loop, assuring its true transmembrane nature [49]. The data have demonstrated that Plg-R_{KT} is highly colocalized with uPAR and also suggested direct interactions with tPA, thereby implicating plasminogen activation through the mechanisms established for other plasminogen receptors. Plg-R_{KT} has been shown to play a critical role in chemotactic migration and Matrigel invasion of macrophages *in vitro* and peritoneal macrophage recruitment in mice [50]. Originally discovered on the surface of monocytic cells, this novel transmembrane plasminogen receptor was found also on neuronal, leukemic, and breast cancer cells. In neuronal cells, this plasminogen receptor stimulates plasminogen activation and modulates catecholamine release and neurosecretory cell functions [51]. The molecular structure of Plg-R_{KT} indicates its translocation to the plasma membrane by one of the known mechanisms established for transmembrane proteins. Therefore, it appears that the transmembrane Plg-R_{KT} is the only member of a redundant family of plasminogen-binding molecules that is "properly" translocated to the cell surface for plasminogen binding. It is also likely that Plg-R_{KT} will be demonstrated to play a major role in plasmin generation *in vivo* and therefore in plasmin-dependent processes involved in tumor cell dissemination.

4. Lung Retention as a Model to Analyze *De Novo* Generation of Plasmin during Tumor-Host Cell Interactions

To discriminate the *in vivo* contribution of individual components of the uPA/plasmin system to plasmin activity, *de novo* generation of which could be quantified and related to survival and colonization potential of tumor cells, we have recently introduced a mouse lung retention model. This model is based on intravenous inoculations of human tumor cells into mice with defined genetic backgrounds, for example, wild-type *versus* uPA, tPA, or plasminogen knock-out mice. Complementing or contrasting the different host backgrounds, inoculated tumor cells can either express or lack specific components of the PA/plasmin system; for example, the tumor cells can be positive or negative for uPAR, or express only single-chain uPA zymogen or both single- and two-chain uPA. Different amounts of plasmin are generated, depending on the presence of distinct components of the uPA/plasmin system provided either by the donor cells or the host microenvironment. Furthermore, the levels of *de novo* generated plasmin positively correlate with the actual numbers of survived tumor cells in the lung tissue, thereby predicting relative levels of long-term colonization by tumor cells with different phenotypes.

Our lung retention model is depicted in Figure 1. Briefly, tumor cells are inoculated into the tail vein of mice. Within 2 hr following cell inoculations, the majority of tumor cells are arrested in the capillary network of the lungs. Because of cell clearance and proapoptotic pressure, only a fraction of injected cells survives in the lungs by 24 hr. The mice are sacrificed at indicated time points by an overdose of anesthetics, the blood is collected into heparin-containing tubes to provide the source of plasma for measuring plasmin activity,

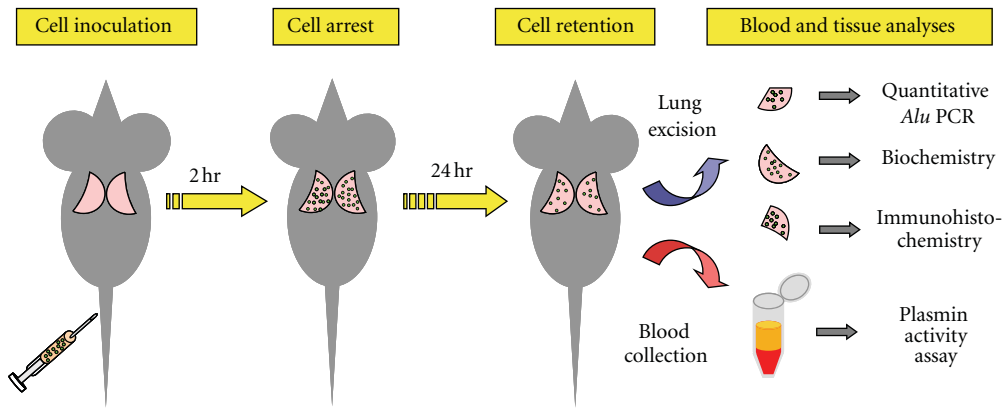

Figure 1: Lung retention model to study *in vivo* tumor cell survival and signal transduction as they relate to generation of plasmin activity.

while the lungs are excised for further quantitative human-specific *Alu* PCR analysis of actual tumor cell numbers. Lung tissue can also be analyzed biochemically for expression of human or host proteins, for example, for activation of signal transduction molecules, and analyzed by immunohistochemistry for tissue localization of tumor cells relative to the lung endothelial, stromal, and inflammatory cells.

The collected cell-free plasma is analyzed for plasmin activity by S2251 peptide cleavage assay (Figure 2(a)). To determine the levels of *de novo* generated plasmin, the background levels of peptide cleavage activity in plasma collected from control mice that did not receive any tumor cells are subtracted from the data obtained for mice inoculated with human tumor cells. By using this model, we have demonstrated that intravenously injected aggressive prostate carcinoma cells, PC-hi/diss, expressing activated two-chain uPA [28], facilitate generation of plasmin in plasminogen-competent mice [52]. As a proof of principle, the lack of the plasmin precursor in plasminogen knock-out mice completely abrogates *de novo* generation of plasmin, whereas supplementing of plasminogen knock-out recipients with plasmin [52] or plasminogen (Figure 2(a)) almost completely restores plasmin generation, indicating that the activity of plasmin detected in the plasma of wild-type mice indeed reflects *newly* generated active enzyme.

In addition to monitoring plasmin generation, the levels of tumor cell vascular arrest and lung retention have been determined by *Alu* PCR of lung tissue collected, respectively, at 2 hr and 24 hrs, after cell inoculations (Figure 2(b)). It is important that the levels of vascular arrest in the lungs determined at 2 hr after cell inoculations are similar between experimental cell variants and not affected by genetic backgrounds of either recipients or donor cells. Meeting this criterion is required to properly correlate the differentials in lung retention at 24 hr to the differences in cell survival rather than cell clearance. Thus, there is no difference in 2 hr vascular arrest of tumor cells regardless of plasminogen competence of recipients (Figure 2(b)). However, the genetic ablation of plasminogen results in diminishment of cell retention levels measured at 24 hr in the lung tissue. Concomitant with the rescue of plasmin production, supplementation of plasminogen-deficient mice with plasmin [52] or plasminogen (Figure 2(b)) rescues the low lung retention of tumor cells, bringing the numbers of survived cells close to those observed in wild-type mice.

Our recent unpublished findings also indicate that low metastatic prostate carcinoma cells, PC-lo/diss, which express little or no active uPA, induce lower levels of newly generated plasmin as compared with their high disseminating counterparts, PC-hi/diss, which express active uPA. Furthermore, we have also demonstrated recently that *de novo* generation of plasmin activity, occurring in response to tumor cell inoculations, depends on the host Plg-R_{KT} as it was substantially diminished by anti Plg-R_{KT} mAb 7H1 (Casar et al., manuscript in preparation). It appears that the monitoring of plasmin generation and tumor cell retention in the lungs at 24 hr, that is, well before any immunological reactions are initiated because of histoincompatibility, provides an ideal model to analyze functionality of distinct molecules of the uPA/plasmin system expressed by human tumor cells *versus* host cells, such as murine vascular endothelium or inflammatory monocytes/macrophages. Finally, combined with the use of immunodeficient mice receiving human tumor cells or congenic hosts receiving murine tumor cells, our 24 hr lung retention model allows to delineate initial contribution of individual components of the plasminogen system and *de novo* generated plasmin to long-term colonization which would be manifested several weeks after tumor cell inoculations.

5. Specific Functions of *In Vivo* Generated Plasmin in the Tumor Microenvironment

The elegant mechanism of uPA-mediated plasmin generation has been demonstrated to operate within the tumor environment. Hence, strong positive correlations exist between the levels of uPA expression in tumor cells and their migration, invasion, colonization, and metastasis, processes that are enhanced at least in part by tumor uPA-generated plasmin [53, 54]. On the other hand, a specific role of host uPA in tumor development has been indicated by the retarded

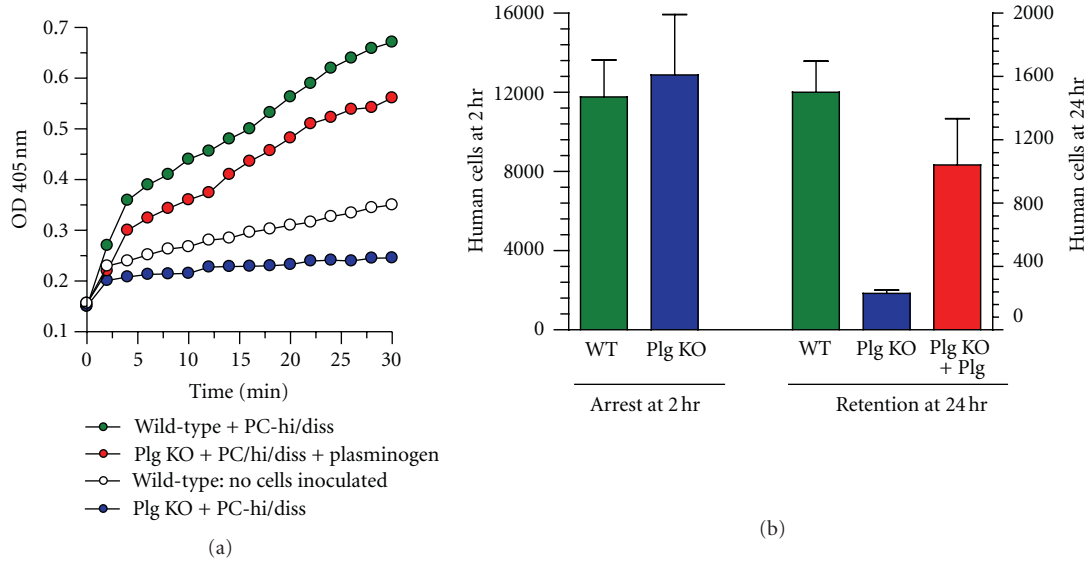

Figure 2: Analysis of plasma plasmin activity, tumor cell vascular arrest, and lung retention in mice after inoculation of human tumor cells. Prostate carcinoma PC-hi/diss cells were injected i.v. into wild-type mice (WT) or knock-out mice (KO) lacking plasminogen (Plg). Control wild-type mice did not receive any tumor cells. A group of Plg knock-out (KO) mice received purified plasminogen 1 hr after cell inoculations. Plasma was collected at 24 hr; lung tissue was collected at 2 hr and 24 hr after cell inoculations.

development and diminished vascularity of primary tumors originating from uPA-competent tumor cells implanted into uPA-deficient mice compared to implants in wild-type recipients [55]. The activity of host uPA also regulates inflammatory cell influx into primary tumors. For example, the levels of macrophage infiltration in mouse prostate tumors were significantly higher in uPA-competent hosts compared with uPA-deficient counterparts [56].

Comparative investigations performed in plasminogen null mice *versus* their plasminogen-expressing littermates allowed to more directly ascertain specific effects of plasmin in different aspects of normal physiology and cancer progression. Functional cooperation between host plasminogen and tumor uPA was shown to promote tumor growth and angiogenesis in the study employing implantation of uPA-competent T241 fibrosarcoma cells into wild-type or plasminogen knock-out mice [57]. In the MMTV-PymT model of mammary gland carcinogenesis, the deficiency in plasminogen or uPA significantly decreased metastatic potential and levels of spontaneous lung metastases, indicating the *in vivo* role of plasmin generation [58, 59]. The dependence of tumor growth on host plasminogen was demonstrated also in the foot pad model with Lewis lung carcinoma and T241 fibrosarcoma [60]. Surprisingly, no quantitative differences were observed in lung metastasis between plasminogen knock-out and control mice if the Lewis lung carcinoma cells were implanted subcutaneously [61], pointing to complexity of plasminogen functions, manifestation of which in cancer can depend on the site of tumor development. Plasminogen-deficient mice also manifest a significantly decreased angiogenic response to vascular endothelial growth factor (VEGF) or basic fibroblast growth factor (bFGF or FGF-2), which clearly indicates the importance of the plasmin system in angiogenesis *in vivo* [62]. The crossing of the blood-brain barrier by tumor cells also appears to depend on plasmin generation since plasminogen-deficient mice demonstrate lower levels of metastasis after intracarotid inoculations of melanoma cells compared to wild-type control [63]. An important physiological process as the recruitment of inflammatory cells also requires the availability of plasminogen, although the outcome of such influx critically depends on the model system. Thus, impaired accumulation of macrophages in the peritoneal cavity was observed in plasminogen knock-out mice [64, 65]. In contrast, plasminogen suppressed the influx of tumor-infiltrating macrophages into fibrosarcoma tumors and inhibited tumor progression and angiogenesis [57].

In the following sections, we will discuss enzymatic functions of plasmin, focusing mainly on those studies, which have implicated plasmin in physiological processes *in vivo* and emphasizing a clear distinction between the suggestive nature of *in vitro* findings and conclusiveness of *in vivo* results demonstrating the proteolytic roles of plasmin in a live animal.

5.1. Plasmin-Mediated Activation of Growth Factors and Cytokines and Their Release from the ECM. By proteolytic processing and activation of nonenzymatic protein precursors and releasing them from the matrix, plasmin has been indirectly implicated in the downstream functional effects of several chemokines, hormones, and growth factors, including transforming growth factor β (TGF-β), fibroblast growth factor-2 (FGF-2), and hepatocyte growth factor (HGF).

TGFβ is a multifunctional cytokine that exerts diverse effects on virtually all cell types and plays a key role during embryo development and tissue homeostasis [66]. The TGF-β isoform, TGF-β1, was one of the first cytokines shown to require proteolytic activation by plasmin to exert its

biological functions [67]. Unlike most propeptides, the propeptide cleaved from the TGF proprotein has a high affinity for TGF-β and assembles intracellularly into a noncovalent complex, composed of homodimers of the propeptide and the active TGF-β molecule. After secretion from the cell, these large latent complexes covalently bind to various ECM molecules, including fibronectin, or to cell surface receptors [68]. The activation mechanism of latent TGF-β1 involves the proteolytic cleavage within the N-terminal glycopeptides, which causes a disruption of tertiary structure and noncovalent bonds and results in the release of active, mature TGF-β1 [69]. Plasmin was initially shown to release TGF-β1 from the pericellular matrix deposited by cultured fibroblasts and fibrosarcoma cells [70]. Pointing to a role in atherosclerosis, plasmin generated through uPA-mediated conversion of exogenous plasminogen induced release of TFG-β protein and TGF-β activity in cultures of macrophages and foam cells [71]. Plasmin also releases TGF-β from activated platelets during *in vitro* dissolution of blood clots [72]. In addition, the ability of cultured alveolar macrophages to generate plasmin suggested its involvement in TGF-β1 activation in the mice after lung injury [73].

During the last 2 decades of investigations, many mechanisms of TGF-β activation have been described *in vitro* and comprehensively summarized in several reviews [68, 74]. However, a gap remains in the demonstration and understanding of TGF-β activation *in vivo*. Since TGF-β activation is the rate-limiting step in TGF-β bioavailability, it is surprising that the functional role of plasmin has not been resolved by using mice genetically devoid of critical members of the PA system. Thus, neither plasminogen-deficient mice nor uPA or tPA knockouts [75–78] exhibit phenotypic similarities with TGF-β null mice [68], casting some doubt on all the *in vitro* data extolling the role of plasmin-mediated activation of TGF-β. Thus far, the most compelling, albeit indirect, result indicating a contribution of plasmin to *in vivo* activation of TGF-β was obtained in the study where the plasmin inhibitor, aprotinin, inhibited generation of TGF-β1 in mice with IL-13-induced lung fibrosis [79].

The 1990 demonstration that plasmin can liberate FGF-2 [80] represents another early example of protease-mediated release of a growth factor from the ECM. FGF-2 is a potent growth factor, which plays an important role in various cell functions, including cell proliferation, and manifests its activity in a number of physiological processes, including tumor angiogenesis [81, 82]. Similar to studies of TGF-β activation, the plasmin-mediated FGF-2 release from pericellular matrices has been shown mainly *in vitro*. Thus, the release of FGF-2 by plasmin and other proteolytic enzymes was initially demonstrated in endothelial cell cultures [83], suggesting a mechanism for induction of angiogenesis. In an *ex vivo* system, the release of FGF-2 coordinated with FGF-2-dependent induction of smooth muscle cell proliferation was shown in organ cultures employing vein segments [84]. Importantly, this study specifically addressed the contribution of plasmin activity to FGF-2 liberation and FGF-2-mediated cell proliferation. Supporting the notion that plasmin activity indeed released matrix-bound FGF-2, the inhibitory effects of the specific plasmin inhibitor, NALME, or the neutralizing anti-uPA antibody on smooth muscle cell proliferation were reversed by exogenous FGF-2 added to inhibitor-containing cultures [84]. FGF-2 was shown also to increase secretion of uPA by cultured endothelial cells [85], suggesting a putative *in vivo* feedback mechanism, whereby uPA-generated plasmin would induce proteolytic degradation of the ECM, release more FGF-2, and thus indirectly mediate FGF-2-dependent endothelial cell migration and angiogenesis.

The first experimental evidence that plasmin can release FGF-2 *in vivo* was provided in the chick embryo model where exogenous human uPA or uPA-overexpressing human tumor cells induced neovascularization which was attenuated by anti-FGF-2 antibody [86]. However, the data from our laboratory demonstrate that in a collagenous matrix environment, the release of FGF-2 is facilitated by MMP-9 delivered by inflammatory neutrophils [87]. Reconciling an apparent discrepancy of plasmin- *versus* MMP-9-mediated release of angiogenic FGF-2, some recent data suggest cooperation between plasmin and MMP-9 during physiologic and tumor-induced angiogenesis. Thus, plasmin incorporated in low nanomolar concentrations into collagen rafts grafted onto the CAM of chick embryos induces a dose-dependent angiogenic response susceptible to aprotinin inhibition (Ewa Zajac, unpublished observations). Exogenous plasmin also induces the angiogenic potential of low metastatic and low angiogenic prostate carcinoma cells, PC-lo/diss, expressing only single-chain nonactive uPA. While the mechanisms of plasmin-induced angiogenesis in this model system are still under investigation, additional experiments have indicated that angiogenesis induced either by exogenously added purified MMP-9 or endogenously supplied neutrophil MMP-9 is highly sensitive to low concentrations of aprotinin. These results demonstrate that the activity of *de novo* generated plasmin somehow overlaps or converges with the angiogenic functions of MMP-9, suggesting close *in vivo* relationships between these two proteolytic systems, and highlighting functional cooperation between PA/plasmin and MMP-9 in tumor angiogenesis. Consistent with this suggestion, simultaneous downregulation of uPA, uPAR, and MMP-9 significantly inhibited angiogenesis induced by glioma cells in an intracranial tumor model in nude mice [88].

A kringle domain-containing glycoprotein, HGF (or scatter factor), is another growth factor that requires activation and release from the ECM to exert its biological activity through the binding to its cell surface receptor, c-met [89]. HGF is produced as a latent, single-chain precursor, which is tightly bound to heparan sulfate proteoglycans. Internal proteolytic cleavage of the HGF precursor generates the active disulfide-linker dimer that can be released from the ECM and bind the c-met on adjacent target cells. Plasmin-dependent mechanism of HGF release was suggested in a lung injury model, where the plasmin inhibitor, tranexamic acid, blocked release of the factor in the bleomycin-treated mice [90]. This suggestion was later validated in the *in vitro* study, where mouse lung fibroblasts were shown to functionally activate and release ECM-bound HGF in a plasminogen-dependent fashion [91].

Plasmin has been also shown to proteolytically process chromogranin A (CgA), the neuronal prohormone stored in catecholamine vesicles [92]. Since plasmin is present in the local environment of chromaffin cells, it was suggested that it can selectively cleave CgA and generate a bioactive fragment inhibiting nicotinic-mediated catecholamine release. Data from plasminogen-deficient mice have also provided the evidence that plasmin is a major *in vivo* protease that regulates the processing of hormones derived from the pro-opiomelanocortin precursor. Thus, a deficiency of plasminogen reduces processing of β-endorphin and α-melanocyte stimulating hormone, thereby causing behavioral abnormalities in response to stress [93]. Recently another neuronal factor, namely brain-derived neurotrophic factor (BDNF), was demonstrated to be processed and activated specifically by plasmin. Being secreted as an unprocessed proform, this neurotrophin undergoes proteolytic cleavage in the pericellular environment by plasmin, which results in generation of an active form of BDNF capable of stimulating neurite outgrowth [94].

Plasmin has also been shown to induce expression of Cyr61 [95], the angiogenic growth-like factor, which acts as an ECM-associated signaling molecule and regulates proliferation and migration of fibroblasts during wound repair [96]. A pathway was suggested for plasmin-mediated promotion of fibroblast proliferation, which involves two independent steps. In the first step, Cyr61 expression is induced *via* plasmin-mediated PAR1 activation, and then, in the second step, plasmin releases Cyr61, originally deposited in the extracellular matrix, making it ready to act on cells [97]. In addition to overall release of Cyr61 from the matrix deposited by fibroblasts, the authors also showed that Cyr61 itself was a plasmin target since only plasmin-cleaved recombinant Cyr61 was functionally competent to induce cell proliferation. Therefore, this model system provides an example where plasmin-mediated release of a growth factor was accompanied by the direct demonstration of its functional activation.

Recently, Kapoor with coauthors demonstrated that plasmin can activate the cytokine function of the extracellular synthetase, TrpRS, which becomes a potent angiostatic factor acting through the vascular endothelial-cadherin (VE-cadherin) receptor and Akt signaling pathway [98]. *In vitro* cleavage of recombinant TrpRS by plasmin resulting in production of low molecular weight fragments suggests that *in vivo* plasmin localized to the surface of endothelial cells, for example, through plasminogen receptors such as S100A10, could process secreted TrpRS and generate angiostatic active fragments. Since TrpRS is secreted by endothelial cells, this scenario can also provide a possible mechanism for negative feedback regulation of tumor angiogenesis.

5.2. Plasmin-Mediated Activation of MMP Zymogens. Indirectly, through its ability to proteolytically activate *in vitro* many matrix-remodeling proteases, plasmin has been implicated in various aspects of tissue remodeling, including tumor angiogenesis and cancer progression [2, 99, 100]. In cell-free systems and cell cultures, plasmin was shown to activate zymogens of many matrix metalloproteinases (MMPs), including MMP-1 [101–103], MMP-2 [104, 105], MMP-3 [106], MMP-9 [104, 107], MMP-13 [108], and MMP-14 [109]. Further solidifying the notion that plasmin has such a broad-range substrate specificity that renders it a nonspecific proMMP activator, cultures of macrophages isolated from uPA deficient mice and supplemented with purified plasminogen clearly demonstrated accumulation of activated forms for MMP-3, MMP-9, MMP-12, and MMP-13 in the conditioned medium [110].

Activation of proMMP-1, the zymogen of a potent interstitial collagenase, represents a well-documented case for plasmin functioning as an MMP-activating enzyme. In cultures of dermal fibroblasts and keratinocytes, producing proMMP-1, plasmin was shown to induce rapid activation of the proenzyme which occurred through a uPA pathway and was facilitated by exogenously added plasminogen [111]. Strict dependency of proMMP-1 activation on plasmin activity, generated on cell surfaces *via* plasminogen conversion, was demonstrated in alveolar epithelial cell cultures [112]. Plasmin-activated MMP-1 then can perform specific collagenase functions manifested in collagen matrix modifications, for example, contraction of collagen matrices [113].

In HT-1080 fibrosarcoma cell cultures, plasmin was shown to activate two gelatinases, MMP-2 and MMP-9, activation of which required plasminogen conversion through the surface bound uPA [104]. Activation of MMP-2 by plasmin was demonstrated to require the association of MMP-2 zymogen with the membrane-type MMP, MMP-14, but surprisingly, the catalytic activity of MMP-14 was not essential [105]. Though exact mechanisms of proMMP-2 and proMMP-9 activation by plasmin were not revealed in these studies, the processing of both zymogens was documented by zymography and the resulting enzymatic activity was confirmed biochemically by cleavage of collagen IV and fibrin, providing a nice example of cooperation between the serine protease system and MMP system in regulation of gelatinase functions.

Gradual activation of proMMP-9 was observed in cultures of thioglycollate-induced peritoneal macrophages in the presence of exogenously added plasminogen and in wild-type mice during thioglycollate-induced peritonitis, but it was impaired in plasminogen-deficient mice [65]. Plasmin-dependent activation of proMMP-9 was also demonstrated in comparative analysis of *ex vivo* cultures of vascular explants from wild-type *versus* plasminogen knock-out mice [114]. The uPA/plasmin system was functionally implicated in cell migration dependent on active MMP-9; however putative activation of proMMP-9 by plasmin was not manifested by pronounced accumulation of any activated species of MMP-9 in this study [115]. An indirect mechanism of MMP-9 activation was demonstrated in breast carcinoma cell cultures, where plasmin did not directly activate the MMP-9 zymogen but first activated the stromelysin MMP-3, which in turn acted as a potent activator of proMMP-9 [106]. Together, these studies indicate that conversion of plasmin and MMP proteolytic systems is complex and may include several intermediate components.

Functional involvement of the uPA/plasmin cascade in activation of MMP-13, collagenase-3, was demonstrated in our investigation of the pro-angiogenic capacity of MMP-13 [116]. In this study we demonstrated that during growth factor-induced angiogenesis, inflammatory monocytes/macrophages deliver proMMP-13 to collagen-enriched matrices, where this zymogen is activated and then functions in uPA/plasmin-dependent manner. *In vitro*, the MMP-13 proenzyme is rapidly and efficiently activated through the uPA/plasminogen/plasmin cascade into a collagenase capable of cleaving native but not the mutant collagenase-resistant collagen. *In vivo*, purified MMP-13 elicited an angiogenic response at nanomolar concentrations, comparable with angiogenic growth factors FGF-2 and VEGF. Angiogenic responses induced either by FGF-2/VEGF or MMP-13 were abrogated by the plasmin inhibitor aprotinin, not only indicating that plasmin-activated MMP-13 functions as an angiogenic factor *in vivo* but once again pointing to a functional cooperation between two distinct matrix-degrading protease systems.

Despite the fact that plasmin cleavage was well documented *in vitro* for several MMPs, including MMP-1, MMP-3, MMP-9, MMP-12, and MMP-13, the majority of studies did not confirm that the putative active MMPs had actual enzymatic activity. In this regard, we showed that under physiological conditions plasmin is not a direct activator of MMP-9 [106], and recently we also demonstrated that although plasmin is capable of processing 92-kDa MMP-9 zymogen *in vitro* to lower-molecular-weight forms, it does not generate enzymatically active MMP-9 capable of cleaving specific peptides or gelatin. Furthermore, *in vivo* studies from other laboratories employing mice deficient in plasminogen or other components of the plasmin-generating system failed to clearly demonstrate that plasmin-mediated activation of distinct MMPs indeed occurred in live animals during matrix remodeling even when the lack of plasmin functionally interfered with MMP-dependent processes. Usually the impairment in MMP-mediated functions has been liberally attributed to the lack of plasmin-mediated activation and therefore, lack of enzymatic activity of the corresponding MMPs. Alternatively, when plasmin-dependent activation of MMPs has been clearly documented biochemically in tissues or body fluids, physiological consequences of the MMP activity missing in plasmin-deficient mice might not have been followed up.

Importance of plasmin in the regulation of MMP-mediated functions *in vivo* has been demonstrated for several MMPs in studies involving mice deficient in plasmin generation. These studies instigated a general conclusion that plasmin is involved in MMP-dependent matrix proteolysis and cell functions requiring matrix remodeling. Thus, mice lacking either plasminogen or both plasminogen activators, uPA and tPA, manifest significantly delayed wound healing kinetics as compared to wild-type mice and show strict dependency of residual wound healing on the activity of unidentified, GM6001-sensitive MMPs [117]. Several MMPs are expressed in the leading-edge keratinocytes in a skin wound, including MMP-2, MMP-3, MMP-9, and MMP-13. However functional overlap of plasmin and MMP systems in wound healing was indicated thus far for MMP-13 in the double knock-out model [118]. In contrast, mice deficient in both MMP-2 and plasminogen manifested no difference in wound healing compared with singular plasminogen knockouts [119], likely indicating that, in contrast to *in vitro* results, plasmin is either not responsible for MMP-2 activation *in vivo* or that MMP-2 activity is dispensable in this model system.

The synergy between a plasminogen cascade and MMP-9 was also indicated in the mouse model of autoimmune disease where delayed blister formation in plasminogen-deficient or uPA/tPA knockouts was restored by applications of the active form of MMP-9 [107]. Importantly, this rescue of a plasminogen-dependent phenotype was not achieved with the MMP-9 zymogen, strongly implicating plasmin in functional activation of MMP-9 *in vivo*.

Functional overlap between the two matrix-degrading proteolytic systems was definitively demonstrated in the hematopoietic system, where stem cells were shown to require plasmin activity to enter cell cycle and initiate multilineage differentiation since genetic deficiency in plasminogen severely impaired the recovery of the hematopoietic system after chemical ablation [120]. Mechanistically, plasmin activity was linked to the activation of MMP-9 and MMP-9-induced release of Kit ligand, the factor that earlier was shown to be obligatory for immobilization of hematopoietic stem/progenitor cells (HSPCs) from bone marrow in response to myelosuppression [121]. The specific mechanisms whereby the plasmin fibrinolytic and MMP pathways control each other and cooperatively regulate hematopoiesis, hematopoietic regeneration, and angiogenesis driven by myeloid cells are described in detail in reviews by Heissig and coauthors [122, 123]. An important clinical implication of functional activation of MMP-9 by plasmin was recently demonstrated by inhibitory effects of the active-site-directed inhibitor of plasmin, YO-2, on MMP-9-dependent growth of T-cell lymphoma and recruitment of tumor-promoting myeloid cells to the sites of tumor development [124].

5.3. Plasmin-Mediated Modifications of Extracellular Matrix Proteins. A broad range of substrates has been indicated by the ability of plasmin to cleave many purified proteins *in vitro* [100]. However, relatively few studies directly linked *de novo* generated plasmin activity with proteolysis of extracellular matrix proteins in tissues or direct cleavage of specific proteins in the matrix deposited by cells in culture.

More than 30 years ago, Liotta and coauthors demonstrated *in vitro* that plasmin could degrade several purified basement membrane proteins, including laminin and fibronectin, and also degrade native laminin from the amnion basement membrane [125]. Later, a specific form of laminin, laminin 5, found in the epithelial basement membrane and deposited in tumor-produced matrix, was shown to be susceptible to tPA-activated plasmin [126]. In turn, binding of tPA and plasminogen to laminin 5 results in a dramatic, 30-fold enhancement of plasminogen activation [127]. In the nervous system, the positive contribution

of laminin-1 proteolysis by plasmin was suggested in a neuritogenesis study, in which plasmin activity generated from the exogenously added plasminogen-enhanced development of neurites and induced laminin-1 cleavage *in vitro* [128]. However, the cleavage of the specific laminin form, laminin $\gamma 1$, by plasmin-mediated proteolysis in the central nervous system *in vivo*, was shown to cause neuronal degeneration *via* activation of caspase-3 [129]. This neuronal degeneration due to laminin degradation was not observed in plasminogen-deficient or tPA-deficient mice and was prevented by the use of a plasmin inhibitor in plasminogen-competent mice, confirming that plasmin was involved in the observed effects *in vivo* and indicating crucial importance of regulating the generation of plasmin and its activity [130–132].

When subjected to limited proteolytic digestion, fibronectin is cleaved only in specific regions, where the protein is believed to be nonfolded and therefore unprotected from protease attack [133]. Most of fibronectin activities have been ascribed to an insoluble form that exists as part of the ECM, where fibronectin matrix assembly is a tightly regulated cell-mediated process. In fibroblast cultures, plasmin-induced degradation of matrix-bound fibronectin was associated with cell apoptosis [134]. Despite the fact that fibronectin can be digested *in vitro* by multiple proteases, including plasmin, the role of proteolytically modified fibronectin *in vivo* remains underexplored, especially in cancer-related systems. Under normal physiological conditions *in vivo*, adipocyte differentiation was shown to require plasmin-mediated degradation of the fibronectin-rich preadipocyte stromal matrix as it was abolished in plasminogen-deficient mice [135].

Plasmin cleavage of fibronectin and laminin *in vivo* was demonstrated in wild-type mice during bone marrow remodeling and recovery of hematopoietic system after 5-fluorouracil treatment [136]. In contrast, plasminogen knock-out mice manifested substantially larger deposits of fibronectin and higher expression of laminin in bone marrow, indicating that loss of plasmin impaired degradation of these ECM proteins. This conclusion was supported by immunoblotting analysis, which demonstrated less low-molecular-weight fragments of fibronectin and laminin in bone marrow plasma samples from plasminogen-deficient mice as compared to wild-type counterparts. However, it was not clarified whether these cleavage products of fibronectin originated from nonsoluble ECM of bone marrow or directly related to soluble plasma fibronectin, one of the most abundant adhesion proteins in the blood [137].

In our CAM model of spontaneous metastasis, inhibition of uPA-generated plasmin with the serine protease inhibitor aprotinin resulted in accumulation of fibronectin at the tumor border and within primary tumors, suggesting that fibronectin undergoes plasmin-mediated proteolysis in nontreated control tumors [28]. Since inhibition of plasmin generation or abrogation of plasmin activity was concomitant with significantly diminished tumor cell escape from primary tumors and reduced stromal invasion, we attempted to directly link cellular uPA-generated plasmin to plasmin-executed fibronectin proteolysis to enhanced migration of tumor cells on the plasmin-modified fibronectin. Thus, when presented with fibronectin cleaved by plasmin, tumor cells increased their migration by 2.5-fold over the levels induced by nontreated fibronectin. Combined with the demonstration that escape from primary tumors and stromal invasion of active uPA-expressing prostate cancer cells were inhibited by function-blocking antibody against $\alpha 5$ integrin, these findings suggested a motility-involving mechanism, whereby tumor cell uPA-generated plasmin cleaves tumor-associated fibronectin and enhances $\alpha 5\beta 1$ integrin-mediated cell motility of tumor cells *in vivo* [28].

A negative feedback mechanism for abrogation of plasmin generation was proposed whereby the ECM protein vitronectin was shown to be specifically cleaved by plasmin *in vitro,* significantly reducing the ability of the cleaved vitronectin to bind PAI-1, thus making PAI-1 available to inhibit plasmin-generating uPA [138]. In addition, *in vitro* cleavage of vitronectin by plasmin can diminish uPAR-dependent cell adhesion and therefore provide a mechanism for regulation of cell motility [139]. Noteworthy, neither of these early demonstrations of vitronectin cleavage by plasmin was followed up to demonstrate that such cleavage occurs and has a functional importance *in vivo*.

Tenascin C represents another matrix protein that was shown to be cleaved by plasmin *in vitro*, converting it from a non-adhesive to adhesive substrate for T lymphocytes [140]. In tumors, tenascin C is produced by both tumor-associated fibroblasts, cancer cells, and cancer stem-like cells and interacts with various membrane receptors and ECM proteins to promote metastatic colonization [141]. Furthermore, stromal tenascin C was shown to be produced by cells expressing the plasminogen receptor S100A4 [141], suggesting that these cells can also facilitate plasmin production at the sites of metastatic colonization. Recently, tenascin C has also been found in stem cell niches, where along with other matrix components it supports the survival of breast cancer cells responsible for lung metastases [142]. Therefore, plasmin-mediated cleavage of tenascin C inducing its adhesive functions may represent a putative proteolytic mechanism for regulation of the biochemical and functional properties of this important matrix protein in stem cell niches.

Recently, osteopontin has been shown to be a novel ECM substrate for plasmin [143]. Osteopontin, a secreted non-collagenous, sialic-acid-rich, chemokine-like protein, plays a crucial role in determining the oncogenic potential of various cancers. Osteopontin has a protease-hypersensitive site that separates the integrin- and CD44-binding domains. Osteopontin has been previously shown to be cleaved *in vitro* by thrombin and MMPs, which modulate osteopontin functions and enhance integrin-binding properties in cell function assays [144, 145]. Plasmin-mediated cleavage of osteopontin, occurring at multiple sites near integrin-binding motifs, also results in increase of cell adhesion mediated by $\alpha v\beta 3$ or $\alpha 5\beta 1$ integrins [143]. Therefore, plasmin can be a potent regulator of osteopontin functions at the sites of primary tumor development as well as at the secondary sites, for example, in metastatic niches, where osteopontin might be an important constituent. Noteworthy,

osteopontin is a key component of hematopoietic niches, where it regulates physical location and proliferation of hematopoietic stem cells [146]. It might also be noted that plasmin cleavage of osteopontin enchancing integrin-mediated adhesion contrasts plasmin cleavage of fibronectin enhancing integrin-mediated cell migration [28].

In conclusion of this section, it is worth to mention that the notion that plasmin executes cleavage of a large number of distinct ECM proteins *in vivo* in live animals appears not to be well substantiated by rigorous experimental evidence. Studies performed in plasminogen-deficient mice or mice lacking uPA and/or tPA have provided such evidence thus far for laminin and fibronectin, conclusively demonstrating the biological significance of plasmin in physiological processes critically dependent on limited proteolysis of these ECM molecules. The recent generation of transgenic mice, expressing two alleles of plasminogen gene with an S743A inactivating mutation in the latent active site, allows for selective elimination of all *in vivo* proteolytic activity of plasmin while preserving all effects associated with the presence of plasminogen and its binding to plasminogen-receptor-like molecules [147], making this newly developed $Plg^{S743A/S743A}$ mouse line especially valuable for cancer progression studies.

6. Cell Surface Remodeling by *In Vivo* Generated Plasmin

The above-reviewed evidence strongly implicates plasmin as an extracellular proteinase that modulates the tumor microenvironment by cleaving proteinaceous substrates, although they often have been suggested by *in vitro* cleavage products rather than conclusively demonstrated as plasmin-cleaved proteins *in vivo* within tumor tissues. However, several studies illuminate another side of plasmin functionality, namely, its ability to cleave specific membrane-anchored receptors and transmembrane proteins and to attenuate or trigger outside-in signaling cascades through distinct signaling partners or through direct activation of signal transduction initiated by the cytoplasmic domain of a membrane-retained cleaved fragment.

6.1. Plasmin Processing and Inactivation of Cell Surface Receptors. Four known protease-activated receptors (PARs) represent a unique class of G-protein-coupled receptors that play critical roles in thrombosis, inflammation, vascular biology and cancer [148, 149]. Among PARs, proteolytic cleavage of PAR1 by thrombin represents the first example of signal transduction through a transmembrane receptor, proteolytically modified by serine proteases [150]. Thrombin-induced activation of PAR1 involves cleavage of the full-length molecule at the R41-S42 site and release of an amino-terminal fragment, resulting in a reduction of molecular weight of the membrane-retained fragment [151]. This cleavage generates a new amino terminus that serves as a tethered ligand domain, which binds to the second loop of the cleaved receptor, resulting in the initiation of signal transduction. Thrombin-induced activation of PARs and PAR signaling has been extensively investigated in studies on hemostasis, blood vessel development, and various cancer-related processes [148, 152–156].

In contrast to thrombin, plasmin-mediated cleavage of PAR1 and its putative implications to cell physiology have not been followed up extensively and precise downstream signaling induced by plasmin-cleaved PAR1 has not been investigated in detail in cancer biology, possibly because plasmin was initially reported to desensitize PAR1 due to the cleavage within the tethered ligand [157]. MALDI-TOF mass spectrometry of fragments generated by plasmin proteolysis of the exodomain of recombinant PARs also predicted that plasmin would inactivate PAR1 [158]. However, by cleaving PAR1 at the R41 thrombin cleavage site, plasmin can generate an activated exodomain without desensitization of Ca^{2+} signaling [157], suggesting that plasmin cleavage of PAR1 can produce dual, activating and attenuating, effects on PAR1 signaling. Furthermore, aprotinin has been demonstrated to inhibit PAR1-specific functions *in vitro* and also during cardiothoracic surgery [159], indicating that plasmin might be involved in PAR1 activation. Consistent with an activating role of plasmin cleavage of PAR1, uPA-mediated conversion of plasminogen into plasmin and plasmin-mediated cleavage of PAR1 have been shown to prevent apoptosis induced in monocytic cells by TNFα or cycloheximdie [160]. Functional involvement of PAR1 in mediating antiapoptotic signals was confirmed by the use of specific anti-PAR1 antibodies. Moreover, apoptosis resistance required the involvement of exogenous plasminogen with an intact protease domain for further proteolytic activity and the presence of unoccupied lysine binding sites within the kringle domains for binding to cell surface plasminogen receptors [160]. Overall, these findings suggest that in specific *in vivo* locales enriched in active uPA, such as the tumor microenvironment, newly generated plasmin activity may regulate cell death through induction of PAR1 survival signaling during crosstalk between tumor cells and peripheral blood monocytes.

Depending on tissue environment, PAR1 activation and PAR1-induced signaling can be elicited not only by thrombin or plasmin, but by a member of a different class of proteases. Thus in certain tumor-stromal microenvironments, MMP-1 has been shown to function as a protease agonist of PAR1, directly cleaving the receptor and generating PAR1-dependent cell signaling and migration [161]. Moreover, MMP-1 activity was shown to derive from fibroblasts and not from the breast cancer cells, strengthening the importance of crosstalk and cooperation of host and tumor cells during cancer progression. Since PAR1 cleavage by thrombin and MMP1 appears to occur at the identical site, namely, between R41 and S42 residues, it is likely that PAR1 cleavage by any of these proteases would induce identical signaling cascades. Thus, both proteases were shown to induce PAR1-dependent Ca^{2+} signaling [148, 161]. However, signaling by PAR1-associated partners has not been compared extensively, leaving a possibility that cleavage by thrombin, plasmin or MMP-1, may induce unique signaling cascades differentially regulating cell functions in a specific, microenvironment-dependent manner. Consistent with this notion, MMP-1-mediated cleavage of PAR1 in breast cancer cells was shown

to activate Akt survival pathway, attenuation of which by respective inhibitors of MMP-1 activity and PAR1 signaling resulted in the induction of cell apoptosis and inhibition of lung metastasis [162]. Therefore, it is plausible to link the redundancy in PAR1 agonists to the possibility that cell locale enriched in proteolytic enzymes belonging to one or another class of proteases would dictate the specific choice of the receptor-activating protease(s).

Investigations of the mechanisms underlying the impairment of hematopoietic recovery in uPA and tPA knock-out mice indicated that, in addition to activation of MMP-9 and induction of MMP-9-mediated release of kit ligand [120], plasmin can be involved in an additional mechanisms of HSC mobilization. Namely, plasmin was shown to proteolytically cleave and functionally inactivate uPAR in HSPCs, enabling them to detach from bone marrow matrix and egress into the circulation [163]. Being a GPI-anchored molecule, uPAR signals through other accessory molecules such as $\alpha 4\beta 1$ integrin but only if uPAR is intact, and therefore plasmin cleavage can functionally inactivate uPAR and abrogate uPAR-mediated signaling. Cleavage of uPAR, indicated in this study by a reduced binding of antibodies recognizing only intact uPAR, represents an example of proteolytic modification of a plasma-membrane-associated molecule, modification of which by plasmin does not directly induce downstream signaling cascades.

6.2. Plasmin Cleavage of Neuronal Receptors. In the central nervous system, plasmin has been shown to cleave *N*-methyl-D-aspartate (NMDA) receptors, increasing their functional responses [164]. NMDA receptors are transmembrane glutamate receptors responsible for neurotransmission and synaptic plasticity. The functional NMDA receptor is a heterotetramer consisting of two NR1 subunits and two NR2 subunits, which bind specific ligands: the coagonist glycine binds to the NR1 subunit, whereas NR2 subunit binds the neurotransmitter glutamate. Both NR subunits have a modular design, and each subunit has an extracellular module, a complex transmembrane domain, and an extensive cytoplasmic domain. The NR2 subunit controls the electrophysiological properties of the NMDA receptor and is represented by four distinct isoforms, which are expressed differentially in various cell types. The intracellular C-terminal domains of individual NR2 isoforms can interact with different sets of signaling messengers. The NMDA receptors serve as Ca^{2+} cation channels that can be blocked by several divalent cations, including Mg^{2+} and Zn^{2+} [165, 166].

By proteolytically cleaving the NR2A subunit amino-terminal domain and removing the Zn^{2+} binding site, plasmin was shown to prevent Zn^{2+}-mediated inhibition of NR2A-containing NDMA receptors. Plasmin cleavage of full-length 180 kDa NR2A subunit occurs at Lys^{317}, producing a 40 kDa N-terminal fragment and leaving a truncated 140 kDa transmembrane C-terminal fragment. Noteworthy, plasmin-mediated relief of the Zn^{2+} inhibition *via* removal of the Zn^{2+} binding site increases responses of NMDA receptors in neurons independently of a plasmin activation of PAR1 [164]. Since NMDA receptors have cytoplasmic domains, harboring residues that can be modified by protein kinases and phosphatases, as well as residues that can interact with various structural, adaptor, and scaffolding proteins, it is possible that plasmin-activated NMDA receptor could induce antiapoptotic pathways to play a critical role in regulation of neuronal cell death.

6.3. Plasmin Cleavage of Cell Surface CDCP1 and Its Functional Activation. Recently, we have presented direct evidence that plasmin cleavage of a single-pass transmembrane molecule can trigger its functional activation through the phosphorylation signaling initiated at the cytoplasmic domain of the membrane-retained fragment [52]. Specifically, we have demonstrated that plasmin executes *in vivo* cleavage of a cancer-related molecule, CUB domain-containing protein 1 (CDCP1), and showed that CDCP1 cleavage leads to outside-in signaling involving activation of Akt and suppression of PARP1-induced cell apoptosis. This signaling cascade ultimately regulates the survival potential of tumor cells in the late stages of the metastatic cascade, namely, during extravasation and early tissue colonization [52].

CDCP1 is a transmembrane glycoprotein highly expressed in a number of malignancies, including cancer of colon, breast, prostate, stomach, lung, kidney, pancreas, and skin [167–171], making CDCP1 an attractive prognostic marker and putative therapeutic target. Functional importance of CDCP1 in cancer is supported by experimental findings demonstrating that CDCP1-positive tumor cells have advantage in metastasis xenograft model systems of lung and gastric cancers [172, 173]. Our unpublished findings in mouse models of human prostate cancer also indicate that high levels of CDCP1 in prostate carcinoma PC-hi/diss cells positively correlate with high levels of secondary organ colonization compared to the cell counterparts where expression of CDCP1 was reduced by RNA interference. Confirming the notion that CDCP1 is a prometastatic molecule, *de novo* expression of CDCP1 in HeLa and HEK cell lines, both completely devoid of CDCP1, dramatically increased their potential to colonize internal organs of immunodeficient mice and chick embryos [52, 174]. Furthermore, our specific anti-CDCP1 mAbs, uniquely generated by a process of subtractive immunization [175], significantly reduced colonization and spontaneous dissemination of prostate carcinoma PC-hi/diss cells naturally expressing CDCP1 in the chick embryo metastasis models [174].

The full-length CDCP1 is 135 kDa molecule containing the extracellular N-terminus with 3 CUB domains, the single-pass transmembrane domain, and the C-terminal domain with several tyrosine residues [175]. Phosphorylation of C-terminal tyrosines by Src family kinases was shown to lead to docking of PKCδ and its Src-dependent phosphorylation [176]. This PKCδ signal transduction facilitates anoikis resistance of human tumor cells [172], indicating that CDCP1 can act as an important cell surface signal transducer and regulator of multiple cell functions. In search

of the triggers of such outside-in signal transduction, we explored the possibility whether cleavage of the CDCP1 molecule, previously demonstrated in cell cultures *in vitro*, would be responsible for initiation of CDCP1 phosphorylation and downstream signaling cascades *in vivo*.

CDCP1 is resistant to proteolytic attacks of MMP-1, MMP-3, and MMP-9. However, several serine proteases, including trypsin, plasmin, and matriptase, can cleave CDCP1 at R368 and K369, generating the 70 kDa membrane-retained fragment [52, 177]. *In vitro* studies confirmed that CDCP1 cleavage was associated with phosphorylation of the 70 kDa fragment and Src and PKCδ recruitment specifically to the cleaved CDCP1 fragment [177]. We further demonstrated that both cleavage by serine proteases and cleavage-induced phosphorylation of cleaved CDCP1 can be completely blocked by the serine protease inhibitor aprotinin or by genetic mutation of the cleavage site. Moreover, we confirmed that our function-blocking anti-CDCP1 mAbs completely prevent proteolytic cleavage of CDCP1, suggesting the *in vivo* mechanism for previously demonstrated inhibitory effects of these antibodies in metastasis [174]. In agreement with our expectations, anti-CDCP1 mAbs completely inhibited CDCP1 cleavage *in vivo*, concomitant with significant inhibition of experimental and spontaneous metastasis of PC-hi/diss cells.

The availability of our high-affinity anti-CDCP1 mAbs allowed us to analyze for the first-time signal transduction elicited *in vivo* by CDCP1 cleavage and establish a signaling cascade required for high levels of tumor cell dissemination. This cascade operating *in vivo* during tumor cell extravasation from the vasculature involves sequential docking of Src, Src-mediated phosphorylation of CDCP1 fragment at Tyr734 and docking of PKCδ phosphorylated in phosphoSrc/phosphoCDCP1-dependent manner. By using the inhibitor of Src activation, dasatinib, we also clarified that both active and nonactive Src can bind to the cleaved CDCP1, but phosphorylation of CDCP1 occurs only if the docked Src has kinase activity.

Specific processing of cell surface CDCP1 by an extracellular serine protease is a prerequisite for CDCP1-induced signaling *in vivo* and CDCP1-mediated survival of cancer cells during metastasis. The *in vivo* cleavage of CDCP1 triggers Src/PKCδ signal transduction, involving downstream activation of Akt and suppression of caspase-induced PARP1 activation. This multistep signaling cascade ultimately increases tumor cell survival and sustain high levels of tumor colonization. Preventing the *in vivo* cleavage of CDCP1 with unique anti-CDCP1 antibodies, serine protease inhibitors or genetic modulation of the cleavage site in the CDCP1 molecule completely abrogated survival signaling and induced PARP1-mediated apoptosis, resulting in a substantial inhibition of metastatic colonization. Finally, by using our lung retention model (Figure 1), we demonstrated complete lack of CDCP1 cleavage in plasminogen knock-out mice, therefore for the first time identifying plasmin as the major serine protease responsible for cleavage of CDCP1 in a live animal. This lack of CDCP1 cleavage in plasminogen-deficient mice was accompanied by reduction in tumor cell survival, which, importantly, could be both rescued by *in vivo* supplied purified plasmin [52] or plasminogen (Figure 2(b)), confirming that plasmin is the crucial serine protease executing cleavage of CDCP1 *in vivo*. Moreover, in this tumor cell *in vivo* setting, we confirmed *de novo* generation of plasmin activity in wild-type mice and lack thereof in plasminogen knock-out recipients unless they were rescued by exogenous plasmin [52] or plasminogen (Figure 2(a)).

Our most recent observations suggest additional mechanisms underscoring the critical role for plasmin-cleaved CDCP1 during spontaneous metastasis. Specifically, we have just shown that plasmin-cleaved CDCP1 engages β1 integrin, resulting in formation of stable complexes which trigger FAK phosphorylation and induce downstream PI3K-Akt signaling. This complexing of β1 integrin with the cleaved CDCP1, followed by phosphorylation-dependent signal transduction, provides a novel mechanism for increased tumor cell motility which is required for cell escape from the primary tumor, invasion of local stroma, and intravasation into tumor-associated vasculature, all processes regulated in part by plasmin-executed cleavage of a transmembrane protein residing at the apex of an outside-in signaling cascade.

Figure 3 summarizes our findings and shows how different components of the uPA/plasmin system, provided either by a tumor cell or a host cell, can contribute to the generation of plasmin, cleavage of tumor cell CDCP1, and signal transduction by the cleaved CDCP1 fragment complexed with β1 integrin. The cell surface of tumor cells expressing uPAR, uPAR-bound pro-uPA, and full-length 135-kDa CDCP1 is depicted on the left. The plasma membrane of host cells represented by vascular cells (endothelium) and inflammatory cells (monocytes/macrophages) is depicted on the right. Although host cells can express uPAR and pro-uPA and various plasminogen-binding molecules, only the transmembrane plasminogen receptor Plg-R_{KT} and Plg-R_{KT}-bound plasminogen (Plg) and plasmin are depicted for clarity of the scheme. Briefly, we propose that tumor cell pro-uPA bound to uPAR is initially activated by trace plasmin, either in solution or bound to one of the plasminogen receptors. The uPAR-associated active uPA then proteolytically activates plasminogen that is bound to Plg-R_{KT} (or other plasminogen-binding molecules). Alternatively, plasminogen can be activated by tPA. Upon activation, receptor-bound plasmin, protected from natural plasmin inhibitors, can cleave transmembrane 135-kDa CDCP1 on the surface of tumor cells and generate plasma membrane-retained 70-kDa fragment and shed 65-kDa fragment. Plasmin can also be released from plasminogen receptor into pericellular milieu and cleave CDCP1 as a soluble enzyme. The C-terminus of cleaved CDCP1 serves as a docking platform for Src. If the Src kinase is functionally active, it phosphorylates C-terminal tyrosine residues of cleaved CDCP1, initiating signal transduction and complex formation with β1 integrin. This signaling cascade involves docking of PKCδ to phosphorylated cleaved CDCP1 and FAK to CDCP1-bound β1 integrin. Both PKCδ and FAK become phosphorylated and functionally active, leading to activation of PI3K and PI3K-mediated activation of Akt. Ultimately, Akt-mediated phosphorylation signaling regulates tumor cell survival, *via*

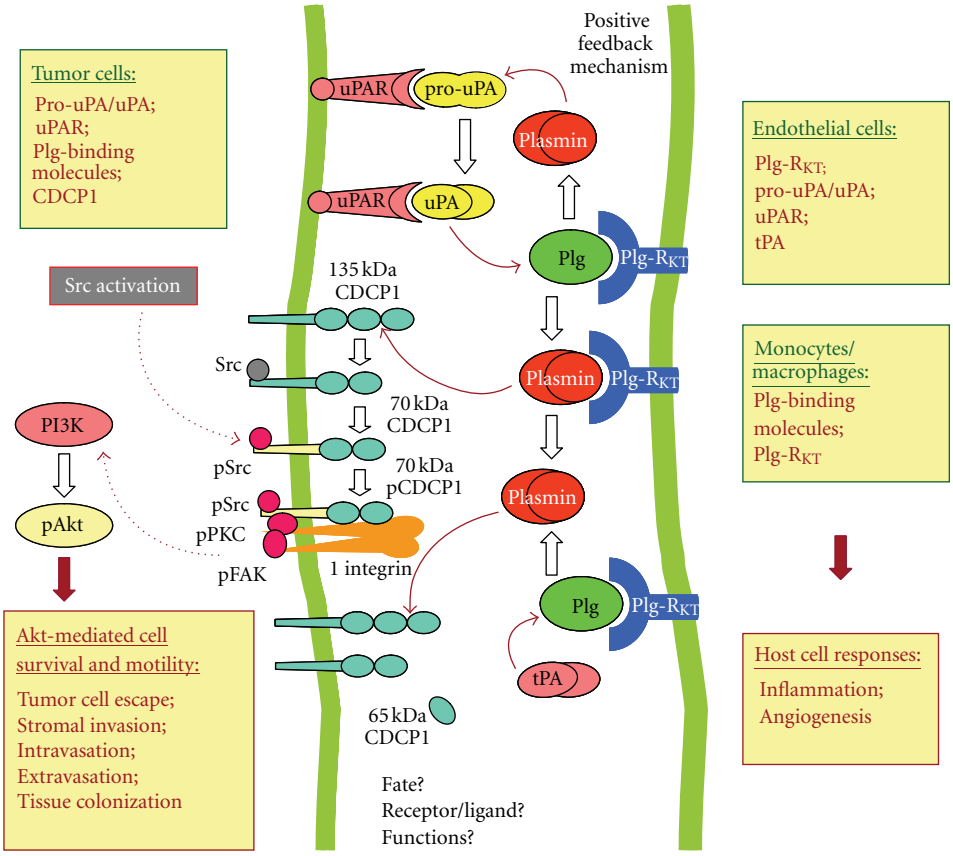

FIGURE 3: Proposed mechanism of proteolytic cleavage of CDCP1 and induction of signal transduction by *de novo* generated plasmin.

suppression of PARP1 cleavage and PARP1-induced apoptosis, and induces cell motility, *via* integrin clustering and activation of FAK. While enhanced integrin-mediated cell motility allows tumor cells to escape from the primary tumor, invade the adjacent stroma, and intravasate into the host vasculature, suppressed apoptosis allows tumor cells to survive during circulation and vascular arrest and efficiently extravasate and colonize secondary tissues to establish metastatic foci. Our findings indicate that *in vivo* blocking of CDCP1 cleavage upstream of CDCP1-induced prosurvival signaling provides a potential mechanism for therapeutic intervention into metastatic disease. Discovery of additional plasma membrane proteins, cleavage of which by plasmin would induce signal transduction that might regulate tumor cell fate, constitutes an exciting area of future cancer research.

6.4. Putative Transmembrane Substrates of Plasmin. In a cell-free *in vitro* system, plasmin and also thrombin were shown to shed the ectodomain of syndecan-4 [178]. Syndecans are single transmembrane domain proteins that through their glycosaminoglycan chains can interact with a wide array of ligands regulating many vital functions of normal and cancerous cells [179]. Syndecan ligands include growth factors such as FGF-2, VEGF, TGF-β, and also ECM molecules such as fibronectin and tenascin C. This combination of ligand diversity and the possibility that serine proteases could be involved in the shedding of syndecans ectodomains makes it tempting to speculate that plasmin-mediated cleavage of syndecans might modulate such cell functions as adherence to the ECM or recognition of specific matrix components. However, in contrast to clear activation-inducing effects of plasmin cleavage demonstrated for CDCP1, it is possible that plasmin-mediated shedding of syndecans *in vivo* would attenuate syndecan functions due to truncation of their extracellular domains and inhibition of intracellular signaling. Therefore, syndecans, including syndecans-4, may represent novel transmembrane targets, proteolytic modification of which by plasmin might induce a negative feed back mechanism.

Another putative transmembrane substrate of plasmin has been suggested by the study of the mechanisms involved in *Streptococcus pneumoniae* spreading [180], which demonstrated that aprotinin-sensitive plasmin activity was responsible for disruption of endothelial monolayers. Endothelial transmigration *in vitro* was facilitated by precoating of bacterial cells with plasminogen, allowing for efficient generation of plasmin, likely through the streptococcal streptokinase, a potent bacterial plasminogen activator. The authors showed that *in vitro* plasmin effectively cleaved recombinant VE-cadherin, the main component of endothelial adherens complexes, and proposed that endothelial monolayers in their model system were disrupted through plasmin-mediated cleavage of intercellular junctions [180]. This finding points

to VE-cadherin as a putative transmembrane molecule, the function of which could be regulated by *de novo* generated plasmin at the sites of tumor cell-host vasculature interactions.

Finally, the study of fibrinolysis-independent role of plasmin during recovery of hematopoiesis after chemical ablation with 5-fluorouracil indicated that plasmin may have yet another plasma membrane-bound target, namely, vascular adhesion molecule 1, VCAM-1 [136]. The hematopoietic recovery in wild-type mice is accompanied by transient increase of soluble VCAM in the bone marrow plasma. However, such an increase did not occur in plasminogen-deficient mice, implicating plasmin in VCAM shedding. Supporting this mechanism, direct treatment of a VCAM-1-positive murine stromal cell line with purified plasmin *in vitro* increased the levels of soluble VCAM-1 accumulated in the conditioned medium [136]. VCAM-1 (CD106) is a type I transmembrane protein which serves as an adhesion molecule, namely, as an endothelial cell ligand for $\alpha 4\beta 1$ or $\alpha 4\beta 7$ integrins expressed on various blood leukocytes. VCAM-1 has been implicated in signal transduction elicited by crosstalk between endothelial cells and leukocytes, making plasmin cleavage of this molecule and its putative plasmin-induced signal transduction an attractive aim for further investigations of tumor cell functions under specific physiological conditions, which facilitate *de novo* generation of plasmin activity, for example, during extravasation of tumor cells from the capillary bed of secondary organs.

7. Plasmin-Mediated Cleavage of Cell Surface Molecules as a Target for Cancer Therapy

Plasmin-mediated proteolytic modification of cell surface molecules, causing their functional activation, points to an aspect of plasmin functionality that has been overlooked or underappreciated by mainstream cancer research but likely deserves more attention and consideration. The latter becomes especially important in view of a preconceived notion that therapeutic inhibition of the proteolytic activity of plasmin, including plasmin-induced activation of certain prometastatic MMPs, could be an efficient means to control metastatic spread in cancer patients undergoing surgical removal of solid tumors. However, the broad use of antiplasmin inhibitors, resulting in the inhibition of plasmin-mediated fibrinolysis, can actually increase formation of microthrombi and therefore inadvertently facilitate thrombi-dependent survival of tumor cells [181]. In agreement with this scenario, the size of thrombi and levels of lung colonization were significantly increased in an experimental metastasis model when melanoma cells were inoculated into mice treated with aprotinin [182]. Furthermore, aprotinin treatment significantly increased spontaneous metastasis to the lung from primary tumors generated by subcutaneously implanted Lewis lung carcinoma cells [183]. The effects of genetic deficiency in plasminogen also indicate that fibrinolytic defects could constitute the main "side effects" caused by a lack of plasmin activity in mice [184]. These experimental studies and also clinical reports about termination of aprotinin trials for patients with lung, esophageal, or breast cancer (see NCT00306137 and NCT00354900 at http://clinicaltrials.gov), all, caution against indiscriminate targeting of plasmin-like serine proteases for cancer therapy.

For a long time, targeting those components of the PA system, which are responsible for activation of plasminogen or pro-uPA or inhibition of uPA activity, were considered as more promising strategies for anticancer therapy than direct inhibition of plasmin-mediated proteolysis. An emerging opinion, however, is that little progress has been made in translating the findings from test tubes, cell cultures, and even animal models into effective therapeutic approaches in patients, resulting in a paucity of clinical trials evaluating modulators of the uPA system [7, 16]. Instead, the inhibition of proteolysis mediated by uPA-activated plasmin could be focused on preventing proteolytic modifications of specific transmembrane or cell-membrane-associated proteins, cleavage of which triggers cancer-promoting signaling in tumor and/or host cells. Such proteolysis-induced signaling can be blocked at the apex of corresponding signal transduction cascades by functionally targeting cleavable molecules, rather than by inhibiting overall activity of cleavage-executing enzymes. In this regard, we have demonstrated that ligation of CDCP1 with specific cleavage-blocking antibodies completely abrogates critical cleavage-dependent signaling, which would otherwise enhance $\beta 1$ integrin-mediated motility, increase tumor cell escape, invasion, and intravasation at the primary tumor site, and help to resist apoptosis and improve survival of successfully intravasated tumor cells [52, 174]. Furthermore, targeting the cleavage-activating step of cell surface-bound pro-uPA with a specific activation-preventing mAb, mAb-112, was shown by us to effectively decrease metastasis of prostate carcinoma cells in several independent models [28, 185–187].

Implemented together, specifically tailored antimetastatic tools targeting distinct transmembrane and plasma membrane-associated molecules, which are activated by limited plasmin cleavage, for example, CDCP1 [52] or pro-uPA [28, 185, 187], can dramatically impede cancer cells from accomplishing different steps of the metastatic cascade and ultimately reduce spontaneous dissemination and tissue colonization. Finally, plasmin-cleaved portions of transmembrane molecules could also serve as biomarkers of a pathological process. Thus, just as biochemical detection of a plasmin-cleaved amino-terminal domain of the NMDA receptor can indicate an injury of the central nervous system [164], the detection of the plasmin-cleaved 65 kDa fragment of CDCP1 may indicate ongoing dissemination of cancer cells.

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Review Article

So Many Plasminogen Receptors: Why?

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Plasminogen and plasmin tether to cell surfaces through ubiquitously expressed and structurally quite dissimilar family of proteins, as well as some nonproteins, that are collectively referred to as plasminogen receptors. Of the more than one dozen plasminogen receptors that have been identified, many have been shown to facilitate plasminogen activation to plasmin and to protect bound plasmin from inactivation by inhibitors. The generation of such localized and sustained protease activity is utilized to facilitate numerous cellular responses, including responses that depend on cellular migration. However, many cells express multiple plasminogen receptors and numerous plasminogen receptors are expressed on many different cell types. Furthermore, several different plasminogen receptors can be used to support the same cellular response, such as inflammatory cell migration. Here, we discuss the perplexing issue: why are there so many different Plg-Rs?

1. Introduction

Plasminogen receptors (Plg-Rs) are a broadly distributed and heterogeneous group of cell surface proteins that share a common feature, the ability to interact with plasminogen (Plg) and plasmin. The list in Table 1, not necessarily all inclusive, identifies 12 different Plg-Rs. Many of these Plg-Rs are expressed by many different cell types, and many are present on the same cell type. Indeed, the number of Plg binding sites on any particular cell type can be extraordinarily high (range from 10^5 to 10^7 Plg binding sites per cell). The similarities among these Plg-Rs are very limited and appear to rest only on their ability to be expressed at cell surfaces where they can display their Plg and Plm binding function. Nevertheless, this binding function allows many different Plg-Rs to orchestrate diverse biological responses including fibrinolysis, inflammation, wound healing, and angiogenesis. The question then arises as to why there are so many Plg-Rs and whether there is a plausible explanation for this extensive functional redundancy? This paper will consider these basic questions. As a forewarning, we do not purport to provide clear answers to these questions but hopefully our speculations will be challenging and stimulating.

2. So Many Plg-Rs: Do Different Plg-Rs Bind Plg Differently?

Almost all of the Plg-Rs listed in Table 1 engage the lysine binding sites (LBS) of Plg and Plm by virtue of a C-terminal lysine or by presenting an internal amino acid residue in a context that mimics a C-terminal lysine. As a consequence of a common mechanism of engagement, Plg-Rs are projected to enhance Plg activation by either urokinase plasminogen activator (uPA) or tissue plasminogen activator (tPA), to enhance the catalytic activity of plasmin and to protect bound plasmin from inactivation by plasmin inhibitors [1–3]. Indeed, several Plg-Rs have been reported to have one or more of these functional attributes [4–6]. Also, with a similar mechanism of binding, the affinities of the various Plg-Rs for Plg should be similar. The context of the LBS binding residue within a Plg-R might be influenced by adjacent amino acids or local conformation and thereby influence the affinity of specific subset of Plg-Rs for Plg. However, even for Plg-Rs that utilize an internal residue rather than a C-terminal lysine to engage Plg, affinities for the ligand appear to be similar ($\sim 1\,\mu$M), [7]. One potential exception to this assertion could be the annexin A2/p11

Table 1: Plg-Rs on various cell types.

Plg-Rs	Cell types	C-terminal lysine	Major cellular localization	Secretory pathways
(1) Annexin A2	Endothelial cells, monocytoid lineage	Absent*	Cytosol and or nucleus	Translocation depends on p11 and phosphorylation; activity of L-type like Ca^{2+} channels and intracellular Ca^{2+}; associates with plasma membrane via phosphatidylserine.
(2) Actin	Endothelial cells, carcinoma, catecholaminergic cells, PC-3, HT1080	Absent	Cytoskeleton	Not known
(3) Amphoterin	Neuronal cells	Absent	Cytoplasmic and extracellular	Not known
(4) $\alpha V\beta_3$	Endothelial cells	Absent	Integral membrane protein	Classical endoplasmic reticulum and Golgi pathway
(5) $\alpha M\beta_2$	Neutrophils, monocytes, macrophages	Absent	Integral membrane protein	Classical endoplasmic reticulum and Golgi pathway
(6) $\alpha IIb\beta_3$	Platelets, RA synovial fibroblasts	Absent	Integral membrane protein	Classical endoplasmic reticulum and Golgi pathway
(7) Cytokeratin 8	Hepatocellular, breast carcinoma	Present	Cytoskeleton	Not known
(8) α-Enolase	Monocytes, neutrophils, carcinoma, lymphoid, myoblast neurons	Present	Cytosol	L-type-like Ca^{2+} channel and intracellular Ca^{2+}
(9) Histone 2B	Neutrophils, monocytoid cells, endothelial cells	Present	Nucleus	L-type-like Ca^{2+} channel and intracellular Ca^{2+}. Associates with plasma membrane via phosphatidylserine and heparin sulfate
(10) P11	Endothelial cells, HT1080 cells	Present	Cytosol and or nucleus	L-type-like Ca^{2+} channel and intracellular Ca^{2+}. Associates with multiple plasma membrane binding partners, including annexin 2
(11) Plg-RKT	Monocytes, macrophages, neuronal cells	Present	Integral membrane protein	Classical endoplasmic reticulum and Golgi pathway
(12) TATA-binding protein-interacting protein	Monocytoid cells	Present	Nucleus	Not known

* requires cleavage to bind Plg [8].

heterotetramer, where the proximity of multiple Plg binding sites within a single molecular species could enhance affinity substantially. To support this possibility or other reports of higher-affinity Plg-Rs, variability in ligand preparations used (e.g., presence of Lys-Plg in Glu-Plg preparation) must be controlled. Furthermore, since ligand availability seems not to be limiting (Plg is present at high concentrations), differences in apparent affinity may have less impact than anticipated.

3. So Many Plg-Rs: Do Different Cell Types Use Different Plg-Rs?

Not all Plg-Rs are expressed on all cell types. As an example of a Plg-R with a restricted cellular distribution, integrin $\alpha M\beta 2$ is a Plg-R [7] and its expression is confined to leukocytes. However, leukocytes express many other Plg-Rs, including annexin A2/p11, which has long been promulgated as the major Plg-R on endothelial cells (ECs). Indeed, inactivation of either the annexin A2 or p11 genes does affect EC-dependent responses, including angiogenesis, tumorogenesis, fibrinolysis, and inflammation [9–12]. However, ECs do express other Plg-Rs. As an illustrative example, histone H2B, a high-abundance Plg-R on monocytoid cells, is also readily detected on the surface of HUVEC (Figure 1). In Figure 1, H2B was detected on the surface of HUVEC by a cell-surface biotinylation approach [13] in which the cells were surface labeled with biotin, lysed, and the biotinylated proteins were isolated on streptavidin beads and then identified by western blotting with specific antibodies (see Figure 1 and its legend for details). H2B was labeled with biotin, whereas p65, a control intracellular protein, was not even though both H2B and p65 were readily detected in the whole cell lysates of HUVEC. H2B associates with the surface of monocytoid cells by binding to phosphatidylserine (PS) [14]. Annexin V, another PS binding protein displaces the H2B from the surface of monocytoid cells [14] and also chases biotin labeled H2B from the surface of HUVEC (see

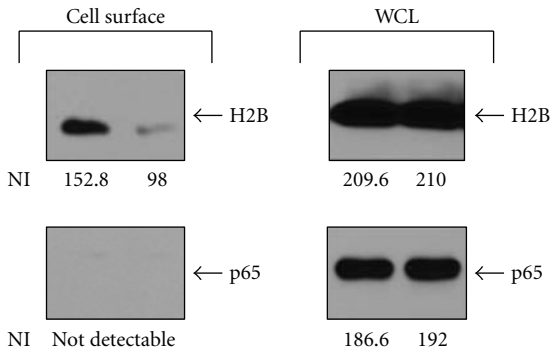

Figure 1: H2B exposure on the surface of endothelial cells. Human umbilical vein endothelial cells (HUVECs) were either untreated or treated with annexin V (250 nM) for 48 hr. Cells were surface labeled with biotin, and the biotinylated proteins were isolated using streptavidin-conjugated beads. H2B and p65 (a transcription factor with a cytosolic and nuclear localization) that were bound and eluted from the streptavidin beads were detected by western blotting with a rabbit anti-H2B or rabbit anti-p65. The absence of biotinylated p65 serves as a control for surface labeling of H2B. Band intensities of the western blots were analyzed using Kodak ID 3.6 software, and net intensity (NI) of each band is indicated below each lane. In each set of two lanes, the right-hand lane is in the presence of annexin V and the left-hand lane in its absence. (WCL: whole cell lysates).

Figure 1). Biotinylation also labeled H2B on the surface of microvascular endothelial cells as well as on large-vessel endothelial cells (not shown). As an independent approach, we confirmed the presence of H2B on the surface of HUVECs by flow cytometry. Also, α-enolase, the first identified Plg-R, has been implicated in the binding of Plg to microparticles released from ECs [15]. Thus, in addition to annexin A2/p11, other Plg-Rs have been detected on endothelial cells. Hence, the notion of the preeminence of a specific Plg-R on a particular cell type does not seem tenable.

The compartmentalization of specific Plg-Rs to select locations on the cell surface could provide a mechanism to distinguish the function of one Plg-R from another. Several integrins serve as Plg-Rs (Table 1) and integrins do localize to the leading edge of migrating cells [16], and uPAR also localizes to the leading edge of migrating cells [17]. Furthermore, annexin 2 has been localized to the leading edge of migrating retinal glial cells and malignant glioma cell [18, 19]. Thus, an advantageous microenvironment may be created in which one Plg-R is particularly proficient in Plg activation. However, recent data have suggested that cell-surface-bound Plg can be efficiently activated or even more efficiently activated by uPA bound to another cell than that on the same cell [20]. The boost in efficiency of Plg activation gained by localization on a single cell may be offset by the restricted diffusion or orientation of the Plg activator on the cell surface. Thus, localization of certain Plg-Rs to a specific microdomain on the cell surface and the functional advantage of such localization remain a possibility. We did note a uniform distribution of several Plg-Rs, as well as bound Plg, on monocytoid cells by confocal microscopy although changes in distribution under stimulated conditions were not tested [13]. A common mechanism dependent on L-type like calcium channels has been implicated in translocation of several Plg-Rs to the surface of monocytoid cells [21], but the mechanisms by which these Plg-R tether to the cell surface are distinct [14]. Hence, Plg-Rs could compartmentalize on the cell surface.

4. So Many Plg-Rs: Are Plg-Rs Differentially Regulated on Cells?

It is well established that Plg binding to cells can be markedly modulated; changes in Plg binding capacity of specific cell types can increase 3- to 20-fold in response to specific stimuli. Cellular events and responses that can induce such changes include oncogenic transformation (breast and adenocarcinoma cancer) [22, 23] differentiation (monocytes, adipocytes) [21, 24], agonist stimulation, (leukocytes, endothelial cells, platelets) [7, 25–27], adhesion (monocytoid cells) [28], and apoptosis (monocytoid cells) [14]. In addition, Plg binding can be enhanced by proteolysis of existing cell surface proteins to generate new C-terminal lysines [29, 30]. This latter mechanism for exposing new Plg-Rs can be triggered by plasmin itself and depends on the availability of uPA on the cell surface [31]. Thus, even though a cell type can express multiple Plg-Rs, a subset of Plg-Rs may be differentially upregulated and utilized to mediate a specific cellular response.

The data in Figure 2 provides an illustrative example of how different Plg-Rs maybe utilized by the same cell in responding to different stimuli. THP-1 monocytoid cells were either stimulated to undergo differentiation using vitamin D3 + IFNγ or apoptosis using camptothecin. Consistent with our prior report [13, 14], the cells respond to these stimuli by markedly upregulating their Plg binding capacity. In association with differentiation, Plg binding increased by 3.3-fold. Of the Plg-Rs analyzed by FACS, enolase, annexin2, p11, and H2B, surface expression increased most markedly for H2B (4.7-fold) in response to differentiation. In response to apoptosis induced by camptothecin, Plg binding increased by 10-fold. While surface localization of H2B did increase significantly (4.6-fold), much more striking was the 20-fold upregulation of p11 in the camptothecin-treated THP-1 cells. This pattern of enhanced p11 expression was also observed in U937 monocytoid cells treated with camptothecin, where 5.8-fold increase of Plg binding was associated with 6.3-fold increase in p11 expression. Of note, these increases in p11 expression on apoptotic cells were not paralleled by substantial increases of the annexinA2 subunit. In the camptothecin-treated THP-1 cells, surface expression of the annexinA2 subunit increased by 2.8-fold and for U397 cells, the increase was 2.3-fold. As explanations for this disproportional upregulation of p11, the subpopulation of annexinA2 molecules that escort p11 to the cell surface may not react with the antibody used in this analysis, or the anti-p11 may selectively penetrate apoptotic cells, which are known to be leaky [32]. A more interesting possibility is that a portion of the p11 that becomes surface expressed is in a free form or is associated with other binding partners.

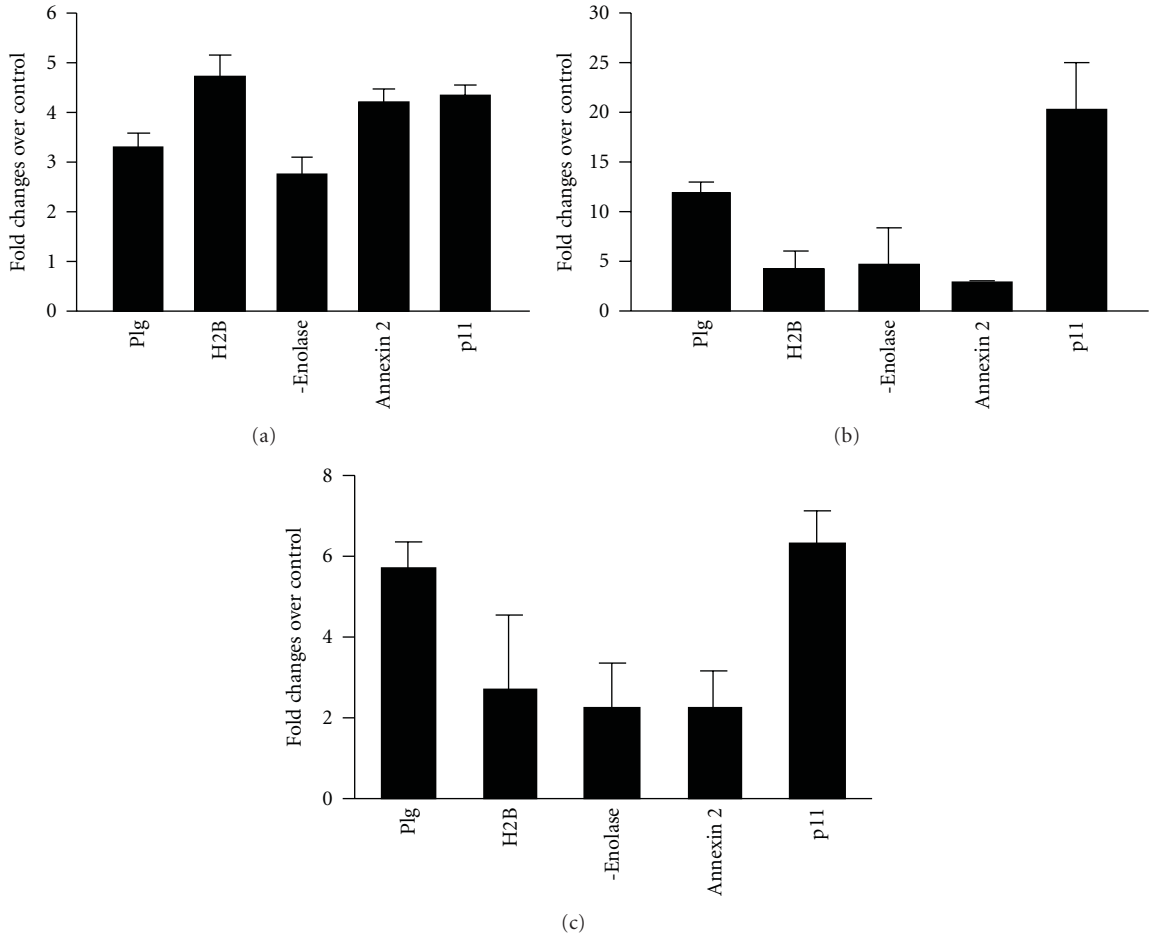

Figure 2: THP-1 (a), (b) and U937 (c) cells were either differentiated with IFNγ + VD3 for 48 h (a) or induced to undergo apoptosis with camptothecin for 24 h (b), (c). Cells are labeled with Alexa-488-Plg or anti-Plg-Rs antibodies against H2B, α-enolase, annexin A2, and p11 followed by Alexa-488-ant-rabbit IgG (c) and analyzed by FACS. Early apoptotic populations are used to analyze the data. Data are means ± SD of two to three independent experiments and presented as the fold change relative to untreated THP-1 or U937 cells.

Besides annexin2, other plasma membrane proteins, NaV1.8 sodium channel, TASK1 potassium channel, TRPV5/TRPV6 channels, and cathepsin B [33] have been shown to interact with p11, could assist in its transport to the cell surface, and may still further extend the repertoire of Plg-Rs expressed by monocytoid cells.

In vivo data also support the proposition that different Plg-Rs mediate the response of the same cell type to different stimuli. In a thioglycollate-induced peritonitis model, an antibody to H2B that blocks Plg binding inhibited macrophage recruitment by ~50% while an antibody to α-enolase that also blocks Plg binding to its target produced less than 25% inhibition of macrophage recruitment [13]. In contrast, in an LPS-induced lung inflammation model, Plg binding to α-enolase overexpressing U937 cells produced a substantial enhancement of macrophage migration [34].

5. So, Why So Many Plg-Rs?

While the utilization of different Plg-Rs to orchestrate different cellular responses is supported by data cited above, blocking of several different Plg-Rs has been shown to markedly suppress what appears to be the same inflammatory response thioglycollate=induced peritonitis. The contribution of H2B (45% [13]), p11 (53% [12]), and Plg-RKT (58% [35]), either with antibodies or gene inactivation, exceeds 100%. Such extensive inhibition becomes even more incomprehensible since macrophage recruitment is decreased by only 65% in $Plg^{-/-}$ mice compared to wild-type littermates [36]. At least four explanations can be considered to explain such observations. First, these various Plg-Rs may exert an effect on macrophage recruitment unrelated to Plg. The effect of blockade of individual Plg-Rs in a $Plg^{-/-}$ background could be used to identify such functions. Second, a threshold of bound Plg must be attained in order for Plg to facilitate cell migration. No single Plg-R may harness sufficient Plg to reach this threshold, and, hence, cooperation among several Plg-Rs is required. Third, while many different Plg-Rs enhance Plg activation, the intracellular signaling responses that they elicit may be distinct. Cellular recruitment is a complex response requiring activation of many different intracellular signaling pathways. Different Plg-Rs may trigger distinct signaling events, and these pathways may need to cooperate to yield efficient migration. Blunting the signaling

response elicited by occupancy of any one Plg-R may lead to suppressed signaling and diminished migration. Fourth, recruitment into the peritoneum is a temporally extended and multi-step response, and different Plg-Rs may come into play at different times and stages during the response. Hence, difference Plg-Rs may be utilized to achieve specific steps in the recruitment cascade.

6. Summary and Concluding Remarks

In this brief discussion, we have raised the question as to why there are so many Plg-Rs. With so many different receptors frequently, with many of them expressed on the same cell type, it is difficult to envision how the cell would prioritize its utilization among these multiple Plg-R. Affinity differences between Plg-Rs for their Plg and plasmin ligands could distinguish one receptor from another, but this can only be tested by direct comparisons among Plg-Rs. Utilization of specific Plg-Rs to mediate tissue specific or cell specific responses can also be envisioned, but such analyses again mandate comparative studies. In fact, in each of the explanations suggested above, to account for the profound role of many different Plg-Rs in what is globally visualized as a single cellular response, macrophage recruitment into the peritoneum, comparative studies are again needed. The goal of such comparative studies is not to prove that one particular Plg-R is *better* than another, but rather to help dissect the ways in which Plg orchestrates cell migration and other cellular responses in vivo.

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Review Article

The Plasminogen Receptor, Plg-R_{KT}, and Macrophage Function

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When plasminogen binds to cells its activation to plasmin is markedly enhanced compared to the reaction in solution. Thus, cells become armed with the broad spectrum proteolytic activity of plasmin. Cell-surface plasmin plays a key role in macrophage recruitment during the inflammatory response. Proteins exposing basic residues on the cell surface promote plasminogen activation on eukaryotic cells. We have used a proteomics approach combining targeted proteolysis with carboxypeptidase B and multidimensional protein identification technology, MudPIT, and a monocyte progenitor cell line to identify a novel transmembrane protein, the plasminogen receptor, Plg-R_{KT}. Plg-R_{KT} exposes a C-terminal lysine on the cell surface in an orientation to bind plasminogen and promote plasminogen activation. Here we review the characteristics of this new protein, with regard to membrane topology, conservation of sequence across species, the role of its C-terminus in plasminogen binding, its function in plasminogen activation, cell migration, and its role in macrophage recruitment in the inflammatory response.

1. Introduction

When plasminogen binds to cells its activation is markedly enhanced, compared to the reaction in the solution phase [1–7]. Active plasmin remains associated with the cell surface where its activity is protected from inhibitors [8, 9]. Localization of plasminogen on cell surfaces is a crucial control point for positive regulation of cell surface plasmin proteolytic activity that facilitates both physiological and pathological processes [10, 11], notably, macrophage recruitment during the inflammatory response [12–15]. Studies in plasminogen deficient mice have demonstrated that plasminogen plays a key role in macrophage recruitment in response to inflammatory stimuli. Plasmin-dependent cell migration is accomplished by direct degradation of extracellular matrix components by plasmin and also by activation of matrix metalloproteinases for further degradation of extracellular matrices [12–14, 16].

Among plasminogen-binding proteins, those exposing C-terminal basic residues on cell surfaces are predominantly responsible for the ability of eukaryotic cells to enhance plasminogen activation because carboxypeptidase B (CpB) treatment abrogates cell-surface-dependent plasminogen activation [7]. Furthermore, plasminogen-dependent macrophage recruitment is mediated by CpB-sensitive plasminogen-binding sites [21].

Several plasminogen-binding proteins with established intracellular functions that are synthesized with C-terminal

lysines are known to associate with the monocytoid cell surface (e.g., α-enolase [22, 23], TIP49a [24], histone H2B, and p11 [25]). Other functional plasminogen binding proteins that are not synthesized with C-terminal basic residues are present on monocytoid cells, including annexin II [26], amphoterin [27], tissue factor [28] and $\alpha_M\beta_2$ [29]. However, no integral membrane plasminogen binding proteins synthesized with C-terminal basic residues had previously been identified. The identification of a receptor with such a structure would constitute a novel mechanism for stimulating plasminogen activation because its induction would endow cells with the ability to bind plasminogen and promote plasminogen activation, without requiring release and re-binding of intracellular proteins or proteolytic cleavage of a membrane protein to reveal C-terminal basic residues.

2. Rationale for the Use of a Proteomics Approach to Identify Integral Membrane Plasminogen Receptor(s) with C-Terminal Basic Residues

Previous characteristics of plasminogen binding proteins and the methods used for their identification may have precluded identification of an integral membrane plasminogen binding protein with a C-terminal basic residue. Previously, the identification of plasminogen receptors has relied on cell surface labeling followed by affinity chromatography on plasminogen-Sepharose columns and N-terminal sequencing of fractions eluted from SDS gels. Thus, many intracellular proteins that are also present on the cell surface were readily identified because protein fractions that bound to plasminogen Sepharose included the labeled, surface-associated protein, as well as nonlabeled and relatively abundant intracellular protein. Thus, a lower abundance integral membrane plasminogen-binding protein might not have been detectable.

Previously, we used a proteomics approach to examine monocytoid cell membranes for the presence of proteins exposing carboxyl terminal lysines on the extracellular side of the cell membrane [30]. We compared plasminogen ligand blots of 2D gels of membrane fractions of intact cells treated with CpB with untreated membranes. We eluted a prominent CpB-sensitive protein from the gels and obtained two peptide sequences using tandem mass spectrometry. Both peptide sequences were contained within TATA-binding protein-interacting protein (TIP49a) [24]. However, TIP49a is a member of the class of cell surface plasminogen-binding proteins synthesized with a C-terminal lysine and also having intracellular functions and is not an integral membrane protein.

The method used to identify TIP49a and other plasminogen receptors required elution of candidate proteins from 2D SDS polyacrylamide gels. However, many membrane proteins do not resolve well on SDS polyacrylamide gels. Therefore, recently we used an isolation method that used column chromatography instead of SDS polyacrylamide gels. We took advantage of the exquisite sensitivity of multidimensional protein identification technology (MudPIT) [31] to search for integral membrane plasminogen receptor(s) exposing a C-terminal basic residue on the cell surface.

3. Discovery of a Regulated Integral Membrane Plasminogen Receptor Exposing a C-Terminal Basic Amino Acid on the Cell Surface

First, to establish a system in which plasminogen receptors could be actively induced, we tested the effect of a differentiation-inducing agonist, macrophage colony stimulating factor (M-CSF) on plasminogen binding to a mouse monocyte progenitor cell line, Hoxa9-ER4. The Hoxa9-ER4 cell line is derived from primary murine bone marrow myeloid precursors immortalized with an estrogen-regulated conditional oncoprotein, HoxA94-ER [32]. The Hoxa9-ER4 line is factor dependent (GM-CSF) and differentiates to monocytes when estrogen is removed from the medium, thereby inactivating the Hoxa9-ER protein. The mature monocytes respond to M-CSF [33]. We could not detect specific plasminogen binding to undifferentiated Hoxa9-ER4 progenitor cells. However, as the cells differentiated along the monocytic pathway in response to M-CSF, specific plasminogen binding to the cells was observed [18].

We used specific proteolysis followed by MudPIT to probe the membrane proteome of differentiated, M-CSF-treated Hoxa9-ER4 cells for the presence of integral membrane plasminogen receptor(s) exposing a C-terminal basic residue on the cell surface, as outlined in Figure 1. First, the Hoxa9-ER4 monocyte progenitor cells were differentiated with M-CSF to induce plasminogen receptor expression [18]. Then intact cells were biotinylated using a biotinylation reagent that reacts with carboxyl groups, rather than basic groups (that would interfere with the plasminogen-binding function of C-terminal basic residues). Because early apoptotic and nonviable/necrotic cells exhibit markedly enhanced plasminogen-binding ability [34–36] we wished to focus on plasminogen receptors on viable cells and, therefore, passed the biotinylated cells over a dead cell removal column to enrich for live cells. The cells were then lysed and membrane fractions prepared and passed over a plasminogen-Sepharose affinity column and specifically eluted with ε-aminocaproic acid (EACA), a lysine analog that blocks the binding of plasminogen to cells [1]. Biotinylated cell surface proteins bound to the avidin column and were digested with trypsin while still on the column. The peptide digest was then subjected to MudPIT.

In MudPIT, the peptide mixtures were first resolved by strong cation exchange liquid chromatography upstream of reversed phase liquid chromatography. The eluting peptides were electrosprayed onto an LTQ ion trap mass spectrometer and full MS spectra were recorded over a 400–1600 m/z range, followed by three tandem mass events. The resulting spectra were searched against a mouse protein database. Only one protein with a predicted transmembrane sequence and

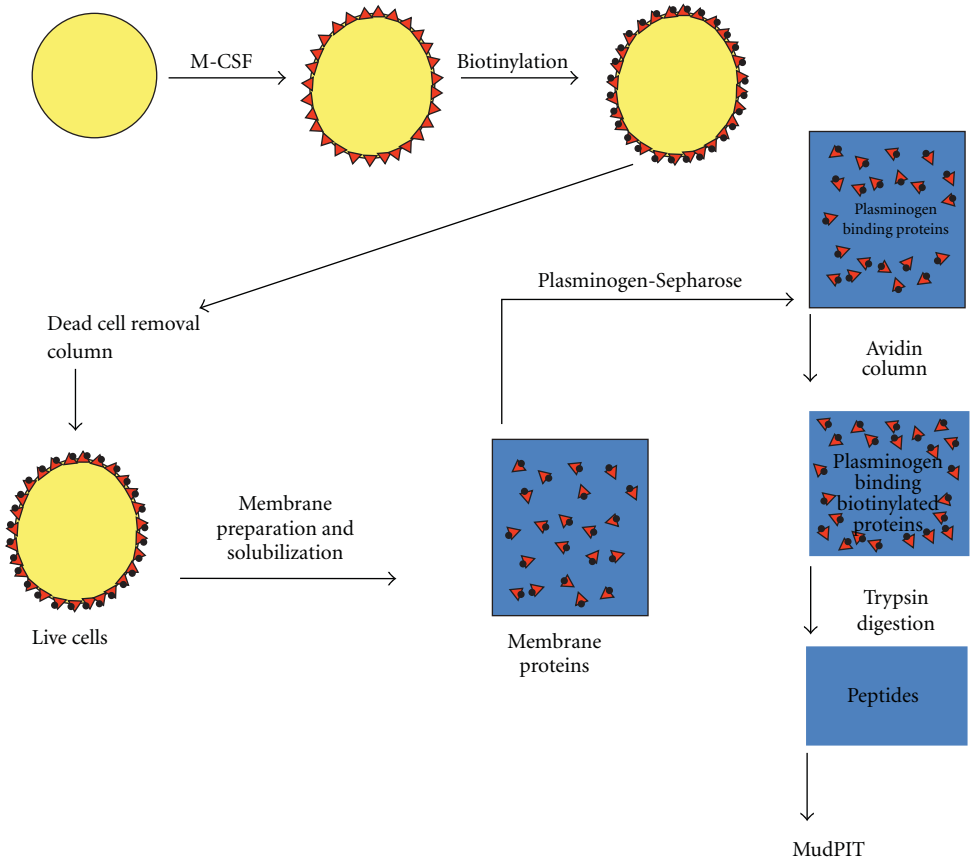

FIGURE 1: Isolation of plasminogen receptors. Monocyte (Hoxa9-ER4) progenitor cells were differentiated with macrophage colony stimulating factor (M-CSF), which induces plasminogen receptors (▲) on these cells. Then intact cells were biotinylated (•) and passed over a dead cell removal column. Live cells were then lysed and membrane fractions prepared and passed over a plasminogen-Sepharose affinity column and specifically eluted. Biotinylated plasminogen receptors (▲•) were then bound to an avidin column and digested with trypsin. This figure was originally published in [17].

a C-terminal basic residue was identified: the hypothetical protein, C9orf46 homolog (IPI00136293), homologous to the protein predicted to be encoded by human chromosome 9, open reading frame 46. We have designated the protein, Plg-R_{KT}, to indicate a plasminogen receptor with a C-terminal lysine and having a transmembrane domain (see below).

A key advantage of MudPIT is that proteins in a given proteome can be identified simultaneously. As proof of principle of our isolation method, peptides corresponding to other proteins previously identified as plasminogen-binding proteins on monocytes were also detected in the membrane preparations: α-enolase, gamma actin, S100A10, annexin 2, histone H2B, and β_2 integrin. (A limitation of shotgun proteomics, such as MudPIT, is that they typically undersample a proteome because they use data-dependent data acquisition (a computer-driven data acquisition approach). This can lead to variations in the proteins identified, particularly among the lower abundance proteins. Thus, we cannot exclude the possibility that other membrane proteins exposing C-terminal basic residues were present in the membrane proteome.)

4. Conservation of the Plg-R_{KT} Sequence

The C9orf46 homolog/Plg-R_{KT} murine DNA sequence encodes a protein of 147 amino acids with a molecular mass of 17,261 Da and a C-terminal lysine (Table 1, first line). We blasted the C9orf46 homolog/Plg-R_{KT} sequence against all species using NCBI Blast and obtained unique human, rat, dog, cow, giant panda, gibbon, horse, pig, rabbit, and rhesus monkey predicted orthologs, with high identity (e.g., human versus chimpanzee = 99% identity) and no gaps in the sequence (Table 1). Of key importance, a C-terminal lysine was predicted for all of the mammalian orthologs obtained in the blast search. In a query of the Ensembl Gene Report, DNA sequences of all 10 other sequenced mammalian orthologs encoded C-terminal lysines (Table 1).

In addition to mammals, the DNA sequences of xenopus and the green lizard also encoded C-terminal lysines (Table 2). Furthermore, Plg-R_{KT} orthologs with 149 amino acids with a C-terminal lysine were encoded in bony fish (e.g., zebrafish) and the high similarity with a mammalian ortholog is illustrated in the alignment with the mouse protein in Table 2.

Table 1: Alignment of Mammalian Orthologs of Plg-R_{KT}.

	10	20	30	40	50	60	70	80
Mouse	MGFIFSKSMN	ENMKNQQEFM	VTHARLQLER	HLTMQNEMRE	RQMAMQIAWS	REFLKYFGTF	FGIATISLAT	GALKRKKPAF
Human	MGFIFSKSMN	ESMKNQKEFM	LMNARLQLER	QLIMQSEMRE	RQMAMQIAWS	REFLKYFGTF	FGLAAISLTA	GAIKKKKPAF
Rat	MGFIFSKSMN	ENMKNQQEFM	VMHARLQLER	QLIMQNEMRE	RQMAMQIAWS	REFLKYFGTF	FGIATISLAA	GAIKRKKPAF
Dog	MGFIFSKSMN	ENMKNQQEFM	LMNARLQMER	QLMMQNEMRE	RQMAMQIAWS	REFLKYFGTF	FGIAAISLTA	GAIRKKKPAF
Cow	MGFIFSKSMN	ENLKSQQEFM	LMNSRLQLER	QLIMQNEMRE	RQMAMQIAWS	REFLKYFGTF	FGITAVSLTA	GAIKGKKPVL
Alpaca	MGFIFSKSMN	ENMKSQQEFM	LMNARLQLER	QLMMQNEMRE	RQMAMQIAWS	REFLKYFGTF	FGIAAISLTA	GAIKRKKPAF
Chimpanzee	MGFIFSKSMN	ESMKNQKEFM	LMNARLQLER	QLIMQSEMRE	RQMAMQIAWS	REFLKYFGTF	FGLAAISLTA	GAIKKKKPAF
Dolphin	MGFIFSKSMN	ENMKSQQEFM	LMNARLQLER	QLMMQNETRE	RQMAMQIAWS	REFLKYFGTF	FGIAAISLTA	GAIKKKKPAF
Gibbon	MGFIFSKSMN	ESMKNQKEFM	LMNARLQLER	QLIMQSEMRE	RQMAMQIAWS	REFLKYFGTF	FGLAAISLTA	GAIKKKKPAF
Guinea Pig	MGFMLSKSMN	ENMKNQQEFM	LMNARLQLER	QLLLQNEMRE	RQMAMQIAWS	REFLKYFGTF	FGISAISLTA	RAIKQKKPAF
Horse	MGFIFSKSMN	ENMKNQQEFM	LMNARLQLER	QLTMQNEMRE	RQMAMQIAWS	REFLKYFGTF	FGIAAISLTA	GALKRKKPAF
Lemur	MGFIFSKSMK	ENAQNQQEFM	LMNARLQLER	QLTMQNEMRE	RQMAMQIAWS	REFMKYFGTF	FGITAISLTA	GAIKSKKPGF
Opossum	MGFLFSKHMN	ENMKQQQEFM	LMNARLQMER	QLTIQNEMRE	RQMAMQIAWT	REFLKYFGTF	FGIAAISLTA	GAIKKKQPGL
Orangutan	MGFIFSKSMN	ESMKNQKEFM	LMNARLQLER	QLIMQSEMRE	RQMAMQIAWS	REFLKYFGTF	FGLAAISLTA	GAIKKKKPAF
Panda (Giant)	MGFIFSKSMS	ENMKNQQEFM	LMNARLQLER	QLMMQNEMRE	RQMALQIAWS	REFLKYFGTF	FGITAISLTA	GAIRRKKPAF
Pig	MGFIFSKSMN	ENMKRQQEFM	LMNTRLQLER	QLIMQNEMRE	RQMAMQIAWS	REFLKYFGTF	FGIASVALTA	GAIKRKKPAF
Rabbit	MGFIFSKSMN	ENLKNQQEFM	LMNARLQLER	QLMLQNEMRE	RQMAMQIAWS	REFLKYFGTF	FGVATISLTA	GAMRRKKPAF
Rhesus Monkey	MGFIFSKSMN	ESMKNQKEFM	LMSARLQLER	QLIMQSEMRE	RQMAMQIAWS	REFLKYFGTF	FGFAAISLTA	GAIKKKKPAF
Tarsier	MGFIF-KSMN	ENMKHQQEFM	LMNAQLQLER	QLTMQNEMRE	RQMAMQIAWS	REFLKYFGTF	FGITAISLTA	GAIKRKKPAL
Tree Shrew	MGFIFSKSMN	ENMKNQQEFM	LMNARLQLER	QLMMQNEMRE	RQMAMQIAWS	REFLKYFGTF	FGIAAISLTA	GAIKKKNPAF
	90	100	110	120	130	140	147	
Mouse	LVPIVPLSFI	FTYQYDLGYG	TLLQRMKSEA	EDILETEKTK	LELPKGLITF	ESLEKARREQ	SKLFSDK	
Human	LVPIVPLSFI	LTYQYDLGYG	TLLERMKGEA	EDILETEKSK	LQLPRGMITF	ESIEKARKEQ	SRFFIDK	
Rat	LIPIVPLSFI	FTYQYDLGYG	TLLQRMKSEA	EDILETEKTK	LELPKGLITF	ESLEKARREQ	SKFFSDK	
Dog	LFPIIPLSFI	FTYQYDLGYG	TLLQRMKGEA	ENILETEKSK	LQLPRGMITF	ESLEKARREQ	SKFFIDK	
Cow	IFPIVPLGFV	LAYQYDMGYG	TLIHRMKGEA	ENILETEKSK	LQLPKGMITF	ESLEKARKEQ	SKFFIDK	
Alpaca	IFPIVPLGFV	LTYQFDLGYG	TLLQRMKGEA	ENILETEKSK	LQLPKGIITF	ESLEKARKEQ	SKFFIDK	
Chimpanzee	LVPIVPLSFI	LTYQYDLGYG	TLLERMKGEA	EDILETEKSK	LQLPRGMITF	ESIEKARKEQ	SRFFIDK	
Dolphin	VFPIVPLGFV	LAYQYDMGYG	TLIQRMKGEA	DNILETEKSK	LQLPKGMITF	ENLEKARREQ	SKFFIDK	
Gibbon	LVPIVPLSFI	LTYQYDLGYG	TLLERMKGEA	EDILETEKSK	LQLPRGMITF	ESIEKARKEQ	SKFFIDK	
Guinea Pig	FIPIVPLSFV	LAYQYDLGYG	TLLQRMKGEA	EDILETEKNK	LELPKGVITF	ESLEKARREQ	SKFFLGK	
Horse	LFPIVPLGFV	LTYQYDLGYG	TLLQRMKGEA	ENILETEKSK	LQLPKGMITF	ESLEKARREQ	SKFFIDK	
Lemur	LFPIVPLSFV	LAYQYDLGYG	TLLQRMKGEA	EDILETEKSK	LQLPKGMITF	ESLEKARREQ	SKFFIEK	
Opossum	FFPIVPLSFI	LAYQYDMGYG	TLLQRMKGEA	ENILETENSK	LQLPRGSITF	ETLEKARKAQ	SKFFIEK	
Orangutan	LVPIVPLSFI	LTYQYDLGYG	TLLERMKGEA	EDILETEKSK	LQLPRGMITF	ESIEKARKEQ	SRFFIDK	
Panda (Giant)	LFPIIPLSFI	FTYQYDLGYG	TLLQRMKGEA	ENILETEKSK	LQLPRGMITF	ENLEKARREQ	SKFFIDK	
Pig	FLPIIPLGFV	FTYQYDLGYG	TLLQRMKGEA	ENILETETSK	LQLPKGMITF	EGLEKARREQ	SKFFIDK	
Rabbit	LLPIVPLSFI	FIYQCDLGYG	TLLQRMKGEA	EDILETEKSK	LQLPGGMITF	ESLEKARREQ	SKFFIDK	
Rhesus Monkey	LVPIVPLSFI	LTYQYDLGYG	TLLERMKGEA	EDILETEKSK	LQLPRGMITF	ESIEKARKEQ	SKFFIDK	
Tarsier	LLPIVPLSFI	FTYQYDLGYG	TLLERMKGEA	EEILEAEKNM	LQLPKGMITF	ESLEKTRREQ	SKFFTDK	
Tree Shrew	FFPIVPLSFI	LTYQYDLGYG	TLLPRMKSEA	EDILETEKSK	LELPRGMITF	ESLEKARREQ	SKFFVDK	

The Plg-R_{KT} sequence also encodes a putative conserved DUF2368 domain (encompassing amino acids 1–135), an uncharacterized protein with unknown function conserved from nematodes to humans. Notably, the DNA sequences of Plg-R_{KT} orthologs of lower organisms predicted proteins of different lengths and did not consistently predict C-terminal lysines. It is interesting to note that the evolutionary origin of plasminogen is currently believed to originate with protochordates [37], so that lower organisms without plasminogen would not need the C-terminal lysine of Plg-R_{KT} to bind plasminogen.

Within species, it is noteworthy that the primary sequence of C9orf46/Plg-R_{KT} is apparently tightly conserved in humans, with no validated polymorphisms (cSNPs) within the 6 exons encoded by the gene (on chromosome 9p24.1) in the NCBI human genome sequence variation database (dbSNP, http://www.ncbi.nlm.nih.gov/SNP/).

Table 2: Alignment of Mouse, Lizard (arborial), Frog (xenopus), and Zebrafish Plg-R_{KT} Sequences.

Mouse	1	MGFIFSKSMNENMKNQQEFMVTHARLQLERHLTMQNEMRERQMAMQIAWSREFLKYFGTF	60
Lizard	1	MGFIFSKSMNENLKNQQEFMIMNSRLQLERQLLMQNQMRERQMAMQIAWTREFLKYFGAF	60
Frog	1	MGSLISKATETQMKKQQELMQMNAQIQLERQIIMQNMMRERQMAMQIAWSREFLKYYGSF	60
Zebrafish	1	MGFVLSKGMEQNFQKQQEFMLLNARLQLERQLAMQNQMRERQMAMQLAWSREFLKYFGSF	60
Mouse	61	FGIATISLATGALKRKKPAFLVPIVPLSFIFTYQYDLGYGTLLQRMKSEAEDILETEKTK	120
Lizard	61	SGLAAVGLTVGAIKRKKPAFFLPMVPLSFILAYQYDMGYGSLLKRMKSEAESILDTESTT	120
Frog	61	FSLAVIGLTVGAVKNKKPALFTPVIPLTFVFAYQFDMGYGTLVTRMKGEAENILEKEHIL	120
Zebrafish	61	FGLATLGLTVGAVKKRKPALLAPVIPLSFILVYQMDAAYGTMLQRMRAEAESIMVSECER	120
Mouse	121	LELPKGLITFESLEKARREQSKL--FSDK	147
Lizard	121	LEMPKGPLTFESIEKARRAQSKF--FIEK	147
Frog	121	LEMPQGLPTFEGIEKTRKAHRSLLL---K	147
Zebrafish	121	LDVPHGMPTFESIEKSRRAKAHLTTLTEK	149

5. Membrane Topology of Plg-R_{KT}

We analyzed the C9orf46 homolog/Plg-R_{KT} sequence in the TMpred site (http://sourceforge.net/projects/tmpred/files/). The strongly preferred model included two transmembrane helices extending from F_{53}-L_{73} (secondary helix, oriented from outside the cell to inside the cell) and P_{78}-Y_{99}(primary helix, oriented from inside the cell to outside the cell) (Figure 2). Thus, a 52 amino acid N-terminal region and a 48 amino acid C-terminal tail with a C-terminal lysine were predicted to be exposed on the cell surface.

We experimentally tested predictions of the topology model. First, we raised a monoclonal antibody against a synthetic peptide, CEQSKLFSDK (corresponding to the nine C-terminal amino acids of murine Plg-R_{KT} with an amino terminal cysteine added for coupling). The mAb reacted with the C-terminal peptide of murine Plg-R_{KT} and blocked plasminogen binding to CEQSKLFSDK. To examine subcellular localization, membrane and cytoplasmic fractions from progenitor and differentiated Hoxa9-ER4 monocyte progenitor cells were electrophoresed and western blotted with anti-Plg-R_{KT} mAb7H1 or isotype control mAb. A specific immunoreactive band migrating with an Mr_{app} of ~17,000 was detected in membrane fractions of differentiated monocyte progenitor cells, clearly demonstrating the existence of this new protein (Figure 3(a)). Plg-R_{KT} was not detected in undifferentiated cells or in the cytoplasmic fraction of the differentiated cells, also demonstrating that Plg-R_{KT} was an M-CSF-inducible protein.

Because we had initially found Plg-R_{KT} in murine monocyte progenitor cells, we also examined the expression and subcellular localization of Plg-R_{KT} in human peripheral blood monocytes and human monocytoid cells using an anti-Plg-R_{KT} mAb7H1. Plg-R_{KT} (17,261 Da) was also observed in the membrane fractions of human peripheral blood monocytes, U937 cells and THP-1 cells, but was not detected in the cytoplasmic fractions of these cells [20]. Thus, Plg-R_{KT} is markedly expressed in membranes of both normal human peripheral blood monocytes and human monocytoid cell lines. Plg-R_{KT} was detected in other human peripheral blood cells, also. Plg-R_{KT} was highly expressed in lymphocytes and less strongly expressed by granulocytes and was not detectable in rbc [20].

To further test the prediction that Plg-R_{KT} is an integral membrane protein, membranes from differentiated monocyte progenitor cells were subjected to phase separation in Triton X-114 as described [38, 39]. In this method, integral membrane proteins form mixed micelles with the nonionic detergent and are subsequently recovered in the Triton X-114 detergent phase, whereas hydrophilic proteins remain in the aqueous phase. Plg-R_{KT} was detected in the detergent phase in western blotting with anti-Plg-R_{KT} mAb7H1, but was not detected in the aqueous phase (Figure 3(b)). These data support the prediction that Plg-R_{KT} is an integral membrane protein.

To experimentally test whether the C-terminal lysine of Plg-R_{KT} was exposed on the cell surface, we treated intact biotinylated cells with carboxypeptidase B prior to performing our isolation procedure. Under this condition, C-terminal lysines exposed on the cell surface are removed but intracellular C-terminal lysines are protected. Under this condition, no peptides corresponding to Plg-R_{KT} were obtained in the MudPIT analysis, consistent with cell surface exposure of the C-terminal lysine of Plg-R_{KT}.

Other key aspects of our experimental paradigm support the topological model shown in Figure 2. First, recovery of peptides corresponding to C9orf46 homolog relied on accessibility to the biotinylation reagent in the context of intact cells, supporting the exposure of Plg-R_{KT} domains on the cell surface. The biotinylation reagent we used reacts with the carboxyl moieties of the R-groups of either Asp or Glu. Thus, biotinylation of Plg-R_{KT} on intact cells would not occur if the highly basic loop between the transmembrane helices (K_{74}–K_{77}) was exposed on the cell surface. Hence, a model in which the amino and C-termini are both on the cytoplasmic face of the membrane can be excluded.

In order to experimentally evaluate whether the N-terminus as well as the C-terminus of Plg-R_{KT} was exposed on the cell surface, PC12 (rat pheochromocytoma) cells were stably transfected with V5-pCIneo-Plg-R_{KT} that expressed a V5 tag at the N-terminus of Plg-R_{KT}. A specific band migrating with a Mr_{app} of 17,000 was detected in cell membranes of the stably transfected cells with both anti-V5 mAb, which reacts with the N-terminus and anti-Plg-R_{KT} mAb7H1, which reacts with the C-terminus of Plg-R_{KT}

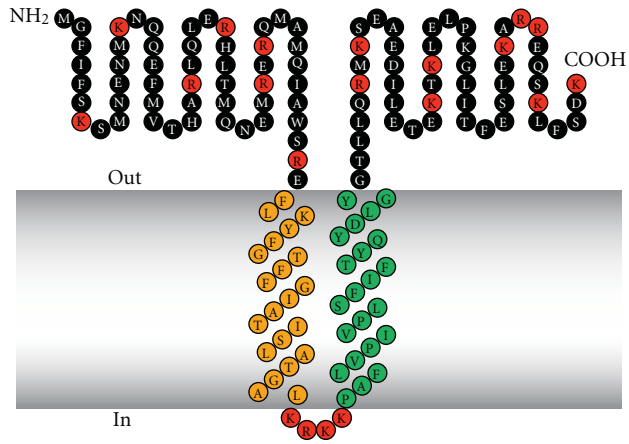

FIGURE 2: Structural model of Plg-R_{KT}. Green indicates amino acids within the predicted primary transmembrane helix. Orange indicates amino acids within the predicted secondary transmembrane helix. Red indicates basic amino acids. This research was originally published in [18].

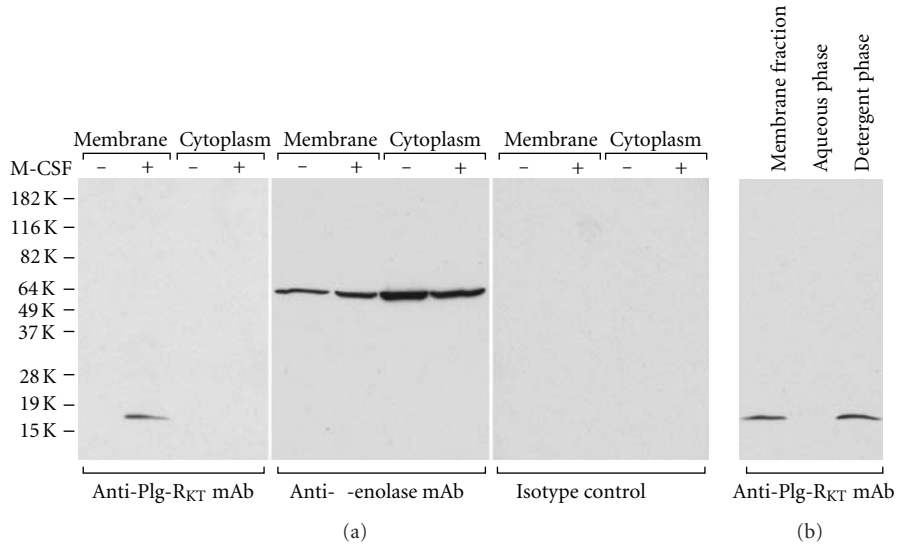

FIGURE 3: Plg-R_{KT} behaves as a regulated integral membrane protein. (a) Membrane fractions or cytoplasmic fractions from either undifferentiated or M-CSF-treated Hoxa9-ER4 cells were electrophoresed on 12% sodium dodecyl sulfate polyacrylamide gels under reducing conditions and western blotted with either anti-Plg-R_{KT} mAb, anti-α-enolase mAb as a loading control, or isotype control mAb. (b) M-CSF-treated Hoxa9-ER4 cell membranes were solubilized in 3% Triton X-114. After heating at 37°C and separation of the phases by centrifugation, an aliquot of both phases was electrophoresed and western blotted with anti-Plg-R_{KT} mAb. This research was originally published in [18].

(Figure 4, lane 1). The band was not detected by either mAb after trypsin digestion of isolated membrane fractions (lane 2). When intact cells were incubated with trypsin and the trypsin neutralized with SBTI prior to preparation of the membrane fraction, the majority of the band detectable with either anti-V5 or anti-Plg-R_{KT} was lost (lane 4). (In controls, treatment with soybean trypsin inhibitor (SBTI) fully neutralized the ability of trypsin to degrade the V5-tagged Plg-R_{KT} in purified membrane fractions (lane 3), demonstrating that the trypsin had been neutralized prior to membrane fractionation of the treated cells.) These results suggest that both the N-terminus and C-terminus of Plg-R_{KT} are accessible to trypsin proteolysis of intact cells and are, therefore, exposed on the extracellular face.

6. Role of the C-Terminal Lysine of Plg-R_{KT} in Plasminogen Binding

We further addressed the exposure of the C-terminus of Plg-R_{KT} on the cell surface using confocal microscopy with anti-Plg-R_{KT} mAb7H1 raised against the Plg-R_{KT} C-terminal peptide. When cells were incubated with anti-Plg-R_{KT} mAb and a polyclonal antiplasminogen antibody, Plg-R_{KT} and plasminogen were both immunodetected in small aggregates dispersed over the cell surface [18], in a similar distribution to that published for confocal analyses of monocyte-associated plasminogen [15]. Most importantly, after preincubation of monocytes with plasminogen, immunodetection of Plg-R_{KT} was reduced by half [18].

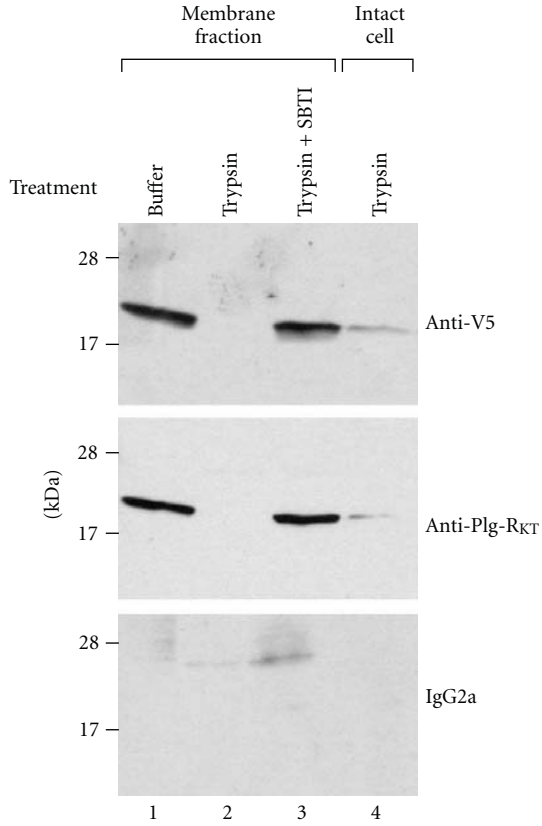

Figure 4: The N-termini and C-termini of Plg-R_{KT} are exposed on the cell surface. Membrane fractions of PC12 cells stably transfected with V5-pCIneo-Plg-R_{KT} were incubated with either buffer (lane 1), trypsin (1 mg/mL) (lane 2), or trypsin 1 mg/mL + soybean trypsin inhibitor (SBTI) (2 mg/mL) (lane 3) for 30 minutes at 37°C or intact PC12 cells were incubated with 1 mg/mL trypsin for 2 hr at 37°C, followed by 2 mg/mL SBTI for 15 min. Following neutralization of trypsin with SBTI, the membrane fraction was prepared from the treated, intact cells (lane 4). 30 μg/lane of membrane fractions was electrophoresed on 18% SDS page under reducing conditions and western blotted with either anti-V5, anti-Plg-R_{KT} mAb, or isotype control. This research was originally published in [17].

These results demonstrate that the C-terminus of Plg-R_{KT} is exposed on the cell surface. Furthermore, these results show that plasminogen binds to the C-terminal domain of Plg-R_{KT} on the cell surface.

To further explore the functional importance of the C-terminal lysine, we tested whether a synthetic peptide, corresponding to the C-terminus of Plg-R_{KT} could interact with plasminogen. The peptide, CEQSKLFSDK, was coupled to BSA and coated onto wells of microtiter plates. Glu-plasminogen was incubated with the wells, followed by antiplasminogen mAb [19, 40] and detection with HRP-conjugated goat anti-mouse IgG. Glu-plasminogen bound to the peptide in a concentration-dependent manner, reaching half saturation at a concentration of 7.6 nM (Figure 5(a)). The binding was specific because it was blocked in the presence of EACA, consistent with the ability of EACA to inhibit plasminogen binding to cells. Lys-plasminogen also bound specifically to the peptide (Figure 5(b)) and the concentration for half saturation was $\leq$2.7 nM, consistent with the higher affinity of Lys-plasminogen compared to Glu-plasminogen for cell surfaces [41, 42]. We also investigated the interaction of t-PA with the C-terminal peptide because t-PA and plasminogen share binding sites on monocytoid cells and t-PA binding to monocytoid cells is sensitive to CpB [43]. Concentration-dependent binding of t-PA to the peptide was observed (Figure 5(c)) and the concentration for 50% saturation was 3.2 nM, consistent with the relative affinities of Glu-plasminogen and t-PA for the cell surface [44]. We noticed that the concentration for 50% saturation of plasminogen binding to immobilized CEQSKLFSDK-coupled to BSA was much greater than the Kd value we had determined for plasminogen binding to cells. The differences in apparent affinity when plasminogen bound to the immobilized peptide compared to the cell surface may have been due to our use of BSA-conjugated peptide to coat the plates. If multiple peptides were conjugated to the BSA that could have provided a higher affinity surface than the cell surface receptor, since plasminogen has multiple kringle domains that may interact in a cooperative lysine-dependent manner with several Plg-R_{KT} peptides on a single BSA molecule. To resolve this issue, we tested the ability of the soluble C-terminal peptide to inhibit Glu-plasminogen binding under solution phase equilibrium conditions. The soluble peptide competed for Glu-plasminogen binding in a dose-dependent

manner with an IC_{50} of 2 μM (Figure 5(d)), similar to the Kd values we had determined for Glu-plasminogen binding to M-CSF-treated Hoxa9-ER4 cells [18]. Furthermore, a mutated peptide with the C-terminal lysine substituted with alanine did not compete for plasminogen binding at concentrations up to 1 mM (Figure 7(d)), further supporting the role of the C-terminal lysine in the interaction of Plg-R_{KT} with plasminogen.

7. Plg-R_{KT} Regulates Cell Surface Plasminogen Activation

Plasminogen activation was stimulated 12.7-fold in the presence of M-CSF-treated Hox9-ER4 cells, compared to the reaction in the absence of cells and cell-dependent plasminogen activation was stimulated 4.4-fold on differentiated cells, compared to undifferentiated cells (Figure 6(a)). In order to verify the role of Plg-R_{KT} in plasminogen activation, we tested the effect of a monoclonal antibody (anti-Plg-R_{KT} mAb35B10) raised in rats against the synthetic peptide, CEQSKLFSDK. The IgG fraction reacted with the C-terminal peptide of murine Plg-R_{KT} and blocked plasminogen binding to CEQSKLFSDK (Figure 5(e)). Anti-Plg-R_{KT} mAb35B10 substantially suppressed cell-dependent plasminogen activation by 46% and suppressed cell differentiation-dependent plasminogen activation by 58% (Figure 6(a)). In controls, plasminogen activation in the absence of cells or on undifferentiated cells was not affected by anti-Plg-R_{KT} mAb.

We examined whether Plg-R_{KT} colocalized with uPAR, an additional key component of the cell-surface plasminogen activation system. Plg-R_{KT} was markedly colocalized with uPAR on the surfaces of M-CSF-differentiated monocyte progenitor Hoxa9-ER4 cells, as revealed by merged confocal images. The extent of colocalization of Plg-R_{KT} with uPAR was 73 ± 3% [18]. These results suggest that Plg-R_{KT} and uPAR are present in very close proximity on the cell surface in an orientation to promote plasminogen activation.

The kinetically favored substrate for uPAR-bound uPA is cell associated, rather than solution phase plasminogen [4]. Therefore, we tested whether Plg-R_{KT} regulates uPA-dependent cell surface plasminogen activation using an antibody inhibition approach with anti-Plg-R_{KT} mAb7H1. U937 human monocytoid cells were preincubated with either mAb7H1 or IgG2a isotype control, followed by incubation with plasminogen for 30 minutes. Plasmin generation was measured after addition of uPA and a chromogenic substrate for plasmin. Treatment with mAb7H1 markedly suppressed cell-dependent plasminogen activation by uPA and the extent of suppression was cell concentration-dependent. At a concentration of 3×10^5 cells/mL, cell surface dependent plasminogen activation by uPA was suppressed by 39% (Figure 6(b)). Therefore, Plg-R_{KT} plays a major role in uPA-dependent plasminogen activation on monocytoid cells.

8. Regulation of Cell Migration by Plg-R_{KT}

Because Plg-R_{KT} promoted plasminogen activation and cell-associated plasmin plays a role in cell migration, we investigated the role of Plg-R_{KT} in cell migration. Invasion of Matrigel by monocytoid cells in response to the chemotactic stimulus, MCP-1, is enhanced in the presence of plasminogen and also requires active plasmin [15, 45] and uPA [15] and is markedly suppressed in the presence of EACA, suggesting a key role of plasminogen receptors in this function [15, 45]. Plg-R_{KT} played a major role in Matrigel invasion. Treatment of U937 cells or human peripheral blood monocytes with anti-Plg-R_{KT} mAb markedly decreased migration of the cells through Matrigel (by 54% and 48%, respectively) compared to isotype control [20].

Plasmin promotes chemotactic cell migration across polycarbonate membranes in the absence of extracellular matrix [46]. We found that chemotactic migration in the absence of Matrigel was markedly regulated by Plg-R_{KT}. Chemotactic migration was enhanced in the presence of plasminogen [20]. Migration was inhibited 65% by EACA consistent with plasminogen-receptor dependence of this function [20]. Cell migration was also inhibited by amiloride and aprotinin, consistent with a requirement for active plasmin and uPA in chemotactic migration [20]. Chemotactic migration of both U937 cells and human peripheral blood monocytes was maximally reduced by 64% and 39%, respectively, by treatment with anti-Plg-R_{KT} mAb, compared to isotype control [20]. Regulation of chemotactic migration in the absence of extracellular matrix appears to be a unique property of Plg-R_{KT} because other plasminogen receptors do not regulate chemotactic migration [15].

We tested whether, in addition to chemotaxis, Plg-R_{KT} was involved in chemokinesis, that is, nondirectional cell motility in response to MCP-1. Checkerboard analysis showed that in the presence of plasminogen, a constant MCP-1 concentration and a positive concentration gradient induced U937 motility. Both processes were inhibited by anti- Plg-R_{KT} antibody 7H1, with chemokinesis being suppressed almost to a background level [20].

9. Regulation of the Inflammatory Response by Plg-R_{KT}

Having established the presence of Plg-R_{KT} on human monocytoid cells and demonstrated its role in invasion and chemotactic migration *in vitro*, we examined the role of Plg-R_{KT} in monocyte migration *in vivo*. $Plg^{+/+}$ mice were injected intravenously with either mAb7H1 or IgG2a isotype control. After 30 minutes, the mice were injected intraperitoneally with thioglycollate to induce a sterile inflammatory response and a second dose of antibody was given intravenously 24 hours after the thioglycollate injection. After 72 hours (when most recruited leukocytes are macrophages [12, 47, 48]), cells were collected by peritoneal lavage and macrophages were purified by adherence. Macrophage recruitment was significantly (49%) impaired in the mice that were injected with mAb7H1 compared with the mice injected with the isotype control ($2.46 \times 10^5 \pm 0.28 \times 10^4$ for mice injected with mAb7H1 versus $4.82 \times 10^5 \pm 0.33 \times 10^4$ for mice injected with isotype control, $n = 5$, $P = .00048$) (Figure 7) [20]. In 3 other experiments, C57Bl/6J mice injected with 7H1 had a

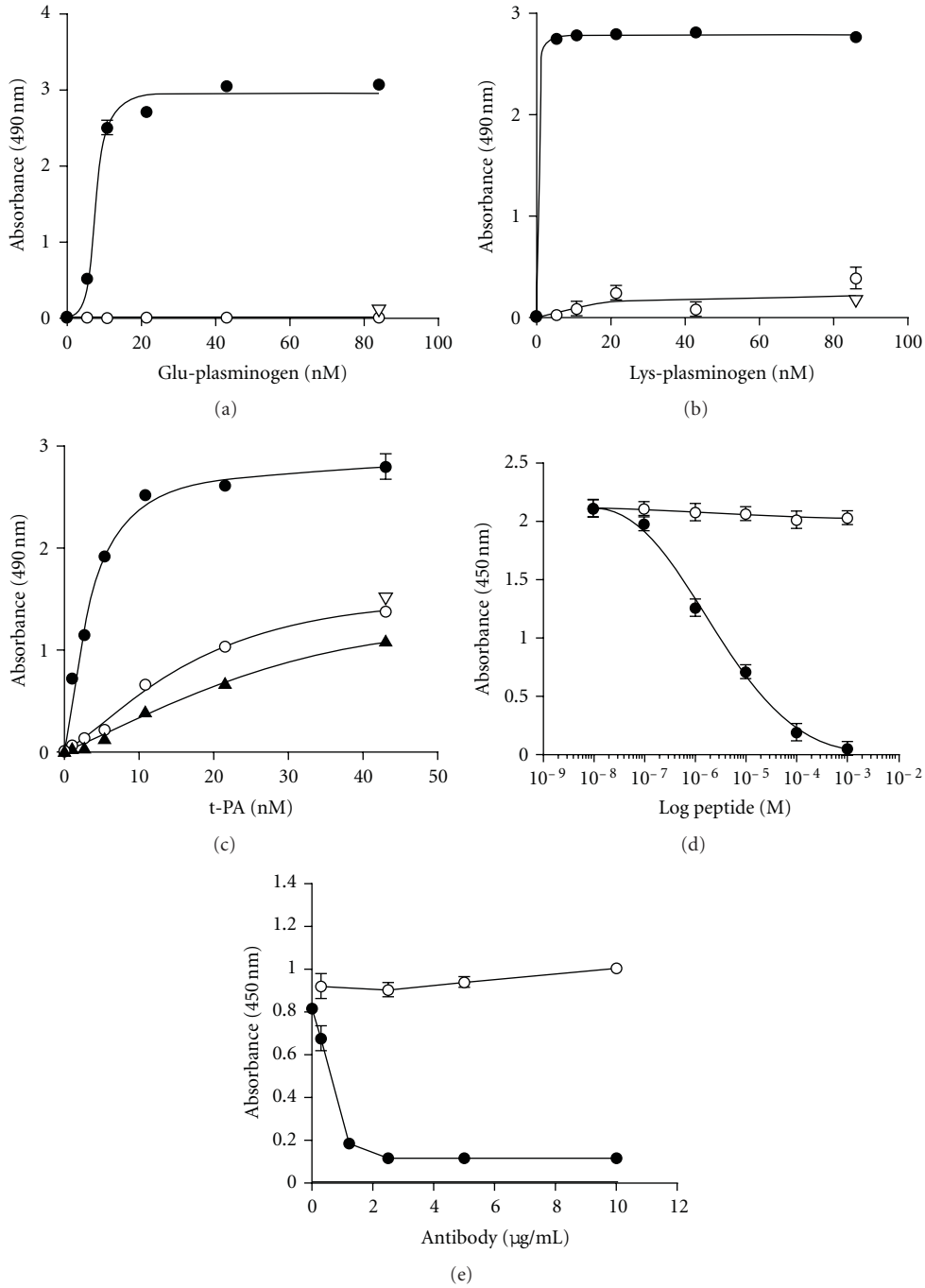

Figure 5: Plasminogen binds to the C-terminal peptide of Plg-R_{KT}. The peptide, CEQSKLFSDK, corresponding to the amino terminus of Plg-R_{KT} was coupled to BSA and coated onto wells of microtiter plates. Either Glu-plasminogen (a) or Lys-plasminogen (b) or t-PA (c) was then incubated with the wells, followed by antiplasminogen mAb [19] (a, b) or anti-t-PA polyclonal mAb (c) and detection with HRP-conjugated goat anti-mouse IgG (closed circles) as described in Materials and Methods. The binding was specific because it was blocked in the presence of 0.2 M EACA (open triangles), consistent with the ability of EACA to inhibit plasminogen binding to differentiated Hoxa9-ER4 cells. In additional controls, nonspecific binding to either BSA (closed triangles) or to the reverse peptide (open circles) was <10% of binding to CEQSKLFSDK. (At high input concentrations of t-PA nonspecific binding increased but was <10% of binding to CEQSKLFSDK at the concentration required for 50% saturation (3.2 nM). In controls for the detection method, $O.D._{490}$ values obtained using an isotype control antibody or in the absence of added plasminogen or t-PA were <5% of the values for plasminogen or t-PA binding to immobilized CEQSKLFSDK. Panels d and e: biotinylated-Glu-plasminogen (25 nM) was incubated with immobilized CEQSKLFSDK in the presence of increasing concentrations of (d) the C-terminal peptide, CEQSKLFSDK (closed circles), or a mutated C-terminal peptide with K147 substituted with alanine, CEQSKLFSDA (open circles) or (e) anti-Plg-R_{KT} mAb35B10 (closed circles) or isotype control (open circles). Biotinylated Glu-plasminogen binding was detected with HRP-streptavidin and was 96% inhibited in the presence of 0.2 M EACA (not shown). Data are as mean ± SEM, $n = 3$, for each determination. This research was originally published in [18].

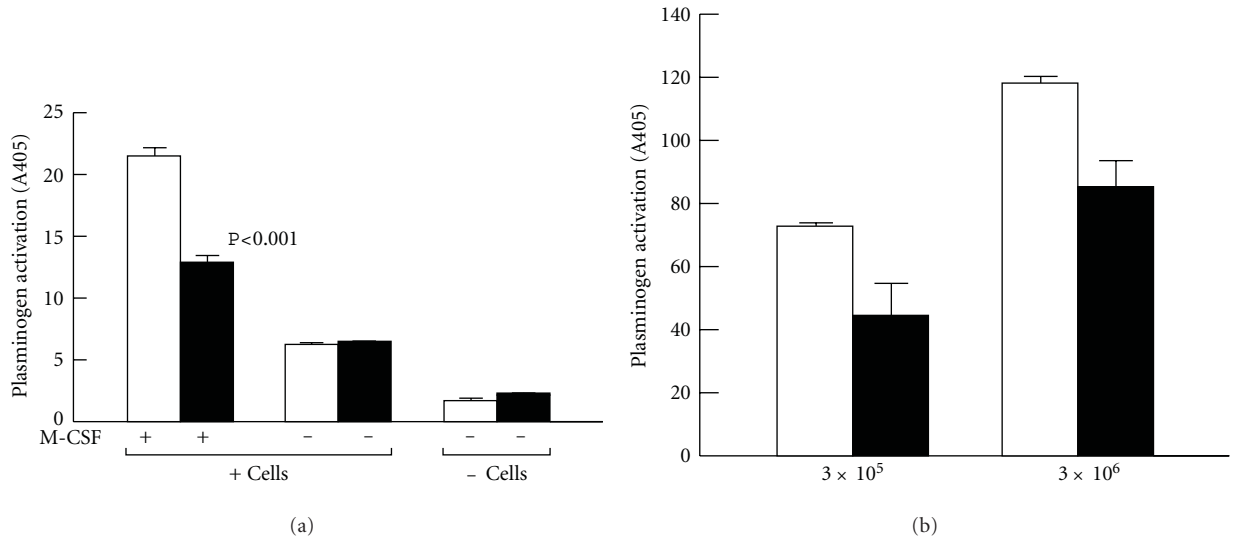

Figure 6: (a) Plg-R_{KT} regulates cell surface plasminogen activation by t-PA. Plasminogen activation was determined after adding 2.7 μM Glu-plasminogen and 20 nM single chain recombinant t-PA in either the presence or absence of either undifferentiated Hoxa9-ER4 progenitor cells or M-CSF-differentiated Hoxa9-ER4 cells and in the presence of either rat anti-Plg-R_{KT} mAb35B10 (filled bars) or isotype control rat IgG2a (open bars). $^{***}P < 0.001$, compared to the corresponding isotype control. This research was originally published in [18]. (b) Plg-R_{KT} regulates cell surface plasminogen activation by uPA. Plasminogen activation was determined in the presence of different concentrations of U937 cells, as indicated, and in the presence of 2.7 μM Glu-Plasminogen and 20 nM uPA and in the presence of 170 nM of either anti-Plg-R_{KT} mAb7H1 (filled bars) or mouse IgG2a isotype control (open bars). Cell-dependent plasminogen activation on the tripeptide substrate, S2251, is shown after subtracting plasminogen activation in the absence of cells. $^{**}P < .001$ compared with the corresponding isotype control. This research was originally published in [20].

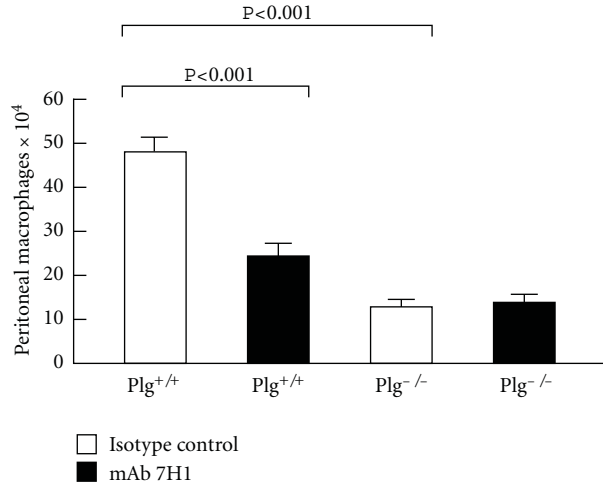

Figure 7: Effect of Plg-R_{KT} on thioglycollate-induced monocyte recruitment. Both plasminogen-deficient ($Plg^{-/-}$) and wild-type littermates ($Plg^{+/+}$) mice were injected intravenously with either mAb7H1 (■) or isotype control (□) (500 μg). After 30 minutes thioglycollate was injected intraperitoneally. A second injection of antibody was given 24 hours later. After 72 hours, thioglycollate-recruited cells were collected by peritoneal lavage and macrophages were purified by adherence. The adherent cells were detached and counted using a hemocytometer. Data represent mean ± S.E.M. $n = 5$/group). This research was originally published in [20].

53 ± 4% reduction in peritoneal macrophages, compared to mice injected with the isotype control.

The decreased response in macrophage recruitment to the peritoneum could not be explained by a decreased level of monocytes in the circulation. Differences in blood levels of monocytes in animals treated with mAb7H1 compared with isotype control were not statistically significant [20].

We also examined the effect of mAb7H1 on recruitment of other leukocytes to the peritoneum. At 6 hr, when recruited granulocyte levels are maximal [12], neutrophil and eosinophil recruitment to the peritoneum was not statistically different in mice treated with mAb7H1 compared with isotype control. Total peritoneal neutrophil recruitment in mice treated with isotype control was $1.73 \pm 0.58 \times 10^6$ and

in mice injected with mAb7H1 was $1.8 \pm 0.66 \times 10^6$ ($n = 5$). Total peritoneal eosinophil recruitment in mice injected with isotype control was $1.08 \pm 0.4 \times 10^6$ and in mice treated with mAb7H1 was $1.45 \pm 0.59 \times 10^6$ ($n = 5$). In contrast, there was an effect of mAb7H1 injection on lymphocyte recruitment. Total recruited peritoneal lymphocytes after 72 hr in mice treated with isotype control were $5.57 \pm 0.63 \times 10^5$ and in mice treated with mAb7H1 were $2.17 \pm 0.22 \times 10^5$ ($n = 4$, $P = 0.002$).

To assess whether the effect of mAb7H1 in the peritonitis model was consistent with the plasminogen binding function of Plg-R_{KT}, we examined the effect of injection of anti-Plg-R_{KT} mAb7H1 in $Plg^{-/-}$ mice. Thioglycollate recruitment in $Plg^{-/-}$ mice injected with isotype control was significantly decreased (by 73%) in $Plg^{-/-}$ compared to $Plg^{+/+}$ littermates, as reported (56% [12]–65% [16]). When $Plg^{-/-}$ mice were treated with mAb7H1, there was no effect on the remaining macrophage recruitment in $Plg^{-/-}$ mice (Figure 7). Thus, the effect of the anti-Plg-R_{KT} mAb7H1 was entirely dependent on plasminogen, consistent with Plg-R_{KT} exhibiting plasminogen receptor function *in vivo*.

It is apparent that the sum of the effects of functional blockade of specific plasminogen receptors, that have been analyzed in the thioglycollate-induced peritonitis model, is greater than a 100% reduction in plasminogen-dependent macrophage recruitment. Treatment with specific antibodies to histone H2B results in 48% less macrophage recruitment [15], and injection of specific antibodies to α-enolase results in 24% less recruitment (compared to injection of nonimmune control) [15]. In $S100A10^{-/-}$ mice, macrophage recruitment in response to thioglycollate is 53% less than in wild-type mice [45]. Injection of mice with anti-Plg-R_{KT} mAb7H1 resulted in 49% less macrophage recruitment compared to mice treated with isotype control [20]. Thus, it is likely that distinct plasminogen receptors may be required at different steps in the inflammatory response, for example chemotactic migration to the peritoneum, or, perhaps, crossing different layers of peritoneal tissue at which different contributions of direct plasmic cleavage of the extracellular matrix may predominate. The contribution of specific plasminogen receptors to macrophage recruitment may also be tissue and stimulus specific. For example in a model of LPS-mediated monocyte recruitment to the alveolar compartment, α-enolase appears to play a predominant role [49].

10. Tissue and Cellular Distribution of the Plg-R_{KT} Transcript

We searched results of gene expression array analyses for possible expression of the C9orf46 homolog/anti-Plg-R_{KT} transcript in other cells and tissues. The transcript is broadly expressed in both normal human and mouse tissues (as determined in high-throughput gene expression profiling in which RNA samples from human and murine tissues were hybridized to high-density gene expression arrays [50, 51]). The C9orf46 homolog/Plg-R_{KT} transcript is present in spleen, lymph node, thymus, bone marrow, lung, intestine, adrenal, pituitary, and other endocrine tissues, vascular tissue, liver, kidney, stomach, bladder, and neuronal tissue (hippocampus, hypothalamus, cerebellum, cerebral cortex, olfactory bulb, and dorsal root ganglion).

In addition, we searched for C9orf46 homolog/Plg-R_{KT} mRNA microarray expression data at http://www.ebi.ac.uk/microarray-as/aew/. C9orf46 homolog/Plg-R_{KT} mRNA is present in monocytes, leukocytes, T cells, natural killer (NK) cells, myeloid, dendritic and plasmacytoid cells, breast cancer, acute lymphoblastic leukemia, and Molt-4 acute lymphoblastic leukemia cells.

These data are consistent with previous reports documenting expression of plasminogen-binding sites on peripheral blood leukocytes [52], breast cancer cells [53, 54], and other tissues (reviewed in [55]).

Other studies of transcript expression provide clues to additional potential functions of Plg-R_{KT}. In a previously published genome-scale quantitative image analysis, overexpression of a cDNA that we now recognize to be the Plg-R_{KT} cDNA resulted in dramatic increases in cell proliferation whereas knockdown of the corresponding mRNA resulted in apoptosis [56]. Consistent with an anti-apoptotic role of Plg-R_{KT}, we have shown that cell-bound plasminogen inhibits TNFα-induced apoptosis [36]. In microarray studies, C9orf46 homolog mRNA expression has a high power to predict cervical lymph node metastasis in oral squamous cell carcinoma [57].

11. Conclusions

In conclusion, MudPIT has allowed us to identify a new protein, Plg-R_{KT}, a novel plasminogen receptor with unique characteristics: integral to the cell membrane and exposing a C-terminal lysine on the cell surface in an orientation to bind plasminogen. Furthermore, the ability of Plg-R_{KT} to bind t-PA, as well as the colocalization of Plg-R_{KT} with uPAR, brings the substrate, plasminogen, and its activators in close proximity on the cell surface in an orientation to promote plasminogen activation as shown in the model in Figure 8. Here we have reviewed emerging data establishing a role for Plg-R_{KT} in plasminogen activation, macrophage invasion and migration, and macrophage recruitment in the inflammatory response.

The broad distribution of the Plg-R_{KT} transcript and its regulation in tissues that have been demonstrated to express plasminogen binding sites suggests that Plg-R_{KT} provides plasminogen receptor function that may serve to modulate plasmin proteolytic functions (both physiologic and pathologic) specific to a large number of tissues. Thus, Plg-R_{KT} is likely to play a key role in plasminogen-dependent functions of cells including inflammation, wound healing, development, metastasis, neurite outgrowth fibrinolysis, myogenesis, and prohormone processing. Furthermore, the potential function of Plg-R_{KT} in the regulation of apoptosis and proliferation may play a key role in cancer and metastasis. Future studies with knockout mice should build on our initial results using MudPIT to elucidate the role of Plg-R_{KT}.

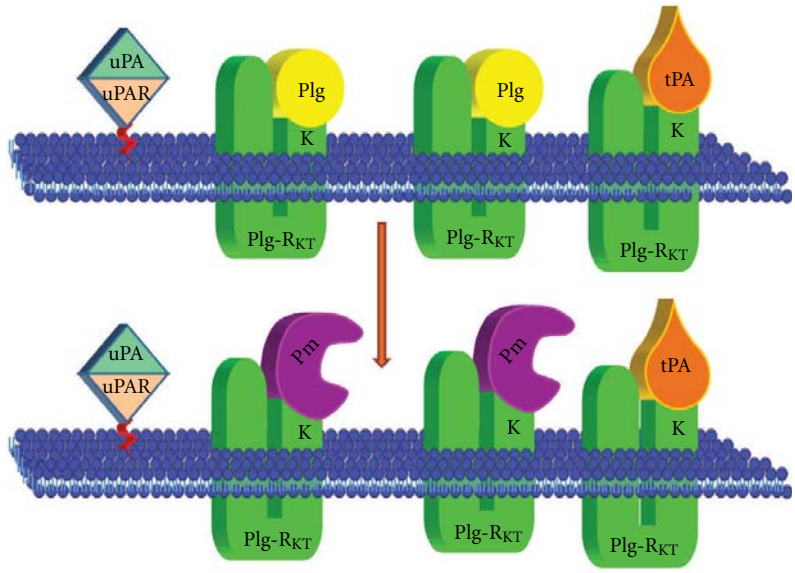

Figure 8: Model for Plg-R_{KT}-dependent cell surface plasminogen activation. Plg-R_{KT} is located on the monocyte surface in close physical proximity to the uPAR. The uPAR brings uPA in close proximity to plasminogen bound to Plg-R_{KT}, thus promoting activation of the bound plasminogen to plasmin. In addition, t-PA also interacts specifically with Plg-R_{KT}, thus mimicking the interaction of t-PA with cellular binding sites. Despite sharing a binding site on Plg-R_{KT}, the relative concentrations of tPA and plasminogen in the circulation should permit simultaneous binding of both ligands to the cell surface, and each tPA molecule should be bound proximally to several plasminogen molecules, thus promoting plasminogen activation to plasmin on the cell surface.

Acknowledgments

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Review Article

The Plasminogen Activation System and the Regulation of Catecholaminergic Function

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The local environment of neurosecretory cells contains the major components of the plasminogen activation system, including the plasminogen activators, tissue plasminogen activator (t-PA) and urokinase-type plasminogen activator (u-PA), as well as binding sites for t-PA, the receptor for u-PA (uPAR), and also the plasminogen activator inhibitor, PAI-1. Furthermore, these cells express specific binding sites for plasminogen, which is available in the circulation and in interstitial fluid. Colocalization of plasminogen and its activators on cell surfaces provides a mechanism for promoting local plasminogen activation. Plasmin is retained on the cell surface where it is protected from its inhibitor, α_2-antiplasmin. In neurosecretory cells, localized plasmin activity provides a mechanism for extracellular processing of secreted hormones. Neurotransmitter release from catecholaminergic cells is negatively regulated by cleavage products formed by plasmin-mediated proteolysis. Recently, we have identified a major plasminogen receptor, Plg-R_{KT}. We have found that Plg-R_{KT} is highly expressed in chromaffin cells of the adrenal medulla as well as in other catecholaminergic cells and tissues. Plg-R_{KT}-dependent plasminogen activation plays a key role in regulating catecholaminergic neurosecretory cell function.

1. Introduction

Chromaffin cells of the adrenal medulla and other neurosecretory cells contain specific binding sites for plasminogen [1, 2], which is available at high concentration in the circulation and in interstitial fluid. Furthermore, these cells secrete major components of the plasminogen activation system, including the plasminogen activators tissue plasminogen activator (t-PA) [3–7] and urokinase-type plasminogen activator (u-PA) [4, 8], and also the plasminogen activator inhibitors, PAI-1 [9] and neuroserpin [10]. Binding sites for t-PA [1, 8] and the receptor for u-PA (uPAR) are also expressed in these cells [8, 11–13]. Thus, the major components of the plasminogen activation system are present in the local neurosecretory cell environment.

Colocalization of plasminogen and plasminogen activators on cell surfaces results in promotion of local plasminogen activation [14]. In addition, cell-associated plasmin is protected from its major inhibitor, α_2-antiplasmin [15, 16]. In neurosecretory cells, localized plasmin activity provides a mechanism for extracellular processing of secreted hormones [1, 2]. For example, neurotransmitter release from catecholaminergic cells is negatively regulated by cleavage products formed by plasmin-mediated proteolysis of the major secretory vesicle core protein, chromogranin A (CgA) [1, 17]. Recently, we have identified a major plasminogen receptor, Plg-R_{KT} [18]. In this paper we summarize the interplay between components of the local neurosecretory cell plasminogen activation system, the functional consequences of the interaction of plasmin with catecholaminergic cells, and our recent studies demonstrating the expression and function of Plg-R_{KT} in catecholaminergic cells.

2. Components of the Local Chromaffin Cell Plasminogen Activation System: Plasminogen Activators, Plasminogen Activator Inhibitors, and Binding Sites for Plasminogen Activators

2.1. t-PA Expression and Its Targeting to the Regulated Secretory Pathway. The expression of t-PA, its subcellular localization, and its release in response to secretagogues have been investigated in several chromaffin cell sources.

t-PA synthesis and expression were demonstrated in rat PC12 cells [4–7], bovine adrenal chromaffin cells [7], human pheochromocytoma (a catecholamine producing tumor of the adrenal gland) [7], and adrenal medulla [3]. A variety of methods including Northern blotting, Western blotting, metabolic labeling and immunoprecipitation with specific anti-t-PA antibody, immunoassays, and enzymatic activity assays have been used to demonstrate the presence of authentic t-PA in these cells [7].

Within neuroendocrine cells, secretory proteins are sorted into one of two pathways, either the regulated secretory pathway or the constitutive secretory pathway [19, 20]. Proteins that enter the constitutive pathway are not stored but are transported directly to the cell surface and secreted in the absence of an extracellular signal. In contrast, proteins that enter the regulated pathway are concentrated and stored in vesicles and subsequently released upon stimulation with a secretagogue or other specific extracellular stimuli. Catecholamine storage vesicles within the chromaffin cell are prototypic examples of regulated secretory vesicles [20–23].

We examined the potential targeting of t-PA to the regulated secretory pathway by evaluating the subcellular localization of chromaffin cell t-PA in functional secretagogue release studies and using subcellular fractionation methods. In functional secretagogue-release studies, in which PC12 cells and bovine adrenal chromaffin cells were stimulated with a panel of secretagogues including nicotine (acting through nicotinic cholinergic receptors), KCl (a membrane depolarizing agent), and $BaCl_2$ (a calcium agonist), each of which causes exocytotic release of catecholamine storage vesicle content, significant increases in t-PA secretion occurred (Figure 1). Moreover, t-PA release occurred in parallel with release of catecholamines, consistent with release from the same subcellular pool, the catecholamine storage vesicle. The release of t-PA from PC12 cells also occurs in response to membrane depolarization with tetraethylammonium chloride and depends on the influx of calcium ion [6]. This rapid release is also consistent with release of presynthesized t-PA from a storage pool because no changes in gene expression or protein synthesis are required [6].

Subcellular fractionation studies using sucrose density gradients also demonstrated trafficking of t-PA to catecholamine storage vesicles [7]. Furthermore, catecholamine storage vesicle lysates isolated from human pheochromocytoma tumors were enriched 30-fold in t-PA antigen, compared with tumor homogenate [7]. The enrichment in t-PA antigen paralleled the enrichment in catecholamines, consistent with colocalization in the same subcellular fraction.

The localization of t-PA in secretory vesicles of PC12 cells has provided a key tool to study the mechanisms and kinetics of exocytosis. GFP-tagged t-PA has been used as a marker to understand axonal transport in nerve-growth-factor-(NGF-)treated PC12 cells. (When exposed to NGF, PC12 cells differentiate into cells that morphologically, biochemically, and electrophysiologically closely resemble sympathetic neurons [26].) Scalettar's group demonstrated that GFP-t-PA was targeted for regulated secretion from growth cones of NGF-differentiated PC12 cells and released in response to the calcium ionophore A23187 or the cholinergic agonist, carbachol [27], and used GFP-t-PA to demonstrate that secretory granules are mobile in growth cones of these cells [28]. In other studies with undifferentiated PC12 cells, fluorophore-tagged t-PA has been used as a marker to demonstrate: that most granules in PC12 cells reseal after exocytosis, resulting in the differential release of cargo [29], that synaptotagmin VII modulates kinetics of dense-core vesicle exocytosis in PC12 cells [30], that actin rearrangement [31] and myosin II [32] influence the time course of secretory granule release, and that newly synthesized dense-core vesicle cargoes are released preferentially compared to aged vesicle cargo [33].

Thus, in response to specific secretagogue stimulation, chromaffin cells release t-PA into the extracellular space. In addition, t-PA can be rapidly released into the circulation in response to stress [34–37]. Studies employing adrenergic stimulation and sympathectomy have demonstrated that sympathoadrenal and sympathoneural tissues may represent substantial sources contributing to changes in plasma t-PA concentrations [38, 39].

2.2. PAI-1 Is also Targeted to Catecholamine Storage Vesicles. As a potential mechanism for regulating t-PA activity, inhibitors of t-PA are present in catecholaminergic cells. We have recently demonstrated that plasminogen activator inhibitor-1 (PAI-1) is present in PC12 cells and bovine adrenal medullary chromaffin cells [9]. Secretagogue stimulation led to co-release of PAI-1 with catecholamines, consistent with storage in the same subcellular vesicle. Furthermore, immunoelectron microscopy and sucrose gradient fractionation studies demonstrated localization of PAI-1 in catecholamine storage vesicles [9]. In addition, parallel increases in plasma PAI-1 and catecholamines were observed in response to acute sympathoadrenal activation by restraint stress in mice *in vivo* [9]. Thus, the overall effect on plasminogen activation and fibrinolysis (both systemically and locally) from catecholamine storage vesicles will depend on a variety of factors that affect local t-PA/inhibitor balance, including the relative rates of synthesis of PAI-1 and t-PA, relative rates of trafficking to the vesicles, and the potential formation of t-PA/inhibitor complexes both within the vesicle and on release. Differential rates of exocytotic release of t-PA compared to PAI-1 could potentially take place as t-PA has been demonstrated to be released more slowly than other granule components [31, 32, 40]. In addition to PAI-1, the t-PA inhibitor, neuroserpin, is present in dense-core secretory vesicles within PC12 cells [10] and a targeting sequence for regulated secretion of neuroserpin has been identified [41].

2.3. t-PA Binding Sites on Catecholaminergic Cells. An additional key mechanism by which local t-PA function is regulated is by the presence of binding sites for t-PA on catecholaminergic cells, as initially demonstrated by Pittman and colleagues [8]. We found that the interaction of t-PA with PC12 cells was saturable and of high capacity [1]. Furthermore, the lysine analog, ε-aminocaproic acid (EACA), also inhibited the interaction [1]. Thus, the recognition

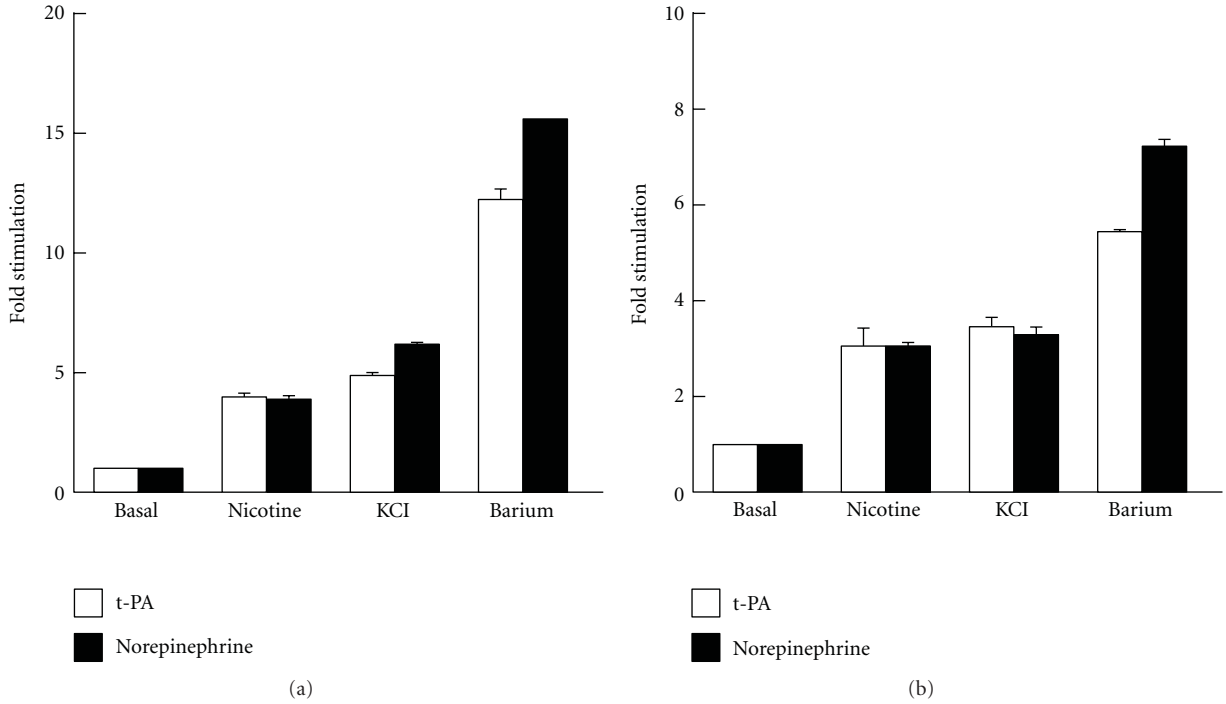

Figure 1: Corelease of t-PA with norepinephrine from catecholaminergic cells. PC12 cells (a) or bovine adrenal chromaffin cells (b) were labeled with [^{3}H]-norepinephrine and incubated at 37°C for 30 min in either the absence or presence of 60 μM nicotine, 55 mM KCl, or 2 mM $BaCl_2$. The release buffer was aspirated, cells were harvested and lysed, and the release of t-PA antigen (open bars) and [^{3}H]-norepinephrine (filled bars) was determined. [7]. ©The American Society for Biochemistry and Molecular Biology.

specificity of t-PA for chromaffin cells is likely to be dependent on receptors that express C-terminal lysines. Thus, t-PA, released in response to secretagogue stimulation, can bind to its receptors for further amplification of plasminogen activator activity in the local environment of the chromaffin cell.

2.4. uPA and uPAR Expression in Chromaffin Cells. The synthesis and secretion of uPA by chromaffin cells has not been addressed extensively in the literature. uPA secretion by PC12 cells has been detected but accounts for only 5% of the plasminogen activator activity in the long-term conditioned media of these cells; the remaining 95% of plasminogen activator activity is contributed by t-PA [4]. In studies of sympathetic neurons, uPA appears to be released as soon as it is synthesized [8]. Thus, the constitutive release of u-PA may be the predominant source of plasminogen activator activity under basal conditions (on unstimulated chromaffin cells) while the action of t-PA, released in response to secretagogue stimulation, may predominate following its exocytotic release and subsequent binding to the chromaffin cell surface.

The urokinase receptor (uPAR) is also present on PC12 cells. We characterized the binding of single chain u-PA (scu-PA) to untreated PC12 cells. Human scu-PA bound specifically and saturably to PC12 cells (Figure 2(a)) with a Kd of 4.3 $\pm$ 0.54 nM and 1.1 $\pm$ 0.26 $\times$ 10^4 sites/cell [11]. A representative binding isotherm and Scatchard analysis are shown in Figures 2(a) and 2(b). Thus, the species specificity that was initially observed (human u-PA does not interact with murine cells and murine u-PA does not interact with human uPAR [42, 43]) does not apply to all interspecies interactions. (Human scu-PA also bound specifically to hamster CHO cells, but minimally to bovine aortic endothelial cells [11, 44]). The affinity of scu-PA for the PC12 cell surface that we determined is consistent with the presence of a functional uPAR on the surface of undifferentiated PC12 cells. In support of this, uPAR mRNA was prominently expressed in PC12 cells as assessed by Northern blotting (Figure 2(c)). Furthermore, we cloned rat uPAR from PC12 cells [11] (Figure 2(d)), and the sequence of the clone we obtained was identical to the sequence published for rat osteoclast uPAR [45]. In addition, using a specific uPAR ELISA, we found substantial expression of uPAR in human pheochromocytoma tissue samples [48 $\pm$ 8.2 pg/mg protein (n = 6)] [11].

With NGF-treated PC12 cells, binding sites for u-PA that did not require an active site in u-PA for the interaction were first detected immunochemically by Pittman and colleagues on the bottom surface of the cells [8]. Subsequently, Herschman and colleagues identified uPAR as a neurotrophin responsive gene (preferentially induced by NGF versus EGF) in PC12 cells [12, 46] and demonstrated that NGF-induced uPAR expression is required for NGF-driven PC12 cell differentiation [12, 46, 47]. Induction of uPAR requires ERK/JNK signaling [48], binding of specific Fos and Jun family members to a specific AP-1 site [49, 50], and is regulated by SH2B1β [51].

Thus, taken together, these studies suggest that multiple plasminogen activator receptors can be present simultaneously on the neurosecretory cell surface.

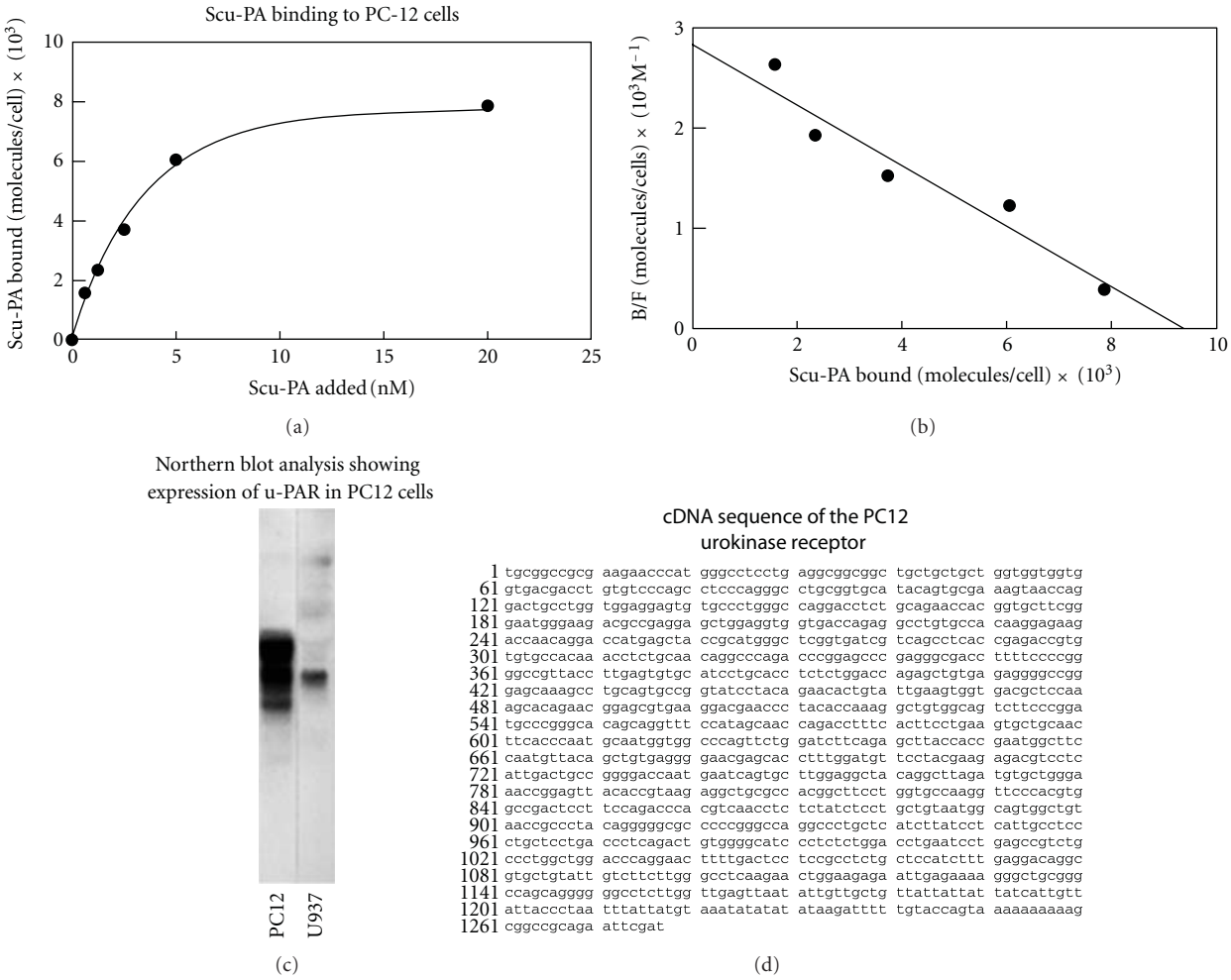

Figure 2: Expression of uPAR by PC12 cells. (a) Binding isotherm of ^{125}I-scu-PA to PC12 cells. Cells in suspension were incubated with ^{125}I-scu-PA, and bound ligand was separated from free by centrifugation over sucrose. Nonspecific binding was determined in the presence of unlabeled scu-PA. (b) Scatchard analysis of the binding isotherm in Panel A. (c) Northern blot analysis showing expression of uPAR in PC12 cells. Poly(A)-enriched RNA was isolated from PC12 cells and Northern blotted with a 458 bp SMaI + BamHI (nuc. 41–493) human uPAR cDNA probe. Northern blotting of U937 cell poly(A) RNA is shown for comparison. (d) cDNA sequence of PC12 uPAR. The rat uPAR sequence was obtained by screening a PC12 cDNA library using a 609 bp EcoRI + NcoI (nuc. 110–718) human uPAR cDNA probe labeled with [α-^{32}P] dCTP.

3. Characteristics of the Interaction of Plasminogen with Chromaffin Cells

As a mechanism for concentrating the activity of plasminogen in the plasminogen activator-rich local environment of the chromaffin cell, we found that plasminogen bound to PC12 and bovine adrenal chromaffin cells in a specific, saturable, and reversible manner [1, 2]. Plasminogen activation is markedly enhanced when plasminogen is bound to chromaffin cells. In the presence of PC12 cells, plasminogen activation by t-PA is markedly increased in a cell-dependent fashion, and at the saturating cell density, the enhancement caused by the presence of cells is 6.5-fold [24]. Primary bovine chromaffin cells also markedly stimulate t-PA-dependent plasminogen activation up to 10-fold in a cell concentration-dependent manner [24].

The interactions of plasminogen with substrates and regulatory molecules depend on the lysine binding sites within the disulfide-bonded kringle structures of plasminogen (reviewed in [52]). The interaction of plasminogen with PC12 and bovine adrenal chromaffin cells is blocked by the lysine analogues, EACA and 8-aminooctanoic acid [1, 2], suggesting that proteins with C-terminal lysines, exposed on the cell surface serve as plasminogen binding sites. To explore the relationship between plasminogen binding and stimulation of plasminogen activation, PC12 cells were treated with increasing concentrations of carboxypeptidase B (CpB) that has a preference for cleavage at the carboxyl side of basic amino acids. A dose-dependent decrease in plasminogen binding to PC12 cells was observed, reaching a plateau at 70% inhibition in the presence of CpB (Figure 3(a)) [24]. In addition,

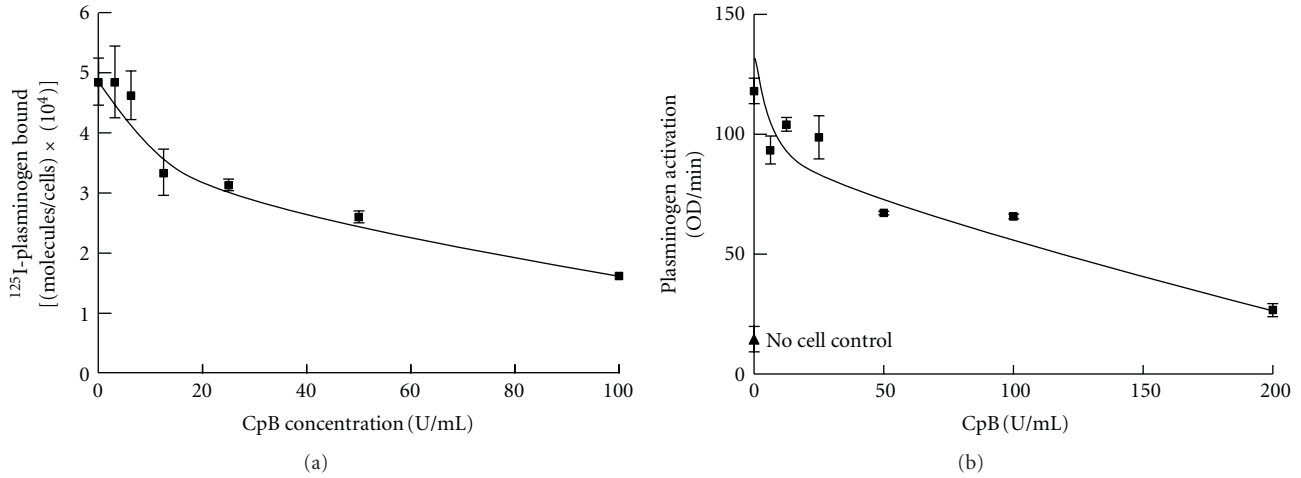

Figure 3: Effect of treatment of PC12 cells with CpB on the interaction with plasminogen. PC12 cells were incubated with increasing concentrations of CpB and specific binding of 0.2 μM ^{125}I-plasminogen (a) and plasminogen activation (b) were assessed as described in [24].

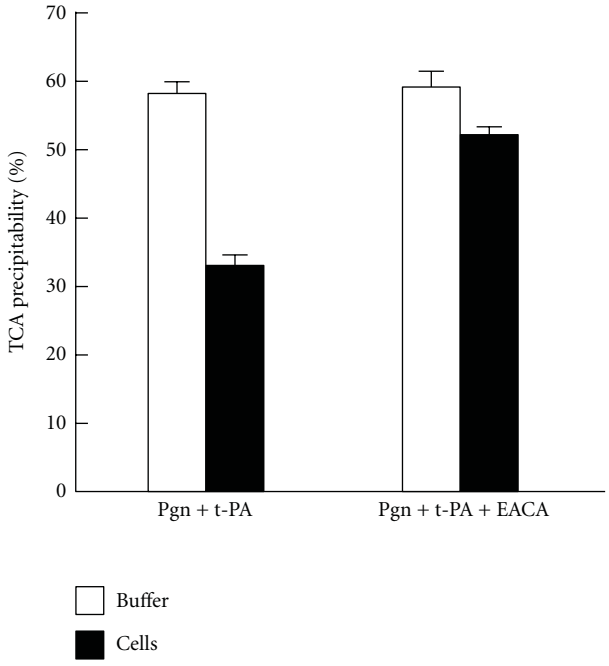

Figure 4: Effect of cells on cleavage of ^{125}I-CgA by plasmin. Either PC12 cells (filled bars) or buffer (open bars) was incubated with unlabeled plasminogen. Then, ^{125}I-CgA and t-PA were added in either the presence or the absence of EACA. The reactions were stopped by addition of aprotinin and centrifuged. The supernatants were analyzed for % TCA precipitability. Results are as mean ± SEM; $n = 6$ for each experimental group. $^{**}P < 0.001$ compared to each of the experimental groups. [1].

CpB treatment decreased the stimulating effect of the cells on plasminogen activation in a dose-dependent manner, approaching a plateau at 88% inhibition (Figure 3(b)) [24]. These results suggest that the chromaffin cell plasminogen receptors that are sensitive to CpB (i.e., proteins exposing C-terminal basic residues on the extracellular face of the cell membrane) are primarily responsible

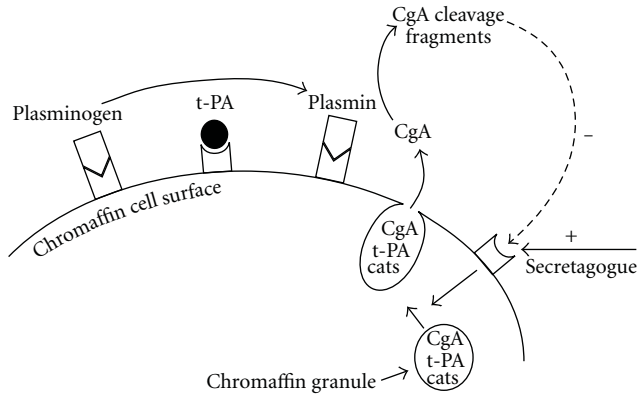

Figure 5: Working model for a local (autocrine/paracrine) chromaffin cell plasminogen/plasmin system and its interactions with CgA. Upon stimulation of the chromaffin cell by a secretagogue, CgA and catecholamines (cats) are coreleased by exocytosis. Plasminogen (from circulating sources) and its activator t-PA (synthesized and secreted from the chromaffin cell) bind to the chromaffin cell surface, with resultant activation of plasminogen to plasmin. CgA subsequently is cleaved by plasmin to liberate peptide fragments, which provides a negative feedback loop to modulate subsequent catecholamine release. [1].

for the promotion of plasminogen activation by these cells.

4. Role of the Local Chromaffin Cell Plasminogen Activation System in Prohormone Processing

Plasmin functions as a prohormone-processing protease within the neuroendocrine system [1, 17, 53–57]. We used the prototypical prohormone chromogranin A (CgA) to study the role of the local chromaffin cell plasminogen activation system in prohormone processing. CgA is the

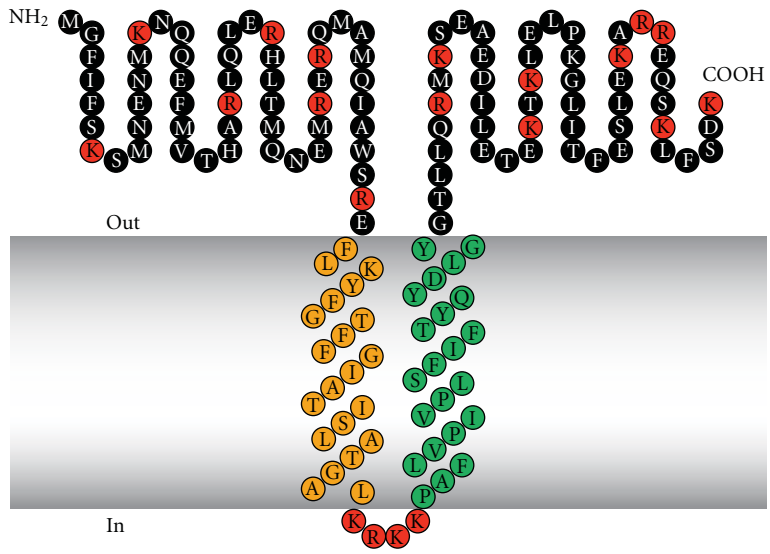

FIGURE 6: Structural model of Plg-R_{KT}. Green indicates amino acids within the predicted primary transmembrane helix. Orange indicates amino acids within the predicted secondary transmembrane helix. Red indicates basic amino acids. [18].

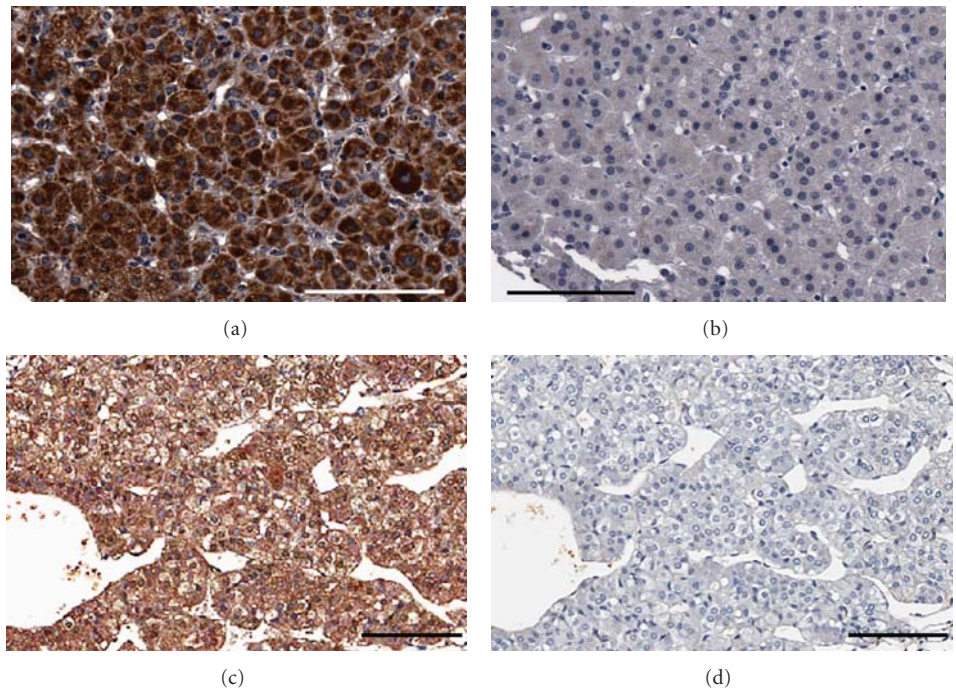

FIGURE 7: Expression of Plg-R_{KT} in adrenal medulla. Human (a, b) and mouse (c, d) adrenal medulla tissue sections were stained with anti-Plg-R_{KT} mAb, in the absence (a, c) or the presence (b, d) of the decapeptide used for immunization, CEQSKFFSDK (corresponding to the nine C-terminal amino acids of rat Plg-R_{KT} with an amino terminal cysteine added for coupling). Positive tissues are indicated by brown staining with DAB. Scale bar = 100 μm. [25]. ©The American Society for Biochemistry and Molecular Biology.

major soluble protein in the core of catecholamine storage vesicles and is released with catecholamines in response to secretagogue stimulation of chromaffin cells of the adrenal medulla and from sympathetic neurons [58]. CgA is present ubiquitously in secretory vesicles of neuroendocrine cells [59] and serves as a precursor of several small, biologically active, secretion-inhibitory peptides that play a key autocrine regulatory role in neuroendocrine secretion from a variety of cell types [60–67]. In the case of chromaffin cells and other catecholaminergic cells, CgA serves as a precursor from which peptides are released that modulate catecholamine secretion, contributing to an autocrine, homeostatic, negative-feedback mechanism for regulating catecholamine responses during stress [65–67].

We found that plasmin processing of CgA produces regulatory secretion-inhibiting peptides. Plasmin specifically

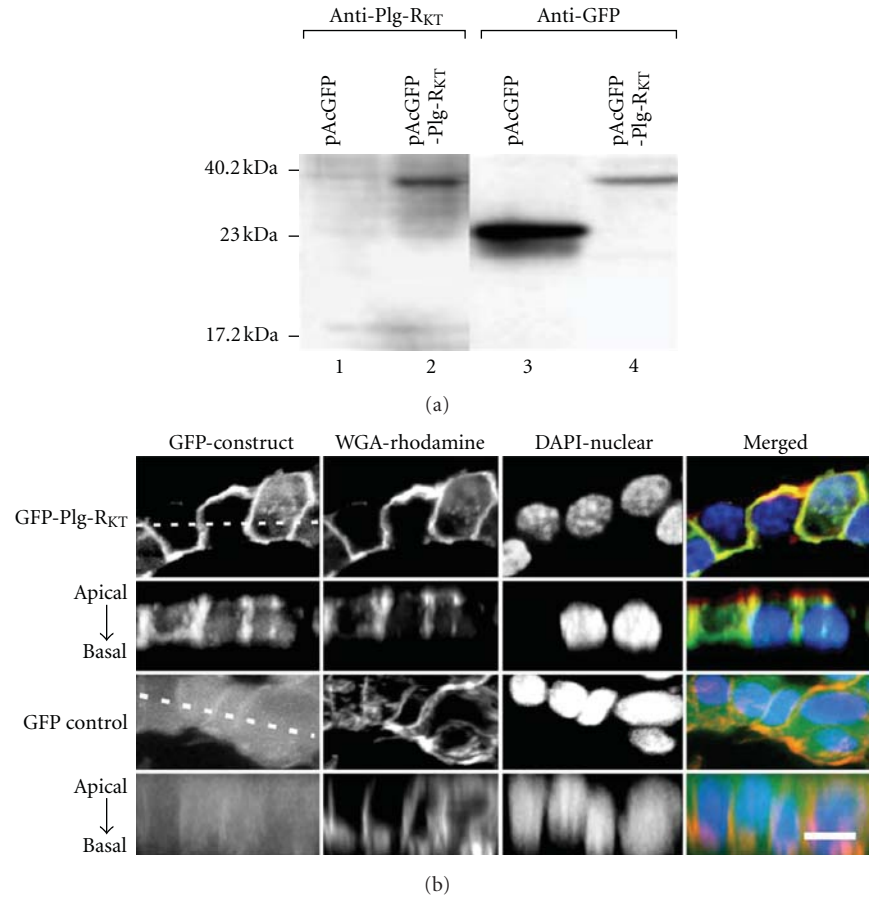

Figure 8: Plg-R$_{KT}$ re-directs GFP to the cell membrane. PC12 cells were transiently transfected with pAcGFP-Plg-R$_{KT}$ (in which the Plg-R$_{KT}$ cDNA was inserted in frame for expression of a GFP-Plg-R$_{KT}$ fusion protein, with Plg-R$_{KT}$ at the C-terminus) or with control pAcGFP vector. (a) Cell lysates were Western blotted with polyclonal anti-Plg-R$_{KT}$ IgG (lanes 1 and 2) or anti-GFP IgG (lanes 3 and 4). (b) The transfected cells were stained with a combination of DAPI and WGA-rhodamine. The first and third panels of images represent maximum projections of a series of optical slices through the cells. The GFP-Plg-R$_{KT}$ signal was localized primarily to the plasma membrane. The second and fourth panels of images represent sagittal (apical-basal) slices through the same cells along the white dotted line indicated. Here the peripheral plasma membrane localization of GFP-Plg-R$_{KT}$ is also evident throughout the vertical stacks of images that were acquired. Scale bar in bottom right corner of image represents 10 microns. [25]. ©The American Society for Biochemistry and Molecular Biology.

cleaved CgA and decreased the trichloroacetic acid (TCA) precipitability of ^{125}I-CgA, indicating that fragments with Mr < 6,000 had been produced [1, 17]. Most importantly, the peptide fragments generated by plasmic cleavage of CgA inhibited secretagogue-stimulated catecholamine release from PC12 and primary bovine adrenal cells [1]. We identified the specific bioactive CgA peptide produced by plasmin proteolysis [using matrix-assisted laser desorption/ionization mass spectrometry (MALDI)] as a major peptide with a mass/charge ratio (*m/z*) of 1546 corresponding uniquely to hCgA-(360–373) [17]. In functional studies the hCgA-(360–373) peptide markedly inhibited nicotine-stimulated catecholamine release from pheochromocytoma cells [17].

Chromaffin cells had a marked effect on CgA cleavage by plasmin [1]. In the presence of PC12 cells, the TCA precipitability of ^{125}I-CgA was markedly decreased, indicative of extensive CgA processing (Figure 4) [1]. EACA inhibited processing in the presence, but not in the absence of cells (Figure 4) [1]. Because EACA inhibits plasminogen binding to chromaffin cells, these data suggest a significant contribution of cellular plasminogen receptors to CgA processing.

We examined the effect of local modulation of the plasminogen activation system on nicotine-mediated catecholamine secretion. Overexpression of t-PA in PC12 cells resulted in a marked (81 ± 2%) inhibition of nicotine-stimulated catecholamine release compared with control cells [1]. In the presence of anticatalytic antiplasminogen mAb, catecholamine secretion was markedly increased, compared with isotype control [1]. Thus, the effect of the anticatalytic anti-plasminogen mAb was to restore nicotine-mediated secretion in these cells.

We present a working model for the function of the local chromaffin cell plasminogen activation system in the processing of CgA in Figure 5 [1]. Upon stimulation of the chromaffin cell by a secretagogue, CgA and catecholamines

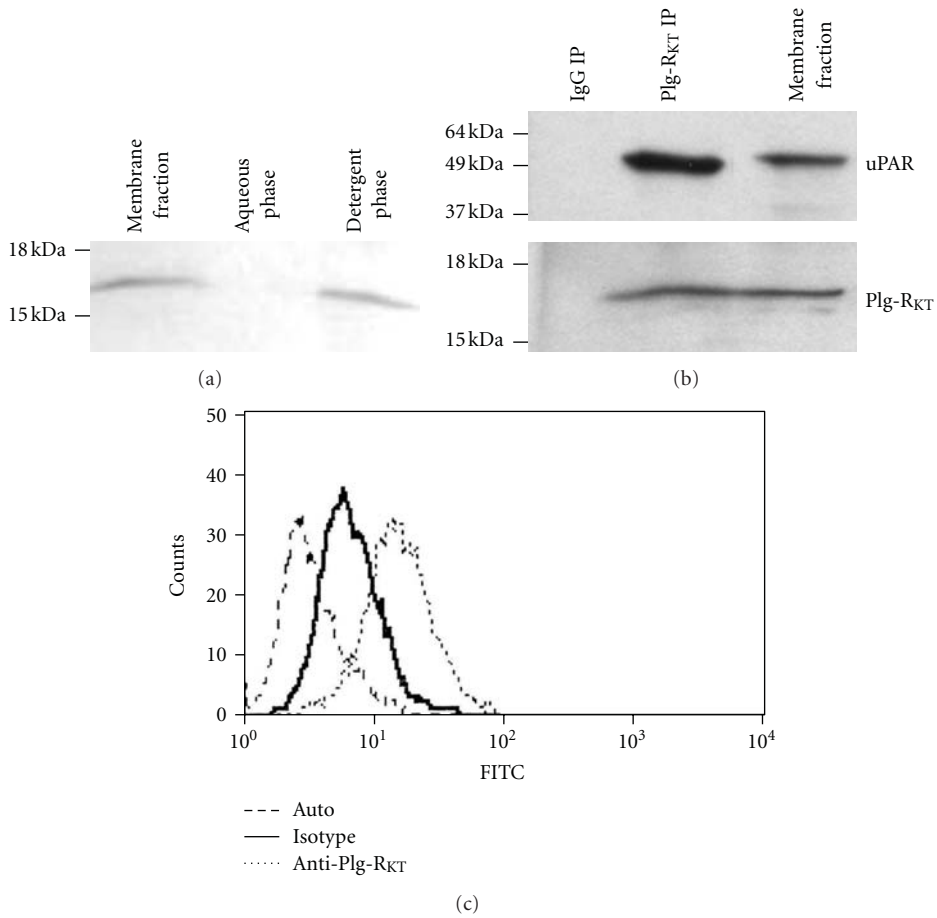

Figure 9: Plg-R$_{KT}$ is an integral plasma membrane cell surface protein. (a) Phase partitioning of Plg-R$_{KT}$. An immunoreactive band corresponding to the Mr$_{app}$ of Plg-R$_{KT}$ was detected in the detergent phase, but not in the aqueous phase. (b) Coimmunoprecipitation of Plg-R$_{KT}$ with uPAR. (c) FACS analysis of Plg-R$_{KT}$ expression on intact PC12 cells. Viable cells were gated from nonviable cells and histogram plots of viable cells are shown. Dotted tracings: anti-Plg-R$_{KT}$ mAb IgG. Black tracings: isotype control IgG. Dashed tracings: autofluorescence. [25]. ©The American Society for Biochemistry and Molecular Biology.

are coreleased by exocytosis. Plasminogen (from circulating sources) and t-PA (synthesized and secreted from the chromaffin cell) bind to the chromaffin cell surface, resulting in activation of plasminogen to plasmin. CgA is subsequently cleaved by plasmin to liberate a peptide fragment that provides a negative feedback loop to modulate subsequent catecholamine release.

5. Expression and Subcellular Localization of Plg-R$_{KT}$ in Chromaffin Cells

In earlier studies of chromaffin cell plasminogen receptors, we identified catecholaminergic plasminogen receptors required for enhancing plasminogen activation using targeted specific proteolysis with CpB and a proteomics approach using two-dimensional gel electrophoresis, radioligand blotting, and tandem mass spectrometry. Two major plasminogen-binding proteins that exposed C-terminal lysines on the cell surface contained amino acid sequences corresponding to β/γ actin [24]. An antiactin monoclonal antibody inhibited cell-dependent plasminogen activation and also enhanced nicotine-dependent catecholamine release [24], suggesting that cell-surface-expressed forms of actin (with a processed C-terminus to generate a C-terminal lysine) bind plasminogen, thereby promoting plasminogen activation and increased prohormone processing, leading to inhibition of neurotransmitter release. Although cell surface actin accounted for a substantial fraction of plasminogen binding and activation, a critical role for other cell surface plasminogen-binding proteins with C-terminal lysines was also suggested on these cells. Notably, in plasminogen ligand blotting of 2D gels of PC12 membrane fractions, we also detected a major unknown CpB-sensitive protein migrating with an M$_{rapp}$ of 17,200 [24].

Recently, we isolated a structurally unique plasminogen receptor from monocyte progenitor cells, the novel protein, Plg-R$_{KT}$ [18]. The Plg-R$_{KT}$ protein is composed of 147 amino acids and has a molecular mass of 17,261 Da. Plg-R$_{KT}$ is synthesized with and exposes a C-terminal lysine on the cell surface, in an orientation to bind plasminogen and to promote plasminogen activation (Figure 6) [18]. Therefore,

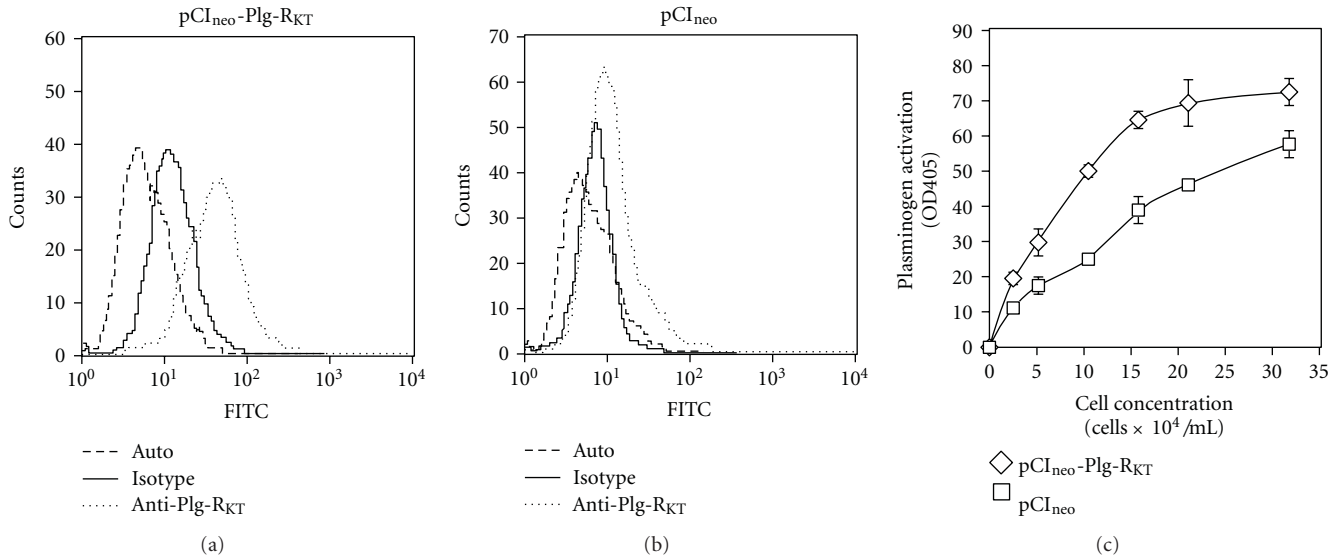

Figure 10: Overexpression of Plg-R$_{KT}$ increases cell surface plasminogen activation. PC12 cells stably overexpressing Plg-R$_{KT}$ (pCIneo-Plg-R$_{KT}$) (a) or vector alone (pCIneo) (b) were analyzed by dual color FACS analysis. Viable cells were gated from nonviable cells and histogram plots of viable cells are shown. Dotted tracings: anti-Plg-R$_{KT}$ mAb IgG. Black tracings: isotype control IgG. Dashed tracings: autofluorescence. (c) PC12 cells stably overexpressing Plg-R$_{KT}$ (pCIneo-Plg-R$_{KT}$) or vector alone (pCIneo) were incubated with plasminogen and t-PA was added and plasminogen activation was measured. Cell-mediated plasminogen activation was substantially greater in Plg-R$_{KT}$ overexpressing cells than in control cells ($P < 0.001$ at each cell concentration tested) ($n = 3$). [25]. ©The American Society for Biochemistry and Molecular Biology.

we investigated expression of Plg-R$_{KT}$ in human and murine adrenal tissues. Prominent staining with anti-Plg-R$_{KT}$ mAb was observed in adrenal medullary chromaffin cells in human (Figure 7(a)) and murine (Figure 7(c)) adrenal tissue [25]. Plg-R$_{KT}$ was also prominently expressed in PC12 cells, bovine adrenal chromaffin cells, and human pheochromocytoma and also in murine hippocampus, representing a nonadrenal source of catecholaminergic cells [25].

To assess the subcellular localization of Plg-R$_{KT}$ in catecholaminergic cells and to determine whether the Plg-R$_{KT}$ protein contains a dominant plasma membrane trafficking signal, we transfected PC12 cells with pAcGFP-Plg-R$_{KT}$ (an expression vector in which the Plg-R$_{KT}$ cDNA was inserted in-frame for expression of a GFP-Plg-R$_{KT}$ fusion protein with Plg-R$_{KT}$ at the C-terminus). In confocal microscopy of nonpermeabilized fixed cells, GFP-Plg-R$_{KT}$ showed membrane localization that was highly colocalized with wheat germ agglutinin (WGA) (a well-established cell surface marker) (Figure 8) [25]. In Triton X-114 phase separation experiments [42, 68] Plg-R$_{KT}$ was detected in the detergent phase, but was not detected in the aqueous phase (Figure 9(a)) [25], consistent with Plg-R$_{KT}$ behaving as an integral membrane protein in these cells. Furthermore, anti-Plg-R$_{KT}$ antibody immunoprecipitated both Plg-R$_{KT}$ and uPAR from membrane fractions of PC12 cells (Figure 9(b)) [25], providing further demonstration of the cell membrane localization of Plg-R$_{KT}$. The physical association of these receptors suggests a key mechanism for promoting plasminogen activation via colocalization of uPA activity (bound to uPAR) with the substrate, plasminogen (bound to Plg-R$_{KT}$). Furthermore, t-PA also binds to the C-terminus of Plg-R$_{KT}$ [18]. Thus, binding of t-PA and plasminogen to adjacent Plg-R$_{KT}$ molecules may also serve as a means to promote plasminogen activation on the neurosecretory cell surface.

In additional studies to address the cell surface orientation of the C-terminus of Plg-R$_{KT}$, fluorescence activated cell surface (FACS) analysis with anti-Plg-R$_{KT}$ mAb (raised against a synthetic peptide corresponding to the C-terminal peptide of Plg-R$_{KT}$) demonstrated prominent and specific binding of the mAb to the PC12 cell surface, indicating both cell membrane localization and exposure of the C-terminus of Plg-R$_{KT}$ on the cell surface (Figure 9(c)).

The above studies do not exclude the possibility that other plasminogen receptors with C-terminal lysines (including α-enolase [69], S100A10 [70, 71], Histone H2B [72], and TIP49a [73]) may participate in plasminogen binding on these cells.

6. Regulation of Catecholaminergic Neurosecretory Cell Function by Plg-R$_{KT}$

Based on our studies reviewed above (Section 3), we investigated whether Plg-R$_{KT}$ played a role in promoting plasminogen activation. We stably overexpressed Plg-R$_{KT}$ in PC12 cells, which resulted in prominent expression of Plg-R$_{KT}$ on the cell surface that was markedly greater than the expression of endogenous Plg-R$_{KT}$ (Figure 10) [25]. When cells were stably transfected with Plg-R$_{KT}$, plasminogen activation was markedly enhanced compared to cells transfected with empty vector (Figure 10(c)) [25]. These results are consistent with a

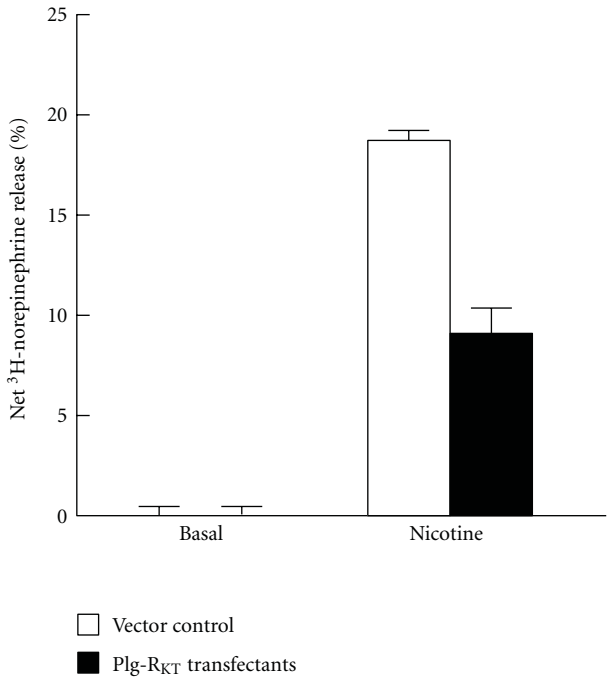

FIGURE 11: Effect of overexpression of Plg-R_{KT} on catecholamine release. PC12 cells stably overexpressing either Plg-R_{KT} (filled bars) or vector alone (open bars) were treated with 60 μM nicotine (Nicotine) or buffer (Basal) at 37°C for 15 minutes and catecholamine release was measured by liquid scintillation counting. Percent release was calculated as percentage of secretion [amount released/(amount released + amount in cell lysate)] and results expressed as net release (% secretagogue-stimulated release minus % basal release). Results are represented as mean ± SEM, $n = 9$ for each experimental group. $^{**}P < 0.001$ for the Plg-R_{KT} transfectants stimulated with nicotine compared with corresponding values for the vector control cells. [25]. ©The American Society for Biochemistry and Molecular Biology.

major role for Plg-R_{KT} in cell surface-dependent stimulation of plasminogen activation.

Based on our studies reviewed above (Section 4), we tested the effect of overexpression of Plg-R_{KT} on secretagogue-stimulated catecholamine release. Norepinephrine release in response to nicotine was markedly sup-pressed in cells overexpressing Plg-R_{KT} when compared with release from control cells (Figure 11) [25]. This result is consistent with processing of prohormones by plasmin to produce peptides that feed back to inhibit catecholamine release as outlined in our model in Figure 5.

7. Conclusions

The plasminogen activation system plays a major role in catecholaminergic cell function by processing secreted hormones that feed back to regulate the neurosecretory characteristics of these cells. In this paper, we summarize results suggesting that Plg-R_{KT} is a crucial molecular focal point in the regulation of the cell-surface-dependent mechanism underlying the ability of catecholaminergic cells to promote local plasminogen activation. Expression of Plg-R_{KT} and additional binding sites for plasminogen [1, 24] and t-PA [1, 8], along with trafficking of t-PA to catecholamine storage vesicles [7, 74], constitute a local catecholaminergic cell plasminogen activation system that regulates cell surface-dependent neuroendocrine prohormone processing (after secretagogue-stimulated storage vesicle exocytosis) that plays a key role in the regulation of neurotransmitter release. In addition, constitutive release of low levels of uPA [4] and subsequent binding to the neurosecretory cell uPAR [11] may also contribute to prohormone processing and to the regulation of differentiation of neuronal cells [12, 46, 47]. These processes are, in turn, locally regulated by the presence of PAI-1 [9] and neuroserpin [10], which are secreted in a regulated fashion, concomitantly with t-PA.

Components of the plasminogen activation system are expressed broadly in neuroendocrine sites, including the cerebral cortex [75], cerebellum [75–77], hippocampus [75, 77–81], sympathetic neurons [39, 82] as well as the adrenal medulla [7, 77]. Notably, the transcript for Plg-R_{KT} is expressed in each of these tissues (http://www.ebi.ac.uk/gxa/). Future studies to develop and characterize Plg-R_{KT} deficient mice and studies with mice deficient in other plasminogen receptors are warranted to address the role of Plg-R_{KT} and other plasminogen receptors in key plasminogen- and t-PA-dependent neuronal/neuroendocrine plasminogen-dependent processes, including neurite outgrowth [5, 83, 84]; synaptic transmission, NMDA receptor-mediated signaling and excitotoxin-induced neuronal degeneration [85, 86]; long-term potentiation, learning, and memory [56, 78, 80, 87–90]; cleavage and activation of other neuroendocrine substrates such as the neurotrophin proBDNF (brain-derived neurotrophic factor) [56], β-endorphin, and α-melanocyte-stimulating hormone [57]; and systemic metabolic and cardiovascular physiologic responses under the control of sympathoadrenal and sympathoneuronal activities [1, 17, 39, 55].

Acknowledgments

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Review Article

The Annexin A2/S100A10 System in Health and Disease: Emerging Paradigms

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Since its discovery as a src kinase substrate more than three decades ago, appreciation for the physiologic functions of annexin A2 and its associated proteins has increased dramatically. With its binding partner S100A10 (p11), A2 forms a cell surface complex that regulates generation of the primary fibrinolytic protease, plasmin, and is dynamically regulated in settings of hemostasis and thrombosis. In addition, the complex is transcriptionally upregulated in hypoxia and promotes pathologic neoangiogenesis in the tissues such as the retina. Dysregulation of both A2 and p11 has been reported in examples of rodent and human cancer. Intracellularly, A2 plays a critical role in endosomal repair in postarthroplastic osteolysis, and intracellular p11 regulates serotonin receptor activity in psychiatric mood disorders. In human studies, the A2 system contributes to the coagulopathy of acute promyelocytic leukemia, and is a target of high-titer autoantibodies in patients with antiphospholipid syndrome, cerebral thrombosis, and possibly preeclampsia. Polymorphisms in the human *ANXA2* gene have been associated with stroke and avascular osteonecrosis of bone, two severe complications of sickle cell disease. Together, these new findings suggest that manipulation of the annexin A2/S100A10 system may offer promising new avenues for treatment of a spectrum of human disorders.

1. The Annexin A2/S100A10 System

1.1. Assembly of the $(A2{\cdot}p11)_2$ Complex. Annexin A2 (A2) belongs to the annexin family of Ca^{2+}-regulated phospholipid binding proteins, which are expressed in plants, animals, and protists throughout the phylogenetic tree [1]. A2 is a 36-kilodalton protein produced by endothelial cells, monocytes, macrophages, trophoblast cells, and some tumor cells and exists both free in the cytoplasm and in association with intracellular and plasma membrane surfaces [2, 3]. The human *ANXA2* gene consists of 13 exons distributed over 40 kb of genomic DNA on chromosome 15 (15q21) [4]. Among mammalian species for which A2 has been sequenced, identity is approximately 98% at the amino acid level.

When A2 is membrane associated, the tightly packed, alpha-helical 33-kDa core domain forms a disk whose convex face is associated with membrane phospholipid and whose concave face is oriented away from the membrane. Membrane binding is mediated by at least two potential Ca^{2+}-binding "annexin" repeats, features common to all annexin family proteins [3]. While the core domains of the annexin

proteins are relatively well conserved, the hydrophilic amino-terminal "tail" or "interaction" domains are highly variable and essentially unique to each family member.

Protein S100A10, also known as p11, is a well-described binding partner of A2 [7, 8]. As a member of the S100 family of proteins, p11 contains Ca^{2+}-binding helix-loop-helix motifs and confers increased phospholipid binding affinity on A2. Typical S100 proteins undergo a conformational change upon Ca^{2+}-binding that places helix III (HIII) in a perpendicular orientation relative to helix IV(HIV), thus forming a cleft that can accept associated target proteins [9]. This calcium activation rule, however, does not apply to p11, which has permanently assumed a "calcium-on" state, due to replacement of the bidentate E^{65} by S^{70}, and the monodentate D^{56} with C^{61} [10]. The published crystal structure of p11 in complex with the N-terminal 13 amino acids of A2 suggests that the basic unit of p11 structure is a noncovalently linked homodimer, each component of which can bind the A2 tail peptide to form a heterotetramer [10]. Upon binding, the A2 tail peptide assumes an α-helical conformation that presents key hydrophobic residues (V^3, I^6, L^7, and L^{10}) within a cleft formed by loop L2 and helix HIV of one monomer and helix HI of the other. The C-terminal region of p11, particularly its hydrophobic residues within the C-terminal extension (Y^{85}FVVHM^{90}), such as Y^{85} and F^{86}, contributes critical contact points for binding to A2 [10].

1.2. The $(A2{\cdot}p11)_2$ Complex and Fibrinolysis. The primary fibrinolytic protease, plasmin, is formed upon cleavage of plasminogen at a single peptide bond at position R^{560}-V^{561} by either of two serine proteases, tissue plasminogen activator (tPA), produced by vascular endothelial cells, or urokinase (uPA) [11–13]. tPA-dependent plasminogen activation is dramatically accelerated in the presence of fibrin, and to a lesser extent by cell surface fibrinolytic receptors. uPAR is expressed by monocyte/macrophages, tumor cells, and activated endothelial cells [14, 15], while the $(A2{\cdot}p11)_2$ complex is expressed on both resting and activated endothelial cells [16, 17]. In addition, an interesting array of plasminogen-binding receptors, including α-enolase [18], TATA-box protein interacting protein (TIP49) [19], histone H2B [20], $\alpha_M\beta_2$ integrin [21], amphoterin [22], and Plg-R_{KT}, have been identified on many cell types [23].

On cell surfaces, the $(A2{\cdot}p11)_2$ complex serves as an assembly site for plasminogen and tPA [16, 17, 25, 26]. Although it is clear that heterotetramer-mediated colocalization of activator and substrate accelerates plasmin generation, there are, interestingly, two main theories as to the exact site of interaction of plasminogen and tPA with components of the heterotetramer complex. While one group suggests p11 as the key ligand interaction site and annexin A2 as the molecule that anchors it to the plasma membrane [26], another proposes annexin A2, in complex with p11, as the ligand binding site [17]. A third group has suggested that, in the context of the cell surface and its proteolytic milieu, both annexin A2 and p11 may have exposed lysine residues that are accessible to the lysine binding "kringle" domains of both tPA and plasminogen [27]. Detailed evidence for each view is outlined in the works cited above.

Translocation of A2 to the outer leaflet of the plasma membrane of the endothelial cell is a key regulatory step governing vascular fibrinolysis [16, 17]. Although cell surface appearance of A2 has been linked to plasma membrane fusion of multivesicular bodies in NIH 3T3 fibroblasts [28], and as a consequence of membrane disruption upon exocytosis of secretory granules in chromaffin cells [29], it is not clear whether similar mechanisms apply to the endothelial cell. Endothelial cell translocation, which can occur within minutes, is initiated by several factors including heat stress, thrombin stimulation, and hypoxia [30–32] and is known to require the presence of adequate p11 as well as src kinase phosphorylation at Y^{23}. A2 was originally identified as a src kinase substrate [33], and translocation is driven by activation of pp60src [31].

1.3. Regulation at the Cell Surface. Translocation of A2 to the cell surface is dependent upon the abundance of p11. In the endothelial cell, p11 is stabilized by A2, which, upon binding, masks a critical "degron," or polyubiquitination site on p11. In the absence of sufficient A2, p11 is polyubiquitinated and targeted to the proteasome for degradation [34]. In $AnxA2^{-/-}$ mice, which demonstrate low to nondetectable p11 expression, treatment with bortezomib, a proteasome inhibitor, restored p11 expression, verifying its regulation via a proteasome-linked pathway *in vivo*. In nonendothelial cells, p11 may be stabilized by one or more of its other partner proteins, which include a number of transmembrane channels and membrane receptors, such as the tetrodotoxin-resistant sodium channel $Na_v1.8$ [35], the two predomain K^+ channel TASK-1 [36], the acid-sensing ion channel ASIC1a [37], the transient receptor potential channels TRP5 and TRP6 [38], and the 5HT-1B serotonin receptor [39].

Protein kinase C-(PKC-) mediated phosphorylation of S^{11} or S^{25} residues on A2 appears to represent an additional regulatory pathway. Serine phosphorylation within the tail domain of A2 dissociates the heterotetramer complex, preventing further translocation to the cell surface by allowing polyubiquitination of p11 and its degradation in the proteasome [40, 41]. This event appears to be initiated by plasmin, which, once generated, signals activation of conventional PKC and thus limits its own generation. This mechanism appears to require cleavage of A2 by plasmin as well as activation of toll-like receptor 4.

In this paper, we summarize evidence from both animal models and human studies on the *in vivo* functions of the annexin A2/S100A10 system. The concept of the "annexinopathy" was first proposed in 1999 [42], and expanded in several subsequent reviews [43–47]. Here, we focus exclusively on the growing body of evidence that annexin A2 and its partner protein p11 contribute to human health and disease.

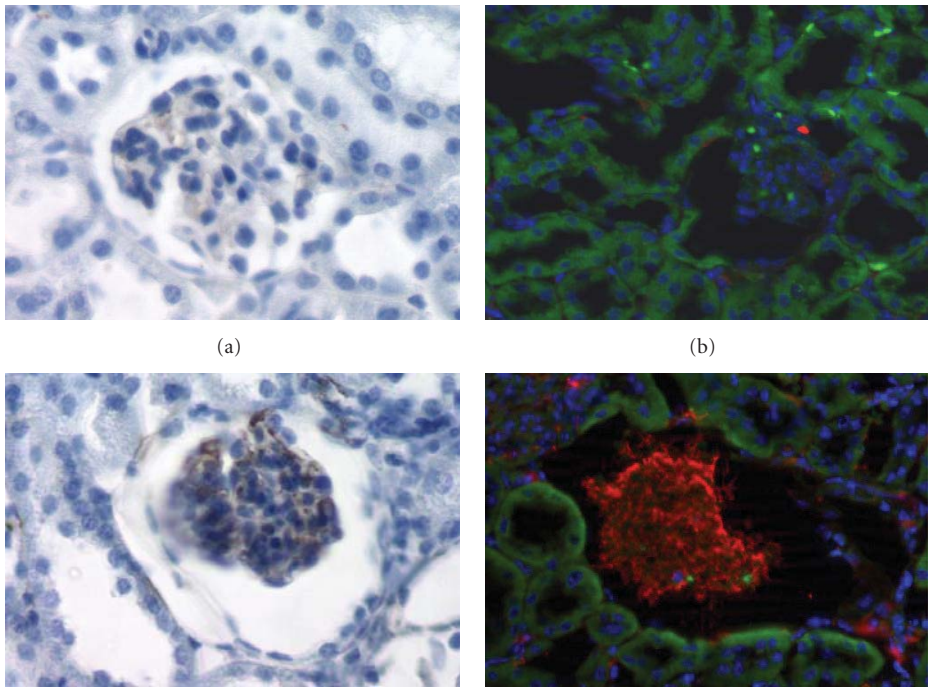


Figure 1: Fibrin accumulation in both $AnxA2^{-/-}$ and hyperhomocysteinemic mice. Kidney sections from $AnxA2^{+/+}$ mice on normal chow (a and b), from $AnxA2^{-/-}$ mice on normal chow (c), or from $AnxA2^{+/+}$ mice on a high methionine diet (d). Sections from heparin-treated, highly perfused animals were stained with a polyclonal rabbit antifibrin(ogen)-specific antibody using either immunoperoxidase plus diaminobenzidine (brown immunoreaction product) with hematoxylin counterstaining [5] (a and c), or biotinylated goat anti-rabbit IgG followed by Alexa 568-conjugated streptavidin (b and d); red staining represents Alexa 568, blue represents DAPI nuclear staining, and green is tissue autofluorescence [6].

2. *In Vivo* Animal Studies

2.1. Fibrin Balance and Models of Thrombosis. The $AnxA2^{-/-}$ mouse has been highly informative in investigating the role of the annexin A2 system in vascular homeostasis *in vivo*. Although A2-deficient mice display normal development, fertility, and lifespan, fibrin accumulation is evident in both intravascular and extravascular locations within the lungs, spleen, small intestine, liver, and kidney (Figure 1) [5]. Microvascular endothelial cells isolated from $AnxA2^{-/-}$ mice, moreover, lack the ability to support tPA-dependent plasmin generation *in vitro*, and arterial injury *in vivo* leads to an increased rate and severity of vascular occlusion in the $AnxA2^{-/-}$ mouse. Recently, fibrinolysis was also assessed in p11-null mice, which also displayed increased vascular fibrin, reduced clearance of thrombi, and impaired neovascularization of Matrigel thrombi [48].

Interestingly, mice with diet-induced hyperhomocysteinemia share this phenotypic feature with the $AnxA2^{-/-}$ mouse (Figure 1) [6]. Homocysteine (HC) is a thiol-containing amino acid that is generated during the conversion of methionine to cysteine [50]. Elevated levels of circulating HC have been associated with both thrombotic and atherosclerotic vascular disease [51], although therapies that lower plasma HC have not been shown to reduce the risk of recurrent cardiovascular disease [52]. Pretreatment, but not cotreatment, of endothelial cells with HC blocks their ability to bind tPA and inhibits endothelial cell-related, tPA-dependent plasminogen activation [53]. Incubation of purified A2 with HC, moreover, interferes with its ability to bind tPA [54]. When wild type mice were subjected to diet-induced hyperhomocysteinemia, fibrin accumulated in multiple tissues (Figure 1(d)), and extracted A2 failed to support tPA binding or tPA-dependent plasmin generation, revealing that HC-induced blockade of the cell surface A2 pathway can occur *in vivo*.

The potential clinical utility of recombinant annexin A2 protein (rA2) in ischemic stroke has emerged from thrombosis models in rats. Animals were treated with low-dose tPA with or without rA2 at 2 or 4 hours following the initiation of focal embolic stroke [55]. Those receiving both agents had a significantly lower infarct size and greater cerebral blood flow compared to animals treated with low-dose tPA alone. In similar experiments, in which animals underwent middle cerebral artery embolization with autologous clot, pretreatment with rA2 not only improved blood flow but also reduced infarct size compared to saline-treated controls [56]. These findings are significant in view of reported neurotoxicity and cerebral hemorrhage associated with the use of tPA in the treatment of thrombotic stroke in

humans [57–59]. Thus, rA2 or related agents may constitute a useful adjunct to tPA alone for the restoration of cerebral blow flow [60].

A2 or its analogs might also prove efficacious in the treatment of peripheral arterial occlusion. When carotid artery thrombosis was induced by adventitial application of $FeCl_3$, administration of recombinant full length, but not truncated, A2 was associated with improved cerebral blood flow and reduced thrombus size in comparison with untreated control animals [61]. This treatment had no effect on bleeding time, prothrombin time, or activated partial thromboplastin time, indicating that global clotting parameters remained intact. Thus, A2 or its analogs may constitute a useful adjuvant to conventional thrombolytic treatment by reducing the effective dose of tPA, thereby limiting its potential toxicity.

2.2. A Model of Hypercholesterolemic Atherosclerosis. The fibrinolytic system appears to modulate the development of plaque-like vascular lesions in mouse models of atherosclerosis in a complex fashion. Mice deficient in both plasminogen and apolipoprotein E (ApoE), for example, display an enhanced tendency toward atherosclerosis compared to those lacking ApoE alone, suggesting that plasminogen protects against lesion formation [62]. On the other hand, when macrophages overexpressed uPA in $ApoE^{-/-}$ mice, plaque development was accelerated through a plasminogen-dependent pathway [63]. When ApoE deficiency is combined with global deficiency of either uPA or tPA, however, the predilection for early fatty streaks and advanced plaque development was similar to that seen in mice with isolated ApoE deficiency [64]. These data suggest that the fibrinolytic system acts at multiple levels in the regulation atherogenesis.

In order to determine whether blocking plasmin(ogen) binding to A2 on the surface of macrophages is an effective strategy to reduce the development of atherosclerosis, $ApoE^{-/-}$ mice were crossed with $AnxA2^{-/-}$ mice to generate double nulls (Figure 2). Following weaning, $ApoE^{-/-}AnxA2^{-/-}$ mice were placed on a Western chow diet (30% fat) and sacrificed at 12 or 24 wks. Aortas were removed and evaluated for lesion development by *en face* Oil Red O staining and morphometry of histologic sections taken through the aortic root at the base of the heart. There was no difference in *en face* lesion area or lesion size in $ApoE^{-/-}AnxA2^{+/+}$ mice as compared to $ApoE^{-/-}AnxA2^{-/-}$ mice. Therefore, we conclude that the redundant nature of plasmin(ogen)-binding sites on macrophages renders targeting a single binding site ineffective in modulating lesion development in this model system.

2.3. Angiogenesis and Oxygen-Induced Retinopathy. Although embryonic vasculogenesis appears to be normal, $AnxA2^{-/-}$ mice display diminished neovascularization in several *in vivo* assays, including Matrigel implant, corneal pocket, and oxygen-induced retinopathy (OIR) models [5]. Mice with diet-induced hyperhomocysteinemia also display impaired corneal neoangiogenesis, which can be corrected upon intravenous injection of recombinant annexin A2 [6]. Microvascular endothelial cells from $AnxA2^{-/-}$ mice, as well as HC-treated human endothelial cells, moreover, migrate less efficiently in growth factor-enriched Matrigel. Together, these data suggest that absence of A2, or its modification by HC, leads to impairment of angiogenesis-related endothelial cell function.

Interestingly, annexin A2 is upregulated in OIR (Figure 3). In this model, newborn mouse pups are transitioned to room air after 5 days in a 75% oxygen environment, whereupon relative hypoxia initiates a robust vascular proliferative response in the retina [66]. The return to 21% oxygen also triggers A2 synthesis out of proportion to the increase in vascular endothelial cell abundance [32]. The retinal neoangiogenic response is inhibited by about 50% in the $AnxA2^{-/-}$ mouse. A2 expression is also increased in the endothelial cell under true hypoxia through the direct action of hypoxia-inducible factor-1 (HIF-1) with the A2 promoter. Electrophoretic mobility shift experiments, chromatin immunoprecipitation studies, and luciferase promoter reporter assays all indicate binding of HIF-1α and HIF-1β to a hypoxia-responsive element within the promoter region of the human A2 gene, leading to its activation.

Although OIR-associated retinal neovascularization is impaired in the $AnxA2^{-/-}$ mouse, it can be reestablished upon treatment of $AnxA2^{-/-}$ mice with a subretinal injection of an A2-encoding adenovirus, which restores A2 expression [32]. In addition, neovascularization of the hyperoxia-treated $AnxA2^{-/-}$ retina can be repaired upon treatment with the defibrinating agent ancrod, which depletes fibrinogen, thereby preventing fibrin formation. Together, these findings provide a link between fibrin accumulation and diminished neoangiogenesis and imply that new therapeutic avenues for proliferative retinal vascular disorders, such as retinopathy of prematurity or diabetic retinopathy, could involve blockade of A2 in addition to inhibition of angiogenic growth factors.

2.4. Models of Cancer. In the central nervous system, glioblastomas, malignant tumors derived from glial cells, are usually highly aggressive and refractory to treatment due to the early development of widespread infiltrative loci [67]. Glioma-generated proteases, such as plasminogen activators and matrix metalloproteinases, contribute substantially to glioma cell invasion [67, 68]. High concentrations of annexin A2, similarly, are associated with the pseudopodia of invasive glioma cells [69], and knockdown of A2 reduces their migratory capacity *in vitro* [70]. In both mouse and rat *in vivo* models, stable knockdown of A2 expression in glioblastoma cells retarded overall tumor progression upon implantation of the cells into rodent brains; cellular invasion, proliferation, apoptosis, and angiogenesis were all inhibited [49]. Interestingly, when A2 expression was stably reduced by transfection of RNAi directed against A2 in rat GL261 glioma cells, tumor growth and invasiveness were reduced,

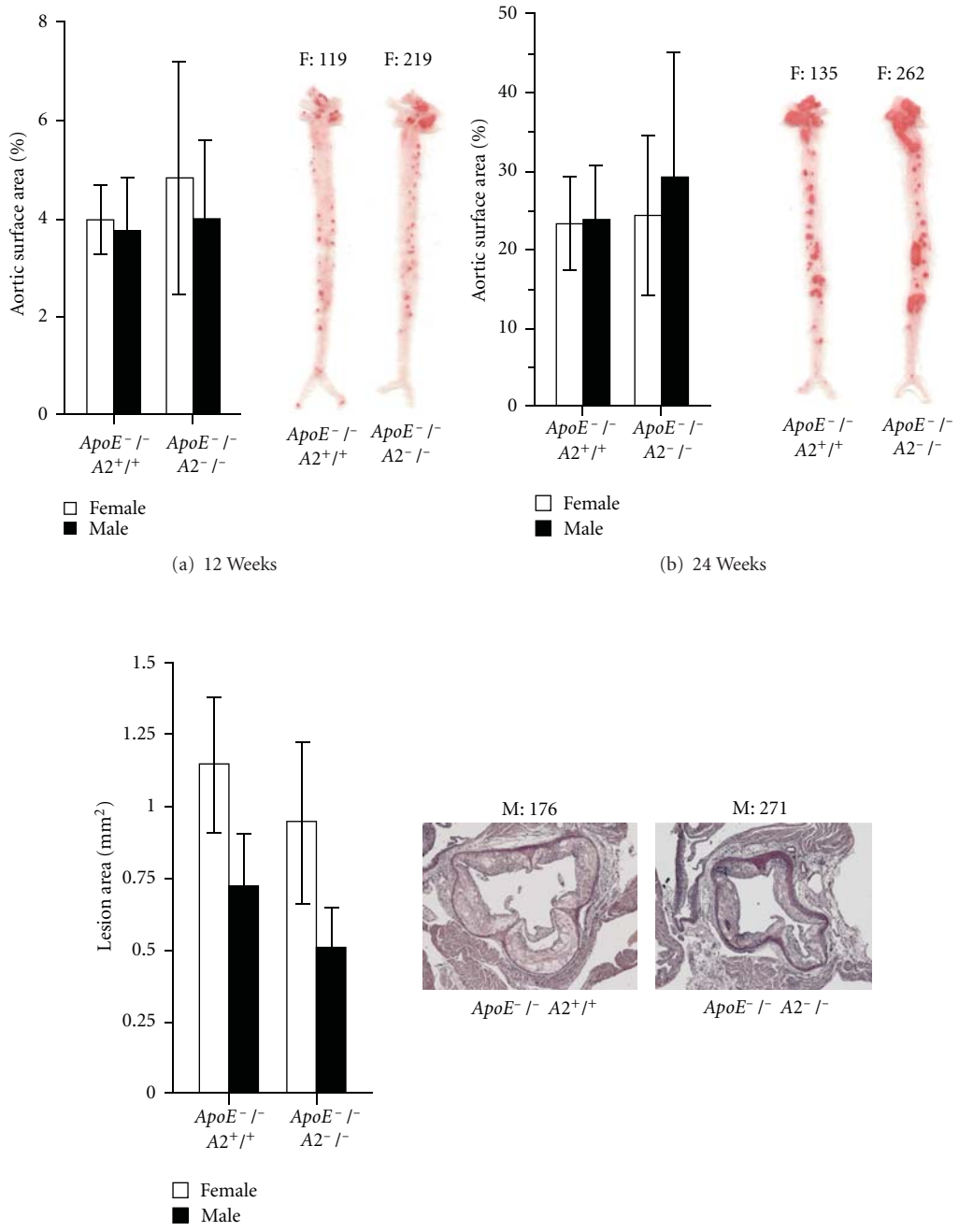
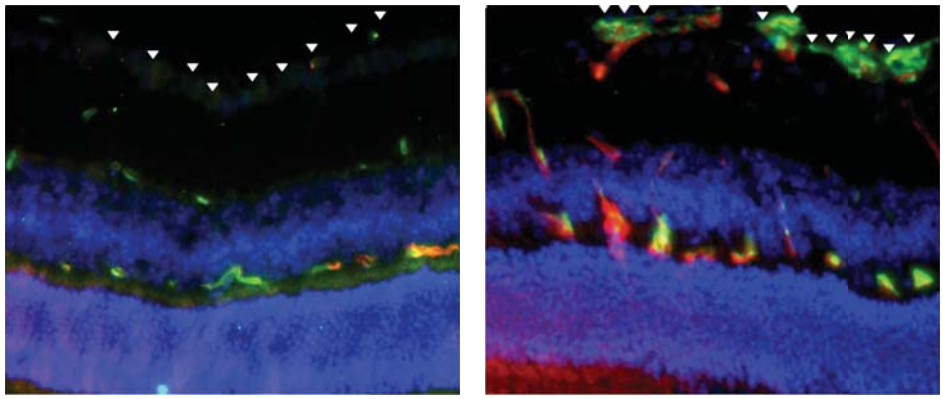
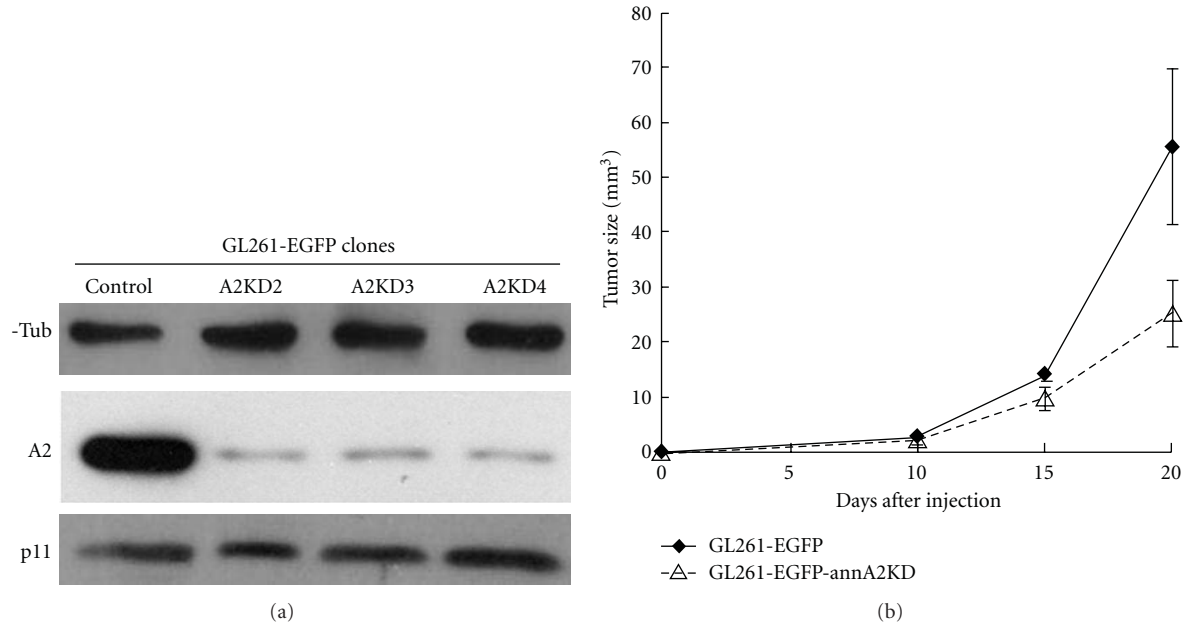


Figure 2: Diet-induced atherogenesis in $AnxA2^{+/+}$ and $AnxA2^{-/-}$ mice. $AnxA2^{-/-}$ mice on the C57Bl/6 background were backcrossed to $ApoE^{-/-}$ mice (Jackson Laboratories) to generate $AnxA2^{-/-}ApoE^{-/-}$ double knockout mice. At 3 weeks of age, both $AnxA2^{+/+}ApoE^{-/-}$ and $AnxA2^{-/-}ApoE^{-/-}$ mice male and female mice were placed on a western diet, composed of 1% (wt/wt) adjusted calories from fat and 0.15% (wt/wt) cholesterol (TD88137, Harland Tekland Laboratory) *ad libitum*, for 12 (n = 14–20 mice/group) or 24 weeks (n = 17–23 mice/group). At 12 or 24 weeks (a and b, resp.), the animals were perfused with PBS for 20 minutes, whereupon the entire aorta from the heart to 5–10 mm below the bifurcation of the iliac arteries was removed and fixed in 4% paraformaldehyde. The aorta was evaluated for lesion development by *en face* Oil red O staining, and morphometry of digital images of the stained aortas was performed using Adobe Photoshop 7.0 software [24]. For aortic root analyses, hearts removed at 24 weeks (c) were fixed in 3% PFA and paraffin embedded. Serial sections, 10 microns in thickness, obtained from the region of the proximal aorta to the level of the aortic leaflet, were stained with hematoxylin and eosin. Digital microscopic images were analyzed using image analysis software (NIH Image 1.63). Lesion size for each mouse was calculated as the average lesion size in 10–15 sections over a distance of 200 to 300 microns in the aortic root. Representative lesions are shown.



(a) (b)

Figure 3: A2-expressing neovessels in oxygen-induced retinopathy in the mouse. Sections through retinas of wild type mice maintained in room air (a) or treated for five days with 75% oxygen to initiate oxygen-induced retinopathy (b) were stained with an endothelial cell-specific lectin (isolectin B4—green), 4′,6-diamidino-2-phenylindole for nuclei (DAPI—blue), and anti-A2 (red) as described [32]. Note A2-positive neovascular tufts arrayed at the inner limiting membrane of the oxygen-treated retina (b, arrowheads) versus control (a, arrowheads).



(a) (b)

Figure 4: The glioblastoma-promoting effect of A2 is p11 independent. (a) When A2 expression in GL261 murine glioma cells was suppressed with siRNA, p11 expression remained intact. (b) Suppression of A2 expression significantly dampened the growth of intracranial gliomas derived from stereotactically implanted cells. Reproduced with permission [49].

even though p11 expression persisted; this result indicated that the contribution of A2 to tumor invasiveness was p11 independent (Figure 4). These data suggest that A2-directed treatment could offer a new therapeutic modality for human glioblastoma.

In a xenograft model in which highly invasive and metastatic breast cancer cells were implanted into nude mice, both tumor growth and vascular density were blocked by administration of anti-A2 monoclonal antibody [71]. The tumor cells employed in this experiment expressed abundant A2, strongly supported tPA binding and tPA-dependent plasmin generation and exhibited plasmin- and A2-dependent cellular matrix invasion [72, 73]. These studies suggest that A2 may contribute to aggressive breast cancer cellular invasion and tumor angiogenesis through production of localized protease activity.

In a third *in vivo* model, growth of Lewis lung and T241 sarcoma tumors implanted into p11-deficient mice was markedly reduced compared to wild type controls [74]. Impaired tumor growth was correlated with diminished

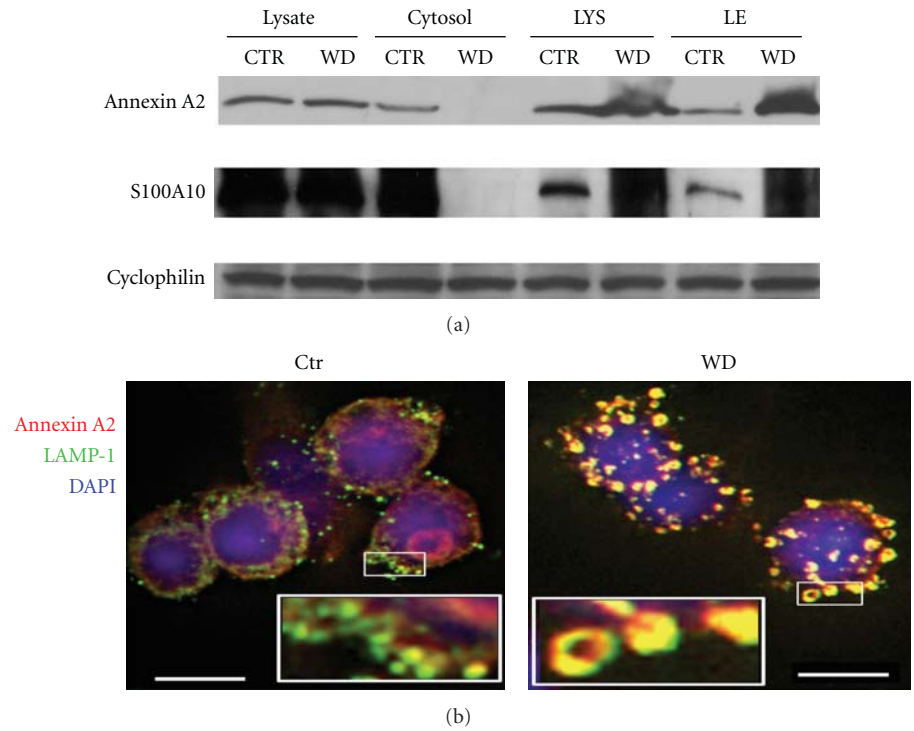


Figure 5: Intracellular A2 redistributes to lysosomal and late endosomal membranes in dendritic cells upon endocytosis of wear debris particles (WDP). (a) Western blot analysis of A2 and p11 localization in late endosomes (LE) and lysosomes (LYS) isolated from WDP-treated dendritic cells. (b) Confocal analysis of untreated and 12 h WDP-treated dendritic cells stained with anti-LAMP1 (green) and anti-A2 (red) revealed well-delineated $LAMP^+$ endosomal compartments with cytosolic distribution of A2 in control cells. Cells cultured with WDP for 12 h (b) demonstrated an extensive increase in the number and size of the endosomal compartments with the subsequent loss of the cytosolic distribution of A2. Bar corresponds to 10 mm. Reproduced with permission [65].

macrophage density within the tumors, and clodronate-mediated depletion of macrophages in wild type mice led to a similar reduction in tumor size. This study recapitulates the finding that thioglycollate-induced macrophage invasion into the peritoneum, and macrophage invasion of subcutaneous Matrigel plugs, were also impaired in the p11 knockout mouse [75]. A related study reveals that soluble $(A2{\cdot}p11)_2$ tetramer activates human and murine monocyte-derived macrophages, that this activation requires toll-like receptor 4 (TLR-4), and that the tetramer modulates cytokine production in the macrophage [76]. Thus, tumor infiltration by macrophages may in part be due to $(A2{\cdot}p11)_2$ tetramer signaling.

2.5. Aseptic Osteolysis. Arthroplastic surgery has revolutionized the treatment of arthritis and related rheumatologic disorders. Nevertheless, aseptic osteolysis, due to generation of wear debris particles (WDP), is an emerging problem that leads to failure of 10–30% of all joint replacements [77]. Recent investigations have thus focused on the mechanism by which WDP induce joint inflammation. Alkane polymers, 8–12 carbon atoms in length and derived from the breakdown of WDP, bind directly to toll-like receptors 1 and 2 and activate the downstream signaling pathway [78]. In addition, endocytosed WDP can induce endosomal membrane damage and disruption in phagocytic cells, and this process is associated with dramatic recruitment of cytoplasmic annexin A2 to the endosomal membrane (Figure 5). In the absence of A2, endosomal disruption leads to leakage of lysosomal cathepsins and H^+ ions into the cytosol with subsequent activation of the NLRP inflammasome and an accelerated inflammatory response [65].

2.6. Neuropsychiatric Disease. A large body of work has focused on the role of p11 in neuropsychiatric function. p11 binds to both the serotonin 1B and serotonin 4 receptors, suggesting a role for p11 in regulation of mood [39]. p11-deleted mice show depression-like behavior, characterized by increased immobility in the tail suspension test, increased thigmotaxis, and decreased responsiveness to a sucrose reward. In wild type mice, adenovirus-mediated deletion of p11 specifically within the nucleus accumbens (NA) resulted in depressive behavior, indistinguishable from that seen in mice with global p11 deficiency; exogenous administration of p11 within the NA of p11-deleted mice restored normal behavior [79]. These data correlate with findings in human depression, in which p11 protein levels were reduced in the NA. Furthermore, reduced p11 mRNA levels in peripheral blood mononuclear cells may serve as a potential biomarker for patients at high risk of suicide [80]. These studies raise the possibility that some forms of human depression may be reversible by augmentation of p11 expression [81]. Indeed,

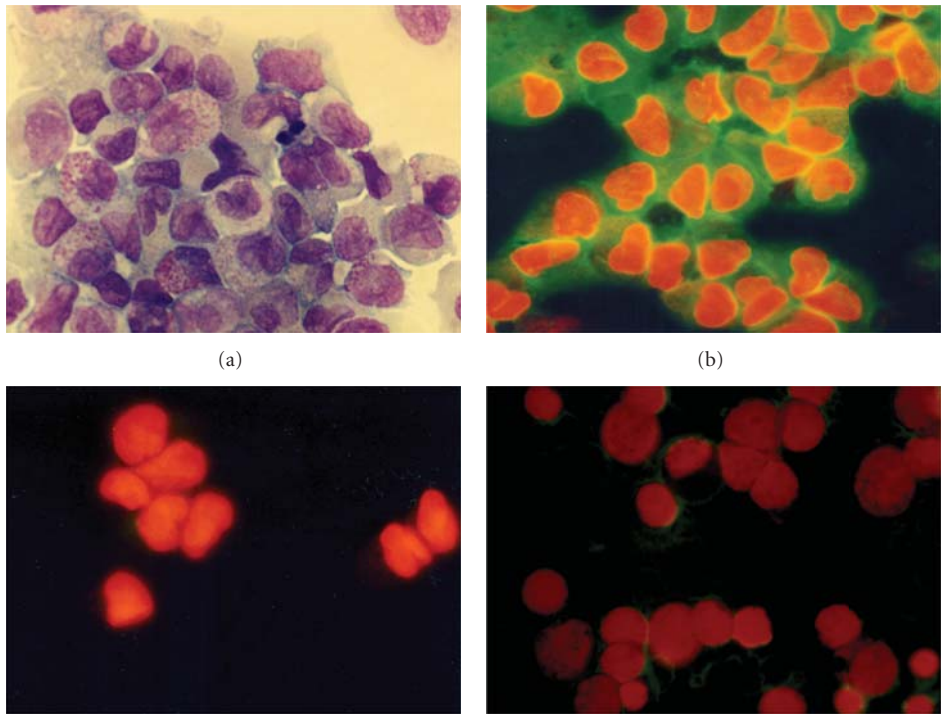


Figure 6: Expression of A2 is specifically elevated in acute promyelocytic leukemia cells. (a) Wright-Giemsa stain of human acute promyelocytic leukemia (APL) cells (M3). (b) APL cells stained with anti-A2 antibody (green fluorescence) and counterstained with ethidium bromide to visualize nuclei. (c) APL cells stained with nonimmune IgG and ethidium bromide. (d) Undifferentiated leukemia cells (M0) stained with anti-A2 antibody and ethidium bromide.

commonly used anti-inflammatory drugs that attenuate the antidepressive effects of serotonin reuptake inhibitors may do so by inhibiting the effects of interferonγ, a known inducer of p11 [82, 83].

p11 also potentiates nociception. The tetrodotoxin-resistant sodium channel ($Na_v1.8$/SNS), whose expression is restricted to sensory neurons, is the major pain perception receptor and is expressed in 85% of neurons emerging from the dorsal route ganglia. p11 binds to the amino terminus of the $Na_v1.8$ protein and promotes its translocation to the plasma membrane to produce functional channels [35]. Deletion of p11 specifically in primary nociceptor sensory neurons was achieved using $Na_v1.8$ promoter-directed Cre recombinase and led to a loss of tetrodotoxin-resistant sodium current density, and severe compromise of noxious coding in sensory neurons from the dorsal root horn [84]. Thus, directed p11 targeting may prove useful in the treatment of refractory pain disorders.

3. Lessons from the Clinic

3.1. Acute Promyelocytic Leukemia. At diagnosis, acute promyelocytic leukemia (APL) is commonly associated with life-threatening hemorrhage [85]. In APL, clonal expansion of immature promyelocytes harboring a balanced chromosomal translocation (t(15; 17)(q22–24; q12–21)) gives rise to the transcriptionally active promyelocytic leukemia-retinoic acid receptor α (PML-RARα) fusion protein. APL frequently responds to differentiation therapy with all-trans retinoic acid (ATRA), which triggers degradation of PML-RARα [86]. While disseminated intravascular coagulation promotes coagulopathy in APL, consumption of the plasmin inhibitor $alpha_2$-antiplasmin and development of a hyperfibrinolytic state due to excessive plasmin generation is also a primary driver.

High-level expression of annexin A2 occurs specifically in APL blast cells (Figure 6). A2 was detected in blast cells recovered from 6 of 6 APL patients, all of whom had evidence of hyperfibrinolysis, as evidenced by elevated circulating fibrin degradation products and D-dimer and depletion of plasma fibrinogen [87]. NB4 cells, which carry the t(15; 17) translocation and express the PML-RARα fusion protein, displayed steady state A2 mRNA levels that were approximately 10-fold higher than those found on leukemia cells that lacked the fusion protein. Treatment of NB4 cells with the retinoic acid receptor ligand, all-trans retinoic acid (ATRA), attenuated A2 expression in a time frame associated with clinical resolution of bleeding [87]. Intracranial bleeding, an unusually frequent problem in APL, may be due to the relatively high level expression of the A2

system on cerebral microvascular endothelial cells compared to those of other vascular beds [85]. Elevated expression of p11 in NB4 cells was also recently demonstrated and shown to respond to treatment with ATRA [88].

In a second clinical study, a cohort of 26 patients were studied prospectively and found to have enhanced fibrinolysis at diagnosis, despite normal tPA levels and increased PAI-1 [89]. APL cells harvested from these subjects expressed 3-fold higher levels of A2, and their rate of tPA-dependent plasmin generation was similarly elevated over that seen in the presence of M1, M2, M4, or acute lymphoblastic leukemia cells. Both elevated A2 expression in blast cells and hyperfibrinolytic hemorrhage corrected in 23 patients upon treatment with differentiation therapy, consisting of all-trans retinoic acid (ATRA) or ATRA plus arsenic trioxide. This study confirms the role of the A2 system in fibrinolytic bleeding in patients with APL.

3.2. Solid Tumors. Expression levels of annexin A2 have been examined in a variety of human malignancies. In some, such as renal cell [90–92], gastric [93], prostate [94], pancreatic [95], breast [72] carcinoma, and osteogenic sarcoma [96], increased expression levels appear to correlate with higher histologic grade and/or development of distant metastases. In human glioblastoma, A2 expression correlates with histologic grade and CNS dissemination [49, 97–99]. In human breast cancer, A2 appears to be associated with the surface of invasive, malignant cells, but not normal ductal or acinar epithelial cells, and expression correlated with neoangiogenic activity [73]. Proteomic profiling of colorectal cancer, moreover, revealed differentially increased expression of A2 in tumors that had progressed to lymph node metastases versus localized tumors [100]. Primary multiple myeloma cells harvested from a cohort of patients displayed 10-fold higher cell surface A2 expression than that observed on normal plasma cells; silencing of A2 in related cell lines suppressed expression of proangiogenic genes [101]. A2 and a related A2-binding "receptor" has been reported to promote myeloma cell adhesion and growth in the bone marrow [102]. On the other hand, A2 expression in oral squamous cell carcinoma [103] or sinonasal adenocarcinoma [104] was found to be inversely related to histopathologic grade. These studies suggest that expression levels of A2 may have prognostic value in malignancy, but would need to be validated for each specific tumor.

Expression of p11 in distinct tumor types has been less extensively studied. High levels of both p11 and A2 were found in 100% of anaplastic thyroid carcinomas, and correlated with their aggressive behavior [105]. In a comprehensive study of S100 gene expression in over 300 primary breast cancers, both p11 (S100A10) and S100A11 were selectively upregulated in basal versus nonbasal breast cancer subtypes, but did not predict overall survival [106]. Among 62 cases of human esophageal squamous cell carcinoma, 11 of 12 S100 genes, including p11, were downregulated, based on reverse transcription-polymerase chain reaction assays [107]. p11 transcripts have also been reported to be increased in both renal cell and gastric carcinomas [92, 108, 109]. Further studies may define p11 expression as a viable biomarker or prognostic indicator in selected tumors.

3.3. Antiphospholipid Syndrome. Antiphospholipid syndrome (APS) is a major cause of acquired thrombophilia [110]. It is characterized by thrombosis and recurrent fetal loss in association with circulating antiphospholipid antibodies. The latter are distinct, often coexisting antibodies directed against either β2-glycoprotein I or other intravascular proteins, which may be found in complex with anionic membrane phospholipids [111]. A2 has been identified as a prominent target of autoantibodies arising specifically in patients with APS with severe thrombosis and/or pregnancy morbidity [112, 113]. *In vitro*, patient-derived antiannexin A2 antibodies blocked endothelial surface tPA-dependent plasmin generation, and also "activated" cultured endothelial cells, inciting them to express elevated levels of the prothrombotic agent, tissue factor [112]. Other groups have noted that A2 can serve as a binding site for $\beta(2)$-glycoprotein I [114] in APS and can initiate A2-dependent endothelial cell activation [115]. Together, these data implicate A2 in the pathogenesis of APS-associated thrombosis through several possible mechanisms [116].

3.4. Cerebral Thrombosis. Cerebral venous thrombosis is a rare disorder of unknown etiology that mainly affects children and young adults [117]. Among a cohort of 40 consecutive patients studied 2 to 6 months following the index thrombotic event, 12.5% were found to have high titer anti-A2 antibodies compared to 2.1% in healthy subjects [118]. Thus, anti-A2 may define a new subset of individuals with immune-mediated thrombosis, play a role in the pathogenesis of this disorder, and/or offer novel therapeutic targets.

3.5. Preeclampsia and Pregnancy Loss. Both A2 and p11 are expressed on the brush border of the placental syncytiotrophoblast [119]. In 60 patients with preeclampsia, A2 mRNA and protein levels in placenta were significantly reduced compared to those in placentas from 30 matched normal pregnancy controls [120]. High titer anti-A2 antibodies, moreover, were detected more frequently in sera from subjects in the pre-eclamptic group and associated with increased placental vascular thrombosis. Impaired local fibrinolytic function due to blunted A2 expression may contribute to the pathogenesis of pre-eclampsia and maternal and perinatal infant morbidity.

3.6. Sickle Hemoglobinopathy. Sickle hemoglobinopathy arises from a point mutation within the 6th codon of the human β-globin chain [121]. Polymerization of abnormal hemoglobin under deoxygenating conditions induces erythrocyte shape change and non deformability, which leads to vascular occlusion, impaired vasodilatation, distal ischemia, and endothelial cell activation with adhesion of leukocytes. In children, sickle cell disease is complicated by stroke in 6–8% of patients. A recent analysis of

108 single nucleotide polymorphisms in 39 candidate genes revealed that variations in the annexin A2 (*ANXA2*) gene, among several others, were associated with increased risk of stroke [122]. A second independent study linked *ANXA2* polymorphisms to increased risk for stroke in sickle cell disease [123], while additional *ANXA2* SNPs have been associated with avascular necrosis of bone (osteonecrosis) in sickle hemoglobinopathy [124]. These data suggest that annexin A2 may represent a significant modifier gene that shapes the clinical expression and natural history of sickle cell disease.

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Review Article

The Plasminogen System in Regulating Stem Cell Mobilization

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The treatment of patients with hematopoietic progenitor and stem cells (HPSCs) to reconstitute hematopoiesis after myeloablative therapy or to repair ischemia after myocardial infarction has significantly improved clinical outcomes. Successful blood or bone marrow transplants require a sufficient number of HPSCs capable of homing to the injured site to regenerate tissue. Granulocyte-colony stimulating factor (G-CSF) is widely used clinically for stem cell mobilization. However, in some patients the response is poor, thus a better understanding of the mechanisms underlying G-CSF-regulated stem cell mobilization is needed. The pasminogen (Plg) system is the primary fibrinolytic pathway responsible for clot dissolution after thrombosis. Recent evidence suggests that Plg plays a pivotal role in stem cell mobilization from the bone marrow to the peripheral circulation, particularly in HPSC mobilization in response to G-CSF. This paper will discuss the potential mechanisms by which the Plg system regulates stem cell mobilization, focusing on stepwise proteolysis and signal transduction during HPSC egress from their bone marrow niche. Clear elucidation of the underlying mechanisms may lead to the development of new Plg-based therapeutic strategies to improve stem cell mobilization in treating hematological and cardiovascular diseases.

1. Introduction

Recruitment of hematopoietic progenitor and stem cells (HPSCs) to the blood followed by chemotherapy or cytokine treatment is a clinical process termed stem cell mobilization. This process mimics enhancement of the physiological release of stem cells and progenitors from the bone marrow (BM) reservoir in response to stress signals during injury and inflammation [1]. Currently, stem cells mobilized to the peripheral blood after treatment are the preferable source of HPSCs harvested for stem cell transplantation because of faster engraftment and reduced procedural risks compared with the direct harvest of the BM cells [2, 3]. Successful stem cell therapy, both autologous and allogeneic, requires the infusion of a sufficient number of HPSCs capable of homing to the injured site to promote tissue repair. Granulocyte-colony stimulating factor (G-CSF) is the most commonly used mobilizing agent to recruit HPSC from the BM; however, impaired response to G-CSF is observed in 25% of patients and 10% to 20% of healthy donors [4–6]. This has led to studies to identify the mechanisms underlying cytokine-induced stem cell mobilization that could offer better strategies to enhance stem cell mobilization.

Plasmin, a serine protease, degrades fibrin and is the primary enzyme for clot lysis, a process called fibrinolysis [7]. Plg, the zymogen of plasmin, can be converted to plasmin at different locations by tissue Plg activator (tPA) or urokinase Plg activator (uPA). While tPA is distributed in the ECM of most cell types, uPA is mainly localized on the cell surface via its highly specific cell surface receptor, uPAR [8]. The activities of uPA and tPA are regulated by Plg activator inhibitor-1 (PAI-1). In addition, plasmin activity can be inhibited directly by α_2-antiplasmin and α_2-macroglobulin [9]. Several studies [10, 11] have reported a marked increase in Plg binding to differentiated cells compared to undifferentiated cells, suggesting an important role for Plg activation for cell differentiation that requires the release and migration of cells from microenvironments. Recent evidence reveals that the Plg system is crucial for cytokine- or chemotherapy-induced stem cell mobilization.

Here, we will discuss the role of the Plg system in regulating stem cell mobilization.

2. Regulation of Stem Cell Mobilization via a Plg-Dependent Proteolytic Pathway

2.1. Stem Cell Mobilization and Plg Function. The egress of stem cells from the BM involves interfering with the physiological interaction between stromal cells and hematopoietic cells, which regulate both cell transmigration and the BM remodeling processes. The dynamic secretion and inactivation of the chemokines, stromal cell derived factor-1 (SDF-1), membrane-bound Kit ligand (KitL), and interleukin-8 (IL-8), which anchor HPSCs in the BM microenvironment, are critical for establishing the chemoattractant gradient between the BM and the peripheral blood for stem cell mobilization. Degradation and functional inactivation of the BM extracellular matrix proteins (ECM) by proteases such as elastase, cathepsin G and MMP-9, are also indicated as major players in stem cell mobilization [1, 12].

In addition to its role in fibrinolysis [13], Plg has many other functions. Interaction of Plg with its cell surface receptors and activation to plasmin, results in degradation of matrix proteins and activation of cytokines [14]. Plg directly binds to the ECM and upon its conversion to plasmin, degrades multiple ECM proteins including fibrin, laminin, and fibronectin [15–17]. Plasmin can also activate other proteases, such as MMP-3, MMP-9, MMP-12, and MMP-13 [18, 19] to degrade other matrix components such as collagens [20].

2.2. Role of Plg in Chemotherapy-Induced Stem Cell Mobilization. Many studies indicate that the Plg system facilitates HPSC mobilization through plasmin-mediated proteolytic mechanisms, by which plasmin inactivates chemotactic cytokines and degrades ECM in the BM compartment. Heissig et al. [21] have shown that deletion of Plg prevents hematopoietic stem cells from entering the cell cycle and undergoing multilineage differentiation after 5-FU treatment, causing lethality in mice. Activation of Plg by administration of tPA promoted cleavage of KitL mediated by MMP-9 secreted from stromal cells, subsequently enhancing HPSC proliferation, differentiation, and mobilization. This data suggests that Plg regulates HPSC function via MMP-9-mediated KitL release. Consistently, Tjwa et al. [22] reported that hematopoietic recovery upon delivery of 5-FU was impaired in Plg, tPA and uPA deficient mice. Moreover, depletion of fibrinogen in Plg deficient ($Plg^{-/-}$) mice did not restore hematopoietic recovery, indicating that Plg-regulated stem cell mobilization is fibrinolysis-independent. Instead, Plg deficiency inhibited breakdown of the BM matrix proteins fibronectin, VCAM-1, and laminin, which are required for adhesion of HPSCs to their BM microenvironment and also in transendothelial migration of HPSCs. These studies indicate that Plg and its activators are required for hematopoietic regeneration by regulating HPSC mobilization, proliferation, differentiation through MMP-9-mediated release of KitL, and plasmin-mediated degradation of ECM in the BM.

2.3. Role of Plg in G-CSF-Induced Stem Cell Mobilization. Plg is also necessary for HPSC mobilization in response to G-CSF. Tjwa et al. [23, 24] have found that genetic loss of PAI-1 or plasmin inhibitor α_2-antiplasmin, which enhances plasmin generation, increased HPSC mobilization in response to G-CSF, and thrombolytic agents such as tenecteplase and microplasmin, enhanced HPSC mobilization in mice and humans. Tenecteplase is a mutant of recombinant human tPA, which has a prolonged half-life and is used for treatment of acute cardiovascular and cerebrovascular syndromes. Microplasmin is a truncated form of plasmin that has an improved safety profile (less bleeding) and is easier to produce as a recombinant protein than is plasmin. Studies of $Plg^{-/-}$ mice [24, 25] validated that Plg is required for G-CSF-induced stem cell mobilization. Fewer HPSCs in the peripheral blood were detected in $Plg^{-/-}$ mice compared with wild-type (WT) mice after treatment with G-CSF. Similarly, WT mice treated with the Plg inhibitor tranexamic acid also impaired HPSC mobilization into the circulation. Furthermore, cleavage of the uPA receptor, (uPAR), by plasmin may account for Plg-regulated stem cell mobilization. Studies suggested that cleavage of the receptor uPAR is an important factor in regulating stem cell function [24]. First, uPAR was found to be expressed on a subpopulation of HPSCs, and HPSC mobilization was impaired in uPAR-deficient mice ($uPAR^{-/-}$). Second, intact uPAR is required for adhesion of HPSCs to the BM as well as homing and engraftment of HPSCs. During stem cell mobilization, uPAR is cleaved and subsequently stem cells are released from the BM to the circulation. Cleavage of uPAR was detected only in WT mice but not in $Plg^{-/-}$ mice during stem cell mobilization, suggesting that plasmin regulates stem cell mobilization by inactivating uPAR via proteolytic cleavage [24]. These findings suggest that uPAR is essential for Plg-regulated stem cell mobilization. However, less inhibition in HPSC mobilization was observed in $uPAR^{-/-}$ mice compared to $Plg^{-/-}$ mice [24], suggesting that there are other mechanisms for Plg regulation of G-CSF induced HPSC mobilization besides uPAR cleavage. While kitL is important in myelosuppression-induced HPSC mobilization, it does not seem to be involved in G-CSF-induced HPSC mobilization since G-CSF does not affect its levels [25, 26].

2.4. Role of Plg in the Regulation of SDF-1/CXCR4 Pathway. Another crucial pathway controlling stem cell mobilization is the SDF-1/CXCR4 signal. Under basal conditions, SDF-1/CXCR4 anchor HPSC in the BM and keep them in a quiescent state. During stem cell mobilization, SDF-1 in the BM is downregulated and HPSCs are released and mobilized into the circulation in response to the higher SDF-1 concentration in the peripheral blood [27, 28]. Our recent study has established the interplay between Plg and SDF-1/CXCR4 signals. Our data have shown that

Plg is required for G-CSF-induced HPSC egress to sinusoidal capillaries in the BM and subsequent mobilization to peripheral circulation. G-CSF induced Plg-dependent activation of MMP-9 in the BM, and MMP-9 neutralization or deficiency suppressed HPSC migration and mobilization. Reconstitution of MMP-9 activity by the BM transplantation after lentiviral overexpression rescued HPSC mobilization in $Plg^{-/-}$ mice, indicating that MMP-9 activation is required for Plg-mediated HPSC mobilization. Interestingly, after G-CSF simulation, Plg downregulated SDF-1 in the BM and spatiotemporally regulated the expression of CXCR4 on mobilized HPSC. Reconstitution of MMP-9 activity in $Plg^{-/-}$ mice reversed CXCR4 expression on HPSC in plasma and the BM, suggesting that CXCR4 serves as a newly identified downstream signal of Plg/MMP-9 in HPSC mobilization [25].

Taken together, these data indicate that the Plg system plays a crucial role in chemotherapy- or cytokine-induced stem cell mobilization. It functions through activating plasmin-mediated proteolytic activity to degrade the ECM in the BM (such as fibronectin or laminin) or by inactivating some key cytokines in the BM niches, such as KitL/c-Kit (KitL receptor), uPAR, and SDF-1/CXCR4, thus eventually leading to the release of HPSCs and the facilitation of their egress from the BM to the circulation (see proposed pathway in Figure 1).

3. Regulation of Stem Cell Mobilization in the Plg-Independent Signaling Pathway

3.1. Urokinase Plg Activator Receptor, uPAR. In addition to proteolytic activity, other regulatory pathways are involved in the Plg system-mediated HPSC mobilization. uPAR was originally identified as a key factor for the activation of Plg to plasmin and thereby the regulation of cell surface proteolysis in space and time [29]. The structure of uPAR consists of three homologous domains of ~90 amino acids each (D_1, D_2, and D_3 as numbered from the NH_2 terminus) and is anchored to the cell membrane through a glycosyl-phosphatidylinositol tail, attached to the C terminal D_3 domain [30]. Interestingly, uPAR, independent of proteolytic activity, regulates migration and adhesion of cells through binding to integrins and G-protein-coupled receptors and initiates intercellular signaling cascades [31]. Previous reports [32–34] have shown that uPAR regulates cell adhesion and migration by activating its downstream intracellular signaling pathways in various cell types [35].

3.2. Stem Cell Mobilization and uPAR. Recently, a critical role of uPAR in stem cell mobilization has also been documented [24, 36–39]. During G-CSF-induced HPSC mobilization in humans, uPAR expression significantly increased on peripheral blood mononuclear cells (PBMNCs), in particular on $CD33^+$ myeloid precursors and on $CD14^+$ monocytic cells released from the BM into the circulation. By contrast, $CD34^+$ cells and T and B lymphocytes were uPAR-negative, suggesting that uPAR may play a selective role in stem cell mobilization [36]. In $uPAR^{-/-}$ mice, stem cell mobilization induced by G-CSF treatment was impaired [24]. A very recent study [39] has demonstrated that uPAR is required to mobilize mesenchymal stem cells (MSC) from the BM of mice stimulated with G-CSF *in vivo*. Down- and up-regulation of uPAR inhibited and stimulated MSC differentiation into vascular smooth muscle cells, respectively. Consistently, infusion of MSCs isolated from $uPAR^{-/-}$ mice impaired its engraftment to injured femoral artery. These data indicate a role of uPAR in stem cell mobilization and engraftment. Additional evidence suggests several mechanisms by which uPAR plays a role in stem cell mobilization: chemotactic role of cleaved uPAR, regulation of integrins, and regulation of CXCR4 signaling.

3.3. The Chemotactic Role of Cleaved uPAR in Stem Cell Mobilization. Proteolytic cleavage of membrane bound uPAR in the linker region between D_1 and D_2 and at the juxtamembrane domain from the cell surface by plasmin or other proteases releases truncated uPAR (suPAR) into the extracellular space, where it may be proteolytically cleaved into smaller fragments (c-suPAR) [40–43]. uPAR fragments generated from uPAR cleavage are essential for uPAR-regulated stem cell mobilization. Previous studies have shown [36] that G-CSF treatment induced an increase in uPAR as well as suPAR. c-suPAR were released *in vitro* by the PBMNCs and were also detectable in the serum of G-CSF-treated donors. Fietz et al. [44] have confirmed that both uPAR and cleaved forms of uPAR are increased in HPSC donors following G-CSF treatment. Moreover, c-suPAR and its derived peptide ($uPAR_{84–95}$) induce *in vitro* migration of bone marrow HPSCs towards SDF-1. Furthermore, the chemotactic human c-suPAR peptide has been shown to mobilize HPSC in mice. Similarly, administration of human $uPAR_{84–95}$ peptides induced mobilization of $CD34^+$ HPSCs into the circulation to an extent similar to that observed in G-CSF in mice [36].

In agreement with these findings, utilizing mice with deleted uPA, tPA, uPAR, and Plg genes, Tjwa, et al. [33] have found that uPAR is expressed on the BM cells that are in close contact with osteoblasts as well as a subset of HPSCs. At steady state, $uPAR^{-/-}$ mice are partially depleted of HPSCs in the BM with a decrease of cell cycle quiescence and chemoprotection. In addition, $uPAR^{-/-}$ mice are impaired in HPSC mobilization, homing, and short-term engraftment. The membrane-anchored uPAR retention signal on HPSCs is inactivated by plasmin via proteolytic cleavage to a c-uPAR truncated product, which stimulates HPSC mobilization. These studies suggest that uPAR serves as a new anchor factor, similar to KitL/c-Kit to maintain HPSC retention in the BM, while cleaved soluble uPAR is a new chemoattractant and mobilizer of stem cell egress from the BM to the circulation.

3.4. The Role of uPAR in the Regulation of Integrins. Lacking transmembrane and intracellular domains, uPAR must cooperate with transmembrane receptors to activate intracellular signaling. Extensive studies suggest that integrins, a major family of ECM receptors are signaling coreceptors

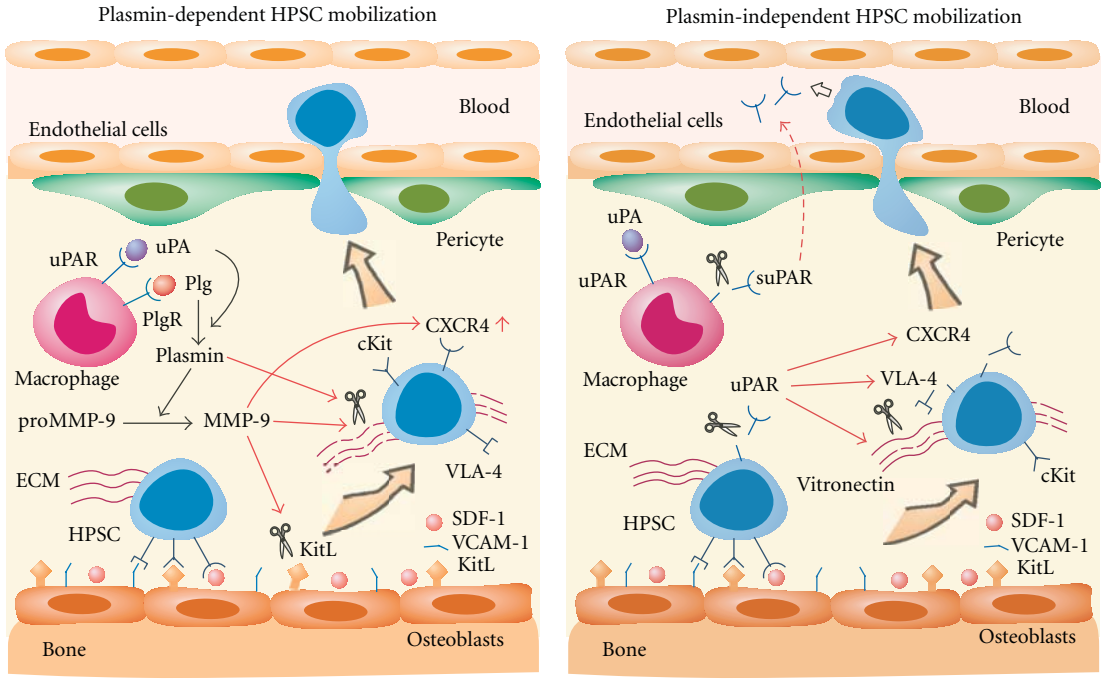

Figure 1: Plasmin-dependent and -independent mechanisms for HPSC mobilization from the BM to circulation. (a) Plasmin-mediated proteolysis induces HPSC mobilization. uPA converts Plg into protease-active plasmin that activates pro-MMP-9. Active MMP-9 cleaves KitL and upregulates CXCR4, and MMP-9 and plasmin degrade ECM, both of which release HPSC from the BM, leading to HPSC egress to circulation. (b) Plasmin-independent proteolysis and chemotaxis induce HPSC mobilization. uPAR is cleaved to chemotactic suPAR that drives HPSC migration to circulation. Cleavage of membrane uPAR also directly disrupts the interaction between uPAR and VLA-4, degrades vitronectin, and desensitizes the CXCR4 signal, which leads to HPSC mobilization.

of uPAR [45–49]. Besides uPA, the ECM glycoprotein vitronectin has been identified as a second ligand for uPAR [50]. It is reported that blocking uPAR-vitronectin binding impaired uPAR-regulated cell morphology, adhesion, and migration [45, 46]. Recent studies suggest that uPAR binding to vitronectin activates integrin signaling by simply increasing plasma membrane—ECM contact, facilitating integrin—ligand interactions [45]. Namely, vitronectin may act as an adaptor for the interaction of uPAR and integrins. The major integrins that uPAR interacts with are $\alpha_1\beta_1$ and $\alpha_4\beta_1$ (very late antigen-4, VLA-4) [51, 52]. The integrin $\alpha_4\beta_1$ regulates the migration and adhesion of HPSCs to fibronectin and VCAM-1 during their homing and engraftment in the BM [53–56]. The function of $\alpha_4\beta_1$ also depends on the presence of intact uPAR, as only intact uPAR interacts with the integrin [43, 56]. Removal of D_I from uPAR reduces $\alpha_4\beta_1$-mediated cellular adhesion *in vitro* [56]. Thus, when uPAR is depleted, such as in $uPAR^{-/-}$ mice, or inactive, such as after preincubation of WT HPSCs with anti-uPAR antibody, adhesion of $\alpha_4\beta_1$ on HPSCs to the BM matrix is reduced, likely explaining why homing and engraftment of HPSCs are impaired [24]. Furthermore, anti-$\alpha_4\beta_1$ antibodies could not further aggravate the adhesion defects of HPSCs when uPAR was absent or blocked. Likewise, homing and engraftment defects were similar in mice lacking functional uPAR or $\alpha_4\beta_1$ [24]. These data suggest that cooperation of uPAR with $\alpha_4\beta_1$ may partially contribute to the uPAR-mediated HPSC mobilization.

3.5. The Role of uPAR in the Regulation of CXCR4 Signal. Recent studies have revealed that functional interactions between the uPA-uPAR system and receptors for N-formylated peptides, such as the fMet-Leu-Phe (fMLP), are important for leukocyte chemotaxis [57]. The peptide, c-suPAR and its derived chemotactic peptide $uPAR_{84–95}$, corresponding to the uPAR chemotactic region unmasked by D_1-D_2 cleavage, can induce monocyte chemotaxis by FPRL1 activation [58]. FPRL1 belongs to the family of fMLP receptors; the other 2 members are FPR and FPRL2 [59]. Interestingly, activation of both FPR and FPRL1 can lead to the desensitization of other chemokine receptors, such as CXCR4 [60, 61]. CXCR4 and its specific ligand SDF-1 strongly contribute to retention of HPSCs in the BM since the downregulation of the CXCR4/SDF1 signal pathway increases HPSC mobilization [27, 28, 62]. Several studies have investigated whether suPAR is also able to interfere with the CXCR4/SDF-1 axis through fMLP receptors [36, 37]. The results have shown that SDF-1-dependent BM HPSC *in vitro* migration was impaired by $uPAR_{84–95}$ through the activation of FPR. Serum c-suPAR *in vivo* can also regulate $CD34^+$ HPSC mobilization by downmodulating CXCR4 activity

[37]. SDF-1 also induced chemotaxis of the BM $CD34^+$ HPSCs isolated from 3 donors, and pretreatment with fMLP or $uPAR_{84–95}$ completely abolished SDF1-dependent migration [36]. These data indicate that uPAR may regulate HPSC migration through FPR-mediated CXCR4 desensitization.

Altogether, these data suggest that uPAR regulates stem cell mobilization through several possible mechanisms. During G-CSF-induced HPSC mobilization, uPAR expression is first upregulated on $CD33^+$ and $CD14^+$ cells and is then cleaved, thus generating chemotactic forms of suPAR that present in the serum of G-CSF-treated donors. In the first case, cleavage of uPAR may disrupt the interaction between uPAR and $\alpha_4\beta_1$ integrin to release HPSC from their osteoblast niche. In the second case, suPAR may inactivate CXCR4 by heterologous desensitization and further promote HPSC release from the BM. Most importantly, c-suPAR may act as a chemoattractant for the BM HPSCs and stimulate their mobilization from the BM to the circulation (see proposed pathway in Figure 1).

4. Conclusion

In multiple pathological settings, including stroke and myocardial infarction, HPSCs are mobilized from the BM to sites of injury to promote tissue repair and regeneration. Stem cell therapy, including direct transplantation of stem cells, stimulation of stem cell mobilization and homing by cytokines, for example G-CSF, has emerged as a promising approach to promote tissue repair and regeneration after ischemia. The studies on Plg have revealed an essential role of the Plg system in cytokine-induced stem cell mobilization and have elucidated the molecular mechanisms regulating Plg-mediated stem cell mobilization. This will potentially contribute to the development of new therapeutic strategies, for example, targeting Plg/MMP-9 for strengthening the established G-CSF treatment for ischemia disease. More importantly, the proposed experimental therapy with μPlm (a truncated form of plasmin with fewer side-effects) or chemotactic peptide ($uPAR_{84–95}$) to promote HPSC recruitment to the damaged cardiac tissue, will confer clinical therapeutic potentials of plasmin in stem cell-mediated treatment, especially given the verified safety and efficiency of plasmin therapy (e.g., tPA) in MI treatment.

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Review Article

Ocriplasmin for Vitreoretinal Diseases

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Fibronectin and laminin are clinically relevant plasmin receptors in the eye. Located at the vitreoretinal interface, they are cleaved by ocriplasmin (Microplasmin, ThromboGenics, Iselin, NJ), a novel ophthalmic medication. A series of clinical trials to study ocriplasmin for the treatment of vitreoretinal diseases such as vitreomacular traction, macular hole, and exudative age-related macular degeneration are underway. The results are promising and may impact patient care.

1. Introduction

The vitreous occupies approximately 80% of the eye and is composed of water, collagen fibers, and hyaluronic acid [1]. In children, the vitreous is normally attached to the retinal surface and relatively innocuous. With aging, the vitreous physiologically liquefies and separates from the retina in a process called posterior vitreous detachment (PVD). The strongest points of vitreoretinal attachment are at the optic nerve, central retina (macula), blood vessels, and ora serrata.

At any age, the vitreous can be abnormally adherent to the macula, leading to sight-threatening diseases such as vitreomacular traction and macular hole [2, 3]. Vitreomacular traction is also implicated in the worsening of diabetic retinopathy and exudative age-related macular degeneration (AMD). Traditionally, diseases of the vitreoretinal interface have been treated with surgery to mechanically detach the vitreous from the retina and improve vision.

Vitreous surgery carries inherent risks such as bleeding, infection, retinal detachment, and accelerated cataract formation. Furthermore, inducing vitreous separation from the retina, particularly in the setting of an abnormal vitreoretinal interface, is among the most technically challenging and dangerous steps of vitreous surgery. Therefore, pharmacological vitreolysis has been an important research goal in ophthalmology [4].

Ocriplasmin (Microplasmin, ThromboGenics, Iselin, NJ) is a medicine that may be injected into the vitreous and administered in an office setting. It is a new technique to pharmacologically induce a posterior vitreous detachment by cleaving the extracellular matrix that adheres the vitreous to the internal limiting membrane of the retina [5–7]. The biomedical rationale and status of ocriplasmin for vitreoretinal diseases are discussed herein.

2. Plasmin Receptors in the Eye

Plasmin, the key enzyme of the fibrinolytic cascade, is also known to cleave other extracellular matrix components, specifically laminin and fibronectin [8–10]. In the eye, both molecules localize to the vitreoretinal interface, where they are postulated to play a central role in the adherence of collagen fibers between the vitreous and the internal limiting membrane (ILM) [11–17].

Early work showing the efficacy of plasmin in inducing a posterior vitreous detachment (PVD) was largely performed in rabbit eyes. Verstraeten et al. [18] initially demonstrated that intravitreal injection of plasmin followed by mechanical core vitrectomy successfully induced a PVD, as was later confirmed by histologic analysis. Hikichi et al. [19] aiming to avert the need for vitrectomy, subsequently combined intravitreal injection of plasmin with SF6 gas into rabbit eyes to successfully create a PVD without any signs of retinal toxicity. Interestingly, plasmin given alone was not sufficient to induce PVD in either study [18, 19]. In contrast to the previous reports, which investigated the intraocular effects of plasmin after only 1 week, Kim et al. [20] followed plasmin-injected rabbit eyes for 4 months, with no significant toxicity

observed. Additionally, they showed that plasmin alone was sufficient to produce a clean separation between the vitreous cortex and retina [20].

Gandorfer et al. [21] demonstrated that the degree of vitreoretinal separation induced by plasmin directly correlates with the concentration as well as length of exposure to the enzyme. Porcine eyes exposed to 1 unit of plasmin for 30 minutes had a dense network of residual collagen fibrils covering the ILM, while those exposed to 1 unit of plasmin for 60 minutes had only sparse collagen fibrils remaining. Furthermore, eyes treated with 2 units of plasmin for 60 minutes had a smooth retinal surface on postmortem examinations, consistent with a bare ILM. A later study by the same group was the first to duplicate these results in human cadaver eyes, and without any evidence of induced retinal damage [22].

Li et al. [23] separately investigated administration of intravitreal plasmin injections in human cadaver eyes. Using electron microscopy, they observed progressively less evidence of vitreous collagen fibers on the retinal surface with increasing doses of plasmin administered (1, 2, and 3 units) without producing morphological changes or acute toxicity to the inner retina. Through immunocytochemical labeling techniques, they were also able to demonstrate that treatment with plasmin dramatically decreased the density of fibronectin and laminin at the ILM. Uemura et al. [24] additionally confirmed through Western blot analyses that fibronectin and laminin were degraded by plasmin to several fragments of lower molecular weight in the ILMs collected from patients with macular holes or cystoid macular edema who underwent vitrectomy.

Cleavage of fibronectin and laminin may actually offer only a partial explanation to the molecular basis of pharmacologic vitreous detachment. Given that laminin and fibronectin are present at other ocular tissues beyond the vitreoretinal interface, such as the lens, ciliary body, retinal vessels, and lamina cribrosa [14], how is it that intravitreal plasmin injection can induce a PVD without adversely affecting these other structures? The answer may lie in plasmin's additional ability to activate endogenous matrix metalloproteinases (MMP), namely MMP-2 (gelatinase A), which normally reside within the vitreous in their proenzyme state [25–30]. Due to its affinity for various collagens, notably basement membrane type IV, activation of MMP-2 by exogenous plasmin likely contributes to the formation of PVD [26].

Beyond creating a PVD, Brown et al. [27] showed that experimentally injected active MMP-2-cleaved bovine vitreous collagen and concluded that MMP-2 activity could be considered a potential mechanism for the vitreous liquefaction seen in aging as well as various pathologic states. Animal studies suggest that plasmin, likely through activation of MMP-2, may also liquefy the vitreous and be of particular benefit as an adjunct to small-gauge vitrectomy systems. This, in turn, may facilitate both easier and increased vitreous removal during vitrectomy, while shortening duration of surgery. Staubach et al. [31] measured a greater reduction in the wet weight of enucleated porcine eyes injected with plasmin compared with controls once the vitreous was removed by core vitrectomy. Corroborating these findings, Hermel et al. [32] observed a 27% increase in rate of vitreous removal through a 25-gauge cutting system in rabbit eyes injected with plasmin as compared with no injection.

Clinically, autologous plasmin has been utilized as an adjunct to vitrectomy in numerous patient cohorts. Given the robust vitreoretinal adhesion in pediatric patients, Trese and colleagues investigated the utility of plasmin-assisted vitrectomy in the repair of traumatic macular holes [33, 34], stage 5 retinopathy of prematurity [35], and complicated X-linked retinoschisis [36], reporting successful anatomic outcomes in all groups. The use of plasmin-assisted vitrectomy to treat stage 3 full-thickness macular holes has revealed higher rates of spontaneous PVD noted intraoperatively in conjunction with reduction in overall surgery time [24, 37–39]. Other investigators employing preoperative plasmin in cases of tractional diabetic macular edema found higher incidences of spontaneous PVD at the time of surgery [40, 41], less suction required to create a PVD when needed [42], and improved postoperative visual outcomes compared with controls [41]. Hirata et al. [43] observed that plasmin pretreatment in patients with proliferative diabetic retinopathy resulted in significantly less surgical time and a decreased risk for iatrogenic retinal breaks.

Unfortunately, autologous plasmin has several shortcomings which limit its feasibility for routine clinical practice. First, it is not readily available, and the process to obtain it is time-consuming and expensive. Autologous plasminogen must be harvested from the patient's own blood, then converted by streptokinase to plasmin *in vitro* prior to use. Second, this procedure must be done immediately before surgery as plasmin is exceedingly unstable and rapidly inactivates itself via autolysis and binding to α2-antiplasmin.

Recent advances in pharmaceutical drug development led to the discovery of ocriplasmin (Microplasmin), a recombinant product of only the catalytic domain of human plasmin [44]. Distinct advantages of ocriplasmin over plasmin include. (1) it is approximately one-fourth the size of plasmin (22-kDa versus 88-kDa) which is thought to facilitate greater penetration of vitreous and epiretinal tissues; (2) generation by recombinant techniques ensures product sterility and eliminates the risk of microbial contamination associated with blood derivatives; (3) when commercially available, it will allow investigators to avoid the rigorous preparation of autologous plasmin; (4) it is more stable than plasmin which simplifies storage and timing of administration [45].

Intravitreal ocriplasmin has been evaluated in several preclinical studies utilizing porcine, rat, rabbit, feline, and human cadaver eyes as the experimental model [46–48]. Gandorfer et al. initially reported a dose- and time-dependent cleavage between the posterior hyaloid and the ILM created by ocriplasmin without any adverse effects on retinal structure, in both human cadaver and feline eyes. Doses greater than or equal to 125.0 μg (equivalent to 2 units of plasmin (Sigma-Aldrich, Poole, United Kingdom)) produced a complete PVD with bare ILM in the human eyes, as demonstrated by electron microscopy.

De Smet et al. [49] confirmed these findings in a porcine eye model, observing that microplasmin caused vitreolysis

and PVD in a dose- and time-dependent fashion. The minimal effective dose also appeared to be 125 μg [49]. Sakuma et al. [50] corroborated these findings as well in rabbit eyes using doses of microplasmin ranging from 12.5 to 250 μg. They, too, found that 125 μg of microplasmin or greater successfully induced a complete PVD, while lower doses only induced a partial PVD. In all treated eyes, there was a temporary reduction in the a- and b-wave amplitudes on electroretinography, which recovered by 14 days after injection all groups except the 250 μg treatment group. In this higher dose faction, while the b-wave eventually recovered, a-wave alterations persisted at 90 days. A mild, transient vitreous haze was also noted within the first day after injection in this and other studies [12, 50, 51].

Most recently, Chen et al. [12] were able to show through immunofluorescence histochemistry that intravitreal microplasmin degraded fibronectin and laminin not only at the vitreoretinal interface, but also at the level of the photoreceptor layer in the outer retina of rats. Theoretically, the smaller molecular weight of ocriplasmin facilitates deeper penetration of retinal tissue but it is uncertain if this holds true in humans.

3. Clinical Trials with Ocriplasmin

In 2004, a series of clinical trials sponsored by ThromboGenics were initiated and collectively called Microplasmin for IntraVitreous Injection- Tractional Release without Surgical Treatment (MIVI-TRUST). To date, there are 14 studies involving intravitreal administration of ocriplasmin (ClinicalTrials.gov). Of these, 9 are included in the MIVI series. Results of the first three clinical trials (MIVI-I, MIVI-IIT, and MIVI-III) are published [7, 18, 19].

In each of these clinical trials, all patients with prior vitreous surgery and/or history of retinal detachments were excluded. Adverse events were also recorded and none of these studies have thus far shown an increased rate of retinal detachment, a known complication of posterior vitreous detachment, after ocriplasmin therapy.

3.1. MIVI-I: A Dose-Escalation Clinical Trial of Intravitreal Microplasmin in Patients Undergoing Surgical Vitrectomy for Vitreomacular Traction Maculopathy. MIVI-I was a Phase I/II safety study with dose escalation (25–125 micrograms) and increasing exposure time (1 hour–1 week) [7]. Sixty patients were enrolled in 6 successive cohorts. All patients had vitreomacular traction (VMT) maculopathy for which vitrectomy was indicated, including macular edema associated with VMT, stage II-III macular hole of <6 months duration since symptom onset, demonstration of vitreomacular adhesion (VMA) based on preoperative optic coherence tomography (OCT), or an OCT finding of posterior hyaloid membrane inserting onto the macula but with some area of clear separation visible between the retina and the posterior hyaloid. Results demonstrated that intravitreal ocriplasmin was well tolerated and capable of inducing a pharmacologic PVD in some patients.

3.2. MIVI-II: A Randomized, Sham-Injection-Controlled, Double-Masked, Ascending-Dose, Dose-Range-Finding Trial of Microplasmin Intravitreal Injection for Nonsurgical PVD Induction for Treatment of Diabetic Macular Edema. MIVI-II was a Phase II trial evaluating PVD induction in patients with diabetic macular edema (DME) 14 days after injection of intravitreal ocriplasmin versus sham. Disease status and safety at 6 months were also evaluated. The study was completed in 2010; however, results have not yet been published at the time of this paper.

3.3. MIVI-IIT: A Randomized, Sham-Injection-Controlled, Double-Masked, Ascending-Dose, Dose-Range-Finding Trial of Microplasmin Intravitreal Injection for Nonsurgical PVD Induction for Treatment of Vitreomacular Traction. MIVI-IIT was a randomized, double-masked Phase II trial with a control sham injection [18]. Sixty patients were enrolled in 4 cohorts. Patients in each of the cohorts were randomized to active treatment or sham injection. In the first 3 cohorts, increasing doses of ocriplasmin (75, 125, and 175 micrograms) were administered. In the fourth cohort, patients received 125 micrograms of intravitreal ocriplasmin monthly until the VMA was released, up to a total of 3 doses.

The first 3 cohorts had a nonsurgical resolution of VMA in 8, 25, 44, and 27% of the patients who received sham, 75, 125, and 175 micrograms of ocriplasmin, respectively. In the fourth cohort, ocriplasmin caused a PVD in 58% of patients at one month after the last treatment.

The MIVI-IIT trial provides support for the potential use of ocriplasmin in the nonsurgical treatment of VMA.

3.4. MIVI-III: A Multicenter, Randomized, Placebo-Controlled, Double-Masked, Parallel-Group, Dose-Ranging Clinical Trial of Intravitreal Microplasmin in Patients Undergoing Surgical Vitrectomy The MIVI III (Microplasmin for Vitreous Injection III) Trial. MIVI–III evaluated the safety and efficacy of a preoperative intravitreal injection of ocriplasmin in patients already scheduled for vitreous surgery [19]. One hundred twenty-five patients scheduled for pars plana vitrectomy (PPV) for the treatment of either VMT or macular hole were enrolled in this Phase II placebo-controlled double-masked dose-ranging clinical trial. A single intravitreal injection of ocriplasmin (25, 75, or 125 micrograms) or placebo was administered 7 days prior to PPV. The presence or absence of PVD at baseline, injection day, operative day, and postinjection day 90 and 180 were evaluated.

Rates of PVD observed at the time of surgery were 10, 14, 18, and 31% in the placebo, 25-, 75-, and 125-microgram ocriplasmin groups, respectively. The rates of resolution of VMT precluding the need for PPV at day 35 were 3, 10, 15, and 31% for the placebo, 25-, 75-, and 125-microgram ocriplasmin groups, respectively. At day 180, these rates were 3%, 7%, 15%, and 28%. At both day 35 and day 180, the rates of canceled vitrectomy in the 125-microgram ocriplasmin group were statistically significant when compared to the placebo group ($P < 0.01$ and $P = 0.01$, resp.).

MIVI-III concluded that ocriplasmin injection at a dose of 125 micrograms led to a greater likelihood of induction

and progression of PVD than placebo injection. This study also suggested that patients receiving ocriplasmin were more likely to not require vitrectomy surgery and that further trials were warranted.

3.5. MIVI-5: A Randomized, Sham-Injection-Controlled, Double-Masked, Multicenter Trial of Ocriplasmin Intravitreal Injection for Treatment of Focal Vitreomacular Adhesion in Subjects with Exudative Age-Related Macular Degeneration (AMD). Exudative AMD is a serious cause of blindness in elderly patients, and current standard of care includes monthly intravitreal antivascular endothelial growth factor (VEGF) injections [52]. Vitreomacular traction is thought to exacerbate AMD by exerting tractional forces on the macula, hypothetically stimulating abnormal blood vessel growth.

MIVI-5 is an ongoing clinical trial evaluating the safety and efficacy of intravitreal ocriplasmin in patients diagnosed with exudative AMD with focal VMA. Patients enrolled in this study have active subfoveal choroidal neovascular membrane and have received at least 3 antiangiogenic intravitreal injections, with evidence of focal VMA on OCT. Patients who have previously received more than 9 antiangiogenic intravitreal injections are excluded.

The primary outcome measure is the proportion of patients with release of focal VMA by day 28 as determined by a masked central reading center. MIVI-5 started in early 2010 and completion is anticipated in late 2012.

3.6. MIVI-TRUST (TG-MV-006): A Randomized, Placebo-Controlled, Double-Masked, Multicenter Trial of Microplasmin Intravitreal Injection for Nonsurgical Treatment of Focal Vitreomacular Adhesion and MIVI-TRUST (TG-MV-007): A Randomized, Placebo-Controlled, Double-Masked, Multicenter Trial of Microplasmin Intravitreal Injection for Nonsurgical Treatment of Focal Vitreomacular Adhesion. MIVI-TRUST TG-MV-006 and TG-MV-007 are both Phase III clinical trials evaluating the safety and efficacy of a 125-microgram dose of intravitreal ocriplasmin in patients with focal VMA. The primary outcome measure was the nonsurgical resolution of focal VMA at postinjection day 28. Both studies began late 2008 and were completed in 2010. Final published results from these 2 studies are not yet available.

3.7. MIVI-8: An Open-Label, Single-Centre Trial of Microplasmin Intravitreal Injection for Nonsurgical Treatment of Focal Vitreomacular Adhesion. MIVI-8 is a Phase II clinical trial assessing the safety and efficacy of 125-microgram ocriplasmin administered as an intravitreal injection in patients with focal VMA. Primary outcome measures include full ophthalmologic examination at baseline, postinjection days 7, 14, 28, and months 3 and 6. A secondary outcome is the proportion of patients with nonsurgical resolution of focal VMA at study visits other than the 28-day postinjection visit. MIVI-8 completed in April 2011 and published results are currently pending.

3.8. MIVI-10: An Open-Label, Ascending-Exposure-Time, Single-Center Trial to Evaluate the Pharmacokinetic Properties of Ocriplasmin (Generic Name of the Molecule Microplasmin) Intravitreal Injection in Subjects Scheduled for Primary Pars Plana Vitrectomy. The purpose of MIVI-10 is to evaluate the pharmacokinetic properties of intravitreal ocriplasmin when administered at different time points prior to planned PPV. In this Phase II clinical trial, 38 patients undergoing primary PPV received an intravitreal injection of 125-microgram ocriplasmin 5 minutes to 7 days prior to surgery. Ocriplasmin activity levels in the vitreous samples were evaluated. The study completed in early 2011 and final results have not yet been published.

3.9. Non-MIVI Trials. In addition to the MIVI-TRUST trials, there are 4 other trials that are currently evaluating the use of intravitreal ocriplasmin(ClinicalTrials.gov). A single-center, placebo-controlled Phase II clinical trial is actively enrolling patients to assess the efficacy of a high-dose (1.875 milligram) intravitreal injection of ocriplasmin in the treatment of focal VMA in patients with exudative AMD. Besides improving AMD by eliminating vitreomacular traction, ocriplasmin may also affect the pharmacokinetics and efficacy of anti-VEGF agents. A study in rabbits showed that bevacizumab (an anti-VEGF agent) in combination with ocriplasmin facilitated the penetration of bevacizumab into the retina [53]. The secondary endpoint of this clinical trial involving AMD and ocriplasmin is a decrease in subsequently required anti-VEGF injections.

Another study recruiting patients is Ocriplasmin for Treatment of Symptomatic Vitreomacular Adhesion Including Macular Hole (OASIS), a Phase II clinical trial evaluating the treatment of symptomatic vitreomacular adhesion including macular hole with a single 125-microgram intravitreal injection of ocriplasmin.

Additionally, the Microplasmin Intravitreal Administration in Participants with Uveitic Macular Edema is an ongoing Phase I/II trial investigating the safety and potential efficacy of intravitreal ocriplasmin as a possible treatment for macular edema secondary to uveitis. This study was initiated in 2010 and is anticipated to conclude in early 2012.

Finally, the Microplasmin in Children (MIC) Trial is also recruiting patients to assess the safety and efficacy of intravitreal ocriplasmin as an adjunct to conventional vitrectomy for the treatment of pediatric patients under 16 years of age. The vitreous in children is denser and more adherent to the retina as compared with that in adults, and the safety and efficacy profile of ocriplasmin may differ in children and adults. This Phase II, placebo-controlled, double-masked trial will evaluate a 175-microgram dose of ocriplasmin in pediatric patients undergoing a standard 2-port or 3-port PPV. Any child diagnosed with Stage 1, 2, 3, or 5 retinopathy of prematurity (ROP) at the time of the surgery is excluded.

In summary, the safety and efficacy of ocriplasmin for vitreoretinal diseases are being systematically evaluated in over a dozen clinical trials. The medicine is meant to help some patients avoid surgery or at least make vitreous surgery safer in others.

4. Federal Drug Administration Approval

In December 2011, a Federal Drug Administration (FDA) application was submitted for the use of ocriplasmin 2.5 mg/mL in adults. It was withdrawn and resubmitted for Priority Review in April 2012. If FDA approved, future applications for ocriplasmin would include its use in more common vitreomacular diseases such as diabetic retinopathy and vein occlusions.

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Review Article

α-Enolase, a Multifunctional Protein: Its Role on Pathophysiological Situations

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α-Enolase is a key glycolytic enzyme in the cytoplasm of prokaryotic and eukaryotic cells and is considered a multifunctional protein. α-enolase is expressed on the surface of several cell types, where it acts as a plasminogen receptor, concentrating proteolytic plasmin activity on the cell surface. In addition to glycolytic enzyme and plasminogen receptor functions, α-Enolase appears to have other cellular functions and subcellular localizations that are distinct from its well-established function in glycolysis. Furthermore, differential expression of α-enolase has been related to several pathologies, such as cancer, Alzheimer's disease, and rheumatoid arthritis, among others. We have identified α-enolase as a plasminogen receptor in several cell types. In particular, we have analyzed its role in myogenesis, as an example of extracellular remodelling process. We have shown that α-enolase is expressed on the cell surface of differentiating myocytes, and that inhibitors of α-enolase/plasminogen binding block myogenic fusion *in vitro* and skeletal muscle regeneration in mice. α-Enolase could be considered as a marker of pathological stress in a high number of diseases, performing several of its multiple functions, mainly as plasminogen receptor. This paper is focused on the multiple roles of the α-enolase/plasminogen axis, related to several pathologies.

1. Introduction

Enolase, also known as phosphopyruvate hydratase, was discovered in 1934 by Lohman and Mayerhof. It is one of the most abundantly expressed cytosolic proteins in many organisms. It is a key glycolytic enzyme that catalyzes the dehydratation of 2-phosphoglycerate to phosphoenolpyruvate, in the last steps of the catabolic glycolytic pathway [1] (Figure 1). It is a metalloenzyme that requires the metal ion magnesium (Mg^{2+}) to be catalytically active. Enolase is found from archaebacteria to mammals, and its sequence is highly conserved [2]. In vertebrates, the enzyme occurs as three isoforms: α-enolase (*Eno1*) is found in almost all human tissues, whereas β-enolase (*Eno3*) is predominantly found in muscle tissues, and γ-enolase (*Eno2*) is only found in neuron and neuroendocrine tissues [3]. The three enolase isoforms share high-sequence identity and kinetic properties [4–6]. Enzymatically active enolase which exists in a dimeric (homo- or heterodimers) form is composed of two subunits facing each other in an antiparallel fashion [6, 7]. The crystal structure of enolase from yeast and human has been determined and catalytic mechanisms have been proposed [8–10].

Although it is expressed in most of the cells, the gene that encodes enolase is not considered a housekeeping gene since its expression varies according to the pathophysiological, metabolic, or developmental conditions of cells [11]. α-Enolase mRNA translation which is primarily under developmental control is significantly upregulated during cellular growth and practically undetectable during quiescent phases [12, 13].

Recent accumulation of evidence revealed that, in addition to its innate glycolytic function, α-enolase plays an important role in several biological and pathophysiological processes: by using an alternative stop codon, the α-enolase mRNA can be translated into a 37 kDa protein which lacks

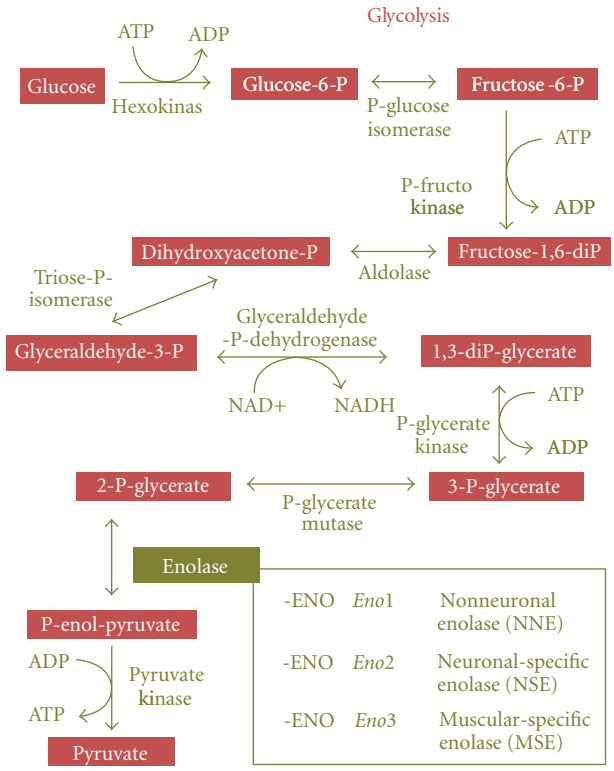

Figure 1: Summary of glycolytic metabolic pathway. Metabolic chain reactions of glycolysis, the central pathway for the catabolism of carbohydrates that takes place in the cytoplasm of almost all prokaryotic and eukaryotic cells. The insert shows different enolase isoenzymes in vertebrates.

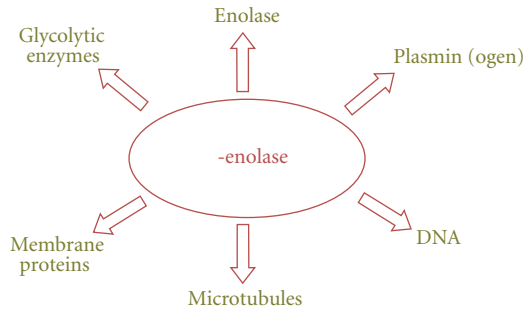

Figure 2: Interaction of α-enolase with other nuclear, cytoplasmic, or membrane molecules. α-Enolase can directly interact with other enolase isoforms (α, β, and γ) to form enzymatically active dimers, other glycolytic enzymes as pyruvate kinase, phosphoglycerate mutase and aldolase. It can also bind to microtubules network proteins, as F-actin and tubulin, and it is localized on the cell surface, interacting with other membrane proteins, where it binds to plasminogen and plasmin.

the first 96 amino acid residues. This protein, named c-myc promoter-binding protein 1 (MBP-1) is localized in the nucleus and can bind to the c-myc P2 promoter and negatively regulates transcription of the protooncogene [14]. α-Enolase has been detected on the surface of hematopoietic cells such as monocytes, T cells and B cells, neuronal cells, and endothelial cells as a strong plasminogen receptor, modulating pericellular fibrinolytic activity. The expression of α-enolase on the surface of a variety of eukaryotic cells has been found to be dependent on the pathophysiological conditions of these cells [15–19].

α-Enolase has also been described as a neurotrophic factor [20], a heat-shock protein (HSP48) [21], and a hypoxic stress protein [22]. Furthermore, α-enolase is part of the crystallin lens of vertebrates [23], binds to fragments of F-actin and tubulin [24], and has been detected associated to centrosomes in HeLa cells [25]. α-Enolase also binds with high affinity to other glycolytic enzymes: pyruvate kinase, phosphoglycerate mutase, which are adjacent to enolase in the glycolytic pathway, and to aldolase, which is known to associate with cytoskeletal proteins [26] (Figure 2).

It has also been suggested that upregulation of α-enolase contributes to hypoxia tolerance through nonglycolytic mechanisms [27]. Increased expression of α-enolase has been reported to correlate with progression of tumors, neuroblastoma, and lung cancer, and enolase has been considered to be a potential diagnostic markers for many tumors [28–32].

Thus, α-enolase appears to be a "moonlighting protein," one of a growing list of proteins that are recognized as identical gene products exhibiting multiple functions at distinct cellular sites through "gene sharing" [33, 34]. This paper is focused on the multiple roles of the α-enolase/plasminogen axis, related to several pathologies.

2. The Plasminogen Activation System

In multicellular organisms, extracellular proteolysis is important to many biological processes involving a dynamic rearrangement of cell-cell and cell-matrix interactions, being the plasminogen activation (PA) system among the most important extracellular proteases. The PA system comprises an inactive proenzyme, plasminogen, and ubiquitous in body fluid, that can be converted into the active enzyme, plasmin, by two physiological activators (PAs): tissue-type plasminogen activator (tPA) and urokinase-type plasminogen activator (uPA). Inhibition of the plasminogen system occurs at the level of the PA, by specific inhibitors (PAI-1 and PAI-2), or at the level of plasmin, by α2-antiplasmin (reviewed in [35]). The PA/plasmin system is a key regulator in extracellular matrix (ECM) remodeling directly by its ability to degrade ECM components, such as laminin or fibronectin, and indirectly via activation of matrix metalloproteinases (MMPs), which will degrade collagen(s) subsequently. Furthermore, plasmin is able to activate latent growth factors, such as transforming growth factor β (TGFβ) and basic fibroblast growth factor (bFGF) (reviewed in [35]).

Work from numerous groups has clearly demonstrated that the localization of plasminogen and its activators uPA and tPA on the cell surface, though association to specific cell membrane receptors, provides a mechanism for cells to harness and regulate the activities of these proteases [36, 37]. Binding sites for plasminogen, tPA, and uPA have been identified on a variety of cell types, including monocytes, fibroblasts, and endothelial cells [38, 39]. uPA is recruited

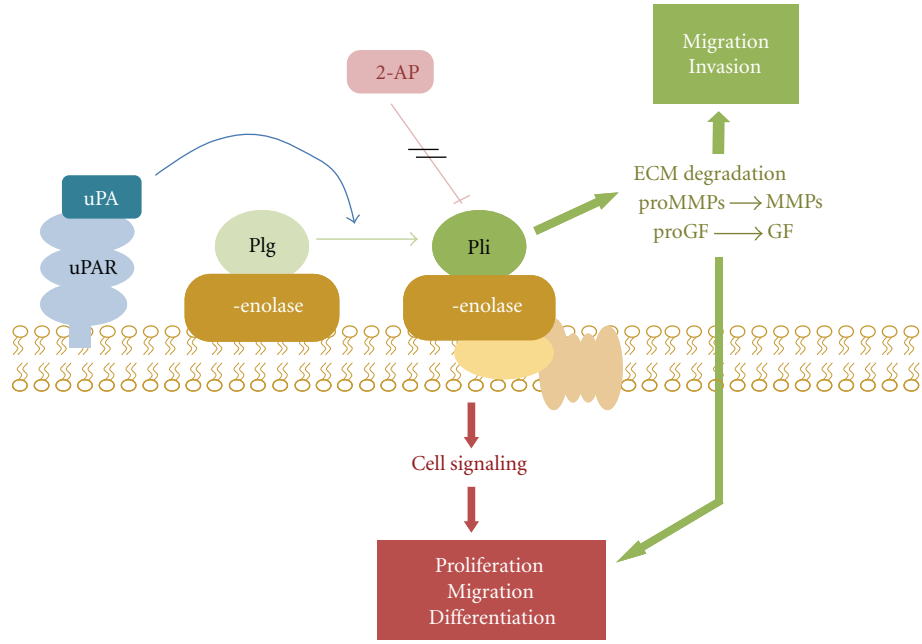

Figure 3: Schematic overview represents α-enolase/plasminogen interaction on the cell surface. α-Enolase enhances plasminogen activation on the cell surface, concentrates plasmin proteolytic activity on the pericellular area and protects plasmin from its inhibitor α2-antiplasmin. Once activated, plasmin can degrade most of the components of the extracellular matrix, directly or indirectly by activating metalloproteases. It is also capable to activate prohormones of progrowing factors. Abbreviations: Plg, plasminogen; Pli, plasmin, α2-AP, α2-antiplasmin; uPA, urokinase-type plasminogen activator, uPAR, urokinase-type plasminogen activator; ECM, extracellular matrix; MMPs, metalloproteases; GF, growing factors.

to the cell membrane immediately after its secretion via a specific uPA receptor (uPAR, CD87), expressed on the cell surface, that localize extracellular proteolysis and induces cell migration, cell adhesion, and proliferation (reviewed in [40, 41]).

Described binding sites for plasminogen include α-enolase [18, 42], annexin A2 [43], p11 [44], histone H2B [45, 46], actin [47], gp330 [48], cytokeratin 8 [49], histidine-proline rich glycoprotein [50], glyceraldehide-3-phosphate dehydrogenase [51] gangliosides [18], and Plg-R_{TK} [52]. α-Enolase and most of these proteins have C-terminal lysines predominantly responsible for plasminogen binding/activation [53]. Notably, most of these proteins have other described functions than plasminogen receptors, and lack a transmembrane domain, Plg-R_{TK} being an exception, as it is a transmembrane receptor [52].

3. α-Enolase as a Plasminogen Receptor

We and others have previously identified α-enolase as a plasminogen receptor on the surfaces of several diverse cell types including carcinoma cells [42], monocytoid cells [15, 18], leukocytic cell lines [54], rat neuronal cells [16], and pathogenic streptococci [1].

On the cell surface, interaction of plasminogen with α-enolase enhances its activation by PAs, concentrates protease activity pericellularly [55–57], and protects plasmin from inhibition by α2-antiplasmin [18, 58] (Figure 3). In order to examine the role of α-enolase in the pericellular generation of plasmin activity, we produced a monoclonal antibody, MAb11G1, that specifically blocked plasminogen binding to purified α-enolase [54]. MAb11G1 allowed us to demonstrate that α-enolase occupancy by plasminogen on leukocytoid cells and on peripheral blood neutrophils is required for pericellular plasminogen activation and plasmin generation [54].

Considering the extraordinarily high number of plasminogen binding sites/cells that have been described in different cell types, no single surface protein can account for all plasminogen binding sites, suggesting that different receptors coexist on the cell surface [18]. Evidence from monocytoid cells suggested that α-enolase was only one of several plasminogen receptors and its contribution to plasmin activation was only modest [18, 54]. Posterior studies have emphasized the role of annexin A2 and histone H2B as plasminogen receptors in the same cells [46], suggesting a minor contribution of α-enolase as plasminogen receptor. In more recent studies, the role of α-enolase has been resurrected, showing a central role for α-enolase in monocyte recruitment in inflammatory lung disease [59]. These results imply that different plasminogen receptors could be targeted to regulate inflammatory cell recruitment in a temporal-specific manner.

The α-enolase-plasminogen interaction is mediated by binding of plasminogen kringle domains to the C-terminal residues of α-enolase (K_{434}) [15, 18]. Furthermore, interaction of plasminogen lysine binding sites with α-enolase depends upon recognition of C-terminal lysines K_{420}, K_{422},

and K_{434}, suggesting that amino acid residues upstream and/or secondary structure may be responsible for the high affinity of α-enolase for plasminogen [15, 18]. Another putative plasminogen-binding motif has been proposed in view of its crystal structure at position, $_{250}$FFRSGKY$_{256}$, that remains exposed when α-enolase forms a dimer, necessary for its glycolytic activity [10]. Human α-enolase structure has been determined and it has been found that it exhibits specific surface properties that are distinct from those of other enolases despite high-sequence similarity. These differences in structure explain its various activities, including plasmin(ogen) and DNA binding [10].

The mechanism by which α-enolase, that lacks a signal sequence, is associated with the cell membrane remains unknown. Some authors have speculated that a hydrophobic domain within α-enolase might serve as an internal signal sequence [60], while others suggest that posttranslational acetylation [61] or phosphorylation [62] may control membrane association. Nevertheless, α-enolase forms part of a growing list of proteins that lack signal sequences, but are transported to the cell surface by a yet unknown mechanism.

4. α-Enolase in Myogenesis and Muscle Regeneration

Proteolysis associated with the cell surface is a usual mechanism in several physiological processes involving tissue remodeling. Myogenesis is an example of tissue remodeling in which massive extracellular matrix degradation takes place. Components of the PA system play important, yet distinct roles in muscle regeneration after injury. Using genetically modified mice for uPA and plasminogen, we and others have shown that loss of uPA-mediated plasmin activity blunts muscle repair *in vivo* [63–66]. In contrast, a negative role for PAI-1 in muscle regeneration was suggested [65]. The PA system has also been shown to have an increasingly important role in muscular dystrophies. For example, greater expression of uPA has been found in *mdx* muscle, the mouse model for Duchenne muscle dystrophy (DMD). Conversely, genetic loss of uPA exacerbated dystrophy and reduced muscle function in *mdx* mice [66]. Satellite cells derived from human DMD patients produce more uPAR and PAI-1 and less uPA than normal satellite cells [67]. uPA and plasmin appear to be required for infiltration of macrophages into the damaged or dystrophic muscle in *mdx* mice. However, an interesting observation underpinning these results was that genetic loss of the uPAR in *mdx* mice failed to exacerbate muscular dystrophy, suggesting that uPA exerts its proteolytic effects independently of its cell surface receptor uPAR [66].

β-enolase is considered the specific muscular enolase isoform, it is expressed in proliferating adult myoblasts as well as in differentiated myotubes [68]. It is upregulated in muscle during embryogenic development and it is considered an early marker of myogenesis [69]. The increase of the β-isoform is accompanied by a decrease of the α and γ isoform; the γ-isoform is completely absent in the adult muscle, but the expression of the α-isoform is maintained in the adult muscle and in muscular cells [70, 71]. Furthermore, we have described that α-enolase is upregulated in murine myoblasts C2C12 differentiation *in vitro* and in muscle regeneration *in vivo* [72], thus raising the question of whether plasminogen receptors may also function in myogenesis and skeletal regeneration as a mechanism for regulating plasmin activity.

We have investigated the role of α-enolase plasminogen receptor in muscle regeneration after injury, a process involving extensive cell infiltration and ECM remodeling. Injured wild-type mice and dystrophic *mdx* mice were treated with inhibitors of α-enolase/plasminogen binding: MAb11G1 (an inhibitory monoclonal antibody against α-enolase) and ε-aminocaproic acid (EACA, a lysine analogue). These treatments had negative impacts on muscle repair by impairing adequate inflammatory cell infiltration and promoting extracellular matrix deposition, which resulted in persistent degeneration. Furthermore, satellite cell-derived myoblasts (i.e., MPCs) expressed α-enolase on the cell surface, and this expression was upregulated during myogenic differentiation, correlating with an increase of plasminogen binding to the cell surface. We found that both MAb11G1 and EACA treatments impaired satellite cell-derived myoblasts functions *in vitro* in agreement with blunted growth of new myofibers *in vivo* (Diaz-Ramos et al., unpublished results).

Loss of uPAR *in vivo* did not affect the degeneration/regeneration process; in addition, cultured myoblasts from uPAR-deficient mice showed efficient myoblast differentiation and fusion [66, 73], indicating that uPAR is dispensable for efficient muscle repair. This reinforces the idea that α-enolase is the main functional plasminogen receptor during muscle tissue remodeling. Altogether, these results demonstrate the novel requirement of α-enolase for restoring homeostasis of injured muscle tissue, by concentrating plasmin activity on the cell surface of inflammatory and myogenic cells.

5. The α-Enolase Expression in Injured Cardiac Muscle

The actuation of the PA system in tissue healing after a cardiac failure, driving the degradation the ECM and scar tissue after an ischemic injury and allowing the inflammatory cell invasion, has been extensively demonstrated.

The regulation of α-enolase in cardiac tissue as regulator of glucose metabolism has been analyzed by several authors. A decrease of α-enolase expression in the aging heart of old male monkeys has been described, paralleling left ventricular dysfunction, and could be involved in the mechanism for the cardiomyopathy of aging [74]. α-Enolase expression has been identified as a strongly induced factor in response to ischemic hypoxia and reoxygenation in rat hearts subjected to ischemia-reperfusion [75]. Furthermore, α-Enolase improved the contractility of cardiomyocytes impaired by ischemic hypoxia [76]. α-Enolase has also been proposed as a marker for early diagnosis for acute myocardial infarction [77].

On the other hand, recent evidences indicates an involvement of proteinases, including the PAs and MMPs systems, in the process of extracellular matrix degradation

and cell migration during cardiac wound healing [78]. In a recent study, Heymans et al. demonstrated that uPA-deficient mice showed impaired infarct healing and were completely protected against cardiac rupture after induction of a myocardial infarction [79]. Wound healing after infarct was abolished in plasminogen-deficient mice, indicating that the plasminogen system is required for the repair process of the heart after infarction. In the absence of plasminogen, inflammatory cells did not migrate into the infarcted myocardium, necrotic cardiomyocytes were not removed and there was no formation of granulation tissue and fibrous tissue [80]. Furthermore, PAI-1, which has been shown to be expressed in mammalian cardiomyocytes [81], has been implicated in the process of the cardiac remodeling by inhibiting activation of MMPs as well as plasmin generation. A dramatic induction of PAI-1 in a mouse model of infarct has been described [82]. Experiments using mice deficient in PAI-1 suggest that increased expression of cardiac PAI-1 may contribute to the development of fibrous change after acute myocardial infarction (AMI). *In vivo* studies also showed that PAI-1 expression was induced in hearts under pathological conditions as ventricular hypertrophy [83].

All these results demonstrate that the PA system plays a role in ECM remodeling after a cardiac injury and allows inflammatory cell invasion. Furthermore, it can also play a role in cardiomyocyte survival. Cardiomyocytes, which are terminally differentiated cells, cannot proliferate, even when they are damaged; the damage can lead to cell death in the case of serious diseases such as acute myocardial infarction and myocarditis [84]. Recent studies have identified myocyte apoptosis in the failing human heart [85, 86]. Plasminogen could also drive cardiomyocyte apoptosis, because plasmin induces cell detachment and apoptosis of smooth muscle cells through its binding to the cell surface, although the receptor responsable for plasminogen binding has not yet been identified [87].

Knowing that the PA system has been associated with cardiac remodeling, and that α-enolase is upregulated in cardiac infarction, it is tempting to speculate that α-enolase could act as plasminogen receptor, regulating PA activity on cardiac cells. Previous results from our laboratory have shown that plasmin activity is concentrated on the cell surface of cardiac fibroblasts in a lysine-dependent manner, and this binding capacity is increased by hypoxic conditions. Furthermore, plasminogen binding drives the activation of fibroblasts to myofibroblasts, the main cells responsible of tissue remodeling after a cardiac injury (Garcia-Melero et al., unpublished results).

6. α-Enolase/Plasmin Role in Apoptosis

It has been described that plasminogen binding to the cell surface and its further activation to plasmin induces cell detachment and apoptosis in smooth muscle cells, neurons and vascular myofibroblasts [88–90], although the molecular responsible for plasminogen interaction with the cell surface has not been identified.

Externalization of glycolytic enzymes is a common and early aspect of cell death in different cell types triggered to die with different suicidal stimuli [91]. Apoptotic cells are recognized by phagocytes and trigger an active immunosuppressive response. The lack of inflammation associated normally with the clearance of apoptotic cells has been linked to inflammatory and autoimmune disease as systemic lupus erythematosis and rheumatic diseases [92–95]. Regarding apoptotic cell surface proteins, a new concept has been defined, SUPER, referring to Surface-exposed (during apoptotic cell death), Ubiquitously expressed, Protease sensitive, Evolutionary-conserved, and Resident normally in viable cells (SUPER), to emphasize defining properties of apoptotic determinants for recognition and immune modulation. Ucker et al. have recently demonstrated that almost all members of the glycolytic pathway are enriched among apoptotic cell membranes, with α-enolase being the more abundant enzyme in the cell membrane, and considered the most paradigmatic SUPER protein [91]. In the cell membrane of apoptotic cells, α-enolase has lost its glycolytic activity, but it acts as plasminogen receptor, coinciding with the description of the association of plasminogen binding with apoptotic cell death [96]. In contrast to α-enolase, other molecular plasminogen receptors as annexin A2 [97] or H2B [46], were not preferentially enriched on the apoptotic cell surface.

7. α-Enolase in Cancer

Several reports have shown an upregulation of α-enolase in several types of cancer [98–100]. The role of α-enolase as a plasminogen receptor on cancer cells has been extensively documented, where it acts as a key protein, promoting cellular metabolism in anaerobic conditions, and driving tumor invasion through plasminogen activation and ECM degradation (reviewed in [101]).

Recently, an analysis of disease-specific gene network identified desmin, interleukin 8, and α-enolase as central elements for colon cancer tumorogenesis [102]. Knockdown of α-enolase expression in different tumor cell lines caused a dramatic increase in their sensitivity to microtubule targeted drugs (e.g., taxanes and vincristine), probably due to α-enolase-tubulin interactions [103], suggesting a role for α-enolase in modulating the microtubule network. Downregulation of α-enolase gene product deceased invasiveness of the follicular thyroid carcinoma cell lines [104]. α-Enolase overexpression has been associated with head and neck cancer cells, and this increase associated not only with cancer progression but also with poor clinical outcomes. Furthermore, exogenous α-enolase expression promoted cell proliferation, migration, invasion, and tumorogenesis [105].

During tumor formation and expansion, tumor cells must increase glucose metabolism [106]. Hypoxia is common feature of solid tumors. Consistent with this, overexpression of glycolytic genes has been found in a myriad of human cancers [107]. In tumor cells, α-enolase is upregulated and supports anaerobic proliferation (Warburg effect), and it is expressed on the cell surface, where it promotes cancer invasion. Thus, it seems that α-enolase is playing a pleitropic role on cancer cell progression. Furthermore, it has been demonstrated that α-enolase is upregulated

in pancreatic ductal adenocarcinoma, where it is subjected to a array of posttranslational modifications, namely acetylation, methylation, and phosphorylation [108]. Both, α-enolase expression and posttranslational modifications could be of diagnostic and prognostic value in cancer (reviewed in [101]).

8. Posttranslational Modifications of α-Enolase

Posttranslational protein modifications, such as phosphorylation, acetylation, and methylation are common and important mechanisms of acute and reversible regulation of protein function in mammalian cells, and largely control cellular signaling events that orchestrate biological functions. Several posttranslational modifications have been described for α-enolase. α-Enolase phosphorylation has been associated with pancreatic cancer, and induces specific autoantibody production in pancreatic ductal adenocarcinoma patients with diagnostic value [109]. Lysine acetylated α-enolase has been detected in mouse brain [110]. Nitration of tyrosine residues in α-enolase has been detected in diabetic rat hearts, contributing to the impaired glycolytic activity in diabetic cardiomyopathy [111]. Phosphorylated α-enolase has been detected in gastrocnemious muscle, and phosphorylation decreased with age [112]. Furthermore, carbonylation of α-enolase has been detected on human myoblasts under oxidative stress [113].

It remains to be determined how the posttranslational modifications of α-enolase can affect its catalytic activity, localization of the cell, protein stability, and the ability to dimerize or form a complex with other molecules. Investigations of these modifications patterns in different pathologies will provide insights into its important role in pathophysiological processes.

9. α-Enolase in Rheumatoid Arthritis

The overexpression of α-enolase has also been found associated with chronic autoimmune diseases like rheumatoid arthritis [19, 114], systemic sclerosis [115], and primary nephropathies [116]. Autoantibodies to α-enolase, are present in the sera of patients with very early rheumatoid arthritis and have potential diagnostic and prognostic value [117]. Recently, citrullinated proteins have been considered the main autoantigen of rheumatoid arhritis. Citrullination, also termed deimination, is a modification of arginine side chains catalyzed by peptidylarginine deaminase. This posttranscriptional modification has the potential to alter the structure, antigenicity, and function of proteins. α-Enolase is abundantly expressed in the sinovial membrane, and antibodies against citrullinated α-enolase were specific for rheumatoid arthritis. Citrullination changes the conformation of α-enolase and interferes with the noncovalent interaction involved in the formation of the enolase dimer, then results in an altered glycolytic activity and plasminogen binding. It is likely that citrullination of cell-surface α-enolase abrogates its plasminogen binding and activating function and contributes to the decreased fibrinolysis observed in rheumatoid arthritis [118]. Curiously, other glycolytic enzymes such as glucose phosphate isomerase and aldolase also promote rheumatoid arthritis autoimmunity by acting as autoantigens [119].

10. α-Enolase in Alzheimer's Disease

Although γ-enolase is the specific neuronal enolase isoform, α-isoform is also present in neurological tissues. Plasmin formation enhanced by α-enolase has been proposed to enhance neuritogenesis [16, 120]. Furthermore, cathepsin X cleavage of C-terminal lysine of α-enolase impaired survival and neuritogenesis of neuronal cells [121]. α-Enolase has been reported as a strong plasminogen receptor within the brain; it is known to be upregulated in the Alzheimer's disease brain and has been proposed as a promising therapeutic target for this disease (reviewed in [122]). Glucose hypometabolism and upregulation of glycolytic enzymes is a predominant feature in Alzheimer's disease [123], but accumulating results suggest that α-enolase may have other functions that just metabolic processing of glucose: plasminogen bound to α-enolase stimulates plasmin activation of mitogen-activated protein kinase (MAPK)/extracellular-signal regulated kinase 1/2 (ERK1/2) prosurvival factor and also can drive plasmin degradation of amyloid-β (Aβ) protein, the main component of amyloid plaques. Thus, α-enolase might play a neuroprotective role through its multiple functions (reviewed in [122]).

Recently, several posttranslational modifications to α-enolase have been found in Alzheimer's disease. Elevated levels of glycosylated-α-enolase [124], oxidized [123], or glutathionylated [125] have been found related to Alzheimer's disease. These modifications would render enolase catallyticaly inactive, related to the metabolic deficit associated to Alzheimer's disease. The effect of these modifications in other multiple functions of α-enolase is a subject of ongoing experiments, but it is possible that α-enolase modifications alter not only glucose metabolism, but also its role as plasminogen receptor, controlling neuronal survival and Aβ degradation.

11. Plasmin and Intracellular Signaling

Other than its role in concentrating proteolytic activity on the cell surface, several recent studies have shown that plasmin is able to activate several intracellular signaling pathways, that led to the activation of several transcription factors, in a cell surface binding dependent way. In most of the cases, the molecular mechanism responsible remains unknown: it could be due to the proteolytic activation of a second factor or due to direct binding of plasmin(ogen) to a specific receptor. Several pieces of work show that the plasmin proteolytic activity is essential for the induction of an intracellular response, as in monocytes, where plasmin bound to the cell surface proteolytically activates annexin A2 and stimulates MMP-1 production through the activation of ERK and p38 pathways [126]. The phosphorylation of Janus Kinase 1 (JAK1)/Tyrosin Kinase 2 (TYK2) that drives to the activation to the transcription factors AP-1 and Nuclear Factor κB (NFκB), and the expression of several

cytokines: interleukin-1α and-1β (IL-1α and IL-1β), tissue factor (TF), and the Tumoral Necrosis Factor-α (TNF-α), are a consequence of plasmin interaction with the cell surface [127–129]. Plasmin promotes p38 and p44/42 MAPK activation and fibroblast proliferation through Protease Activated Receptor-1 (PAR-1) [130, 131]. Other authors have described that plasminogen and plasmin regulate the gene transcription of genes as *c-fos, erg-1*, and *Eno1* in mononucleated blood cells and fibroblasts, by activating the MEK/ERK pathways [132, 133].

In most of the cases, the receptor responsible for this cellular response remains to be identified. Most of the protein candidates for plasminogen receptors are small proteins that lack a transmembrane domain and are not able to induce directly an intracellular response. Some work suggests an association between the plasminogen receptor and other membrane proteins, that could serve as molecular collaborators to induce the activation of intracellular signaling pathways. Several proteins have been identified as such molecular collaborators. For instance, plasmin can activate PAR-1 in fibroblasts, by the phosphorylation of Erk [130]; plasminogen and plasmin activate the expression of several genes in fibroblasts and monocytes through G-Protein Coupled Receptors, (GPCR) [132, 133]; some integrins such as $\alpha 9\beta 1$ integrin in Chinese Hamster Ovary (CHO) cells [134] and $\alpha v\beta 3$ integrin, in vascular endothelial cells [135], participate actively in plasmin-induced cell migration.

In none of these cases, the plasmin receptor associated with these proteins have been identified. Some work have identified annexin A2 as the receptor that concentrates plasmin activity to the cell surface and drives a subsequent intracellular response [127–129]. Other authors have described a collaboration between α-enolase and GPCR in fibroblasts and mononucleated blood cells [132, 133]. Plasmin induces smooth muscle cell proliferation through extracellular transactivation of the epidermal growth factor receptor (EGFR) by a MMP-mediated, heparin binding—epidermal growth factor (HB-EGF-) dependent process [136]. Future studies will be necessary to determine the molecular mechanism of the plasminogen receptor on several cell types and the putative proteins associated with it.

We have shown that plasmin activity is able to activate MAPK/ERK and phosphatidyl-inositol 3-kinase (PI3K)/Akt pathways in C2C12 murine myoblast cell lines and in primary cultures of muscle precursor cells, and that intracellular activation depends on plasmin activity, but also on plasmin(ogen) binding to the cell surface in a lysine binding sites dependent way (Roig-Borrellas et al., unpublished results), although the receptor responsible and the molecular mechanism remains to be elucidated.

12. Concluding Remarks

Recently, a proteomic meta-analysis of 169 published articles, including differently expressed 4700 proteins, based on 2-dimensional electrophoresis analysis of human, mouse, and rat tissues, identified α-enolase as the first protein differentially expressed in mice and the second in human pathologies, regardless of the tissue used and experiment performed [137], suggesting that α-enolase could be part of a group of universal cellular sensors that respond to multiple different stimuli. Thus, α-enolase could be considered as a marker of pathological stress in a high number of diseases. The importance of α-enolase as plasminogen receptor has been determined in several pathologies such as cancer, skeletal myogenesis, Alzheimer's disease, and rheumatoid arthritis, among others. α-Enolase upregulation has also been described in a myriad of other pathologies, as inflammatory bowel disease [138, 139], autoimmune hepatitis [140], or membranous glomerulonephritis [141], not discussed in this paper, although its role on concentrating plasmin activity on the cell surface has not always been stablished. It will not be surprising that in many of these pathologies, α-enolase could exert one of its multiple functions, mainly as a plasminogen receptor, focalizing plasmin activity on the cell membrane and promoting ECM degradation/remodeling, but also activating intracellular survival pathways and controlling survival/apoptosis of cells.

Further studies of posttranslational modifications of α-enolase and its implications on α-enolase subcellular distribution and function, especially interaction with other proteins will be necessary. Also, the role of α-enolase as activator of intracellular signaling pathways, probably in collaboration with other membrane proteins, will serve to elucidate the multiples roles of this functionally complex protein.

Unexpectedly, other glycolytic enzymes have been described as having other nonglycolytic functions in transcriptional regulation (hexokinase-2, HK; lactate dehydrogenase A, LDH; glyceraldehydes-3-phosphate dehydrogenase, GAPDH), stimulation of cell motility (glucose-6-phosphate isomerase), and regulation of apoptosis (glucokinase, HK and GAPDH), indicating that they are more complicated, multifunctional proteins rather than simply components of the glycolytic pathway (reviewed in [142]).

Some of the more interesting and challenging issues, regarding α-enolase multifunction that need to be addressed are (i) the mechanism of its export to the cell surface, (ii) the role of α-enolase as an inductor of intracellular signaling pathways, and (iii) the role of posttranslational modifications of α-enolase and implications on its subcellular distribution and function. Investigations of these subjects in different human pathologies will provide insights into its important role on pathophysiological processes and it would make this protein an interesting drug target for different diseases.

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Review Article

Bacterial Plasminogen Receptors Utilize Host Plasminogen System for Effective Invasion and Dissemination

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In order for invasive pathogens to migrate beyond the site of infection, host physiological barriers such as the extracellular matrix, the basement membrane, and encapsulating fibrin network must be degraded. To circumvent these impediments, proteolytic enzymes facilitate the dissemination of the microorganism. Recruitment of host proteases to the bacterial surface represents a particularly effective mechanism for enhancing invasiveness. Plasmin is a broad spectrum serine protease that degrades fibrin, extracellular matrices, and connective tissue. A large number of pathogens express plasminogen receptors which immobilize plasmin(ogen) on the bacterial surface. Surface-bound plasminogen is then activated by plasminogen activators to plasmin through limited proteolysis thus triggering the development of a proteolytic surface on the bacteria and eventually assisting the spread of bacteria. The host hemostatic system plays an important role in systemic infection. The interplay between hemostatic processes such as coagulation and fibrinolysis and the inflammatory response constitutes essential components of host defense and bacterial invasion. The goal of this paper is to highlight mechanisms whereby pathogenic bacteria, by engaging surface receptors, utilize and exploit the host plasminogen and fibrinolytic system for the successful dissemination within the host.

1. Introduction

Bacterial invasion is generally mediated by the bacterial surface and secreted products which often function to circumvent host innate and acquired defense systems. Evasion of host immune response and production of invasive molecules are often critical first steps for initiating systemic diseases. The host hemostatic system plays an important role in systemic infection and bacterial pathogenesis. Hemostatic processes such as coagulation and fibrin deposition, as a result of inflammation, are an essential part of the host defense system. Invasive bacterial pathogens, however, have developed a variety of strategies to elude the host line of defense and gain entry into the surrounding host tissue. The ability to degrade tissue barriers formed by extracellular matrices (ECM) and basement membranes (BM) is one of the most important factors in the pathogenesis of bacterial infection. Degradation of this network by secreted bacterial proteases leads to tissue and structural damage and thereby enhances bacterial invasiveness into the host body. However, a number of invasive bacteria like *Streptococcus*, *Haemophilus, Neisseria* and most enteric bacteria like *E. coli* are extracellular pathogens and produce low levels of proteases. Consequently, degradation and penetration through this network of membranes require the use of different mechanisms for invasion. A number of these mechanisms rely on the interaction with protease-dependent cascade systems of their host which include fibrinolysis, coagulation, phagocytosis, and complement activation. The mammalian fibrinolytic system which constitutes dissolution of thrombus by the serine proteinase plasmin (Pm) offers a potential proteolytic system that could be utilized by pathogenic bacteria to gain entry into the host system. Plasminogen (Plg) binding to bacteria can almost be considered a universal event [1–3]. Plasmin has been known to play a significant role in several physiological processes apart from degradation of fibrin clot in fibrinolysis and various extracellular matrix and connective tissue components like

laminin and fibronectin. Pm also activates procollagenases to active collagenases and is involved in activation of certain prohormones and growth factors [4–6]. Recently, it has also been shown that blood-brain barrier invasion is enhanced by Pm acquisition [7]. Through activation of matrix metalloproteases (MMPs), Pm can break down extracellular matrices and basement membranes either directly or indirectly and degrade complement and immunoglobulins thereby facilitating the likelihood of bacterial spread (see review [3]) [8, 9]. Therefore, Pm activity must be tightly controlled in order to maintain tissue homeostasis and avoid random tissue damage. Such regulation is achieved by the plasminogen system due to the availability of Plg receptors (PlgRs) and plasminogen activators (PAs).

Bacteria interact with the Plg system by secreting PAs and expressing PlgRs on their surface which direct the Pm activity to locations where proteolytic activity is required. Many of the bacterial PlgRs are critical virulence factors and are among the major targets of vaccine development.

The fibrinolytic system is known to play an important role in the inflammatory response to bacterial infections and host Plg system plays a central role in fibrinolysis. The fibrinolytic system functions to break down the existing fibrin-containing blood clot and is an important constituent of wound-healing mechanisms. Fibrin clots are formed during coagulation and injury to blood vessel walls resulting in fibrin deposition and platelet aggregation. The main active enzyme involved in the fibrinolytic process is Pm. Fibrinolysis is initiated when Plg is converted to Pm by host physiological activators urokinase-type plasminogen activator (uPA) or tissue-type plasminogen activator (tPA). During infection, at the site of local microbial injury, host inflammatory cells in association with bacterial secreted products like endotoxins generate a vigorous response in the surrounding vasculature resulting in local vascular thrombosis. This serves to wall off the site of infection and can, in turn, entrap bacteria and acts as a barrier to prevent bacterial invasion and systemic spread. Most likely this is why fibrin deposits are often seen at the site of infection. The formation of active Pm on the bacterial surface facilitates the degradation of the fibrin layer deposited by the host around the site of local infection promoting release of bacteria from fibrin clot and subsequently assists in ECM degradation thereby facilitating the bacterial dissemination into deeper tissues. Therefore, there is an exquisite mechanistic relationship between the bacterial proteins and host protein which facilitate bacterial dissemination and survival. Through their ability to bind to host Plg directly, PlgRs therefore serve to enhance the activation of surface bound Plg to Pm, creating a proteolytic microenvironment to be employed by pathogens to catalyze degradation of matrix barriers, which then assists the spread of the bacteria [23, 24] (Figure 1). In recent years, studies to understand the mechanisms and the role of the Plg system in bacterial infections have increased significantly, and this paper summarizes our present knowledge of a select group of bacterial pathogens that utilize host Plg/Pm system for the dissemination into surrounding tissue.

2. Key Players in the Plasminogen System

2.1. Plasminogen and Plasmin. Human plasminogen (hPlg) is synthesized in the liver as a 90 kDa, 810 amino acid polypeptide chain. During secretion, a 19 amino acid leader peptide is cleaved generating the mature form of this protein, which comprises 791 amino acids [25, 26]. This native form of Plg is called Glu-Plg due to the presence of an aminoterminal glutamic acid residue. The Plg molecule contains a total of seven structural domains, each with different properties. The N-terminal portion of the molecule consists of an activation peptide (AP) followed by a series of 5 repeating homologous triple-disulfide-linked peptide regions, approximately 80 amino acids in length, termed kringles (K1–K5). Cleavage of the peptide bond between residues 77 and 78 is required for the release of the activation peptide. The truncated form of the zymogens is then designated as Lys-Plg. The cleavage of the Arg561-Val562 peptide bond in hPlg leads to the formation of Pm, which contains a heavy chain of 561 amino acid residues, disulfide linked to a light chain of 230 amino acid residues. The 65-kDa heavy chain comprises the N-terminal portion of the Plg molecule, which consists of AP and 5 kringles (K1–K5) (Figure 2). The function of the five kringles in the heavy chain of Plg is primarily to mediate protein-protein interactions, such as those between binding of Plg or Pm to fibrin, ECM targets, and lysine-containing receptors/analogues. Plg kringles are also independently involved in angiogenesis and inhibition of cell migration [27, 28]. The Pm light chain contains the carboxyl terminus of hPlg, which comprises the catalytic domain that resembles that of the serine protease family. The catalytic triad of amino acids that define serine proteases is present in human Pm and consists of His603, Asp646, and Ser741 [5]. The catalytic domain or serine protease domain (SP) of Pm is a compact module that can recruit adapter molecules, or cofactors, such as the bacterial PAs streptokinase and staphylokinase, which modify the substrate presentation to the enzyme and its specificity [29]. Almost all kringle modules bind to lysine or lysine-like ligands except K3. K1 and K4 exhibit the strongest ligand affinities [30–33] while K2 possesses the weakest affinity [34]. K2 shows strong affinity to a endopolypeptide (VEK-30) derived from *Streptococcal* Plg receptor M protein (PAM) [35]. The binding strength of kringle modules is dependent on the nature of the ligand.

Glu-Plg can adopt two different conformations, T and R, which highly influence their activation capability. The T state (tight conformation) is a compact state seen in full-length hPlg. In this conformational state, Plg is poorly activated [36, 37]. Investigations of recombinant (r) Glu-Plg variants have shown that both negative and positive effector molecules have an influence on the structure and activation of Plg [38, 39]. For example, it has been suggested that in the presence of Cl^-, Lys side chains, and/or pseudo-Lys arrangements in the 77 amino acid NH2-terminal AP interact with ω-amino acid binding sites of K1Plg, K4Plg, and to a lesser extent, K5Plg. In addition, studies have shown that a number of activation peptide residues interact with these kringle sites and participate in the stabilization of the

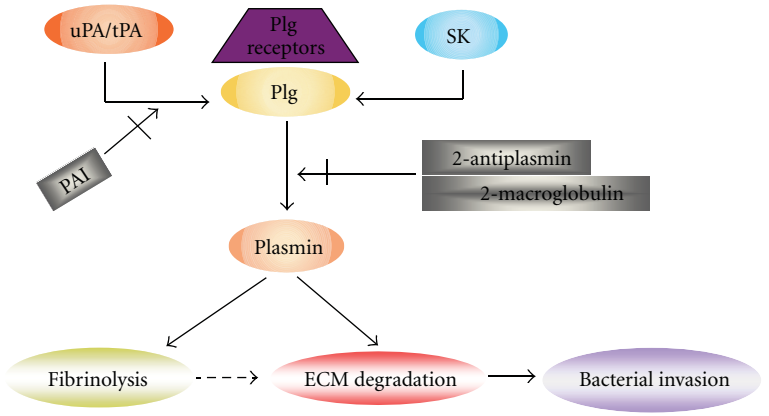

Figure 1: Simplified overview of the mammalian plasminogen system and its utilization by bacteria. Bacterial plasminogen receptors immobilize plasminogen on the surface, which enhances activation of plasminogen to the active serine protease, plasmin, by host plasminogen activators uPA, tPA and/or bacterial plasminogen activators like streptokinase (SK). Both tPA and uPA can be inhibited by plasminogen activator inhibitors (PAI), while plasmin activity is controlled by its major inhibitor, α2-antiplasmin, and to a lesser extent by α2-macroglobulin. Plasmin degrades fibrin clot (fibrinolysis) and various ECM components which enables bacterial migration through tissue barriers.

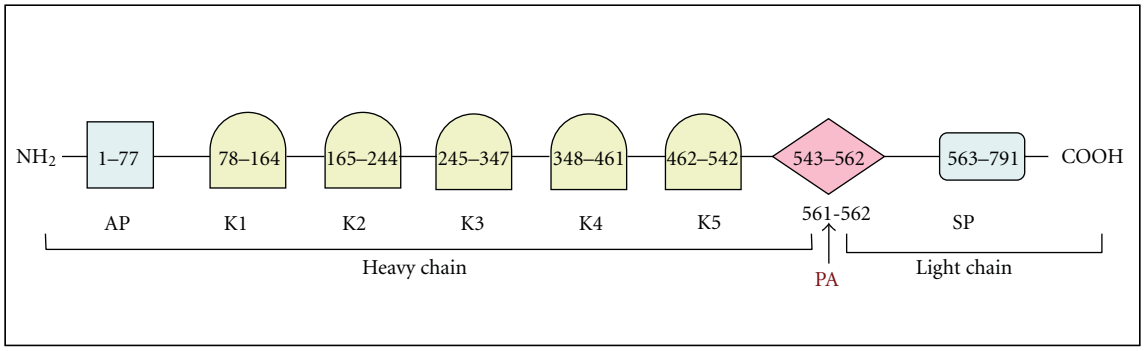

Figure 2: Schematic presentation of the structure of human Plg. From the amino terminus of the mature protein, the 77-residue activation peptide (AP) is followed by 5 kringle domains (K1–K5) containing lysine binding sites and the catalytic SP domain. R561-V562, which is proteolytically cleaved to generate Pm, is shown with an asterisk. The cleavage site for plasminogen activator (PA) is shown by an arrow. Heavy chain and light chains after proteolytic cleavage are marked with lines.

T conformation [40]. On the other hand, the conformation of Glu-Plg changes to a more flexible relaxed state (R state), that is readily activated when the activation peptide (AP) is displaced from its kringle binding sites upon addition of ω-amino acids, for example, ε-aminocaproic acid (EACA). Once Glu-Pm is formed, it acts as a catalyst for cleavage of the AP from Glu-Plg and Glu-Pm, thus catalyzing conversion of Glu-Plg to Lys78-Plg or Glu-Pm to Lys78-Pm. Release of the AP renders Lys78-Plg a more activatable substrate due to its transformation to the R state and transition to a protein that is now insensitive to negative and positive effector molecules [41, 42]. The Lys-Plg conformation enables it to interact with PlgRs and also facilitates ready conversion to Pm on the cell surface [43].

The atomic structures of the kringles as well as a truncated form consisting of a 20 amino acid long fragment of the heavy chain connected to the light chain by two disulfide bridges have been reported [44–47]. The structural changes that the catalytic domain undergoes in association with the activation of Plg have also been identified by crystallography [48].

Recently, the crystal structure of full-length type II human Plg has been solved [49]. The structure shows a closed conformation which reveals that N-terminal AP domain makes extensive contacts with K4 and K5. These interactions, together with SP domain and chloride ions, maintain the T conformation. The Arg561-Val562 peptide bond which is proteolytically cleaved to convert Plg to Pm is shielded by the position of K3/K4 linker which perhaps protects the proenzyme from unwanted activation in the closed state. Position of K3 might also serve as a physical barrier to activating proteases. However, superpositioning SK, which can readily activate the closed state of plasminogen, to the Plg structure revealed that despite these safeguards SK can bind to the SP domain in a way that can cleverly avoid the kringle modules. K3 forms a significant interface (including a disulphide bond) with K2, as previously observed in the structure of angiostatin [50]. These interactions position

K3 next to the SP domain. The authors also noted that differences in glycosylation most likely alter the position of K3. The structure shows that LBS of K1 is exposed and most likely mediates proenzyme recruitment to fibrin or receptors. Previously it was suggested from the structures of isolated kringle domains in the presence or absence of lysine analogs that K1 is most likely not involved in structural rearrangement but another kringle(s) might be. Further analysis of the Plg structure reveals that indeed K5 is the kringle crucial for triggering Plg conformational change and peeling away of K5 from AP domain may initiate that change. Another human plasminogen closed conformation structure published recently also supports these observations and shows that only K1-LBS is readily available for ligand and/or receptor binding while LBS of other kringles, except K1, are involved in intramolecular interactions [51].

2.2. Host Plasminogen Activators. Plasminogen is present in large amount in blood as well as in tissues. Due to the broad biological significance of Plg and Pm in cellular processes, it is therefore of utmost importance that this enormous reservoir of proteolytic activity be tightly regulated. This is achieved by specific PAs, inhibitors, and receptors in order to restrict the generation of Pm, as well as immobilize Plg to its receptors or target molecules.

Mammals have two PAs, tissue-type plasminogen activator (tPA) and urokinase-type plasminogen activator (uPA) which were originally identified in tissue and urine extracts, respectively [52]. They recognize and cleave the R560-V561 peptide bond in Plg generating active Pm. uPA has been identified as a critical trigger for Pm generation during cell migration and invasion, under physiological and pathological conditions and, therefore, appears to be the primary PA in eukaryotic cell migration processes, whereas tPA is the main PA in fibrinolysis and maintenance of vascular patency [4]. The activities of PAs are regulated by specific plasminogen activator inhibitors (PAIs) thereby restricting the generation of Pm for extracellular matrix (ECM) as well as intravascular fibrin degradation [53, 54].

Both PAs are serine proteases that are secreted as low-activity single-chain zymogens and proteolytically processed by plasmin into fully active two-chain molecules held together by a single disulfide bond [52, 55, 56]. Crystal structures of the catalytic domains of tPA and uPA have shown that the active sites are located in the B chain in both PAs and Plg specificity is determined by the insertion loops which are positioned around the active site cleft [57, 58]. In uPA, the A chain consists of one kringle structure and a growth factor-like domain, which contains the receptor-binding amino acid sequence [59]. In tPA, the N-terminal region contains a "fibronectin-type II" finger domain, an "EGF-like" domain and two "kringle" domains. The finger-like domain, together with the second kringle domain, provides tPA with high affinity for fibrin [60]. A large serine protease catalytic domain is located at the carboxyl terminus, similar to uPA.

2.2.1. Urokinase-Type Plasminogen Activator (uPA). uPA is expressed in a variety of tissues as a 411 amino acid glycoprotein with an apparent molecular weight of 53 kDa. uPA-mediated Pm generation results in the proteolytic activation of matrix metalloproteinases (MMP), other ECM proteinases, and growth factors. Therefore, uPA initiates a proteolytic cascade that leads to degradation of the ECM, facilitating processes such as cell attachment and detachment, tissue remodeling, and tumor metastasis. uPA binds to a specific glycoprotein receptor (uPAR) which increases its overall catalytic efficiency [61, 62]. uPAR restricts uPA activity to the cell surface, where Plg is also bound to its cognate sites [63]. Plg and PAs are thus colocalized on cells and tissues thereby reinforcing cell-associated proteolysis. uPA/uPAR interactions account for localized pericellular proteolysis, as opposed to the extracellular and plasma proteolytic activity of tPA. Alternatively, uPA-uPAR binding induces a conformational change in the complex, which initiates signal transduction cascades that affect cell proliferation and survival, cytoskeleton dynamics, and cell adhesion [64].

2.2.2. Tissue-Type Plasminogen Activator (tPA). tPA, a 530 amino acid glycoprotein is primarily secreted by vascular endothelial cells as a prozymogen with very low level activity until proteolytically activated by Pm [65]. A variety of stimuli, such as thrombin, histamine, bradykinin, adrenaline, acetylcholine, and shear stress can govern the release of tPA. The major role of tPA is to degrade fibrin in blood vessels. tPA activity is greatly stimulated by fibrin which interacts with kringle 2 and the finger and EGF-like domains [66]. Accordingly, tPA synthesis is induced under ischemic conditions. In tPA-deficient ($tPA^{-/-}$) mice, clot lysis is strongly impaired whereas, in uPA-deficient mice, there is occasional fibrin deposition [67]. In addition to its role in fibrinolysis, tPA has been shown to have an additional role that is unrelated to its proteolytic activity and independent of Pm generation. tPA can act as a ligand to cell surface proteins and trigger several cellular responses [68, 69]. In $tPA^{-/-}$ mice, cerebellar granule neurons migrate significantly slower than granule neurons from wild-type mice; as a consequence, late arriving neurons are impaired in their synaptic interactions [70].

Annexin 2 has been identified as a plasma membrane receptors for tPA in endothelial cells which also possess binding affinity for Plg but not uPA [71–73]. Annexin 2 is a highly conserved member of the annexin super family of calcium-dependent, phospholipid-binding proteins [74]. Like all annexin family members, annexin 2 has a variable N-terminal "tail" and a conserved C-terminal "core" region. Annexin 2 heterotetramer consists of two annexin 2 monomers dynamically linked by two molecules of p11 (also known as S100A10), a member of the S100 family of calcium-binding proteins. Binding of p11 to annexin 2 is essentially irreversible. This heterotetrameric complex which is the predominant species in most cells is referred to as AIIt and provides a surface for tPA and plasminogen interaction and may even have a greater stimulatory effects on tPA-dependent plasmin generation [75, 76]. Both plasminogen and tPA bind

to distinct annexin 2 domains. As discussed earlier, binding of plasminogen to its cellular receptor is dependent on the interaction of plasminogen with the carboxyl-terminal lysines of the receptor. In a purified-protein system, native human placental annexin 2 conferred an approximately 60-fold increase in catalytic efficiency of tPA-dependent Plg activation which is almost completely negated in the presence of Lys analogues indicating a lysine-dependent interaction [75]. As annexin 2 lacks carboxyl-terminal lysine, it has been proposed that proteolytic processing of annexin 2 at a specific site might expose a carboxyl-terminal lysine, specifically Lys307 which could mediate annexin 2 binding to Plg [73]. However, this has never been demonstrated either *in vitro* or *in vivo*. Mutational study of this lysine did not dramatically alter the stimulatory activity of annexin 2 when compared with the wild-type recombinant annexin 2 [75]. On the other hand, p11 subunit of AIIt has the prerequisite carboxyl-terminal lysines, mutation of which resulted in reduced tPA-dependent Plg activation compared to wild-type p11 subunit [75]. These results suggest that carboxyl-terminal lysine residues of p11 mediate Plg binding and it is probably these lysines which result in the stimulation of tPA-dependent Plg activation of AIIt (reviewed in [77]). P11 subunit binds to the aminoterminal side of annexin 2. It is also the aminoterminal in particular Leu-Cys-Lys-Leu-Ser-Leu hexapeptide within the aminoterminal "tail" domain which mediates tPA binding and Cys^8 of this sequence has been proposed to be essential for the binding [78]. Several studies also suggest a physiological role for annexin 2 in fibrin homeostasis. Annexin 2 and p11 are not only involved in regulation of plasmin generation, fibrin homeostasis, and angiogenesis but are also involved in tumor growth, invasion, and metastasis (reviewed in [79]) [80]. Annexin 2 and p11 could therefore play the role of physiological receptors of plasminogen.

Low-density lipoprotein receptor-related protein (LRP) is a large two-chain scavenger receptor that mediates clearance of tPA-PAI-1 complex, *in vitro* [81, 82]. Both growth factor and the finger domain of tPA are required for this interaction. LRP binds a variety of biologically diverse ligands including the broad spectrum protease inhibitor α2-macroglobulin (α2M) and free and PAI-complexed plasminogen activators [83]. LRP sequesters proteases and/or protease-inhibitor complexes and thereby regulates extracellular proteolysis thus decreasing the overall protease load in the pericellular space [84, 85].

Apart from uPA and tPA, certain proteases belonging to coagulation cascade have also been shown to activate Plg directly. These accessory activators include kallikrein, factor XIa and factor XIIa [86, 87]. Type IV collagen is also known to immobilize Plg and lead to enhanced tPA-mediated Plg activation [88]. Factor VII-activating protease has also been reported to be a potent *in vitro* activator of single-chain Plg activators, but its physiological role is not yet clear [89]. Plasma protein histidine-proline-rich glycoprotein (HPRG) also contains carboxyl-terminal lysines and is known to bind Plg [90, 91].

2.3. Plasmin and Plasminogen Activator Inhibitors. The activation of Plg is negatively modulated by a family of serine protease inhibitors known as serpins [92]. The mode of action of serpin requires formation of a stable complex with active site serine of the target protease, followed by the proteolytic cleavage of the serpin by target protease [93].

The chief serpin PA inhibitors are plasminogen activator inhibitor-1, -2, -3 (PAI-1, PAI-2 and PAI-3) and a broad-spectrum protease inhibitor nexin [94]. PAI-3 can inhibit both uPA and tPA albeit with a much slower rate than PAI-1 and -2 [56]. Out of the two major PAIs, PAI-1 is the most ubiquitous and most rapidly acting physiological inhibitor of both uPA and tPA. PAI-2, on the other hand, is less effective towards single-chain tPA and does not inhibit single-chain uPA but can inhibit both two-chain uPA and two-chain tPA with comparable efficiency.

The chief physiological inhibitor of plasmin is the serpin α2-antiplasmin. This glycoprotein forms a complex with plasmin by binding to kringle 1–3 of plasmin [95]. Since the same lysine-binding kringle sites mediate Plg binding to receptors, receptor-bound plasmin(ogen) is resistant to inhibition by α2-antiplasmin. Another broad-spectrum proteinase inhibitor α2-macroglobulin can also inhibit plasmin. This nonserpin forms a noncovalent complex with plasmin; however, this inhibition takes place only when there is significant decrease in the local or systemic concentration of α2-antiplasmin [96].

3. Bacterial Plasminogen Activators

Several pathogenic bacteria, such as *Streptococcus* and *Staphylococcus*, also produce PAs that are either secreted or surface-bound proteins. Streptokinase (SK) and staphylokinase (SAK) are not enzymes in and of themselves but form 1 : 1 complexes with Plg and Pm, leading to changes in conformation and specificity of Plg. Crystal structures of SK and SAK reveal that they have similar structural fold, although sequence homology is relatively low [97, 98]. The mechanism of Plg activation by SK and SAK is similar but differs in some respects. While SK-Plg is enzymatically active, SAK-Plg is inactive and requires the conversion of Plg to Pm. Another important difference is that SAK primarily activates fibrin-bound Plg. This requirement has encouraged studies with SAK regarding its ability to function as an *in vivo* thrombolytic agent [99].

3.1. Activation of Human Plasminogen by Streptokinase. Invasive bacterial infections caused by *Streptococci* represent one of the most extensively studied models for interactions between pathogens and the Plg system [100]. *Streptococci* have the ability to invade nonphagocytic cells by breaking the host cellular and tissue barriers through interaction with host proteins that facilitate access into the vascular system [101, 102]. Interactions between the Plg system and streptokinase (SK) have been hypothesized to promote the bacterial invasion into tissues [103]. The SK, secreted by β-hemolytic group A, C, and G *streptococci*, is the prototypical bacterial plasminogen activator. It is a 440

amino acid protein containing α, β, and γ domains, with a 26 amino acid N-terminal signal peptide that is cleaved during secretion to yield a 414 amino acid mature protein [104]. Experiments have demonstrated that each individual domain can bind to Plg. However, they cannot activate Plg independently. Mutagenesis studies have shown that the α, β, and γ domains cooperatively induce the formation of an active site within the hPlg activator complex, providing a means for the substrate Plg to be recognized by the activator complex, mainly through interactions mediated by the SK α-domain [105, 106]. Interestingly, unlike other Plg activators which activate Plg by limited proteolysis, SK lacks hydrolytic activity. Thus, in order to activate Plg, SK has to bind to Plg and induce within it an active site through nonproteolytic mechanisms.

The activation of Plg by SK involves two major mechanistic steps: (1) formation of a PA complex and (2) the activation of substrate Plg. The crystal structure of SK from *S. pyogenes* in complex with the catalytic unit of Plg shows that the formation of the activator complex involves interactions between the carboxyl-terminal domain of SK and the catalytic domain of Plg [97]. In the SK-Plg complex, the active site of Plg is exposed and functions without hydrolysis of the Arg560-Val561 peptide bond [107]. Crystallographic studies have also determined the surface area of the respective SK domains available to interact with Plg and found that the interaction sites are located in the loops of α and the γ domains of the SK. In the second step of the activation, SK-Plg complexes become catalytic activators of the remaining Plg. The formation of the complex induces conformational changes, such that Plg is converted to Pm [108]. The Plg activation mechanism is also modulated by lysine-binding site- (LBS-) dependent interactions between SK and the kringle domains of Plg and Pm [109–111] which most likely involves K5 of Plg and β and/or β, γ domains of SK [105, 112].

Although the specific roles of the domains of SK that function in various ways in the activator complex have been revealed [113, 114], the basis for the species selectivity of SK in activation of mammalian Plg is still unclear [115, 116]. Human and nonhuman-derived SKs differ from each other and activate Plg in a species-specific manner. Early studies with rabbit Plg showed weak activation when incubated with SK produced by a strain of human origin. It was found that although a complex was formed between SK and rabbit Plg, SK was rapidly degraded to inactive forms, indicating that the complex was unstable [117]. Investigations with SKs isolated from *streptococcal* strains from equine or porcine origin have shown that although equine/porcine-derived SKs interact with hPlg, no activation was achieved. A similar situation was observed when either equine or porcine Plg was incubated with SK from a bacterial strain of human origin. Comparisons of amino acid sequences of different mammalian-derived SKs have shown that there is low sequence homology. These data indicated that the complex formed between SK and Plg could vary in primary structure and conformational properties, affecting Plg activation [118]. This remarkable species specificity is believed to originate from the species-specific interaction between SK and the preferred host's Plg. Using transgenic mice expressing human Plg it has been shown that a marked increase in the susceptibility of mice to group A *streptococcus* (GAS) was observed which is largely abrogated by deletion of the SK gene. These results demonstrate that SK is a key determinant for host specificity of *streptococcal* infection [119].

3.2. Activation of hPg by Staphylokinase. Staphylokinase (SAK) is a 136 amino acid protein produced by strains of *S. aureus*. SAK does not possess protease activity by itself. Instead, hPlg activation by SAK depends on the formation of a stoichiometric complex between SAK and Pm. The SAK-Pm is formed from SAK-hPlg in the presence of other PAs for example, tPA [120]. The SAK-Pm complex modifies SAK within the complex by cleavage of the Lys10-Lys11 peptide bond and subsequently converts SAK-Plg to SAK-Pm, which in turn converts free Plg to Pm. The kringle domains of Plg are not involved in the interaction with SAK, and there is evidence that Arg719 in Plg and Met26 in SAK are important for the binding [98, 121]. Moreover the NH_2-terminal region of SAK is important for active site formation in the Pm molecule in the binary complex [99, 121, 122].

Plg activation mediated by SAK differs from SK-mediated Plg activation in some major aspects. (i) The SAK-Plg complex is enzymatically inactive and requires conversion of Plg to plasmin. (ii) SAK requires fibrin as a cofactor. (iii) Active SAK-plasmin complex is efficiently inhibited by α_2-antiplasmin. However, binding of Plg to fibrin or bacterial cell surfaces protects against inactivation by α_2-antiplasmin and, more importantly, enhances the SAK-induced Plg activation [123]. (iv) Binding of α_2-antiplasmin to the SAK-plasmin complex releases SAK from the complex allowing it to interact with other plasmin(ogen) molecules [121, 124, 125]. SAK also binds much more efficiently to substrate-bound Plg than to soluble Plg [126]. Other than Plg activation, SAK can also induce immunogenic antibody responses and proliferation of SAK-specific T lymphocytes [127]. Furthermore, SAK can directly interact with the host innate immune system and inhibit the bactericidal effect of α-defensins, thereby reducing the rate of Plg activation. As a result, the functional activity of SAK promotes the bacterial infection process [128]. The observation that SAK primarily activates Plg bound to fibrin without causing systemic Plg activation has raised interest in the clinical use of SAK as a thrombolytic agent to dissolve the fibrin component of blood clots [99].

4. Bacterial Plasminogen Receptors

Plasminogen activator production is not universal amongst pathogenic bacteria but many bacteria express PlgRs on their surface [1, 129]. Bacterial PlgRs capture Plg on the bacterial surface which in turn enhances its activation to Pm by SK or host plasminogen activators uPA or tPA [130]. Consequently bacteria become proteolytic organisms using the host-derived system and thus facilitate bacterial penetration of endothelial cell layers and degradation of extracellular matrix

Table 1: Bacterial plasminogen receptors.

Receptor	Primary bacteria	Possible physiological function	Reference
Enolase/SEN	*Streptococcus*	Plg binding and uPA/tPA-mediated activation Fibrinolysis, subsequent extracellular matrix degradation, and transmigration. Involved in tumorigenesis, cancer proliferation, invasion, specific humoral and cellular immune response, myogenesis as well as hypoxic stress response	[10]
GAPDH/SDH/Plr	*Streptococcus*	Plg binding and activation by uPA/tPA. Adhesion to uPAR, fibronectin	[11, 12]
M proteins, PAM	*Streptococcus*	Major virulence factor, promote fibrinolysis, antiphagocytosis, vascular leakage and tissue injury, proinflammatory properties, adhesion to host cell and tissues, transmigration and deep tissue invasion, immunoglobulin binding, acute rheumatic fever	[13, 14]
OspA, OspC	*Borrelia*	Plg activation by host uPA, increased MMP expression, degradation of soluble and insoluble ECM components, endothelial monolayer penetration including blood-brain barrier, effective dissemination in host	[15–17]
HP-NAP	*Helicobacter pylori*	Fibrin clot stabilization, inhibition of fibrinolysis, possibly antiphagocytic, tissue factor synthesis and PAI2 stimulation	[18]
PgbA/PgbB	*Helicobacter pylori*	Lysine-dependent Plg binding, tPA-mediated Plg activation	[19]
DnaK	*Bifidobacterium animalis* subsp. *lactis*	Plg binding; upregulated in response to bile salts	[20]
Flagella	*Escherichia coli*	Plg binding and activation by host PAs	[21]
Fimbriae	*Escherichia coli Salmonella*	Fibronectin binding, adhesion to ECM components/BM, facilitate penetration	[22]

components. Additionally, immobilization of Pm protects it against the serine protease inhibitor α_2-antiplasmin [131]. Several bacterial PlgR molecules have been identified and characterized (Table 1). Most of the identified bacterial PlgRs have other important functions as well. In this paper we will focus only on the well-known and well-characterized bacterial PlgRs.

The best characterized PlgRs have been identified in group A and C *streptococci* from humans. These include α-enolase (SEN) [10] and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; also known as SDH and Plr) [11, 12] as well as the *streptococcal* M-like protein (PAM) [13, 14]. Both GAPDH and enolase are glycolytic enzymes expressed on the bacterial surface [132, 133]. These are anchorless multifunctional proteins which, in addition to their housekeeping functions, are also involved in bacterial-induced fibrinolysis and inflammation through their ability to bind Plg.

Other than *streptococci*, GAPDH is also expressed on the surface of several Gram-positive bacteria including pneumococci as well as pathogenic *Escherichia coli* (*E. coli*) and binds to plasminogen, fibrinogen, and fibronectin binding proteins [133–135]. Enterohemorrhagic and enteropathogenic *E. coli* are reported to secret GAPDH which was found to bind human Plg and fibrinogen therefore suggesting a possible role in bacterial pathogenesis [135]. GAPDH (also known as *streptococcal* surface dehydrogenase—SDH) possesses lower affinity for Glu-Plg than Pm [133]. The C-terminal lysine residue of GAPDH appears to be essential for Pm binding as substitution of this residue with leucine abolishes binding. However, this replacement failed to show any effect on Plg binding indicating possible involvement of other *streptococcal* PlgRs [136]. It was found to be impossible to analyse the effect of GAPDH deletion on Pm binding since GAPDH appears to be essential for viability in GAS. However, analysis of swine pathogen *Streptococcus suis* mutant lacking cell surface GAPDH activity showed reduced Pm affinity. Lysine-dependent Plg binding by recombinant GAPDH isoform at physiological concentrations has been recorded for *Bacillus anthracis* [137]. Immunization of mice with this isoform offered significant protection against *Bacillus anthracis* infection. Taken together, these data establish the significance of GAPDH as a Plg receptor but also indicate that GAS probably expresses multiple PlgRs. In addition to its Plg binding activity, the GAPDH/SDH binds to the ectodomain D1 of uPAR/CD87 on Detroit human pharyngeal cells and mediates bacterial adherence to host cells [138]. SDH has also been shown to be involved in the regulation of phosphorylation of human pharyngeal cells which suggests a role for this enzyme in signal transduction and cell-to-cell signalling between *streptococci* and pharyngeal cells [139, 140]. GAPDH may also act as a virulence factor which could contribute to pathogenesis [135].

Like GAPDH, enolase is a glycolytic enzyme identified in archaebacteria to mammals. It is a highly conserved protein with similar overall fold and identical catalytic residues in

all organisms [141]. There are three isoforms of enolase—α, β, and γ which share high sequence identity. Of these, α-enolase is present in almost all tissues [142–144]. Enolase consists of a relatively small N-terminal domain and a rather large C-terminal domain. In some eubacteria, for example, *streptococci*, α-enolase is octameric [145], whereas in eukaryotes, enzymatically active enolase is present as an antiparallel dimer [146]. Enolase is a ubiquitous surface-associated protein although it is not clear how enolase is exported to the surface because it lacks the N-terminal signal peptide required for surface export as well as the membrane anchorage motif required for cell wall anchorage [10, 132, 145, 147]. Enolase is a multifunctional protein which, apart from being a key player in metabolism, is also involved in tumorigenesis, cancer proliferation, invasion, specific humoral and cellular immune response, and myogenesis, as well as hypoxic stress response (reviewed in [144, 148, 149]). Interestingly, surface-associated enolase serves another important function in both pro- and eukaryotes in that it acts as a PlgR by binding to and activating host Plg [132, 150–155] on cell surfaces and thereby facilitating fibrinolysis and subsequent extracellular matrix remodelling. This may in turn assist in bacterial invasion of host cells. Binding leads to activation of Plg to Pm by the action of tPA or uPA [156, 157]. Enolase binds to kringles of Plg in a lysine-dependent manner. C-terminal lysine residues are critical for binding to Plg as the lysine analog ε-aminocaproic acid significantly inhibits binding as has been shown in *Borrelia* and human pharyngeal cells [158, 159]. However, ionic interaction does not affect this binding [158]. Lysine residues at positions 420 and 427 of enolase were found crucial in Plg-binding activity in *Aeromonas hydrophila* SSU [160]. It was also found that immunization of mice with purified recombinant enolase significantly protected the animals against a lethal challenge dose of wild type (WT) *A. hydrophila* suggesting that enolase could potentially be important for the viability of the pathogen [160]. Similar to GAPDH, α-enolase shows greater affinity for Lys-Plg than to Glu-Plg [161]. Lys-Plg-coated GAS resulted in increased adherence of GAS to human pharyngeal cells (Detroit 562) that have α-enolase expressed on their surface [159]. Additionally, in pneumococcal enolase, a nine-residue motif [FYDKERKVY] has been found to be the key cofactor for Plg binding and degradation of ECM proteins as well as important for dissolution of fibrin or laminin and transmigration of pneumococci through fibrin matrices [162]. Using an intranasal mouse infection model it has been shown that the functional inactivation of the nine-residue motif significantly impairs virulence of *streptococci* [163]. This motif has been found to be pivotal in other pathogens as well [144, 160]. However, the full conservation of the motif does not seem to be necessary for Plg activation [164]. Interestingly, pretreatment of mice with recombinant enolase from *Streptococcus sobrinus* suppressed the primary immune response against T-cell dependent antigens and also induced an early production of the anti-inflammatory cytokine, interleukin-10, thereby suggesting that surface enolase might act as an immunosuppressive agent [165]. Because of its location and possible function, enolase therefore has the potential of being a therapeutic target [149, 166].

Perhaps the best characterized bacterial PlgRs are M proteins secreted by group A *streptococcus* (GAS). GAS is a highly specific human pathogen. Its efficient colonization and dissemination in the host lead to a broad spectrum of diseases that range from simple and uncomplicated pharyngitis, tonsillitis, and skin infections, for example, impetigo, to life-threatening invasive illnesses including pneumonia, bacteremia, necrotizing fasciitis, *streptococcal* toxic shock syndrome, as well as nonsuppurative complications like acute rheumatic fever, and glomerulonephritis [167]. GAS produces a variety of surface-bound and secreted virulence factors which are known to contribute to the severity of their infections [168]. Among these, M- and M-like protein PAM (plasminogen binding M-like protein), which are anchored to the cell wall, play key roles in bacterial resistance to phagocytosis, adherence, invasion, and microcolony formation in tonsillar tissue (reviewed in [168]). M and M-like proteins function through various mechanisms for effective dispersion of the bacteria in the host. M proteins enable GAS to resist host immunity and invade the host by binding to IgG and IgA, as well as other proteins of the innate immune system. Apart from its role as antiphagocytic, M and M-like proteins also interact with and stimulate activation of the host fibrinolytic system [119]. M and M-like proteins of GAS are able to bind to host Plg directly [13, 35] or indirectly via fibrinogen (Fg) and/or fibrin (Fn) [169, 170] and serve to enhance the activation of Plg to Pm. M proteins have been studied extensively since their discovery in 1928 and are now amongst the best studied virulence factors of pathogenic bacteria [167, 171]. M proteins exhibit extensive sequence variations between strains accounting for over 130 distinct serotypes, each one exhibiting unique antigenic properties [172]. This enormous diversity of GAS serotypes is a major obstacle in the development of vaccines against GAS.

M proteins adopt a dimeric α-helical coiled-coil structure [173] tethered to the membrane at their C-terminal end. The hypervariable N-terminal region, which extends into the external environment, varies extensively in sequence between different bacterial strains and gives rise to the so-called antigenic variation in M proteins. M proteins consist of several domains of distinct functionality. The first 40 residues, which form the signal sequence, are absent in the mature protein. Mature PAM proteins begin with a variable P domain that contains two 13 amino acid repeats termed a1 and a2. This is followed by three C repeats and a D domain all of which are highly conserved across the M-protein family. These sequences bind a variety of human plasma proteins including members of the complement system. The highly conserved Pro/Gly domain is inserted in the GAS cell wall and anchors PAM on the bacterial surface. PAM binds to the kringle 2 (K2) domain of hPlg via its a1a2 domain [13, 174] whereas M proteins on other GAS strains bind to Fg through B repeats, which in turn interacts with Plg via K1, K4, and/or K5 domains of Plg [170, 175]. Binding of PAM to hK2/hPlg is mediated mostly by R72, H73, E75, R85, and H86 residues in the a1/a2 repeat of native PAM. Substitution of the central lysine in the a1 repeat sequence DAELQRLKNERHE reduced

Plg binding by 80% [14]. The role of the a1 and a2 regions in Plg binding was further demonstrated by expressing the a1 and the combined a1a2 regions as chimera to Arp4, an M-like protein which lacks Plg binding. Not only did the recombinant chimeric Arp/PAM proteins bind to Plg and Pm but also the expression of these fusion proteins conferred Plg binding on the *streptococcal* host strains lacking the Plg-binding ability [171]. In the presence of Plg, the a1-Arp4 fusion in an SK-producing strain resulted in surface-associated plasmin activity, whereas a host strain with an inactivated SK gene required exogenous SK for Plg activation [142].

PAM is encoded by the emm53 gene, which is associated with the GAS strains that cause skin infections in humans [176, 177]. Inactivation of emm53 leads to a loss of Plg binding *in vitro* and attenuated infection in an experimental model of impetigo as well as in transgenic mice expressing human Plg. The surface-bound Plg can be activated by SK or host PAs. In this way, PAM concentrates Plg on the surface of the bacteria and greatly augments the SK-mediated conversion of hPlg to hPm. PAM appears to work synergistically with SK to yield bacteria-bound Pm *in vivo*.

5. Mechanisms of Pathogen Invasion Using Host and Bacterial Proteins

5.1. Interaction of Bacteria with the Fibrinolytic Cascade via Plasminogen Receptors. The connection between microbial infection and fibrinolytic system has been known since 1933 when haemolytic *streptococci* from human infection samples were found to possess fibrinolytic activity [178]. Speculation was rife when it was reported that injection of *streptococcal* cell wall products triggered activation of the coagulation pathway in rats [179]. The binding of bacterial Plg receptors to Fg has been extensively studied mostly in GAS [180]. Plasminogen deficient ($Plg^{-/-}$) mice have been utilized in several pathogen challenge models. Binding of host Fg to M proteins of GAS enhances SK function in plasma. Using a mouse skin infection model it has been shown that GAS isolates with a surface-bound SK-human Plg-human Fg complex are invasive when injected into the skin of wild-type (WT) mice but not $Plg^{-/-}$ mice [24]. This also demonstrates that PlgR interaction with host Plg facilitates bacterial invasion. This supports the *in vitro* observation that GAS incubated with human plasma acquires Pm activity on its surface [181]. In subcutaneously infected mice, coadministration of human Plg considerably increased the susceptibility to GAS infection which was largely abolished by inactivation of the SK gene [103]. Similarly, inactivation of SK gene leads to a loss of virulence in a human-skin-mouse model for impetigo [182]. In an epidemiological study, GAS strains expressing M proteins isolated from invasive infectious cases were found to bind more Plg than strains isolated from noninvasive infectious cases in the presence of Fg and SK [183]. These results demonstrate the importance of bacterial Plg receptors in host pathogen interaction and indicate that SK and PlgRs probably share a synergistic action. A transgenic line of mice that express only human Plg has been generated to study the role of the host fibrinolytic system in GAS infection [119]. These mice showed increased mortality and increased bacterial dissemination, when subcutaneously infected with GAS. The presence of M protein was found to be required for full scale virulence. In this infection model it was also demonstrated that decreasing Fg levels facilitated bacterial spread thereby suggesting that in the absence of Pm activity, fibrin clot formation can be an effective host-defense mechanism. Fibrin-mediated protection to infection-stimulated hemorrhage has also been documented in mice infected with *Toxoplasma gondii* [184]. In an infection study using $Plg^{-/-}$ mice and mice deficient in Plg activators, it was observed that during infection with *Mycobacterium avium* the liver granulomas in $Plg^{-/-}$ mice had enhanced fibrin and fibronectin deposition, as well as increased neutrophil infiltration suggesting that Pm plays a role in the turnover of extracellular matrix proteins within granulomas, likely limiting the fibrotic response in these lesions [185].

The binding of Fg to M protein may also play a role in modulating bacterial-phagocyte interaction. Fg-M protein complex inhibits complement deposition on the bacterial surface thereby protecting GAS from phagocytosis [169]. M protein-Fg complex can bind to $\beta 2$ integrins, thus activating neutrophils and consequently inducing vascular leakage that could cause extensive pulmonary damage [186]. This further establishes the importance of Fg in pathophysiology of *streptococcal* infection. M protein-Fg interaction and subsequent activation of coagulation can have a profound influence on clot modulation of human plasma [187]. M proteins can target both intrinsic and extrinsic pathways of coagulation which are critical for the induction of fibrin clot formation [179, 188–190]. M proteins can also trigger platelet aggregation *in vitro* and thereby play a role in primary hemostasis. Fg was later found to be required for *streptococcal*-induced platelet aggregation [191]. Severe *streptococcal* infection is associated with M protein-induced platelet activation and thrombus formation [192]. M protein is a multipotent and powerful inducer of inflammation. It can also interact with Toll-like receptor 2 on human blood monocytes [193], resulting in expression of cytokines and tumor necrosis factor-α [194]. These proinflammatory roles of M proteins may explain why aggregates of M protein and platelets are often found at the site of infection in patients with toxic shock syndrome. GAS-associated fibrinolysis is potentially necessary for facilitating bacterial access to the vasculature [119].

5.2. Plasminogen-Mediated Bacterial Invasion. Plasminogen activation is also important for the tissue barrier degradation that allows cell migration. Through activation of metalloproteases, Pm-coated bacteria can break down extracellular matrix and basement membranes either directly or indirectly thereby facilitating bacterial spread [195]. In *Salmonella enterica* it has been demonstrated that Pm activity associated with the bacterial surface is able to degrade laminin as well as mammalian ECM and can potentiate *in vitro* bacterial penetration through a reconstituted BM [196]. Plg binding in *Borrelia* resulted in enhanced penetration of endothelial

cell monolayers [197]. In *S. aureus*, α-enolase has been suggested to play a critical role in bacterial pathogenesis by allowing its adherence to laminin-containing extracellular matrix [198]. In an infection study of bovine mammary epithelial cells by *S. aureus*, it has been reported that addition of Plg to the medium enhanced dissemination and invasiveness of bacteria [199]. Pneumococci also bind to and activate Plg via enolase which mediates transmigration across endothelial and epithelial monolayers by intercellular junction cleavage [200, 201]. α-enolase also showed enhanced monocyte migration through epithelial monolayers and promoted matrix degradation *in vitro*. These effects were abrogated by antibodies directed against the Plg binding site of enolase. Enolase also promotes Plg-mediated recruitment of monocytes to the acutely inflamed lung. These data suggest an important mechanism of inflammatory cell invasion mediated by increased cell-surface expression of enolase [202]. *Bacillus anthracis*, the causative agent of anthrax, is known to circumvent the humoral and innate immune defense of the host to initiate a productive infection. Enolase has been found to be at the helm of Plg binding which makes the Plg-bound spores capable of exhibiting antiopsonic properties by cleaving C3b molecules, *in vitro*, resulting in a decrease in macrophage phagocytosis [203]. This study showed that recruitment of Plg by PlgR can assist in evading host innate immunity also by the enhancement of anticomplement and antiopsonization properties of the pathogen. It is noteworthy that fibrillar surface appendages like fimbriae, flagella that have a morphological similarity to fibrin, form a major class of PlgR molecules in gram-negative bacteria, for example, *E. coli*. Pathogenic *E. coli* and *S. typhimurium* can bind to Plg by their thin aggregative fimbriae (curli) and/or flagellar filaments resulting in Plg activation by tPA [21, 22]. These organelles have been found to adhere to ECM or BM proteins, indicating that these bacterial structures are possibly involved in the invasion of host tissues as well as promotion of bacterial colonization (reviewed in [204, 205]). *E. coli* strains isolated from patients with ulcerative colitis showed higher affinity to various solubilized ECM proteins compared to those from healthy subjects [206]. This may indicate the existence of a positive correlation between PlgR activity and pathogenicity.

5.3. Plasminogen-Dependent Tissue Invasion by Borrelia . The importance of Pm acquisition for invasion and dissemination has been demonstrated for the vector borne spirochetes *Borrelia burgdorferi*. These bacteria are transmitted by ticks to human skin where they invade the outer skin layers and disseminate in the blood to cause Lyme disease [207]. They also migrate to secondary infection sites, such as the joints, the heart, and the central nervous system and, over time, Lyme disease can evolve into erosive arthritis and the destruction of ECM in the joints. *Borrelia* secretes two Plg-binding surface proteins—OspA and OspC [15–17] which are differentially expressed in host and ticks. In unfed ticks, OspA is highly expressed on the surface of spirochetes. After infection in mammals, OspA expression is downregulated and OspC is constitutively produced [208]. *Borrelia*-bound Pm promotes degradation of soluble and insoluble components of ECM [209] and penetrates endothelial monolayers [197] including the blood-brain barrier [210]. A similar observation has been made recently with *Cryptococcus* where blood-brain barrier invasion by the pathogen is enhanced by functional interaction with Pm [7]. Studies in $Plg^{-/-}$ mice demonstrated that Plg is required for effective dissemination of *Borrelia* in ticks and for enhancement of spirochetemia in mice [211]. *Borrelia* do not produce collagenases. However, increased expression of MMPs including MMP-1, MMP-3, and MMP-9 were observed in cell culture infections with *Borrelia* [212–216]. It is noteworthy that surface-bound Pm in *S. aureus* activates the proteolytically active interstitial collagenase MMP-1 in a similar way [217]. Using $Plg^{-/-}$ mice, it has been demonstrated that during relapsing fever from Borrelia infection, a lack of host Plg resulted in decreased spirochetal burden in the hearts, brains, and kidneys of these mice compared to WT mice. Neurological symptoms were also less common in $Plg^{-/-}$ mice [218, 219]. Interestingly, there is no endogenous PA in *Borrelia*; instead, the bacteria use host uPA to activate surface-bound Plg. Results from these studies suggest that receptor-bound Plg promotes tissue invasion in *Borrelia*.

5.4. Interaction of Helicobacter pylori with the Plasminogen System. In vitro binding of Plg has also been reported for *Helicobacter pylori* isolates which is associated with severe gastrointestinal disorders including chronic gastritis, peptic ulcers, and stomach cancer [220, 221]. Binding is sensitive to lysine analogs, and bound Plg is activated by tPA [222]. *H. pylori* produces a dodecameric neutrophil activating protein (HP-NAP) that can potentially inhibit fibrinolytic activities [18]. This study showed that HP-NAP efficiently stimulates human monocytes to synthesize tissue factor (TF) and plasminogen activator inhibitor-2 (PAI-2) without altering uPA production. Clotting assays established that tissue factor (TF) is functionally active thus triggering blood clotting and promoting fibrin deposition. As a result, the coagulation-fibrinolysis balance is shifted towards fibrin formation, prothrombotic events, and fibrin clot stabilization. This is perhaps beneficial for chronic gastritis and tissue disruption which requires tissue healing by means of degradation and removal of fibrin deposits and tissue debris. Fibrin deposition might in turn protect the bacteria against phagocytosis. In gastric cancer cells and in gastric biopsies of *H. pylori* positive patients, an increased level of PAI-2 has been demonstrated [223]. These data were also supported by the observation that in *H. pylori*-infected patients an increased level of PAI-2 and TF expression in monocytes was detected suggesting the involvement of pathogen in the regulation of the fibrinolytic balance and, hence, disease development [224]. Apart from HP-NAP, two more genes (pgbA and pgbB) encoding surface-exposed proteins with Plg receptor activity have been identified and characterized from *H. pylori* [19]. Both bind to Plg specifically in a lysine-dependent manner and enhance tPA-mediated Plg activation. In both cases, Plg binding has been

shown to be located in the conserved C-terminal region which contains two lysine residues.

5.5. Other Bacterial Pathogens and the Plasminogen System. In *Porphyromonas gingivalis* and the plague bacterium *Yersinia pestis*, plasmin inhibitors α_2AP and α_2-macroglobulin (α_2M) are inhibited [225, 226]. Since both of these pathogens also activate Plg, specific inactivation of antiproteases will therefore lead to uncontrolled Pm activity resulting in ECM and BM degradation [195]. Suppressing host antiproteases is therefore another effective mechanism undertaken by bacteria to promote proteolysis and invasion [227]. *Y. pestis*-derived plasminogen activator (Pla) is a surface protease responsible for the invasive character of plague. This pathogen does not express any Plg or Pm receptors; instead Pla acts as a highly efficient Plg activator [226] which proteolytically cleaves Plg similar to host uPA and tPA [3]. It is also an adhesin with affinities for ECM, laminin, and BM [228]. $Plg^{-/-}$ mice were found to be more resistant to plague infection than normal mice therefore validating the involvement of Pla in Plg activation [229].

Together, these observations clearly demonstrate that host Plg plays a critical role in pathogenesis of a broad range of invasive pathogens.

6. Concluding Remarks

The experimental evidence discussed here clearly demonstrates that a vast number of bacterial pathogens express PlgRs and interact with the host Plg system which in turn results in enhanced bacterial virulence. It is also clear that pathogenic bacteria can, and most often do, use the Plg system for migration across tissue barriers. Most of the identified and characterized bacterial PlgRs are multifunctional proteins involved in other important functions as well, such as adhesion, enzymatic activity, movement, interaction with immune system, myogenesis, and antiphagocytosis. It is also noteworthy to mention that a single bacterial species can express multiple PlgRs most likely due to the affinity of Plg for carboxy-terminal and internal Lys residues. Recent reports show that nonpathogenic bacteria can also express PlgRs and bind Plg via internal lysines [20, 230, 231]. Probiotic and commensal lactobacilli express an enolase, which, similar to staphylococcal enolases, interacts effectively with Plg and laminin. [232]. However, the significance of these expressions or the possible effects on colonization are not yet known.

During the last few decades various studies have suggested that there may be a correlation between the pathogenic invasion and the host haemostatic mechanisms, mainly fibrinolysis. Experimental results discussed here also demonstrate the complicated relationships of fibrinogen with invading bacteria and suggest a possible manipulation of the host fibrinolytic cascade and the receptor molecules of the pathogens to evade the host defense system. Not only is the Plg system important in controlling fibrin-containing clot degradation under physiological and pathophysiological conditions but this system can also be hijacked by bacterial pathogens to create exuberant proteolysis resulting in extracellular matrix and fibrin barrier degradation, subsequent tissue damage, and enhanced bacterial migration and invasion into the surrounding host tissue and vasculature. These properties therefore highlight the fibrinolytic system as a novel therapeutic target for controlling pathogenic invasion and dissemination.

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Review Article

The Role of Nephritis-Associated Plasmin Receptor (NAPlr) in Glomerulonephritis Associated with Streptococcal Infection

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It is well known that glomerulonephritis can occur after streptococcal infection, which is classically referred to as acute poststreptococcal glomerulonephritis (APSGN). The pathogenic mechanism of APSGN has been described by so-called immune complex theory, which involves glomerular deposition of nephritogenic streptococcal antigen and subsequent formation of immune complexes *in situ* and/or the deposition of circulating antigen-antibody complexes. However, the exact entity of the causative antigen has remained a matter of debate. We isolated a nephritogenic antigen for APSGN from the cytoplasmic fractions of group A streptococcus (GAS) depending on the affinity for IgG of APSGN patients. The amino acid and the nucleotide sequences of the isolated protein revealed to be highly identical to those of reported plasmin(ogen) receptor of GAS. Thus, we termed this antigen nephritis-associated plasmin receptor (NAPlr). Immunofluorescence staining of the renal biopsy tissues with anti-NAPlr antibody revealed glomerular NAPlr deposition in essentially all patients with early-phase APSGN. Furthermore, glomerular plasmin activity was detected by *in situ* zymography in the distribution almost identical to NAPlr deposition in renal biopsy tissues of APSGN patients. These data suggest that NAPlr has a direct, nonimmunologic function as a plasmin receptor and may contribute to the pathogenesis of APSGN by maintaining plasmin activity.

1. Introduction

Acute poststreptococcal glomerulonephritis (APSGN) develops after streptococcal infection with the obvious latent period of around 10 days. It is mostly accompanied by decrement in serum complement titer and glomerular deposition of C3 and IgG. From these characteristic manifestations, it has been widely accepted that the immunological reaction against streptococcus related antigens is engaged for the initiation of this disease. The most popular theory of the pathogenic mechanism of APSGN has been the immune complex theory, which involves the glomerular deposition of nephritogenic streptococcal antigen and the subsequent formation of immune complexes *in situ* and/or the deposition of circulating antigen-antibody complexes [1, 2]. However, glomerular immunoglobulin deposition is not often prominent in this disease, and the reason for the difference in the site of glomerular cell infiltration and the site of immune complex deposition is unclear; the major site of inflammation in this disease occurs on the inner side of the glomerular tufts (endocapillary site), whereas the immune complex in early phase is localized to the outer side of the glomerular tufts (subepithelial site). Indeed, another type of human glomerulonephritis with subepithelial immune complex deposition, membranous nephropathy, is rarely accompanied by endocapillary cell infiltration. Thus, the actual mechanism of how prominent glomerular endocapillary proliferation occurs in this disease is still unknown, and the most essential and critical issue, "what is the causative entity/antigen," has remained a matter of debate [3–6].

We recently isolated and characterized a nephritogenic antigen from group A streptococcus (GAS) that we call the nephritis-associated plasmin receptor (NAPlr) and is homologous to the streptococcus plasmin(ogen) receptor

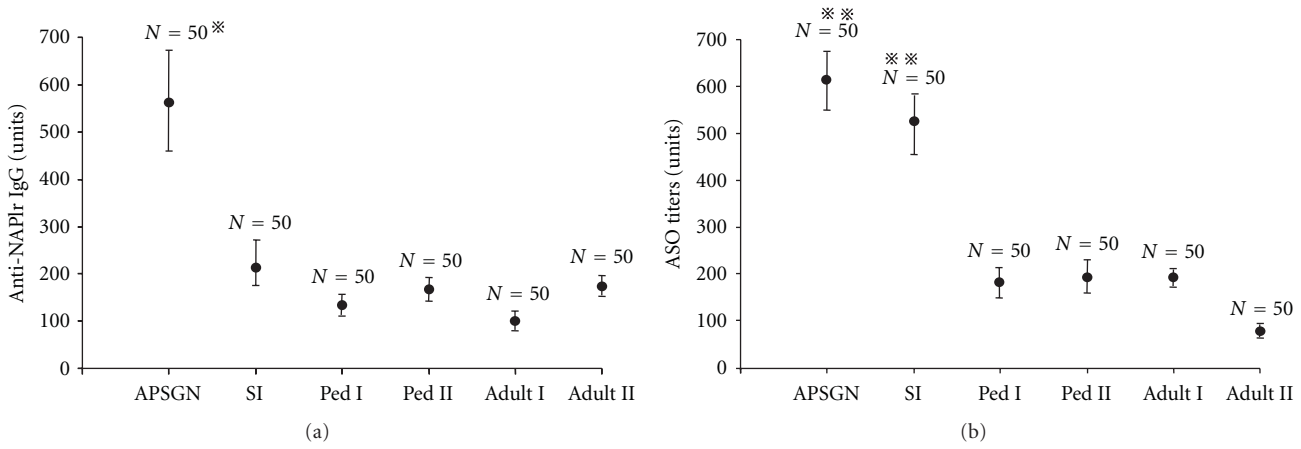

Figure 1: (a) The results of Western blotting assay for anti-NAPlr antibody titers in patients with acute poststreptococcal glomerulonephritis (APSGN), patients with a streptococcal infection (SI) without renal involvement, pediatric patients without renal disease, and normal healthy adults (see Table 1 for ages of subjects in each group). Values are means ± SEM. $^{*}P < 0.05$ for APSGN versus SI, pediatrics, and normal adults by t test. (b) ASO titers for the same groups of patients. The titers in the APSGN and SI groups are significantly higher than those in the normal adults and the pediatric patients without renal disease. Values are means ± SEM. $^{**}P < 0.001$ for APSGN or SI versus those in other groups by t test.

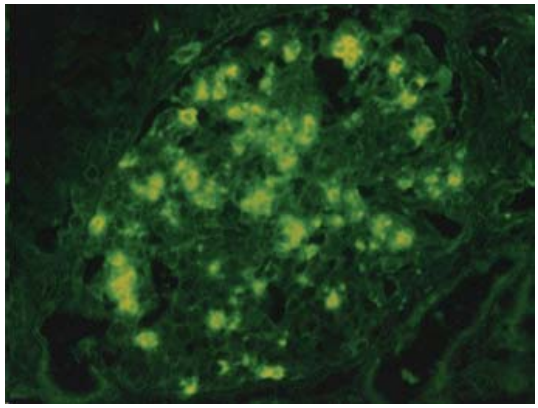

Figure 2: Immunofluorescence staining for NAPlr in tissue sample from a patient with acute poststreptococcal glomerulonephritis. Staining sites, which are thought to represent free antigen, are localized primarily in the mesangium and part of the glomerular basement membrane (GBM), and infiltrating leukocytes show a ring-like granular pattern (original magnification ×200).

(Plr) [7, 8]. The evidence for the important roles of NAPlr and the related plasmin activity in the development of glomerulonephritis associated with streptococcal infection are described.

2. Isolation of Nephritis-Associated Plasmin Receptor (NAPlr)

We postulated that the nephritogenic antigen for APSGN should have affinity for the serum of convalescent APSGN patients. So the fraction from the cytoplasmic proteins of GAS that has high affinity for the IgG of APSGN patients were collected by using affinity chromatography with APSGN patients' IgG-immobilized Sepharose and then purified by ion exchange chromatography. Eventually the 43-kDa protein, a potent nephritogenic antigen for APSGN, was isolated [7, 8]. The amino acid and the nucleotide sequences of the antigen revealed to be highly identical to those of reported Plr, or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) of GAS [7–10]. Thus, we termed this antigen NAPlr. Plr has been shown *in vitro* to bind plasmin and maintain its proteolytic activity by protecting it from physiologic inhibitors like α_2-antiplasmin (α_2-AP) [11]. NAPlr exhibited similar functions as Plr, such as specific binding with plasmin(ogen), and expression of GAPDH activity [7]. Further analysis revealed the nephritogenic characteristics of the isolated antigen as described in the following sections.

3. Antibody Response against NAPlr in APSGN Patients

We analyzed the anti-NAPlr antibody titers by Western blotting in serum samples from 50 APSGN patients, 50 streptococcal infection (SI) patients without nephritis, 50 young pediatric patients (<11 years old), 50 older pediatric patients (11–20 years old), 50 young normal adults (25–35 years old), and 50 older normal adults (52–59 years old). The percentage of those positive for anti-NAPlr antibody was high in APSGN patients, but was also quite high even in healthy older people (Table 1). APSGN patients, however, showed significantly higher antibody titers as demonstrated in Figure 1(a) than other groups, even SI patients without nephritis. Anti-NAPlr antibody titer differed from ASO titer in that the ASO titer was similarly high in both APSGN and SI patients (Figure 1(b)) [8].

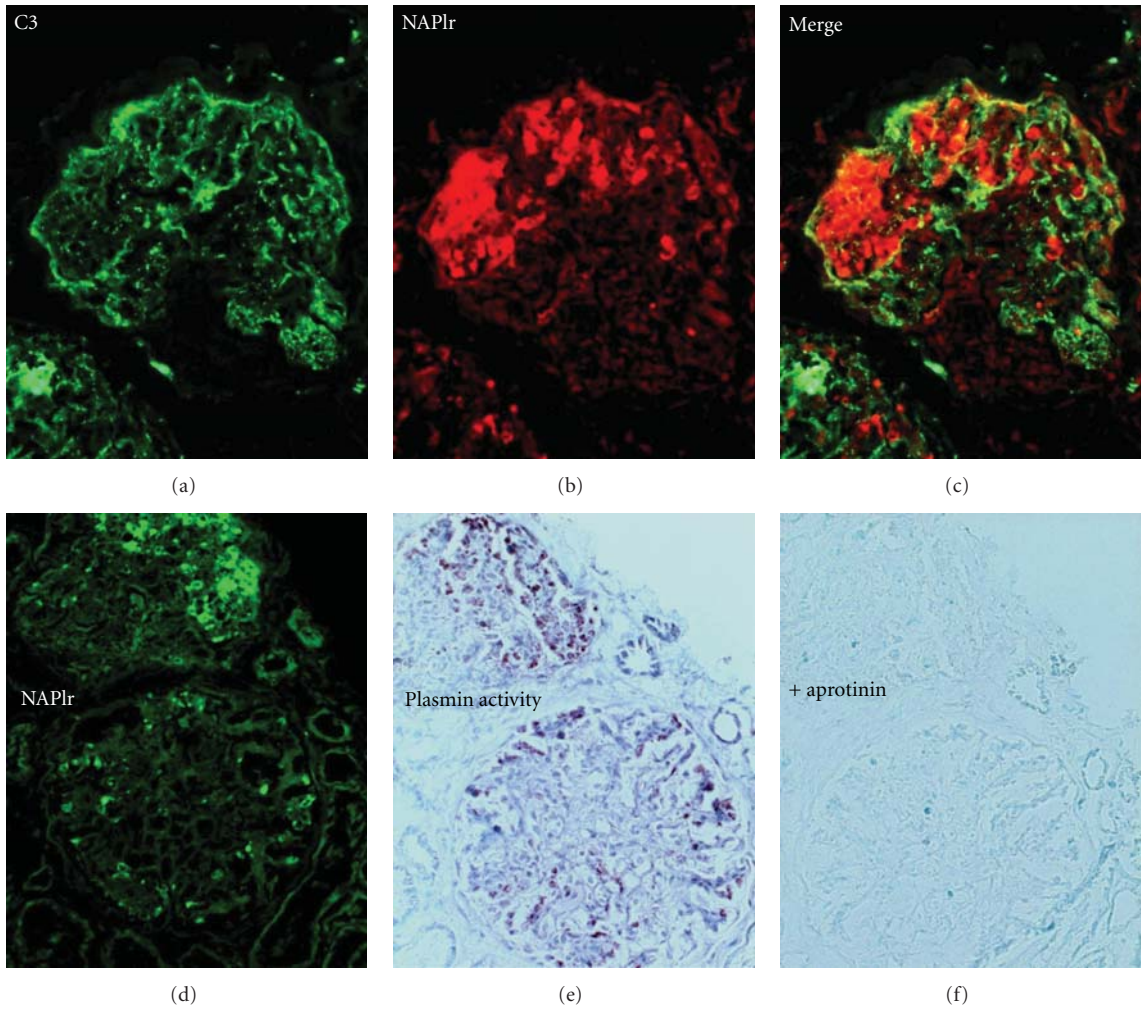

Figure 3: Representative photomicrographs of double immunofluorescence staining for C3 (FITC; green) and NAPlr (Alexa Fluor 594; red) ((a)–(c)). The distributions of C3 (a) and NAPlr (b) were obviously different in the merged image (c). ((d)–(f)). NAPlr IF staining and *in situ* zymography for plasmin activity in serial sections of renal biopsy tissue from an APSGN patient. The distribution of plasmin activity was similar to that of NAPlr deposition ((d) and (e)). Addition of aprotinin inhibited the zymographic activity suggesting the activity to be plasmin (f) (original magnification ×200).

Table 1: Positive rate of serum anti-NAPlr antibody in patients with APSGN, streptococcal infection, pediatric patients, and normal adults.

	Age in years range	Anti-NAPlr antibody (+)
APSGN	5–75 years, mean 29.3	45/50 (90%)
Streptococcal infection	8–64 years, mean 29.0	15/25 (60%)
Pediatric I	0.2–10 years, mean 7.2	13/50 (26%)
Pediatric II	11–20 years, mean 14.1	18/50 (36%)
Normal adults I	25–35 years, mean 30.0	24/50 (48%)
Normal adults II	52–59 years, mean 53.2	36/50 (72%)

4. Glomerular Deposition of NAPlr in APSGN Patients

Direct immunofluorescence staining with rabbit anti-NAPlr antibody in renal biopsy tissue from APSGN patients revealed glomerular NAPlr deposition mainly on mesangial and endocapillary site as ring-like granular pattern (Figure 2). As shown by the values listed in Table 2, glomerular NAPlr deposition was observed in 100% (25/25) of APSGN patients within 2 weeks after disease onset or in 84% (36/43) of APSGN patients within 30 days after disease onset, but the percentage of tissue specimens showing NAPlr deposition decreased over time. On the other hand, no normal kidney was positive for NAPlr and only 4 out of

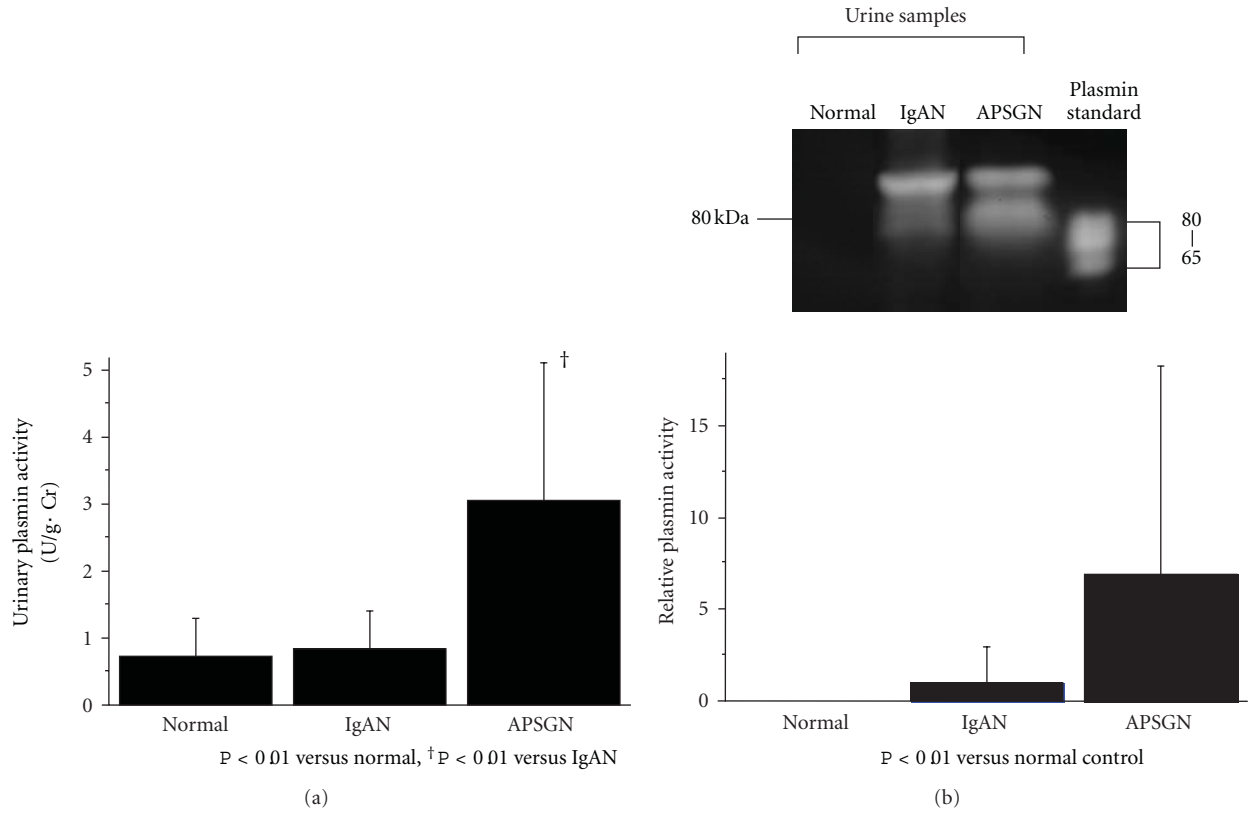

Figure 4: (a) Urinary plasmin activity assessed by chromogenic assay and corrected for urinary creatinine concentration. Results are expressed as corrected mean activity ± SE. $^{*}P < 0.01$ versus normal control; $^{\dagger}P < 0.01$ versus IgAN. (b) Representative casein gel zymography results for a plasmin standard and urine supernatants from normal controls, patients with IgAN, and patients with APSGN. Graph shows mean ± SE of the density of 80-kDa bands in casein gel zymography expressed in arbitrary units. $^{*}P < 0.05$ versus normal controls.

Table 2: Glomerular NAPlr deposition in APSGN, non-APSGN, and normal kidney tissues.

Biopsy specimens	Onset to biopsy	Glomerular NAPlr (+)
APSGN	1–14 days	25/25 (100%)
	15–30 days	11/18 (61%)
	31–90 days	0/7 (0%)
	Total	36/50 (72%)
Non-APSGN		4/100 (4%)
Normal kidneys		0/10 (0%)

100 patients with other glomerulonephritis were positive for NAPlr [7].

5. Localization and Properties of NAPlr, and Pathogenic Mechanism of APSGN

It is worthy of notice that the glomerular distribution of NAPlr was essentially different from that of IgG or C3 in the glomeruli of APSGN patients (Figures 3(a)–3(c)) [8]. In early-phase APSGN, C3 and/or IgG deposits are usually found at a subepithelial site (on the outer side of the glomerular tufts), while NAPlr deposits are always found on the inner side of the glomerular tufts. Using double immunofluorescence staining for more precise analysis, we found NAPlr to be localized mainly on glomerular endocapillary neutrophils, mesangial area, and partially on endothelial cells or glomerular basement membrane [12].

NAPlr is a 43 kD protein with a pI of 4.7, and its most characteristic feature *in vitro* is that it binds to plasmin and maintains the proteolytic activity of plasmin by protecting the enzyme from physiological inhibitors such as α_2-AP. Actually, plasmin is engaged in many physiological phenomenon, such as, fibrinolysis, extracellular-matrix turnover, cell migration, wound healing, angiogenesis, and neoplasia [13–16], but because it is easily inhibited and tightly regulated by physiological inhibitors it is not normally found in an active form *in vivo*. We found significant glomerular NAPlr deposition in the early phase of APSGN, which led us to speculate that deposited NAPlr would trap plasmin and cause glomerular damage by keeping it in an active condition *in vivo*. To evaluate this hypothesis, we performed an *in situ* zymography with a plasmin-sensitive synthetic substrate (*p*-toluenesulfonyl-L-lysine α-naphthyl ester), and found prominent intraglomerular plasmin activity only in NAPlr-positive APSGN patients (Figure 3(e)) [17]. This activity was completely inhibited by aprotinin (Figure 3(f)), a plamin inhibitor, but was resistant to α_2-AP. In contrast to the different distributions of NAPlr and C3 or

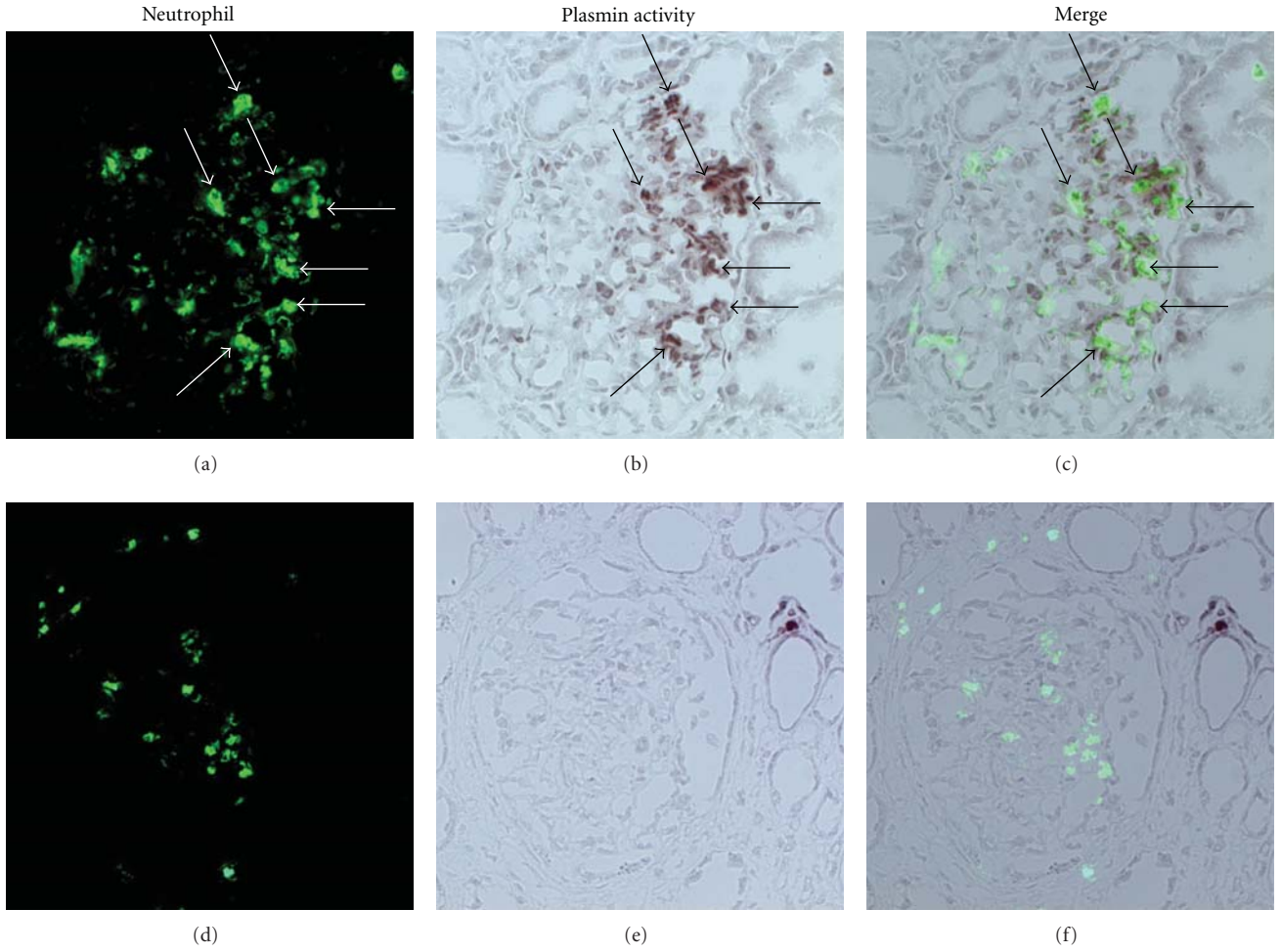

Figure 5: Glomerular infiltrating neutrophils and plasmin activity in APSGN and rapidly progressive glomerulonephritis. Representative photomicrographs of double staining for neutrophil elastase ((a) and (d), indirect immunofluorescence staining) and plasmin activity ((b) and (e), *in situ* zymography) from a patient with APSGN ((a)–(c)) and with rapidly progressive glomerulonephritis ((d)–(f)). The same fields were observed under fluorescence microscopy ((a) and (d)) and light microscopy ((b) and (e)) and were merged ((c) and (f)). The merged image (c) shows upregulated plasmin activity in a large portion of glomerular neutrophils in APSGN patients (indicated by arrows) but not in rapidly progressive glomerulonephritis patients (f) (original magnification ×260).

IgG (Figures 3(a)–3(c)), the glomerular distributions of plasmin activity and NAPlr are essentially identical (Figures 3(d) and 3(e)), suggesting that deposited NAPlr does indeed cause glomerular damage in APSGN by trapping plasmin and maintaining its activity [17]. Plasmin might damage renal tissue directly by degrading extracellular matrix proteins such as fibronectin or laminin but might also exert an indirect effect on variety of extracellular matrix proteins by activating promatrix metalloproteases [13]. Plasmin can also mediate inflammation by activating monocytes and neutrophils and causing their glomerular accumulation [18, 19]. Thus we think that glomerular damage may initially be induced by deposited NAPlr, which can bind plasmin and maintain its proteolytic activity, rather than by subepithelial immune-complexes. In this respect, the finding that NAPlr is localized on the inner side of glomerular tufts (endocapillary) is consistent with the predominantly endocapillary glomerular inflammation in APSGN. In other words, endocapillary localization of NAPlr might account for the different sites of glomerular inflammation and immune-complex deposition in APSGN. NAPlr thus acts not only as a component of the immune complex but also as a plasmin receptor and might contribute to the pathogenesis of APSGN by maintaining proteolytic activity. This is consistent with previous clinical findings that proteinuria and microscopic hematuria are occasionally found in the dormant phase of APSGN, when antibody against the nephritogenic antigen has not yet developed. The incidence of APSGN in streptococcal infection patients with these manifestations (proteinuria and hematuria in the dormant phase) is higher than that in streptococcal infection patients without these symptoms [20]. In keeping with these results, urinary

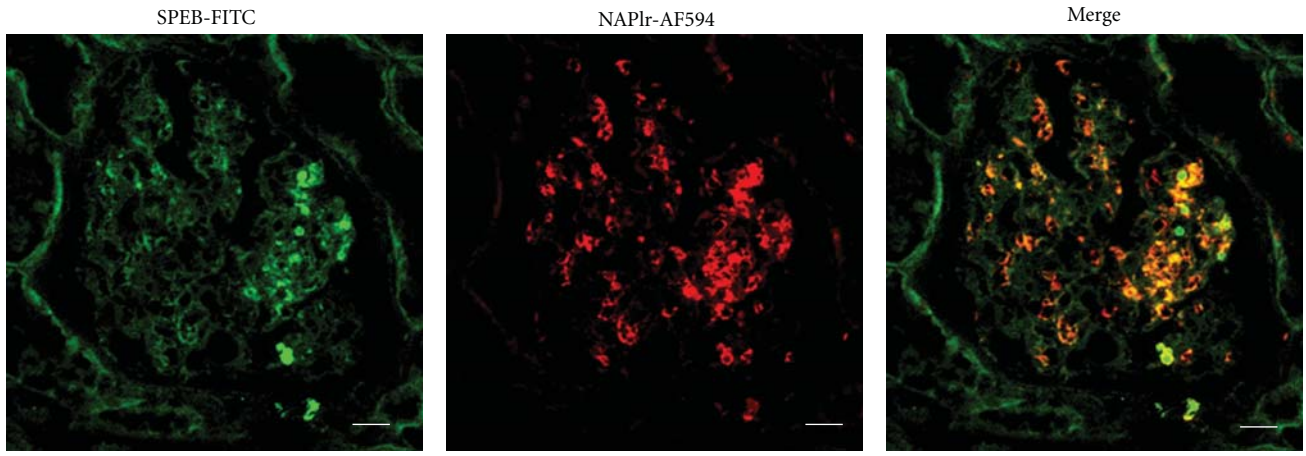

Figure 6: Representative photomicrographs of double IF staining for streptococcal pyrogenic exotoxin B (SPEB) ((a), FITC) and NAPlr ((b), Alexa Fluor 594) in an APSGN patient. A similar but not identical distribution of NAPlr and SPEB was observed in the merged image (c). Generally, NAPlr staining results were rather stronger than SPEB staining results (scale bar = 20 μm).

plasmin activity assessed by chromogenic assay (Figure 4(a)) and casein gel zymography (Figure 4(b)) was upregulated in APSGN patients than that in the urine of healthy subjects and IgA nephropathy patients, which support the pathogenic role of NAPlr and plasmin activity in APSGN [21].

As NAPlr was found to be localized mainly in neutrophils, we examined the plasmin activity of glomerular neutrophils and found that many were positive for plasmin activity in renal tissues from APSGN patients (Figures 5(a)–5(c)). On the other hand, glomerular neutrophils were not positive for plasmin activity in renal tissues from rapidly progressive glomerulonephritis patients (Figures 5(d)–5(f)), which suggests disease specificity of the relationship between plasmin activity and neutrophils [12]. With respect to the pathogenic role of NAPlr on neutrophils, the hyperproteolytic state of NAPlr-positive neutrophils in the present study indicates a possible role of these neutrophils in the induction of proteolytic glomerular damage. Specifically, plasmin activity of NAPlr-positive neutrophils may damage mesangium and glomerular basement membranes from inner side of glomerular tufts by promoting plasmin-catalyzed proteolysis. Regarding the mechanism of localization of NAPlr on neutrophils, we suggest two possibilities. NAPlr may bind the urokinase-type plasminogen activator receptor expressed on neutrophils [22], which has recently been shown to be the receptor for streptococcal GAPDH (NAPlr) [23]. Alternatively, NAPlr may be phagocytosed by neutrophils as a foreign bacterial antigen.

6. Comparison of NAPlr and Streptococcal Pyrogenic Exotoxin B (SPEB) as Nephritogenic Antigens for APSGN

Despite the previously mentioned evidence that NAPlr is a potent nephritogenic antigen that could cause APSGN, Batsford et al. [24] hypothesize that the nephritogenic antigen responsible for APSGN is streptococcal pyrogenic exotoxin B (SPEB). SPEB is a cationic cysteine protease secreted as a 42-kDa zymogen that is subsequently cleaved to a 28-kDa active proteinase. It is a toxin in severe invasive streptococcal infections [25] but has also been suggested by several groups [3, 6, 24, 26] to be a potent nephritogenic antigen of APSGN. Because of its cationic character, it is suspected to pass easily through the glomerular basement membrane and be deposited at a subepithelial site, where it would then induce immune complex formation. Cu et al. [6] were able to demonstrate glomerular localization of SPEB in 67% of APSGN patients by indirect IF staining with polyclonal anti-SPEB antibody. Batsford et al. recently compared NAPlr and SPEB in the renal biopsy tissues from a series of APSGN patients with anti-SPEB and antistreptococcal GAPDH (NAPlr) antibodies that they generated [24]. In contrast to our previous findings [7, 8], they found rare glomerular positivity for streptococcal GAPDH compared to those for SPEB. In contrast, they demonstrate that SPEB is the principal nephritogenic antigen. It is important to note that they used an indirect immunofluorescence staining method with the antistreptococcal GAPDH antibody that they generated, whereas we performed direct immunofluorescence staining with the anti-NAPlr antibody that we generated. Immunostaining results can vary with the use of different antibodies, so the comparison may be inappropriate [27]. Nonetheless, we compared glomerular localization profiles of SPEB and NAPlr by using an SPEB antibody provided by Dr. Batsford (Department of Immunology, Institute of Medical Microbiology, Freiburg, Germany) and our NAPlr antibody. Double staining showed an extremely similar distribution of both antigens in the glomeruli of APSGN tissues, although NAPlr staining appeared to predominate (Figures 6(a)–6(c)) [12, 28]. These results were surprising because many researchers, including us, have been assuming that APSGN is the result of a single nephritogenic streptococcal antigen.

Therefore, we should consider the possibility that two or more antigens interact in the induction of this disease. It is also interesting that NAPlr and SPEB share a common function. Both bind plasmin, thereby protect it from physiological inhibitors, and thus might cause chemotaxis of inflammatory cells and degradation of glomerular basement membranes, due to the activity of plasmin [17, 26]. Plasmin activity may be a common final pathway in APSGN [29], or those antigens may cooperate from different mechanisms for the development of APSGN as recently suggested by Rodríguez-Iturbe and Batsford [30].

7. Glomerular NAPlr Deposition in Other Glomerulonephritis Related with Streptococcal Infection

As described above, NAPlr is originally isolated as the putative nephritogenic antigen for APSGN. Indeed, glomerular NAPlr deposition can be found with extraordinary frequency in early-phase APSGN patients. However, recent observation has revealed that glomerular NAPlr deposition and plasmin activity could be found in a similar fashion also in other glomerular diseases, such as dense deposit disease (DDD) [31, 32], Henoch-Schonlein Purpura nephritis (HSPN) [7, 33] and membranoproliferative glomerulonephritis (MPGN) [7], in which recent streptococcal infection has been suggested by serological tests. The histological characteristics common to these cases are prominent endocapillary proliferation. We believe that there is a subgroup of patients in these diseases (DDD, MPGN, and HSPN) in which glomerulonephritis is induced by streptococcal infection and subsequent glomerular deposition of NAPlr and related plasmin activity. We would like to refer to these diseases collectively as streptococcal-infection related nephritis (SIRN).

8. Conclusion

In light of our recent finding on nephritogenic antigen responsible for APSGN, we propose the following mechanisms for the development of APSGN (Figure 7). Infection of the throat or skin with streptococcus induces the release of a nephritogenic antigen, such as NAPlr, into the circulation. Circulating NAPlr accumulates in the renal glomeruli on the mesangial matrix and glomerular basement membrane, probably by adhesion [7]. NAPlr then traps and maintains the activity of plasmin, which might induce glomerular damage by degrading the glomerular basement membrane by itself or by activating promatrix metalloproteases. Plasmin activity may also mediate neutrophil and macrophage infiltration [18, 19]. Therefore we believe that an antibody-independent direct effect of the nephritogenic antigen is important for the initiation of the disease. Such glomerular damage may induce urine abnormalities during the latent period of the disease. The host immune reaction (both humoral and cell-mediated) against the nephritogenic antigen also develops during this latent period. The circulating antibody forms immune complexes, either *in situ* or in the circulation that can readily pass through the altered glomerular basement membrane and accumulate in the subepithelial space as humps. These final steps of immune cell accumulation and immune complex deposition are accompanied by the activation of complement and lead to the overt disease state. The mechanisms localizing NAPlr specifically to the glomeruli and possible contributions and interactions of other nephritic antigens in APSGN must be elucidated in future studies.

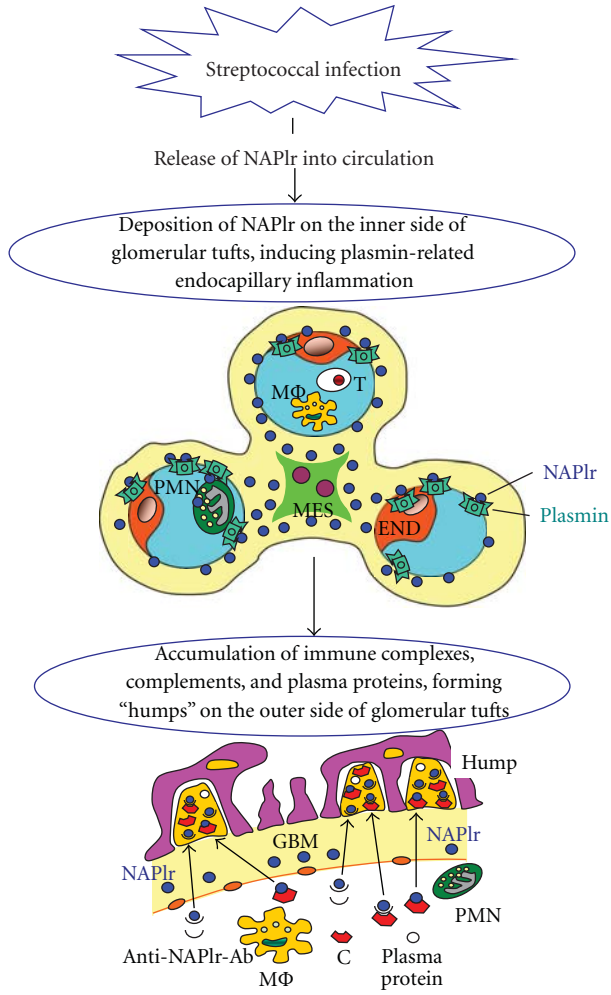

Figure 7: Schematic representation of proposed mechanisms involved in the development of APSGN. MES: mesangial cell; END: endothelial cell; PMN: polymorphonuclear cell; MΦ: macrophage; T: T lymphocyte; GBM: glomerular basement membrane; C: complement; Anti-NAPlr-Ab: Anti-NAPlr-antibody.

Glomerular NAPlr deposition and plasmin activity could be observed in a similar fashion in other glomerular diseases, such as DDD, HSPN, and MPGN, in which recent streptococcal infection has been suggested. We propose to refer to these diseases (glomerulonephritis induced by streptococcal infection and subsequent glomerular deposition of NAPlr and plasmin activity) collectively as SIRN.

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Review Article

Accelerated Fibrinolysis and Its Propagation on Vascular Endothelial Cells by Secreted and Retained tPA

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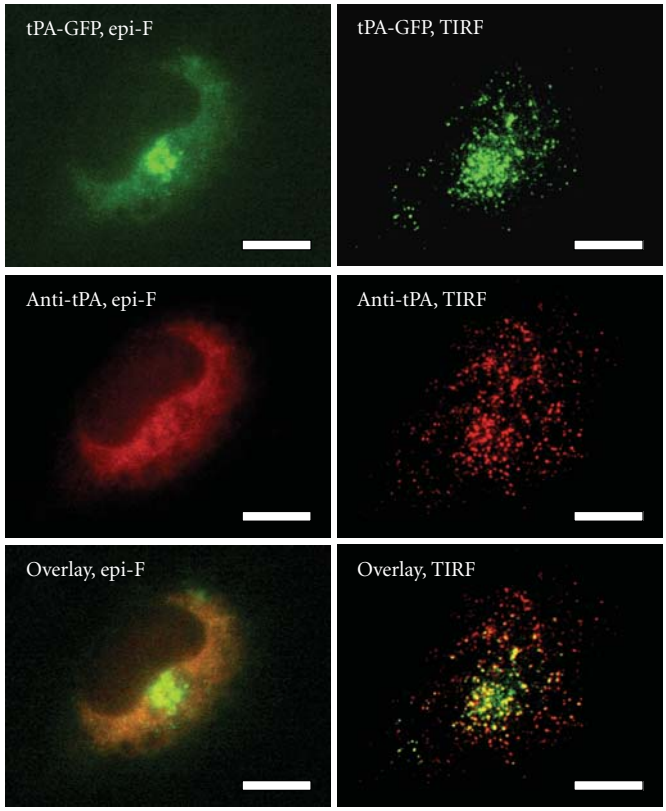
We successfully visualized the secretory dynamics of tissue-type plasminogen activator (tPA) tagged by green fluorescent protein (tPA-GFP) from cultured vascular endothelial cells (VECs) using total internal reflection fluorescence (TIRF) microscopy and demonstrated that tPA-GFP secreted from VECs was retained on cell surfaces in a heavy-chain-dependent manner. Progressive binding of Alexa568-labeled Glu-plasminogen was also observed on the surface of active tPA-GFP expressing cells via lysine binding sites (LBS), which was not observed on inactive mutant tPA-GFP expressing cells. These results suggest that retained tPA on VECs effectively activated plasminogen to plasmin, which then facilitated the binding of additional plasminogen on the cell surface by proteolytically cleaving surface-associated proteins and exposing their C-terminal lysine residues. Thus prolonged retention of tPA appeared to play an important role in initiating and amplifying plasmin generation on VECs. LBS-dependent binding of plasminogen was also observed as a narrow band at the lytic front of the fibrin mesh formed on active tPA-GFP expressing cells, which expanded outward as the lytic area increased. This binding was not observed on inactive mutant tPA-GFP expressing cells or in the presence of aprotinin. The binding of plasminogen to partially digested fibrin appears to be indispensable for spontaneous fibrinolysis.

1. Introduction

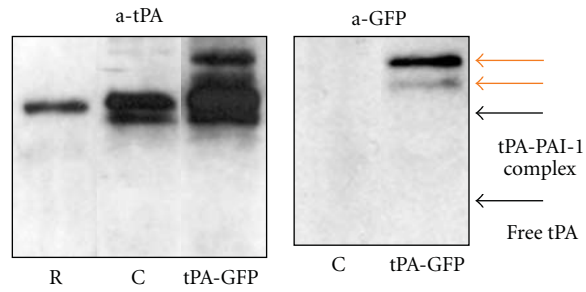
Fibrinolysis takes place when undesirable fibrin is formed or when a hemostatic thrombus becomes unnecessary. Plasminogen activation by plasminogen activators (PAs) is the initial step in fibrinolysis, which is regulated by many physiological and pathological factors [1] so as to quickly dissolve unnecessary thrombi. Tissue-type PA (tPA) and urokinase-type PA (uPA) are the physiological PAs, and the former is synthesized and released from vascular endothelial cells (VECs) as an active form to initiate intravascular thrombolysis. Its characteristic of binding to fibrin is believed essential in the effective activation of plasminogen on the fibrin surface as well as in plasmin-catalyzed fibrin digestion [2]. The binding of plasminogen to fibrin through lysine-binding sites (LBS) existing in kringle domains is also believed to play an essential role in its effective activation on the fibrin surface [1]. In this paper, we introduce our recent findings on tPA secretory dynamics from VECs, and on the subsequent process of secreted tPA catalyzing plasminogen activation and fibrinolysis on vascular endothelial cells. Indispensiveness of the binding of plasminogen to partially digested fibrin in spontaneous fibrinolysis is also demonstrated.

2. Secretion of tPA from VECs and Regulation of Its Activity in Plasma

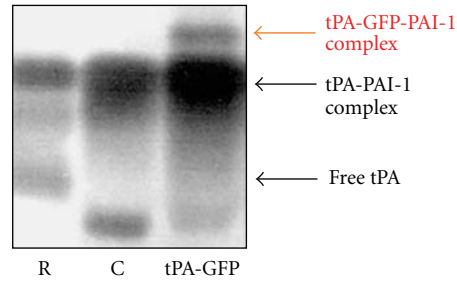
tPA is synthesized and released from VECs as an active form, either constitutively or in a regulated fashion following various kinds of stimuli from several different granules [5], and thus the amount of secreted tPA is a principal determinant of plasminogen activation potential in the vasculature. Another determinant of this potential is plasminogen activator inhibitor type 1 (PAI-1), the primary inhibitor of both tPA and uPA under physiological conditions. Since PAI-1 exists in plasma in molar excess over tPA, most of the tPA circulates in an inactive complexed form with PAI-1, and



(a) Immunofluorescence image of tPA-GFP expressing EA hy926 cells



(b) Western blotting



R: recombinant protein
C: control cells

(c) Fibrin autography

Figure 1: Characteristics of tPA-GFP expressing EA hy926 cells. (a) Immunofluorescence images of tPA-GFP expressing EA hy926 cells were captured by either epifluorescence microscopy (epi-F) or total internal reflection fluorescence microscopy (TIRF), the latter of which more clearly detects tPA-GFP containing granules as dots. (b) Culture media of tPA-GFP expressing EA hy926 cells and control cells were analyzed by western blotting. Additional high-molecular-weight bands detected by both anti-tPA and anti-GFP were observed in tPA-GFP expressing EA hy926 cells (orange arrows), suggesting that tPA-GFP has activity to form high-molecular-weight complex with PAI-1. (c) Fibrin autography of the same culture media. The additional bands detected by western blot analysis showed a lytic band (orange arrow). This figure was originally published in [3].

only a small fraction of this total tPA circulates as an active enzyme [6].

3. tPA Secretory Dynamics

3.1. Slow Kinetics of tPA Secretion. The human vascular endothelial cell line EA.hy926 was transfected with an expression vector encoding tPA tagged by green fluorescent protein (tPA-GFP), and tPA secretory dynamics were analyzed using total internal reflection fluorescence (TIRF) microscopy (Figure 1(a)) [3]. The expressed tPA-GFP was confirmed to be active by the facts that it formed a high-molecular-weight complex with PAI-1 (Figure 1(b)) and that it developed a lytic band on a plasminogen-rich fibrin overlay (Figure 1(c)). The sizes and distribution of tPA-GFP containing granules were indistinguishable from those of intrinsic wild tPA, which was detected using an anti-tPA antibody. TIRF microscopy was used to visualize tPA-GFP-containing granules, which were detected as dots when they appeared in the so-called evanescent field, which is approximately 100 nm from the interface. The secretory dynamics of docking and opening of tPA-GFP containing granules followed by tPA-GFP secretion were appreciated by a sudden increase in fluorescent intensity followed by its gradual decrease. The most striking peculiarity in tPA secretory dynamics was that the release of tPA-GFP from the opened granule was very slow, which was shown by a slow decline in the fluorescent signal as well as by the persistence of an apparent fluorescent spot even 2-3 minutes after the opening of the granule. The mechanism for the long retention of tPA-GFP on membrane surfaces after granular opening was analyzed using several domain-deleted mutants of tPA-GFP. A mutant lacking the entire heavy chain and having only the catalytic domain disappeared quickly after granular opening, similar to the dynamics of a peptide hormone like insulin, suggesting that the prolonged retention of tPA requires the heavy chain.

4. Retention of tPA on the Cell Surface after Exocytosis and Its Release by PAI-1

Heavy-chain-dependent retention of tPA on the surface of VECs seems beneficial as it maintains an elevated plasminogen activation potential on the surface of the vasculature. The facts that in the culture medium of EA.hy926 cells tPA

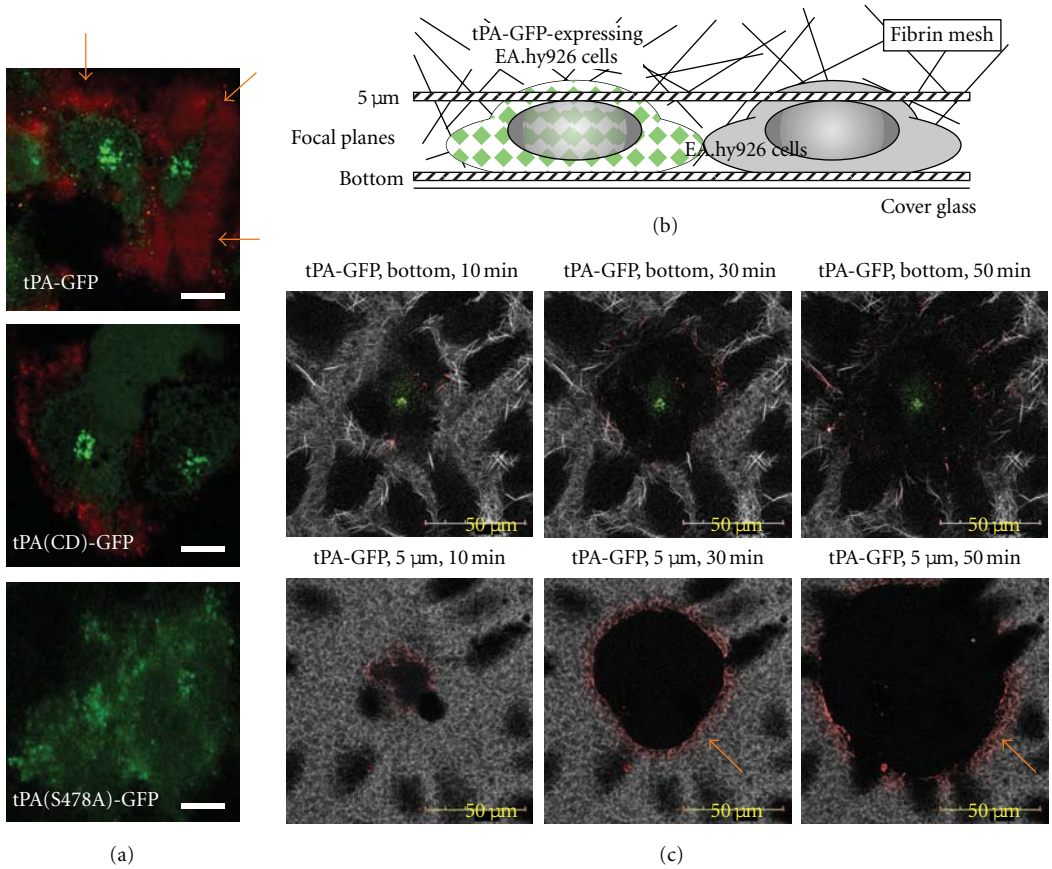


FIGURE 2: Accumulation of plasminogen and effective fibrinolysis on tPA-GFP-expressing EA hy926 cells. (a) Alexa568-labeled Glu-plasminogen (568-Glu-plg) was incubated with EA.hy926 transfected with tPA-GFP, and the binding of plasminogen on the cell surface was observed (orange arrow), which was suppressed when either heavy-chain-deleted tPA-GFP (tPA(CD)-GFP) or catalytically inactive tPA-GFP (tPA(S478A)-GFP) was employed. (b) A fibrin network was formed on EA.hy926 transfected with tPA-GFP using Alexa647-labeled fibrinogen, and its spontaneous lysis was monitored by confocal microscopy. (c) Fibrinolysis initiated by tPA-GFP expressing cells, and its gradual expansion was clearly observed both focal planes of the bottom and 5 μm above the bottom. Linear binding of 568-Glu-plg was also observed at the lytic front (orange arrow), which expanded outward as the lytic area increased. A part of this figure was originally published in [4].

exists mostly as tPA-PAI-1 complex and that free tPA rarely exists prompted us to analyze the potential effect of PAI-1 on tPA retention. The following three results suggested that PAI-1 facilitated the release of tPA from the surface of VECs. First, following suppression of PAI-1 expression in EA.hy926 cells by siRNA, the retention time of tPA-GFP was prolonged. Second, supplementation of recombinant PAI-1, in turn, shortened the retention time and increased the amount of tPA-GFP-PAI-1 complex but not of free tPA-GFP in the supernatant. Finally, a mutant tPA-GFP whose active site serine was substituted by alanine so as not to react with PAI-1 stayed on the cell surface for a longer time, and essentially no tPA-GFP was detected in the supernatant. Thus PAI-1 appeared to attenuate the fibrinolytic potential not only in plasma but also on the surface of VECs.

5. Fibrinolysis Initiated by tPA Secreted from VECs

tPA effectively activates plasminogen when it is bound to solid structures including fibrin [2], denatured proteins having a crossed beta-structure [7], and cell surfaces [2]. To achieve effective fibrinolysis, synchronized binding of plasminogen to these surfaces is necessary. When Alexa568-labeled Glu-plasminogen (568-Glu-plg) was incubated with EA.hy926 transfected with tPA-GFP, progressive binding of plasminogen to the cell surface in an LBS-dependent manner was observed (Figure 2(a)) [4]. This accumulation of plasminogen on the cell surface was strongly attenuated when catalytically inactive mutant tPA-GFP was employed or when inhibitors of tPA or plasmin were supplemented, suggesting that plasmin-catalyzed exposure of new C-terminal lysine residues is involved in this binding process (Figure 3).

To analyze how surface-retained tPA on VECs effectively initiates fibrinolysis, a fibrin network was formed on EA.hy926 transfected with tPA-GFP using Alexa647-labeled fibrinogen (647-fbg) and its spontaneous lysis was monitored using confocal microscopy (Figure 2(b)) [4]. Monitoring at several sequential planes revealed that fibrinolysis began at the surface of tPA-GFP expressing cells and gradually expanded (Figure 2(c)). Importantly, linear binding of 568-Glu-plg was always observed at the lytic

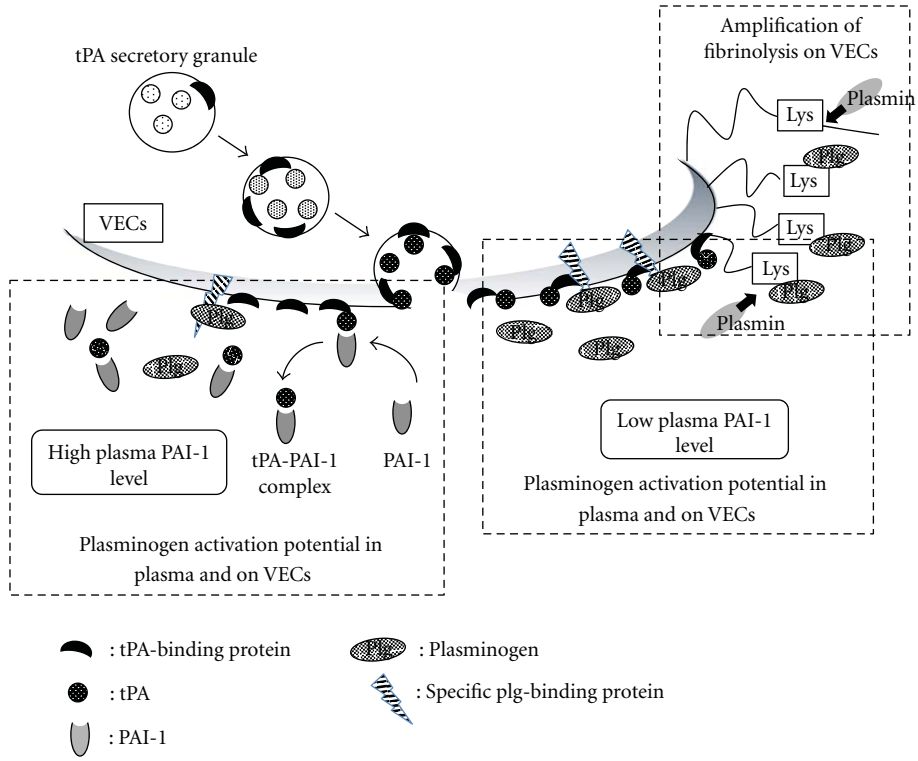


Figure 3: Mechanism to maintain a high fibrinolytic potential on VECs and its modification by PAI-1. Secreted- and retained-tPA readily activates cell-bound plasminogen on VECs (lower right), which amplifies plasminogen accumulation to newly exposed C-terminal lysine by plasmin-dependent cleavage of cell surface proteins (upper right). PAI-1 suppresses these mechanisms by removing retained tPA from VECs by forming high molecular weight complex (left).

front, which expanded outward as the lytic area increased. Presumably the 568-Glu-plg bound preferentially to newly exposed C-terminal lysine residues by plasmin-catalyzed partial digestion of fibrin fibers. As was shown before using many different methods [1] and was recently confirmed by analyzing the crystal structure of plasminogen [8], Glu-plasminogen seems to have undergone a conformational change from its closed conformation to its easily activatable open form, and this likely facilitates clot lysis. Thus, as has been expected for a long time, the binding of Glu-plg to fibrin that has been partially cleaved by plasmin clearly proved essential for spontaneous initiation of clot lysis.

6. Cell-Associated Fibrinolysis and Cell Function

Plasminogen activation and the associated fibrinolysis on cell surfaces are known to play a variety of functions under both physiological and pathological conditions [2, 9, 10]. Plasminogen binding-molecules play central role in these events, and several candidate molecules have been reported such as Plg-R_{KT}, histone H2B, and annexin A2-S100A10 complex as well as alpha-enolase. The binding of plasminogen and/or its activation to plasmin on these molecules has been suggested to possibly transmit signals into the cells and to modify cellular functions [11, 12]. These specific plasminogen binding molecules, therefore, seem to play important roles in the initiation of effective plasmin generation, as well as in cell signaling, both of which are important in modifying cellular functions. Our findings, however, that the accumulation of plasminogen increased during the time course of tPA secretion from VECs suggests that other proteins also function as plasminogen binding molecules on the cell surface by providing newly exposed C-terminal lysine residues after plasmin-catalyzed cleavage (Figure 3). We believe that this augmentation of plasminogen binding by newly exposed C-terminal lysines as well as its effective activation also plays essential roles not only in the degradation of fibrin or matrix proteins, but also in protease-dependent signal transduction.

7. Summary

We have described a unique mechanism of tPA secretion and its role in effective fibrinolysis that takes place on VECs. This mechanism seems to require retention of tPA after secretion as well as the binding of plasminogen to specific molecules on the cell surface. Following the exposure of new C-terminal lysines on membrane proteins by plasmin-catalyzed cleavage, further accumulation of plasminogen on the cell surface was observed. Such initiation and augmentation of plasminogen activation seems to be important in modifying cell functions involved in a variety of physiological and pathological events. Understanding these mechanisms in greater detail will provide us with new strategies to control these events.

Acknowledgments

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