International Journal of Inflammation

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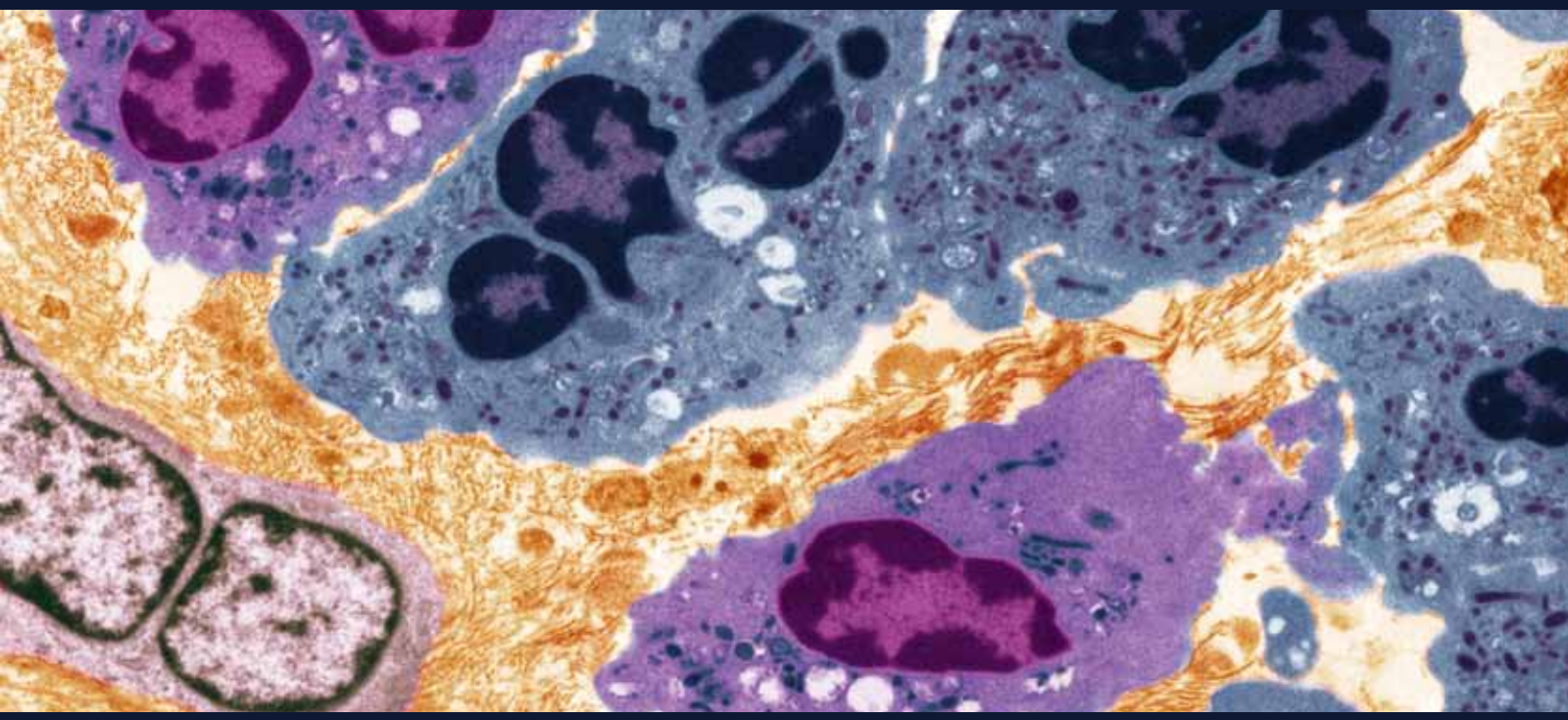
Guest Editors: Jun-Ichi Abe, Ichiro Manabe, Masanori Aikawa, and Elena Aikawa



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Editorial Board

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Editorial

Cardiovascular Inflammation 2012: Reactive Oxygen Species, SUMOylation, and Biomarkers in Cardiovascular Inflammation

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Cardiovascular diseases are growing burden in westernized countries. Cardiovascular diseases, including atherosclerosis, associate with chronic inflammation. The goal of this special issue is to highlight the inflammatory nature of cardiovascular diseases and their association with the production of cytokines, reactive oxygen species (ROS), and SUMOylation in atherosclerotic plaques. In addition, biomarkers that reflect the biological processes of progression and destabilization of atherosclerotic plaques would provide noninvasive means to identify high-risk patients for cardiovascular events. Armed with this understanding, it is hoped that novel therapeutic strategies will aid in the prevention and management of chronic cardiovascular inflammation and benefit the patients afflicted with cardiovascular disease.

Increasing pieces of evidence support the important role of ROS and cytokine production in the process of atherosclerosis via regulating various signaling pathways leading to vascular inflammation. In the present review, N. T. Le et al. focused on ROS-mediated SUMOylation, which is one of the posttranslational modifications, and discussed its possible implications on vascular inflammation. It becomes apparent that ROS production can regulate the process of SUMOylation in both vasculature and heart and mediate a number of biological processes, such as apoptosis and inflammation. The crucial role especially of ERK5, p53, and MK2 SUMOylation in the process of endothelial inflammation has been discussed. Furthermore, a recent study from their group has found a strong correlation between $C_{247}T$ SNP of the NADPH oxidase p22phox subunit and cardiovascular events in postinfarction patients with concurrently high levels of HDL cholesterol and CRP. These data suggest that ROS not only affects the intracellular signaling but also can produce dysfunctional HDL. Since ROS can increase nitration and chlorination of specific tyrosine residues on apolipoproteinA-I, which is a major apolipoprotein component of HDL particles and major mediator of HDL functionalities, it is intriguing to assume that $C_{247}T$ SNP of the NADPH oxidase p22phox subunit relates to this HDL modification, leading to dysfunctional HDL.

R. N. England and M. V. Autieri discussed the role of novel and undercharacterized cytokine IL-19 on vascular inflammation. IL-19 shows pleotropic effects on vascular wall. It can inhibit proliferative and proinflammatory gene expression in vascular smooth muscle cells (VSMCs) via NF-kB independent signaling, which is very different from the effects of IL-10. The possible involvement of the expression of suppressor of cytokine signaling 5 (SOCS5), human R antigen (HuR), and hem oxygenase-1 (HO-1) in this inhibitory effect has been discussed. In contrast to VSMCs, IL-19 showed proliferative, promigratory, and proangiogenic effects in vascular endothelial cells (ECs), although its role in EC inflammation remains unclear. Since IL-19 can inhibit ROS production and atherosclerosis formation in a mouse model, investigation the exact mechanisms of and interplay between IL-19 and ROS in both VSMCs and ECs will be valuable for orchestrating anti-inflammatory therapies for cardiovascular events.

Biomarkers can reflect the biological processes of disease progression. For instance, CRP levels have been shown to

be predictive of cardiovascular risk. However, CRP is a nonspecific inflammatory marker that is affected by any inflammatory conditions. Therefore, it is important to develop biomarkers that specifically report the biological processes in atherosclerosis and other cardiovascular diseases, associated with chronic inflammation. N. Kafkas et al. showed that serum neutrophil gelatinase-associated lipocalin (NGAL) levels were increased in patients with coronary artery disease. They propose that NGAL could be used to discriminate patients with acute coronary syndrome from those who are with stable angina or without coronary artery diseases. V. Panichi et al. review newer biomarkers, including CD40 ligand and pentraxin-3 that reflect chronic inflammation in cardiovascular and renal diseases. D. Vasic and D. Walcher summarize the current knowledge about C-peptide as a biomarker and bioactive molecule. The deposition of C-peptide may promote inflammation.

The docking proteins of the Grb2-associated binder family (Gab1, Gab2, and Gab3) serve as important signaling compartments. They may amplify and integrate signal transduction pathways in response to various stimuli such as cytokines. Interestingly, recent evidence has linked abnormal Gab signaling with human diseases including cancer and cardiovascular disease, for which inflammation plays a key role. Y. Nakaoka and I. Komuro. extensively described molecular structure, recruitment, and phosphorylation of Gab proteins and discussed their role in cardiovascular and cancer inflammation.

An emerging concept suggests that chronic inflammation participates in the pathogenesis of atherosclerosis and other cardiovascular diseases. Thus, controlling proinflammatory pathways such as ROS-mediated SUMOylation may attenuate such diseases. The goal of this thematic series is to highlight emerging proinflammatory mechanisms involved in cardiovascular inflammation. We believe that this issue will offer updated concepts and help readers develop ideas leading to future investigations and new drug development.

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Review Article

Gab Docking Proteins in Cardiovascular Disease, Cancer, and Inflammation

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The docking proteins of the Grb2-associated binder (Gab) family have emerged as crucial signaling compartments in metazoans. In mammals, the Gab proteins, consisting of Gab1, Gab2, and Gab3, are involved in the amplification and integration of signal transduction evoked by a variety of extracellular stimuli, including growth factors, cytokines, antigens, and other molecules. Gab proteins lack the enzymatic activity themselves; however, when phosphorylated on tyrosine residues, they provide binding sites for multiple Src homology-2 (SH2) domain-containing proteins, such as SH2-containing protein tyrosine phosphatase 2 (SHP2), phosphatidylinositol 3-kinase regulatory subunit p85, phospholipase Cγ, Crk, and GC-GAP. Through these interactions, the Gab proteins transduce signals from activated receptors into pathways with distinct biological functions, thereby contributing to signal diversification. They are known to play crucial roles in numerous physiological processes through their associations with SHP2 and p85. In addition, abnormal Gab protein signaling has been linked to human diseases including cancer, cardiovascular disease, and inflammatory disorders. In this paper, we provide an overview of the structure, effector functions, and regulation of the Gab docking proteins, with a special focus on their associations with cardiovascular disease, cancer, and inflammation.

1. Introduction

The mammalian Grb2-associated binder (Gab) proteins are homologs of *Drosophila* DOS (Daughter Of Sevenless) and *Caenorhabditis elegans* SOC-1 (Suppressor Of Clear). These proteins define a family of docking proteins closely related to the insulin receptor substrate (IRS-1, IRS-2, IRS-3), fibroblast growth factor substrate (FRS2), linker of T cell (LAT), and downstream of kinase (Dok) families [1]. In contrast to adaptor proteins such as growth factor receptor bound protein 2 (Grb2) and Shc, which are usually smaller and often function as a molecular bridge between two proteins in the assembly of larger protein complexes, docking proteins contain a membrane-targeting region at the N-terminus, binding sites for src homology 3 (SH3) domain-containing proteins, and multiple tyrosine phosphorylation sites that, when phosphorylated, function as binding sites for the src homology 2 (SH2) domains of a variety of effectors. Consequently, the docking proteins are significantly larger than adaptor proteins. In addition, docking proteins usually contain one or more moieties that mediate their recruitment to plasma membranes through protein-protein or protein-lipid interactions. Their multiple functional domains and large molecular size reflect the docking proteins' function as a platform for the assembly of signaling subsystems. Since there have been several excellent general reviews on Gab proteins to date [1–4], here we will focus on the role of Gab docking proteins in cardiovascular and inflammatory disorders.

2. Identification of Gab Family Docking Proteins

Gab1, the first of the three mammalian *gab* genes cloned to date, was originally identified as a Grb2-binding protein from a human glial tumor expression library and found to undergo tyrosine phosphorylation in response to stimulation by epidermal growth factor (EGF) and insulin [5]. It was also isolated as a c-Met-receptor interacting protein in a yeast two-hybrid screen and as the major tyrosine-phosphorylated

protein in cells transformed by the *Tpr-Met* oncogene [6, 7]. Gab2 was cloned as a binding protein and a substrate of the SH2 domain-containing protein tyrosine phosphatase (SHP2) [8–10]. The *Gab3* cDNA was cloned with the aid of the genome sequencing project, using a search strategy based on sequence similarities to Gab1 [11]. Although a putative *Gab4* gene has been found in the human genome database, its expression pattern, signaling mechanism, and functional roles have not been characterized to date.

DOS is the only Gab homolog in *Drosophila*. It was identified as a potential substrate for the product of *Corkscrew* (*Csw*) [12], the *Drosophila* SHP2 ortholog, and independently in a screen for mutants that suppress the rough-eye phenotype of a hyper-activated *sevenless* allele [13]. SOC-1, the *C. elegans* homolog, was found in a screen for suppressors of hyperactive Egl-15 (an FGF receptor ortholog) signaling [14].

3. Molecular Structure, Recruitment, and Phosphorylation of Gab Docking Proteins

3.1. Molecular Structure. All Gab docking proteins share a highly conserved N-terminal Pleckstrin homology (PH) domain, proline-rich segments in the central region, and multiple tyrosine residues within the potential binding motifs favored by various SH2 domain-containing signaling proteins (Figure 1) [1–4]. Mutagenesis and *in vitro* binding assays have demonstrated that a number of signaling molecules interact with Gab docking proteins (Figure 1).

3.2. Recruitment. Gab docking proteins utilize several different mechanisms to regulate their subcellular localization. First, the PH domain enables Gab proteins to translocate to plasma membrane patches enriched in phosphatidylinositol 3,4,5-triphosphate (PIP3), a product of phosphatidylinositol-3 kinase (PI3K) [15–18]. Besides the PH domain, Gab docking proteins use at least two additional mechanisms for their recruitment to activated plasma membrane-associated receptors. The first mechanism has been demonstrated only for the interaction between Gab1 and c-Met (the receptor for hepatocyte growth factor; HGF) [7]. A region in Gab1 (amino acids 450–532), termed the c-Met binding domain (MBD), interacts directly with the tyrosyl-phosphorylated c-Met in response to stimulation with HGF [7, 19–21]. Both the activated kinase domain of c-Met and the MBD in Gab1 are involved in this direct interaction [19, 20]. The minimal amino acid sequence sufficient for the direct interaction between Gab1 and c-Met, termed the c-Met binding sequence (MBS), consists of 16 amino acids (486–501) [19, 20]. Since no other Gab docking proteins contain the MBS [22, 23], Gab2 interacts with activated receptors via the adaptor protein Grb2, which is also utilized as a secondary mechanism by the c-Met receptor to associate indirectly with Gab1. The importance of this indirect recruitment was revealed in knockout mice expressing a Gab1 mutant incapable of binding with Grb2: the phenotype was lethal [21].

3.3. Phosphorylation. Gab-mediated signal transduction is regulated by the site-specific tyrosine phosphorylation of the Gab proteins. Phosphorylated tyrosine residues provide docking sites for the SH2-domains of SHP2, the Crk adaptor protein, phospholipase C (PLC) γ, and the regulatory subunit of PI3K, p85 [2–4]. By recruiting various effectors with SH2 domains, Gab proteins not only promote signal transduction but also translate the receptor-evoked signals into distinct biological properties. Therefore, Gab family proteins function as a signaling platform for an entire signaling subsystem.

The best-characterized effector signaling pathway of Gab proteins is transmitted via the protein tyrosine phosphatase SHP2. SHP2 has two tandem N-terminal SH2 domains, which confer autoinhibition of the C-terminal phosphatase domain [24]. All mammalian Gab proteins, as well as the *Drosophila* DOS and *C. elegans* SOC-1, bind SHP2 (or its homologs), suggesting that the recruitment of SHP2 is an evolutionarily conserved feature of Gab family proteins [24]. Most Gab proteins contain two SHP2 binding sites, which act as a biphosphoryl tyrosine activation motif (BTAM) and bind both SH2 domains, which releases SHP2's autoinhibition [24, 25]. Therefore, SHP2 binding partners, including Gab proteins, may act not only as signaling platforms, but also as allosteric activators.

The functional significance of the Gab-SHP2 interaction has been extensively studied using mutants of Gab family proteins unable to bind SHP2 or its homologs. Mutant DOS bearing a Y to F mutation at either of the two CSW-binding sites is nonfunctional, and Sevenless signaling cannot rescue the lethal phenotype associated with *DOS* loss-of-function mutations [26, 27]. A Gab1 mutant that is unable to bind SHP2 fails to transduce the signal for c-Met-dependent morphogenesis in MDCK cells and blocks the activation of extracellular signal-regulated kinase 1/2 (ERK1/2), MAP kinase upon stimulation with epidermal growth factor (EGF), HGF, or lysophosphatidic acid (LPA) [23, 25, 28, 29]. In endothelial cells, the recruitment of SHP2 to Gab1 not only regulates vascular endothelial growth factor- (VEGF-) induced migration, but also contributes to HGF-induced migration [30–32]. We also found that the Gab1-SHP2 interaction is involved in the activation of extracellular signal-regulated kinase 5 (ERK5) in gp130-dependent cardiomyocyte hypertrophy [33, 34]. In addition, in certain cellular circumstances, the Gab-SHP2 complex positively regulates other downstream pathways, such as c-Kit-induced Rac activation and β1-integrin-induced PI3K activation [35, 36].

These findings demonstrate that SHP2 is a crucial positive modulator for the activation of ERK1/2. Although the molecular mechanism underlying why the recruitment of SHP2 by Gab1 is required for the full activation of ERK1/2 is still not completely understood, two possible mechanisms have been proposed. First, SHP2 may dephosphorylate the recruitment site for the Src-inactivating kinase Csk on the transmembrane glycoprotein PAG/Cbp and paxillin, resulting in the enhanced activation of Src family kinases [37, 38]. Second, SHP2 may dephosphorylate the binding site for p120Ras-GAP on the activated receptors for EGF and on Gab1, thus inactivating the Ras-dependent signaling pathway [37, 39].

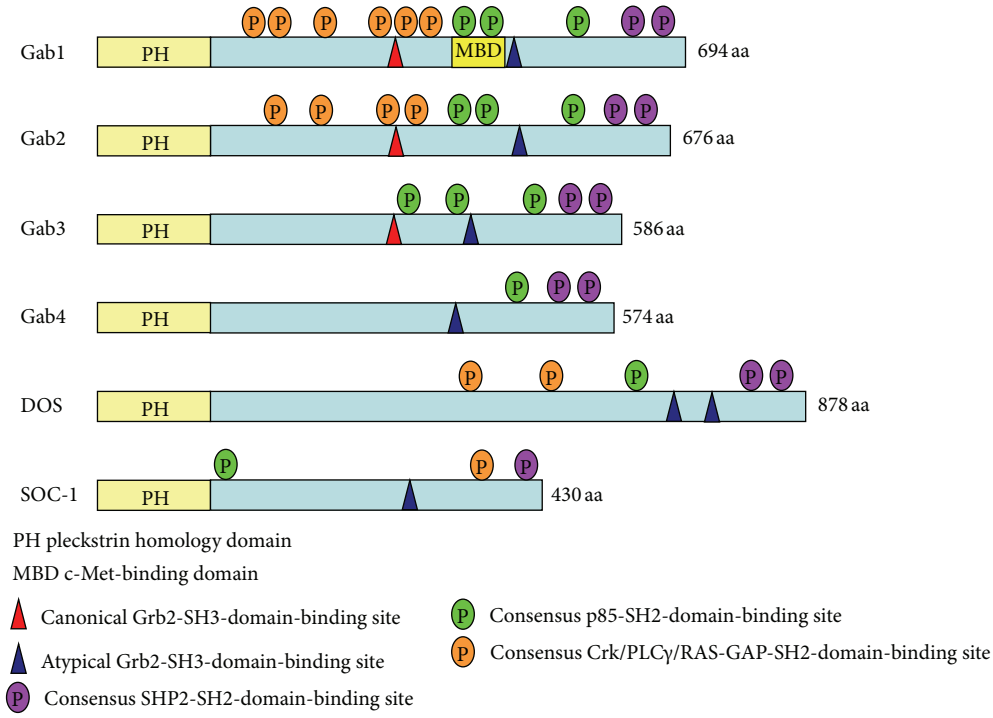

Figure 1: Schematic structures of Gab family docking proteins. Shown are the schematic domain structures of three human Gab proteins (Gab1–3), the putative human Gab4 protein, *Drosophila* DOS, and *C. elegans* SOC-1. All Gab proteins consist of a highly conserved N-terminal PH domain that is involved in membrane targeting. The central proline-rich regions mediate the association with SH3 domain-containing adaptor proteins such as Grb2. Consensus binding motifs favored by various SH2 domain-containing proteins such as SHP2, p85, Crk, and PLCγ are indicated.

4. Physiological Functions of Gab Proteins Revealed by Global Knockout or Knock-In Mice

The presence of multiple *gab* genes in mammals suggests that the function of each Gab protein may be specialized or restricted to certain pathways or tissues. On the other hand, these gene products may be functionally redundant. Extensive analyses of the expression level of the *gab* genes by northern blot and RT-PCR have shed some light on this issue [5, 7, 8, 11]. Gab1 shows the broadest expression and greatest abundance: it is found in almost all tissues examined, including the brain, heart, liver, lung, kidney, pancreas, spleen, thymus, and uterus of the adult mouse, and is expressed at early developmental stages, such as in ES cells [11]. Although Gab2's expression is relatively weak in most tissue samples, compared with Gab1, it is abundantly expressed in hematopoietic progenitor cell lines, such as BAF3 and FDC-P1 [5, 7, 8, 11]. The expression of Gab3 is also confined to the hematopoietic system [5, 7, 8, 11]. Thus, the three mammalian *gab* genes have unique but overlapping expression patterns.

Consistent with Gab1's early and broad expression during development, Gab1-knockout (Gab1$^{-/-}$) mice die *in utero* between embryonic days (E) 13.5 and 18.5 with developmental defects in the heart, placenta, skin, and skeletal muscle [40, 41]. In line with the close relationship between Gab1 and c-Met, Gab1$^{-/-}$ mice phenocopy most of the phenotypes of HGF- and c-Met-knockout mice, such as early embryonic lethality with placental defects, reduced liver size, and defects in the migration of muscle precursor cells [40, 41].

Gab1 knock-in mice carrying mutations in the SHP2 binding site show defects in muscle and placental development presumably directed by HGF/c-Met signaling, demonstrating a specific role for the Gab1-SHP2 complex in the migration of muscle progenitor cells [21]. Consistent with these findings, we found that the myogenic differentiation of C2C12 cells induced by IGF-1 or low-serum conditions was strongly enhanced by the adenovirus-mediated overexpression of a mutated Gab1 (Gab1$^{\Delta SHP2}$) incapable of binding SHP2, but inhibited by the overexpression of wild-type Gab1 [42]. This result suggests that Gab1 negatively regulates myogenic differentiation through its association with SHP2. Taken together, these findings suggest that Gab1 plays a key role not only in the inhibition of myogenesis, but also in the maintenance of the undifferentiated state of mesenchymal cells, effected through the activation of SHP2. On the other hand, Gab1 knock-in mice carrying mutations in the p85 binding site show defects in EGF receptor-mediated embryonic eyelid closure and keratinocyte migration [21], and knock-in mice expressing a Gab1 mutant lacking the Grb2 binding sites display an embryonic lethal phenotype and defects in liver, placenta, and craniofacial development [21]. These results support the idea that Gab1 induces different

biological responses through the recruitment of distinct effectors *in vivo*.

In contrast, Gab2-knockout ($Gab2^{-/-}$) mice are viable, generally healthy, and have an apparently normal life span. Although Gab2 was initially believed to be essential for the development of various hematopoietic lineages through its association with SHP2 [43], steady state hematopoiesis is largely normal in $Gab2^{-/-}$ mice [44, 45]. However, $Gab2^{-/-}$ mice exhibit a drastic phenotype in mast cell functioning [44]. Mast cells are major players in allergic responses, and $Gab2^{-/-}$ mice have severe defects in their response to passive allergic challenge; their mast cells display defects in degranulation and cytokine gene expression in response to the activation of FcεRI, the high-affinity IgE receptor. The defective activation of $Gab2^{-/-}$ mast cells is ascribed mainly to their failure to induce PI3K activation. Furthermore, $Gab2^{-/-}$ mice show decreased numbers of mast cells in various tissues, including the skin and stomach, because of weakened c-Kit signaling [45]. These findings suggest that Gab2, which is often upregulated in inflammatory disease, might be an important target for novel therapies against inflammation and allergy [46].

$Gab2^{-/-}$ mice also exhibit an osteopetrotic phenotype that is attributed to the role of Gab2 in regulating RANK-(receptor activator of nuclear factor-κ-B-) dependent signaling [47]. Gab2 associates with RANK and mediates the RANK-induced activation of NF-κB, AKT, and JNK. Bone homeostasis is determined by an intricate balance between the anabolic action of mesenchymal osteoblasts and the catabolic action of osteoclasts. Consistent with Gab2's pivotal role in the differentiation of a variety of hematopoietic lineages [43, 45], $Gab2^{-/-}$ mice exhibit defective osteoclast differentiation, resulting in decreased bone resorption and a subsequent systemic increase in bone mass [47]. In addition, Gab2 has a crucial role in the differentiation of human progenitor cells into osteoclasts [47].

To dissect the Gab2-dependent signaling pathways required for the degranulation of mast cells *in vivo*, Nishida et al. established knock-in mice that express Gab2 mutated at the binding sites for either the PI3K regulatory subunit p85 or SHP2 [48]. They found that both binding sites of Gab2 are required for degranulation and the anaphylaxis response, but not for cytokine production or contact hypersensitivity. Interestingly, the PI3K, but not the SHP2, binding site turned out to be important for granule translocation during degranulation. In particular, the Fyn/Gab2/PI3K-signaling pathway activates a small GTPase, ADP-ribosylation factor (ARF)1, which regulates granule translocation. These results indicated that Fyn/Gab2/PI3K/ARF1-signaling is specifically required for granule translocation and the anaphylaxis response in mast cells [48].

No specific role has been identified to date for Gab3. $Gab3^{-/-}$ mice are healthy and viable, and no obvious phenotype was detected in $Gab3^{-/-}$ macrophages, despite the strong upregulation of this protein during macrophage differentiation [49].

5. Physiological Functions of Gab Proteins Revealed by Conditional Knockout Mice

5.1. The Roles of Gab Proteins in Cardiomyocytes. Because the $Gab1^{-/-}$ phenotype is embryonically lethal in mice, several groups, including ours, have created conditional Gab1-knockout mice, to determine its physiological functions in adulthood [50–53]. Gab1 is exclusively expressed in the heart from E10.5 to 13.5 [40], indicating that it might have a specific role in the heart. Therefore, we created cardiomyocyte-specific Gab1-knockout (Gab1CKO) mice, but these mice are viable and display no obvious cardiac phenotypes [52].

Since Gab1 and Gab2 are expressed in cardiomyocytes, we hypothesized that Gab2 might complement the loss of Gab1. We therefore created cardiomyocyte-specific Gab1/Gab2 double-knockout (DKO) mice by crossing Gab1CKO mice with $Gab2^{-/-}$ mice [52]. Although the DKO mice were viable, they showed a high postnatal mortality rate with marked ventricular dilatation and reduced contractility. In addition, the DKO mice showed remarkable pathological phenotypes including endocardial fibroelastosis and a large number of abnormally dilated coronary vessels in the ventricles. Neuregulin-1 (NRG-1) and ErbB receptors, including ErbB2 and ErbB4, comprise an important signaling pathway for heart development and the maintenance of heart function in adulthood. The NRG-1-induced activation of ERK1/2 and AKT were observed in the hearts of control, Gab1CKO, and $Gab2^{-/-}$ mice, but not of DKO mice. These results suggest that Gab1 and Gab2 share a critically redundant role in NRG-1-dependent signaling in cardiomyocytes (Figure 2).

To determine the effects of the DKO on gene expression, we performed a DNA microarray analysis of cardiac tissues, and found that NRG-1 upregulates the expression of the endothelium-stabilizing factor, angiopoietin-1 (Ang1), in the control mice, but not in the DKO mice [52]. Conventional Ang1-knockout mice show impaired development of myocardial trabeculae and vessel maturation [54], which are quite similar to the pathological abnormalities in the hearts of the DKO mice. Furthermore, the expression patterns of NRG-1 and ErbB are almost mirrored by those of Ang1 and Tie2, in the heart, suggesting that these two signaling pathways influence each other like a paracrine signaling circuit in the cardiac microenvironment [55]. These results suggest that the contributions of Gab1 and Gab2 to the crosstalk between NRG-1/ErbB and Ang1/Tie2 signaling are required for the maintenance of heart function (Figure 2).

5.2. The Role of Gab1 in Angiogenesis, Vascular Inflammation, and Atherosclerosis. Angiogenesis, the process of new blood vessel formation, is involved in many pathological settings, including ischemia, atherosclerosis, diabetes, and cancer [56]. It has been reported that Gab1 has a role in vascular endothelial growth factor- (VEGF-) dependent signaling in *in vitro* experiments using endothelial cells (ECs) [30, 31]. To reveal the *in vivo* role of Gab proteins in angiogenesis, we created endothelium-specific Gab1 knockout (Gab1ECKO) mice [32]. The Gab1ECKO mice are viable and do not show any obvious defects in vascular development. We then subjected

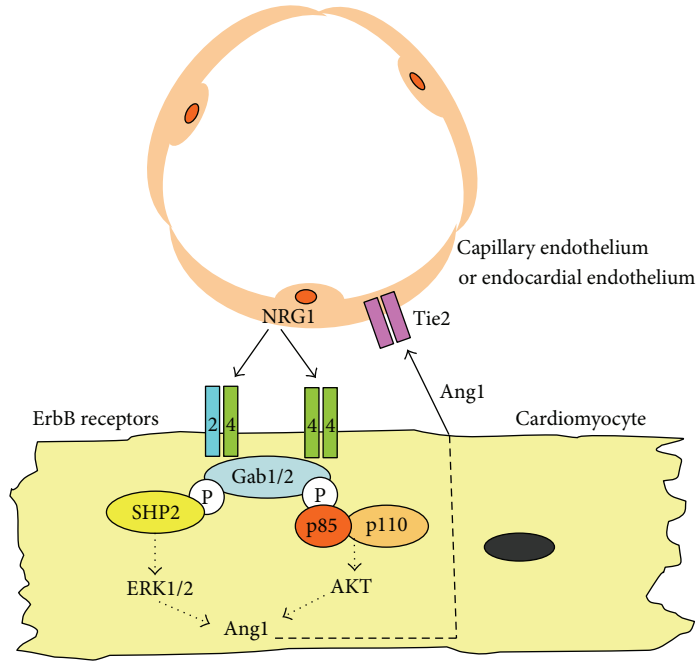

Figure 2: Schematic illustration of the roles of Gab docking proteins in the myocardium. Neuregulin-1 (NRG-1) shed from the capillary or endocardial endothelium in the heart activates the ErbB receptors expressed on cardiomyocytes, leading to the tyrosine phosphorylation of Gab1 and Gab2 and subsequent activation of ERK1/2 and AKT. NRG-1/ErbB-Gab1/Gab2 signaling in the myocardium is directly required for the postnatal maintenance of myocardial function. Furthermore, NRG-1/ErbB-Gab1/Gab2 signaling indirectly contributes to the postnatal stabilization of capillary or endocardial endothelium via the upregulation of angiopoietin-1 (Ang1). Ang1 derived from myocardium activates the Tie2 receptor, which is expressed on the cardiac endothelial cells.

Gab1ECKO and Gab2$^{-/-}$ mice to hindlimb ischemia (HLI) induced by unilateral femoral artery ligation. Intriguingly, impaired blood flow recovery and necrosis in the operated limb was observed in all the Gab1ECKO mice, but not in the control (wild-type) or Gab2KO mice. In human ECs, we compared the effects of several angiogenic growth factors and found that HGF induces the most prominent tyrosine phosphorylation of Gab1 and the greatest subsequent complex formation of Gab1 with both SHP2 and p85 [32]. The Gab1-SHP2 complex was required for both the HGF-induced migration and proliferation of ECs via the ERK1/2 pathway and the HGF-induced stabilization of ECs via ERK5. The Gab1-p85 complex also regulated the migration of ECs after HGF stimulation, and it regulates the activation of AKT [32]. A microarray analysis of HGFs effects on gene expression in ECs demonstrated that it upregulates angiogenesis-related genes such as *Kruppel-like factor 2* (*KLF2*) and *early growth response* 1 via the Gab1-SHP2 complex in human ECs (Figure 3) [32]. Furthermore, gene transfer of VEGF, but not HGF, improved the blood flow recovery and ameliorated the limb necrosis after HLI in the Gab1ECKO mice [32]. These results suggest that Gab1 is essential for postnatal angiogenesis after ischemia via PI3K HGF/c-Met signaling (Figure 3).

At the same time as our study, two other groups reported results on postnatal angiogenesis in Gab1ECKO mice using the HLI model [57, 58]. Whereas Zhao et al. reported that endothelial Gab1 is essential for HGF-dependent postnatal angiogenesis, a finding almost identical to ours [58], Lu et al. reported that Gab1 regulates postnatal VEGF-dependent angiogenesis through the protein kinase A- (PKA-) endothelial NOS (eNOS) pathway [57]. Together, these findings provided by three independent groups show that Gab1 is a crucial signal transducer that unites the HGF-dependent and VEGF-dependent signaling and angiogenesis in endothelial cells (Figure 3) [32, 57, 58].

Since the above findings led us to hypothesize that Gab1 might have a role in endothelial homeostasis, we intercrossed the Gab1ECKO mice with apolipoprotein E (ApoE) knockout (ApoEKO) mice. Six-month-old male ApoEKO/Gab1ECKO and littermate control (ApoEKO) mice were treated with angiotensin II (AngII) via an osmotic infusion minipump for 4 weeks. After the AngII treatment, the ApoEKO/Gab1ECKO mice showed significantly exacerbated atherosclerosis and aneurysm formation compared with control mice [59]. The production of proinflammatory cytokines in the aorta was also significantly greater in the ApoEKO/Gab1ECKO than in the control mice. Furthermore, the expression levels of KLF2 and KLF4, key transcription factors for endothelial homeostasis, were significantly reduced in the aortic endothelium of the ApoEKO/Gab1ECKO mice compared with the control mice [59, 60]. Consistent with the reduced expression of KLF2 and KLF4, both vascular cell adhesion molecule-1 (VCAM-1) expression and macrophage infiltration of the aortic walls were enhanced in ApoEKO/Gab1ECKO mice compared with the control mice [59, 60]. Taken together, these findings show that endothelial Gab1 protects

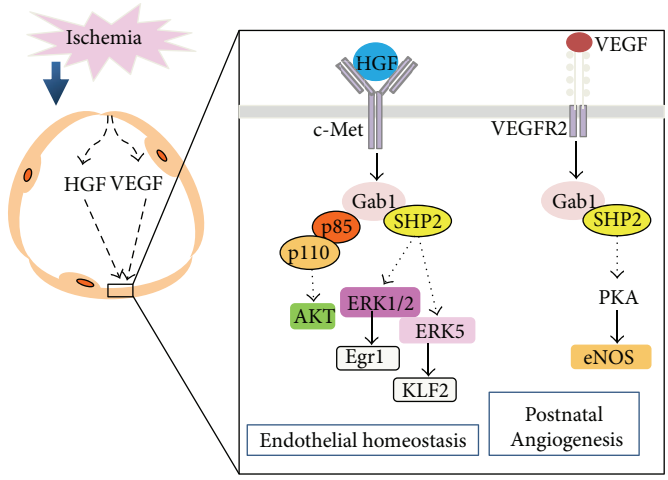

Figure 3: Schematic illustration of the role of Gab1 in postnatal angiogenesis and endothelial homeostasis. Hypoxic tissues secrete growth factors, such as HGF and VEGF, which stimulate their specific receptors on endothelial cells (inset). The activation of c-Met receptors leads to the tyrosine phosphorylation of Gab1 and thereby to the subsequent complex formation of Gab1 with both SHP2 and p85. Whereas the formation of the Gab1-SHP2 complex is required for the activation of ERK1/2 and ERK5, the Gab1-p85 complex is essential for the activation of AKT in response to HGF. The ERK1/2 and ERK5 pathways contribute to the upregulation of early growth response 1 (Egr1) and Kruppel-like factor 2 (KLF2). On the other hand, activation of the VEGFR2 receptors leads to the tyrosine phosphorylation of Gab1, and to the subsequent formation of the Gab1-SHP2 complex, which causes the activation of protein kinase A (PKA) and endothelial nitric oxide synthase (eNOS). Collectively, current findings indicate that Gab1 is an essential component of postnatal angiogenesis after ischemia.

the endothelium from AngII-dependent vascular inflammation and atherosclerosis in the *ApoE*-null background, presumably in association with the downregulation of KLF2 and KLF4 [59].

5.3. The Role of Gab1 in Liver Regeneration. Liver regeneration is a rapid and concerted response to injury, in which growth factor-evoked intracellular signals lead to the activation of various transcriptional factors, DNA synthesis, and hepatocyte proliferation. Liver-specific Gab1 knockout (LGKO) mice exhibit defective liver regeneration after a two-thirds partial hepatectomy [50]. The defects in LGKO mice may be ascribed to the decreased proliferation of hepatocytes, due to the decreased activation of ERK1/2 and attenuated upregulation of immediate-early genes, such as c-fos, c-jun, and c-myc, after liver injury [50]. Interestingly, liver-specific SHP2-knockout mice phenocopy the defective liver regeneration of LGKO mice after partial hepatectomy, suggesting that Gab1 plays a critical role in liver regeneration via its association with SHP2 [50]. In addition, Gab1 negatively regulates the hepatic insulin-induced activation of AKT via the ERK1/2-mediated phosphorylation of IRS-1 on Ser612 [51]. Therefore, Gab1 is required not only for liver regeneration but also for the negative regulation of insulin-mediated hepatic glucose homeostasis.

5.4. The Roles of Gab Proteins in Bone Homeostasis. The analysis of $Gab2^{-/-}$ mice shows that Gab2 couples RANK to the downstream signaling essential for osteoclastogenesis, and that Gab2 has a negative regulatory role in osteoblast differentiation [47, 61]. In contrast, osteoblast-specific Gab1-knockout mice display a low-bone-turnover osteopenic phenotype at 2 months of age, demonstrating an essential role for Gab1 in osteoblast functioning [53]. These results indicate that Gab1 and Gab2 have distinct functions in the maintenance of bone homeostasis: Gab1 in osteoblasts and Gab2 in osteoclasts.

6. Gab Proteins in Human Cancers

Gab proteins have been implicated in several hematological neoplasias and solid cancers, although only a few mutations have been reported in human Gab proteins to date. It is currently established that Gab proteins promote tumorigenesis by functioning as "accomplices" of certain oncoproteins or by amplifying signaling upon the Gab proteins' overexpression.

The chromosomal 11q13-14 locus containing the Gab2 gene is amplified in breast, ovarian, and gastric cancers and in acute myeloid leukemia (AML) [62–65]. Gab2 is overexpressed in estrogen receptor-positive cells [66], and a subset of breast cancers is driven by Gab2 overexpression coupled with RTK ErbB2 (also known as Neu or HER2) receptor signaling [62]. Consistent with these clinical results, Neel's group demonstrated that in the cultured human mammary epithelial cell line MCF-10A, the coexpression of wild-type Gab2, but not $Gab2^{\Delta SHP2}$ (incapable of binding SHP2) with ErbB2/Neu/HER2 resulted in an invasive growth phenotype [62]. They also revealed that NeuNT-transgene-evoked mammary tumorigenesis is potentiated in MMTV-Gab2 transgenic mice and attenuated in Gab2-deficient mice [62]. Similarly, Gab2's overexpression can potentiate metastatic melanomas [67]. Furthermore, myeloid progenitors from $Gab2^{-/-}$ mice are resistant to transformation by Bcr-Abl, indicating that Gab2 is required to sustain the leukemogenesis evoked by this oncogenic fusion protein in

a model of chronic myelogenous leukemia (CML) [68]. The phosphorylation of Y177 within the Bcr moiety results in the recruitment of the Grb2-Gab2 complex and the activation of downstream signaling via SHP2 and PI3K, which is essential for the cancer cells' enhanced proliferation and survival [68]. These results suggest that the Grb2-mediated recruitment of Gab2 to the oncogenic fusion protein Bcr-Abl is a critical event for the induction of a CML-like disease. Gab2 is also important in the progression of other hematological neoplasias, such as juvenile myelomonocytic leukemia (JMML), acute myelocytic leukemia (AML), and acute lymphoblastic leukemia (ALL) [65, 69].

That Gab1 plays a role in tumorigenesis is implied by its strong relationship with c-Met receptor signaling, since c-Met is activated, mutated, or overexpressed in a wide range of cancers [19, 70, 71]. Gab1 is also implicated as a mediator of EGFR-signaling-induced tumorigenesis in glioblastomas and intestinal adenomas [72, 73].

The elucidation of this direct linkage between Gab proteins and human cancers may contribute to the development of novel anticancer drugs in the future.

7. Gab Proteins in Human Cardiovascular Diseases

The neuro-cardiofacial-cutaneous (NCFC) syndromes consist of neurofibromatosis (NF), Noonan syndrome (NS), LEOPARD (multiple lentigines, electrocardiographic conduction abnormalities, ocular hypertelorism, pulmonary stenosis, abnormal genitalia, growth retardation, and sensorineuronal deafness) syndrome (LS), Costello syndrome, and cardiofacial-cutaneous syndrome. All of these syndromes are associated with autosomal-dominant germline mutations within either the core components (Ras, B-Raf, Raf-1, MEK) of the Ras-ERK1/2 pathway or its modulators (NF1, SHP2, SOS, and Spred). The resulting mutant proteins exhibit abnormal activities and disturbed overall fine-tuning of the Ras-ERK1/2 pathway (and to some extent of the Ras-PI3K pathway) [74, 75]. Since the ERK1/2 pathway has a central role in both proliferation and differentiation, many processes in human development and organ maintenance are disturbed by its dysfunction, resulting in various clinical symptoms, such as a distinctive cranio-facial appearance, cardiac defects, musculocutaneous abnormalities, and mental retardation [74, 75]. Germline missense mutations in the SHP2-encoding *PTPN11* gene are seen in approximately 50% of NS cases; this observation contributed to the identification of *PTPN11* as the most common target of somatic mutations in JMML [76, 77]. The most frequent JMML-associated mutation, E76 K, confers an enhanced catalytic activity on SHP2 and requires Gab2 for the transformation of primary murine myeloid progenitors [69]. This result demonstrates that Gab2 is an essential player in JMML and suggests that NS-associated SHP2 mutants may require Gab proteins similarly, as a recruitment tool.

Dominant-negative mutations of SHP2 are reported in LS patients, although NS patients usually carry constitutively active SHP2 mutations [78]. Intriguingly, the expression of LS-associated SHP2 mutants with reduced catalytic activity in cultured cells significantly enhances the EGF-induced association of Gab1 with p85 [79]. This result suggests that LS-associated mutations in SHP2 might potentiate abnormal PI3K activation by blocking SHP2 from dephosphorylating the p85 recruitment sites on the Gab proteins. Collectively, these studies suggest that Gab proteins might exert an important role as "accomplices" of NCFC-associated SHP2 mutants in the pathogenesis of NCFC syndromes.

8. Molecular Mimicry of Gab Proteins by a Bacterial Virulence Factor, CagA

The CagA protein of the gastric pathogen *Helicobacter pylori,* a rod-shaped bacterium that infects the epithelial cells lining the stomach, has been described as functioning as a Gab-like protein [80]. The CagA protein is injected into the cytoplasm of gastric epithelial cells by the bacterium, whereupon it undergoes tyrosine phosphorylation by Src family kinases and c-Abl on E-P-I-Y-A sequence motifs present in its C-terminal region [81, 82]. Subsequently, CagA recruits SH2 domain-containing effector proteins such as SHP2 and Grb2, enabling CagA to effectively take over the signaling pathways that are normally regulated by Gab proteins. This process results in the rearrangement of actin cytoskeleton, cell scattering, and cell elongation, termed the "hummingbird" phenotype, which is reminiscent of the cellular response to Gab activation in cardiomyocytes and other cells [33, 83].

CagA has been categorized as a Gab mimic based on its ability to interact with partners of Gab and exert similar effects in human gastric cells [80]. Intriguingly, this concept of molecular mimicry is strongly supported by transgenic studies in *Drosophila*, demonstrating that a *cagA* transgene can rescue larval viability and photoreceptor development in mutant animals that lack DOS [84]. In addition, an epistasis analysis demonstrated that the complementation of DOS by CagA overexpression requires the expression of the SHP2 ortholog CSW [84]. Thus, these results revealed how CagA can mimic Gab/DOS proteins *in vivo*.

9. Conclusion

Since the discovery of Gab docking proteins, a little more than a decade ago, it has become evident that these proteins play critical roles in a variety of physiological processes as well as in disorders including cancer, inflammation, and cardiovascular diseases. Quite recently, a genome-wide association study conducted by Tamari's group identified Gab1 as a candidate gene for adult asthma in the Japanese population [85]. Whereas the molecular mechanism underlying this association remains unclear, further studies focusing on Gab proteins will aid in elucidating the pathophysiology of this kind of bronchial asthma in the near future. Thus, the versatile functions of Gab docking proteins might extend beyond the original definition of a docking protein. Furthermore, through careful analyses of Gab docking proteins, as shown in this paper, we may be able to obtain a more detailed

understanding of Gab-mediated cardiovascular diseases, cancers, and inflammation.

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Review Article

Reactive Oxygen Species, SUMOylation, and Endothelial Inflammation

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Although the exact mechanism through which NADPH oxidases (Nox's) generate reactive oxygen species (ROS) is still not completely understood, it is widely considered that ROS accumulation is the cause of oxidative stress in endothelial cells. Increasing pieces of evidence strongly indicate the role for ROS in endothelial inflammation and dysfunction and subsequent development of atherosclerotic plaques, which are causes of various pathological cardiac events. An overview for a causative relationship between ROS and endothelial inflammation will be provided in this review. Particularly, a crucial role for specific protein SUMOylation in endothelial inflammation will be presented. Given that SUMOylation of specific proteins leads to increased endothelial inflammation, targeting specific SUMOylated proteins may be an elegant, effective strategy to control inflammation. In addition, the involvement of ROS production in increasing the risk of recurrent coronary events in a sub-group of non-diabetic, post-infarction patients with elevated levels of HDL-cholesterol will be presented with the emphasis that elevated HDL-cholesterol under certain inflammatory conditions can lead to increased incidence of cardiovascular events.

1. Introduction

Small ubiquitin-related modifier (SUMO) proteins are ubiquitously expressed in eukaryotic cells [1–4] and are highly conserved from yeast to human. They are attached to specific lysine residues on their substrates through the SUMOylation process, which is catalyzed by E3-like ligase enzymes (E3 SUMO) ligase enzymes. Interestingly, recent studies have revealed that the protein inhibitor of activated STATs (PIAS) proteins, which are initially identified as negative regulators of cytokine signaling that inhibit the activity of STAT transcription factors, act as E3 SUMO ligase enzymes. Because the SUMO E3 ligase activity and the transcriptional coregulator activity are functionally correlated in most cases, the PIAS/SUMO complex appears to be critical for regulating transcriptional activity. Our group has reported the crucial role of reactive oxygen species (ROS) in SUMOylation and possible effects of protein SUMOylation on endothelial function. In this paper, we will discuss some key findings that have elucidated the role for the PIAS/SUMO complex in the transcriptional regulation. Although SUMOylation is implicated in a variety of cellular processes, this paper will focus on the effect of ROS-mediated SUMOylation on endothelial inflammation. In addition, we will also discuss the clinical evidence for the critical involvement of ROS production on the progress of cardiovascular disease (CVD), especially in the patient population with high levels of HDL cholesterol and C-reactive protein (CRP).

2. SUMOylation

Among posttranslational modifications, ubiquitination and SUMOylation are unique because they require the covalent interaction between ubiquitin (ubiquitination) and SUMO (SUMOylation) to their protein substrates instead of the addition of a functional group such as a phosphate, acetate, lipid, or carbohydrate. Ubiquitination and SUMOylation are

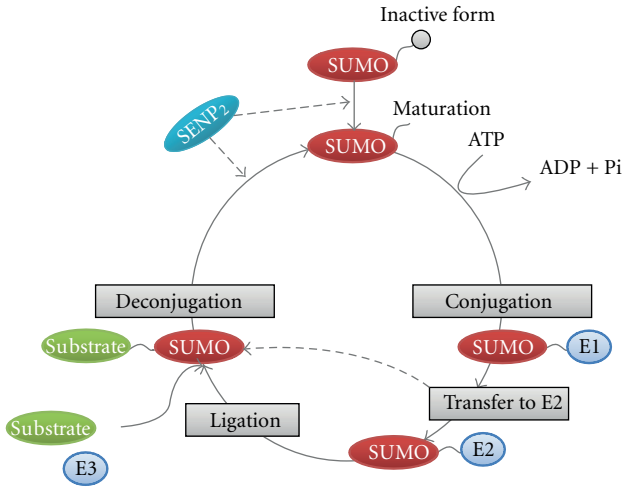

Figure 1: SUMOylation process. Protein SUMOylation consists of deconjugation and conjugation pathways. SUMO-conjugation requires three classes of enzymes (E1 → E2 → E3). SUMO-deconjugation requires the sentrin/SUMO-specific proteases (SENP2).

analogous. Although the structures of ubiquitin (a 76-amino acid polypeptide) and SUMO (a 101-amino acid polypeptide) are similar, they share only ~18% sequence homology [5, 6].

SUMOylation is a dynamic and reversible process regulated by both conjugation and de-conjugation enzymes via a three-step process. First, free SUMO is covalently linked to the E1 activating enzyme in an ATP-dependent reaction. Next, SUMO is transferred from the E1 enzyme to the E2 conjugating enzyme. Finally, interaction between the E2 and the E3 ligase enzymes allows the E3 ligase enzyme to initiate the transfer of SUMO from the E2 enzyme to a lysine residue on the substrate [7, 8]. The regulatory mechanism of SUMOylation is analogous to that of ubiquitination, but the two processes employ different sets of enzymes (Figure 1) [6]. SUMOylation is a part of important regulatory mechanisms that modify proteins in the nucleus and regulate multiple cellular processes such as nucleo-cytoplasmic signal transduction [9], stress responses, subcellular localization of proteins, protein-protein interactions, protein-DNA interactions, and transcriptional activity of transcription factors [10].

3. SUMO E3 Ligase-PIAS Family of Proteins

Attempts to isolate proteins that regulate the signal transducer and activator of transcriptions (STATs) have identified protein inhibitors of the activated STAT (PIAS) family [11, 12]. The PIAS protein family consists of four members: PIAS1, PIAS2 (PIASx), PIAS3, and PIAS4 (PIASy) [13]. STATs and NF-κB, the two important transcription factor families that are activated in response to a wide range of inflammatory stimuli to regulate multiple cellular processes [10, 14, 15], are negatively regulated by both PIAS1 and PIAS4 [16]. In addition, PIAS proteins also display SUMO E3 ligase activity and promote SUMOylation [13].

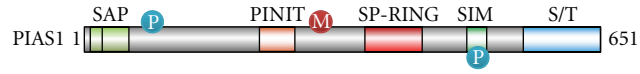

Figure 2: Schematic structure of PIAS1.

3.1. Structure of PIAS Proteins. The size of mammalian PIAS proteins varies from 510 (PIAS4) to 651 (PIAS1) amino acids. They share highly homologous sequences. Overall, five different motifs on PIAS proteins have been characterized: (1) an N-terminus SAP motif (scaffold attachment factor-A/B, acinus and PIAS), (2) a PINIT motif, (3) a RING-type zinc-binding motif (SP-RING), (4) an SIM motif (SUMO-interacting motif), and (5) a serine/threonine-rich C-terminus region (S/T). The N-terminus SAP and the middle SP-RING motif are the most conserved regions. The C-terminus S/T motif is the least conserved region (Figure 2) [13, 17]. The SP-RING motif, although lacking two zinc-coordinating cysteines [18] compared to the classical RING domain, is suggested to resemble the classical RING domain, which has ligase function. The PINIT motif, which regulates PIAS nuclear retention, also plays a role in PIAS SUMO E3 ligase function for some substrates [19, 20]. On the contrary, the SIM motif is not required for PIAS SUMO E3 ligase activity, despite its ability to interact noncovalently with SUMO proteins [13].

3.2. PIAS as a Transcriptional Repressor of NF-κB and STAT1. NF-κB is an important transcription factor that regulates many inflammatory genes such as cytokines, chemokines, and adhesion molecules that play major roles in atherosclerosis. Tumor necrosis factor-α (TNF-α) is a key inflammatory cytokine involved in the progression of atherosclerosis by activating NF-κB signaling [21]. Through the canonical NF-κB pathway, TNF-α activates IKK to phosphorylate and degrade IκB, releasing NF-κB into the nucleus where it can activate the transcription of inflammatory genes. Biochemical and genetic studies have demonstrated that PIAS1 negatively regulates this pathway by interacting with NF-κB-p65 to repress its transcriptional activity, thus downregulating the expression of TNFα-induced genes [22].

PIAS1 has also been shown to bind directly to STAT1 and repress STAT1 transcriptional activity. PIAS1$^{-/-}$ mice are more sensitive to inflammatory responses mediated by interferon-γ (IFN-γ) or interferon-β (IFN-β) [12, 22, 23], and are hypersensitive to lipopolysaccharides (LPS) that induces endotoxic shock [22]. The STAT1 and NF-κB activities are increased in PIAS1$^{-/-}$ mice. In response to inflammatory stimuli, PIAS1 is rapidly phosphorylated at Ser-90, which is required for PIAS1-mediated inhibition of STAT1 or NF-κB. The phosphorylation of PIAS1 at Ser-90 is mediated by IKKα during TNFα-induced inflammation and blocks its NF-κB repressor function, which acts as a negative feedback mechanism on the TNFα-IKKα-NF-κB signaling pathway [24].

3.3. PIAS as a SUMO E3 Ligase. In NF-κB activation, the regulatory subunit NF-κB essential modulator (NEMO/IKKγ)

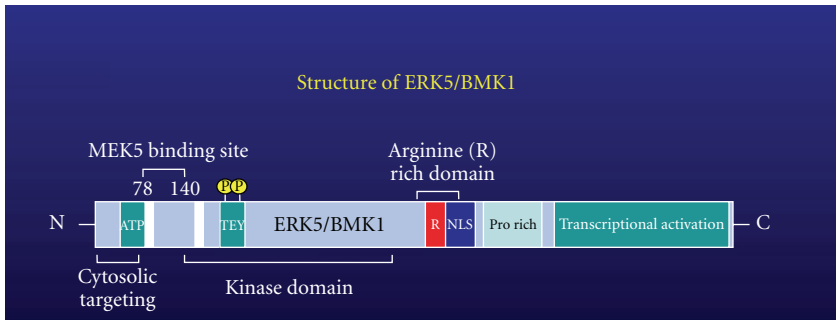

Figure 3: Schematic structure of ERK5.

of the cytoplasmic IκB kinase complex (IKK complex) plays a central role [14]. SUMO-1 modification of NEMO/IKKγ is required for NF-κB activation in response to genotoxic stress inducers [25]. Attempts to identify a SUMO E3 ligase that is critical for the SUMO-1 modification of NEMO have indicated the involvement of PIAS4 in this process. PIAS4 interacts with NEMO and preferentially stimulates site-selective modification of NEMO by SUMO-1. Subsequently, the activation of NF-κB is enhanced [26].

The activation of NF-κB can be antagonized by peroxisome proliferator-activated receptor γ (PPARγ). PPARγ is a target for SUMO-1 modification. PPARγ agonists induce ligand-dependent conjugation of SUMO-1 to PPARγ. SUMOylation of PPARγ mainly occurs at the Lys-107 residue, resulting in significant inhibition of PPARγ transcriptional activity [27]. When adenoviral vector expressing PPARγ-K107R was delivered into the rat carotid arteries after balloon injury, a significant decrease in neointimal formation was noted, compared to arteries treated by wild type or control vector [28]. Thus, PPARγ SUMOylation at Lys-107 residue not only downregulates its transcriptional activity but also increases neointima formation. SUMOylation of PPARγ is mediated by PIAS1 SUMO E3 ligase. Because PIAS1 can participate directly in the inhibition of LPS-induced NF-κB-mediated inflammatory gene activation, PIAS1 has two different pathways to inhibit NF-kB activation [10, 27].

4. ROS-Mediated ERK5 SUMOylation and Inflammation

4.1. ERK5 as a key Factor to Inhibit Endothelial Inflammation. The mitogen-activated protein kinase (MAPK) is protein kinase that is activated by the redox and hyperosmotic stresses, growth factors, and pathways involving certain G-protein-coupled receptors [29]. Extracellular-signal-regulated kinase 5 (ERK5), or BMK1 (big MAPK1), is the newest member of the MAPK family. The human *erk5* gene (or *MAPK7*) is located on chromosome 17p11.2, extends 5.79 kb, and encodes a protein of 816 amino acids with a predicted molecular mass of 98 kDa (Figure 3) [30]. The ERK5 kinase domain (a.a. 78–406) is on its NH_2-terminus. On the ERK5 NH_2-terminus, amino acids 1–77 are important for cytoplasmic targeting, amino acids 78–139 are required for interaction with MEK5, and amino acids 140–406 are important for oligomerization [31, 32]. ERK5 shares approximately 66% sequence homology with ERK1/2 within the kinase domain, which contains the TEY dual phosphorylation motif on its activation loop. However, the long ERK5 COOH-terminus (~400 a.a.) makes ERK5 unique among the MAPK family members. The ERK5 COOH-terminus contains a nuclear localization signal (NLS) (a.a. 505–539) and two proline-rich domains (a.a. 434–465 and 578–701) that are suggested to serve as binding sites for SH3 (Src homology 3)-domain-containing proteins [32, 33]. In addition, the ERK5 COOH-terminus also contains a myocyte enhancer factor 2 (MEF2)-interacting region (a.a. 440–501) and two transcriptional activator domains (a.a. 664–789) that regulate MEF2 transcription factor activity [31]. Therefore, ERK5 has not only kinase but also transcriptional activity. The ERK5 NH2-terminus works as a negative regulator of these transcriptional activator domains. The upstream kinase that phosphorylates ERK5 has been identified as MEK5α [33, 34]. When activated, ERK5 releases its NH_2-terminus inhibitory effect, enabling transcriptional activity of the COOH-terminus. Therefore, ERK5 transcriptional activity is regulated by an intramolecular interaction [35]. However, the ERK5 COOH-terminus tail (a.a. 684–806) also possesses a basal transcriptional activity even without the activation induced by MEK5α kinase. Similar to other MAPK family members, ERK5 plays a significant role in cell growth and differentiation. Nevertheless, emerging evidence suggests ERK5's unique functional characteristics.

It has been well studied that steady laminar flow (s-flow) generates a frictional dragging force on the endothelium surface (called fluid shear stress), which is known to possess anti-inflammatory and antiatherosclerotic effects and to protect endothelial cells (ECs) from becoming dysfunctional [36, 37]. ERK5 is strongly activated by s-flow through the activation of its upstream MEK5α. Once activated, the arginine-rich middle region of ERK5 binds the hinge-helix region of PPARγ1, thus increasing PPARγ1 transcriptional activity [35]. Moreover, the activation of MEK5α/ERK5 increases transcriptional activity of MEF2, a crucial component of the transcriptional machinery required for regulating Krüppel-like factor-2 (KLF2) expression.

KLF2 is a mechanoactivated transcription factor that induces vasoprotective, anti-thrombotic, and anti-inflam-

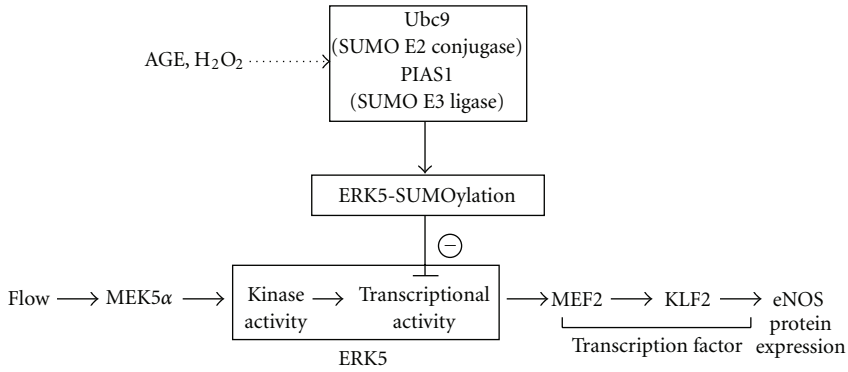

Figure 4: A signaling scheme describing the relationship between the laminar flow-mediated ERK5/MEF2/KLF2/eNOS pathway and H_2O_2 or AGE-mediated ERK5-SUMOylation (License number: 2860201127159, date: Mar 01, 2012).

matory responses to s-flow [38–40]. MEF2 binds the endogenous KLF2 promoter [41] and increases its activity. The increased KLF2 activity results in an orchestrated regulation of endothelial transcriptional programs that control inflammation, thrombosis/hemostasis, vascular tone, and blood vessel development [41]. Because KLF2 enhances the expression of endothelial nitric oxide synthase (eNOS) and reduces the expression of cytokine-mediated adhesion molecules [39, 41, 42], s-flow-mediated ERK5/MEF2/KLF2 induction leads to the upregulation of eNOS and the downregulation of endothelial inflammation [39, 41, 42]. Thus, s-flow-mediated ERK5 activation plays a critical role in regulating PPARγ as well as KLF2, which subsequently inhibits endothelial inflammation and maintains normal vascular reactivity.

The blood flow pattern inside blood vessels is complex, and different flow patterns activate different signaling events. While s-flow is vessel protective, there is a strong correlation between localized atherosclerotic plaque development and regions of the endothelium exposed to disturbed flow (d-flow) that are found at vessel curvatures, bifurcations, and branches. It has been shown that d-flow increases endothelial apoptosis and inflammation by promoting ROS production, which reacts with NO to form peroxynitrite and induces proatherogenic responses in ECs [43, 44]. Previously, we have reported that ROS induces endogenous ERK5 SUMOylation at Lys-6 and Lys-22 and that this SUMOylation inhibits s-flow-mediated ERK5 transcriptional activity in ECs. We have also found that d-flow can increase ERK5 SUMOylation (unpublished data). The inhibition of ERK5 transcriptional activity by ERK5 SUMOylation results in an inhibition of s-flow-mediated KLF2 promoter activity, subsequently inhibiting the KLF2 and eNOS protein expression in ECs (Figure 4) [45]. Interestingly, the negative regulation of ERK5 transcriptional activity by SUMOylation is independent of ERK5 phosphorylation as well as kinase activation [45]. Inhibition of ERK5 SUMOylation by constitutively active (CA)-MEK5α is independent of ERK5 kinase activity, but is dependent on the binding between MEK5 and ERK5 [46]. Thus, our observations imply a crucial role of ERK5 SUMOylation in ROS-mediated ERK5 transcriptional repression, which may contribute to EC inflammation and dysfunction [45].

5. p53-SUMOylation and Inflammation

5.1. D-Flow Induces ROS Production and Increases Endothelial Cell Apoptosis via PKCζ-PIAS4-p53 SUMOylation. ROS functions as a second messenger for various biological responses. NADPH oxidase (Nox) has been identified as the major ROS producing enzyme in blood vessels in response to d-flow [47]. Our recent study has shown that the activation of protein kinase Cζ (PKCζ) by d-flow-mediated ROS induces EC apoptosis by regulating p53 [43]. PKCζ activation has been reported in the lesser curvature of the aortic arch in porcine aorta [48], suggesting a proatherogenic role of PKCζ and a possible correlation between d-flow and the activation of this kinase. To verify the potential effect of shear stress on PKCζ activation in ECs *in vitro*, we exposed ECs to different flow patterns and found increased PKCζ activation by d-flow, but not by s-flow [43]. Indeed, activation of PKCζ mediated by d-flow plays a critical role in endothelial apoptosis *in vitro* [43, 49, 50].

The p53 tumor repressor is activated by various cellular stresses. It is a key regulator of cell death. p53 plays a proapoptotic role in both transcription independent and dependent manners. On one hand, p53 directly interacts with the B cell lymphoma/leukemia-2 (Bcl-2) protein family members, Bcl-xL and Bcl-2, thus antagonizing their anti-apoptotic function by stabilizing the outer mitochondrial membrane [50]. This is a transcription independent mechanism. On the other hand, p53 promotes the transcription of several proapoptotic genes such as p53 upregulated modulator of apoptosis (PUMA) and Bad [50–55]. In most cases, p53 antiapoptotic effect is attributed to its nuclear localization, because nuclear p53 can protect cells from apoptosis, especially under low stress conditions [56, 57]. In our study, we observed the antiapoptotic nuclear localization of p53 in ECs in areas exposed to s-flow. In contrast, d-flow

increases p53 nuclear export, which in turn increases p53-Bcl-2 interaction, and subsequently antagonizes antiapoptotic effect of Bcl-2, resulting in enhanced EC apoptosis [43].

p53 nuclear export is positively regulated by its SUMOylation, which involves PIAS4 as a SUMO E3 ligase enzyme [52]. Interestingly, d-flow-mediated PKCζ activation also regulates p53 nuclear export via SUMOylation. Once activated, the PKCζ C-terminus kinase domain (a.a. 401–587) interacts with PIAS4 at the SP-RING domain. This binding between PKCζ and PIAS4 is required for p53 SUMOylation, which then increases p53 nuclear export, enhances p53-Bcl2 interaction, and consequently EC apoptosis [43]. Although the role of vascular p53 in either promoting or dampening the process of atherosclerosis remains controversial, we suggest that the PKCζ-PIAS4-p53 SUMOylation pathway should be investigated in the context of the pathogenesis of atherosclerosis.

5.2. The Role of p53 in KLF2 Regulation. In several cell types, p53 stimulates inflammatory signaling and inflammatory gene expression [58, 59]. Recently, it has been reported that endothelial p53 promotes EC dysfunction and impairs EC-dependent NO production by suppressing the expression of KLF2. By binding to a 27-bp sequence on the KLF2 promoter, p53 increases the hypoacetylation of histone H3 on KLF2 promoter and thus decreases KLF2 expression [60]. Since p53 SUMOylation can increase p53 expression by decreasing its degradation, it is also possible that p53 SUMOylation can increase EC inflammation via regulating KLF2 expression. However, further studies are necessary to elucidate this hypothesis.

6. MK2-SUMOylation and Inflammation

The MAPK-activated protein kinase 2 (MK2) is a direct substrate of p38 MAPK-α and -β. p38 MAPK binds to a docking site on the C-terminus of MK2 and subsequently phosphorylates MK2 at different regulatory sites [61–63]. The phosphorylation of MK2 mediated by p38 MAPK results in MK2 nuclear export and serves a dual function. First, it leads to an increase in MK2 kinase activity, which in turn results in the phosphorylation of its substrates such as heat shock protein 25 (HSP25), heat shock protein 27 (HSP27), tyrosine hydroxylase, Cdc25B/C, and leukocyte-specific protein 1 [64–68]. Second, it determines the nuclear export of p38 MAPK [69]. In addition to determining the subcellular localization of p38 MAPK, MK2 has a role in stabilizing it. Notably, MK2 kinase activity is not required for p38 MAPK stabilization [70].

TNF-α has also been shown to activate the MK2-HSP27 pathway to induce actin filament remodeling [71, 72]. As a mechanism by which TNF-α mediates actin filament remodeling via MK2-HSP27, we have suggested MK2 SUMOylation, which is a novel mechanism for regulating actin filament dynamics in ECs. The TNF-α mediated-MK2 SUMOylation occurs mainly at lysine (K)-339. The MK2-K339R SUMOylation defective mutant exhibits an increased kinase activity and a sustained phosphorylation level of HSP27 compare to WT-MK2, suggesting the inhibitory effect of MK2 SUMOylation on its kinase activity and subsequent phosphorylation of HSP27. The alignment of ECs in response to laminar flow due to the increase in HSP27 phosphorylation and the subsequent increase in actin filament remodeling is significantly increased in the MK2-K339R SUMOylation defective mutant. In addition, cell elongation with increased cortical actin polymerization which is caused by TNF-α-mediated actin filament remodeling is prominent in cells expressing the MK2-K339R SUMOylation defective mutant, compared to WT-MK2, confirming a negative effect of MK2 SUMOylation on TNF-α-mediated actin filament remodeling and subsequent EC elongation. Therefore, under TNF-α, the decreased actin filament dynamics by sustained inhibition of MK2 kinase activity in the dominant negative (DN)-MK2 and/or the increased actin polymerization by sustained activation of MK2 kinase in the WT-MK2 can inhibit cell movement by deregulating the coordinated "on-off" role of MK2 on actin dynamics [73].

MK2 kinase activity regulates not only cell migration but also cytokine production. Studies using MK2-kinase deficient cells demonstrate a central role of MK2 in the production of inflammatory cytokines such as TNF-α, IL-1β, MIP-1α, IL-8, IL-6, and INF-γ [74–77]. The involvement of MK2 in upregulating NF-κB target genes VCAM-1 and MCP-1 has also been documented [78]. Therefore, the inhibitory effect of MK2 SUMOylation on its kinase activity may have anti-inflammatory effect in ECs.

7. Potential Effects of Medications Targeting Endothelial Inflammation on Protein SUMOylation

7.1. Statins. Statins (HMG-CoA reductase inhibitors) are known to reduce low density lipoprotein cholesterol levels by inhibiting the 3-hydroxy-3-methelglutaryl coenzyme A reductase. Numerous studies on statins have been performed, and numerous pleiotropic effects of statins, beyond their cholesterol reduction properties, have been described [79–82]. The inhibition of NADPH oxidase activity was demonstrated as a major mechanism for statins' pleiotropic effects [79]. In particular, statins can inhibit endothelial inflammation [81, 83, 84]. It has been reported that atorvastatin inhibits inflammation in vascular smooth muscle cells and mononuclear cells through the inhibition of NF-κB activity and chemokine gene expression [85]. In addition, the inhibition of NF-κB activity mediated by atorvastatin can improve PPAR signaling in cardiac hypertrophy [86]. While SUMOylation of PPARγ at Lys-107 inhibits its transcriptional activity and increases NFκB activity, statins can improve PPAR signaling and reduce NF-κB activity. These data open a potential, yet to be elucidated, of the linkage between the statins' pleiotropic effects and endothelial inflammation, possibly, through inhibiting PPAR SUMOylation.

7.2. ACE Inhibitors. The beneficial clinical effects of angiotensin-converting enzyme inhibitors (ACEI) have been indicated in many studies [87]. ACEI improve EC function

through several mechanisms such as lowering Angiotensin II (Ang II), increasing bradykinin [88], and altering mechanisms that regulate NADPH oxidase activity [89]. Advanced Glycation Products (AGE) have been suggested to play a role in NADPH oxidase signaling, which results in the increased levels of ROS, matrix metalloproteinase (MT-MMP1 and MMP9), monocyte chemoattractant protein-1 (MCP-1), as well as plasminogen activator inhibitor-1 (PAI-1) [90, 91]. However, temocaprilat (ACEI) inhibited all of these AGE-mediated effects [91]. Previously, we reported that AGE and H_2O_2 induce endogenous ERK5 SUMOylation [45], suggesting a possible inhibitory effect of ACEI on AGE-induced endogenous ERK5 SUMOylation in reducing endothelial inflammation (Figure 4).

7.3. Antioxidant Vitamins. Although the antioxidant properties of vitamins have been reported both *in vitro* and *in vivo* [92, 93], the beneficial clinical effects of vitamins are contradictory. Stephens et al. demonstrated a significant decrease in cardiovascular incidence in patients received vitamin E in the CHAOS (Cambridge Heart Antioxidant Study) clinical trial [94]. However, Harrison et al. indicated that scavenging ROS by exogenous antioxidants is not effective in preventing cardiovascular disease development [95]. In addition, many clinical studies (HOPE, GISSI, and HPS) have not confirmed the protective effects of vitamin E on major cardiovascular events, which was nicely reviewed and summarized by Schramm [96]. The failed promise of antioxidant vitamins suggests that our current concept of oxidative stress need to be revised, and many aspects need to be taken into account [96] for the implications of antioxidant vitamins.

8. ROS, Inflammation, and Cardiac Events in Clinical Studies

8.1. NADPH Oxidase and Polymorphism. An increasing body of evidence has implicated the role of oxidative stress in atherosclerotic development through regulation of multiple signaling pathways that associates with vascular inflammation [97, 98]. Oxidative stress is the term used to describe the imbalance between producing and removing ROS within a biological system. A major source of ROS within the vasculature is the reduced nicotinamide adenine dinucleotide/nicotinamide adenine dinucleotide phosphate (NAD(P)H) oxidase system. NAD(P)H oxidase is a membrane-associated enzyme, consisting of five subunits that catalyze transfer of an electron to molecular oxygen using NADH or NADPH as the electron donor. Among the five subunits, the 22-kDa NAD(P)H oxidase p22-phox subunit has a polymorphic site on exon 4 which is considered to be the most interesting due to its ability to change NAD(P)H enzyme structure and activity. This polymorphism, $C_{242}T$, is a point of mutation that causes the replacement of histidine by tyrosine at amino acid 72 of the protein, which affects one of the heme binding sites essential for the NAD(P)H enzyme activity [99, 100]. Recent study has found that patients with the NAD(P)H oxidase p22-phox subunit containing T allele on the $C_{242}T$ single-nucleotide polymorphism (SNP) instead of C allele are at lower risk for recurrent coronary events than the patients with the C allele [101, 102]. The T allele has been shown to associate with the reduced NAD(P)H enzyme activity, which results in a decrease in vascular peroxidase production [102]. In addition, the T allele also increases the oxidation of high-density lipoprotein (HDL) by altering the redox state in the vasculature [103].

8.2. Association of the $C_{242}T$ SNP of the NAD(P)H Oxidase p22-phox Subunit with CVD Risk in Postinfarction Patients with Concurrently High Levels of HDL Cholesterol and CRP. High levels of HDL cholesterol (HDL-C) are well known to be inversely related to cardiovascular disease (CVD) risk; however, evidence is accumulating indicating that HDL functionality is also important in the protective effects of HDL particles [104–106]. In addition to important roles in reverse cholesterol transport (RCT), HDL particles have additional protective roles including preservation of endothelial function, protection against thrombotic events, and resistance against the inflammatory and oxidative stress-related injury and alterations to the vascular wall and lipoprotein particles. However at the same time, there is growing recognition that atheroprotective effects of HDL can degrade and actually undergo dysfunctional transformation resulting in proatherogenic HDL especially in the setting of inflammation and oxidative stress [107–111].

To explore manifestations of potential HDL dysfunction in the establishment of CVD risk, we have investigated relationships of HDL-C with CVD risk in human populations. We have performed epidemiologic studies specifically focused on HDL-C in the setting of systemic inflammation. To do this, we have studied individuals with concurrently high levels of HDL-C and CRP. Individuals with high levels of HDL-C were chosen to minimize potential confounding effects related to well-known risk associations with low levels of HDL-C, while high CRP levels were chosen as an indicator of systemic inflammation. In terms of high HDL-C, it is notable that multiple earlier studies have demonstrated such associations with CVD risk [112–123]. Our approach has been to use functional genetic polymorphisms and biomarker levels as probes to assess risk associations connected with aspects of HDL functionality. Thus, we have shown for subgroups with concurrently high levels of HDL-C and CRP risk associations with various aspects of RCT; that is, in postinfarction patients recurrent coronary risk with the TaqIB polymorphism of *CETP* [124], and in healthy subjects incident coronary risk with the D9N polymorphism of *LPL*, the TaqIB polymorphism of *CETP*, and high levels of apolipoprotein E [125, 126]. As noted above, it is becoming increasingly clear that HDL possesses protective functions beyond those connected with RCT. In this vein, we have shown for the same subgroup of postinfarction patients, risk associations connected with thrombogenesis using the A387P polymorphism of *THBS4* (thrombospondin-4) [127]; for oxidative stress, a major cause of endothelial dysfunction, the $C_{242}T$ polymorphism of *CYBA* (p22phox) [101, 127].

In order to perform such studies, we have developed a graphical discovery tool for distinguishing specific high-risk zones of overlap between high HDL-C and high CRP levels

that we call outcome event mapping [128]. Outcome event mapping is an exploratory data analysis approach that generates three-dimensional plots of estimated risk (z-axis) as a function of two biomarker levels (x- and y-axes). Novel aspects of the approach include rank transformation of biomarker levels to more evenly distribute points over the bivariate biomarker risk domain, and the coding of outcome events (0—event absent; 1—event present) with application of a surface-smoothing algorithm to generate a smooth surface over the bivariate risk domain such that the height of the surface at any point in the bivariate risk domain is a measure of the estimated outcome rate at that point. The approach has also been extended to accommodate analyses involving binary variables including single-nucleotide polymorphisms in dichotomized form [126]. We have now used the approach in multiple studies [101, 124–131].

In one such paper, outcome event mapping led to identification at high levels of HDL-C and CRP of a subgroup of postinfarction patients at high risk for recurrent events [127]. Associations of risk with functional genetic polymorphisms connected with HDL activity were then assessed within the subgroup including functionality related to RCT, thrombogenesis, and oxidative stress. Results of multivariable modeling adjusted for significant clinical and laboratory covariates within the subgroup demonstrated significant risk associations for each area; however, results for the $C_{242}T$ polymorphism representative of oxidative stress (*CYBA*, p22phox) demonstrated the strongest association (hazard ratio 2.36, 95% CI 1.30–4.17, $P = 0.004$). Specifically, results for the $C_{242}T$ polymorphism indicated risk association for patients homozygous for the C allele (normal enzyme activity) in comparison to carriers of the T allele (decreased enzyme activity). Figure 1 presents outcome event maps as a function of HDL-C and CRP: in panel A, for T-allele carriers; in panel B, for C homozygotes. High risk for C-homozygotes is clearly demonstrated at concurrently high levels of HDL-C and CRP by the prominent risk peak at this location (Figure 5(b)) and lack thereof for T-allele carriers (Figure 5(a)).

The observed strong association of risk with the p22phox polymorphism is consistent with the major role of oxidative stress in the development of atherosclerosis extending from the earliest stages of endothelial injury to full-fledged endothelial dysfunction and beyond. This derives from the key role of the generation of ROS in the vasculature, especially superoxide ($O_2^{\cdot -}$), by NADPH oxidases [132–135]. This process is facilitated by p22phox as an essential activating subunit of NADPH oxidases in the generation of ROS. In terms of endothelial dysfunction, one pathway of ROS generation involves reactive nitrogen species. This starts with depletion of nitric oxide as NADPH oxidase-generated superoxide reacts with it to form peroxynitrite ($ONOO^-$). This can subsequently can go on to form additional ROS (hydroxyl radical, $HO^{\cdot}$, and nitrogen dioxide radical, $NO_2^{\cdot}$). All of these species are known to be potent nitrating agents capable of oxidative modification of biomolecules, including apolipoproteins that may affect function [132, 136–138].

The superoxide generated by the NADPH oxidase system can be the source of additional oxidants in the vasculature through formation of hydrogen peroxide (H_2O_2) from superoxide as mediated by superoxide dismutase [138]. In a myeloperoxidase pathway, the enzyme, myeloperoxidase (MPO) found predominantly in neutrophils, monocytes, and macrophages and present in atheroma, mediates the reaction of nitric oxide with hydrogen peroxide to also form the nitrogen dioxide radical [138, 139]. Additionally, MPO

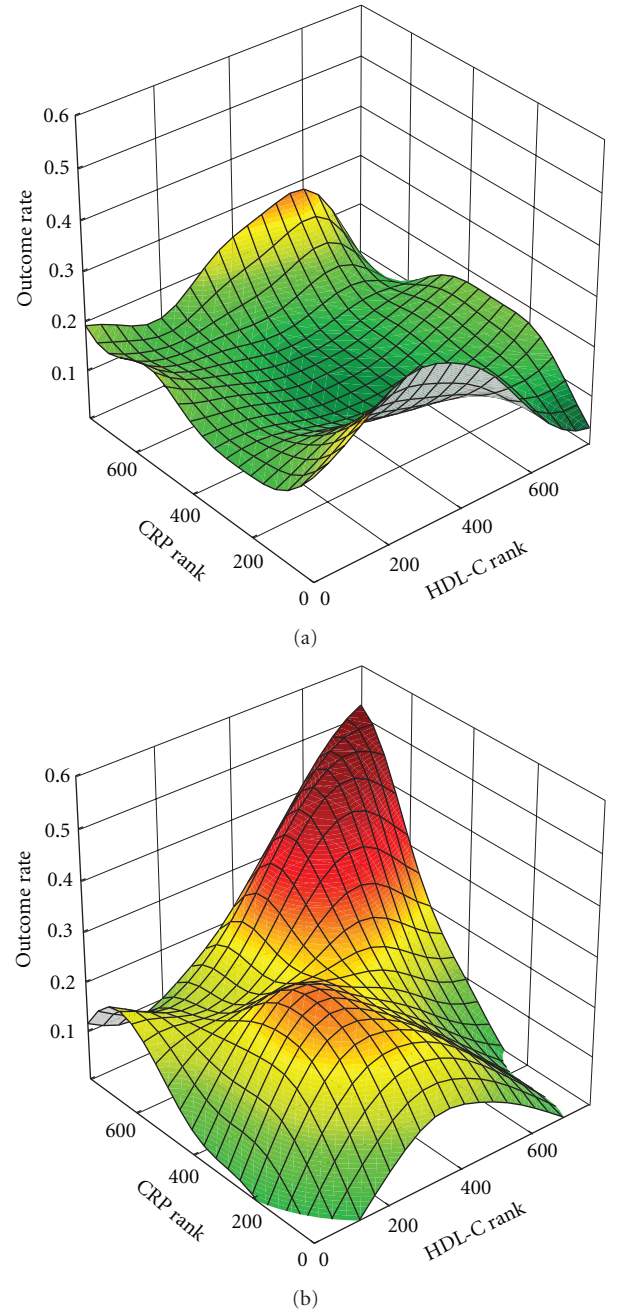

Figure 5: Outcome event mappings in 767 non-diabetic postinfarction patients from the THROMBO study demonstrating estimated outcome event rate as a function of HDL cholesterol and CRP levels (rank-transformed) for (a) carriers of the lower activity T allele (CT plus TT) and (b) homozygotes for the higher activity C allele.

mediates the reaction of hydrogen peroxide with chloride ion to form hypochlorous acid (HOCl), another oxidant species potentially affecting molecular function through chlorination. The relevance of these processes to endothelial dysfunction is underscored by the finding that MPO avidly binds to endothelial cells and is subsequently transcytosed to the subendothelial space where its actions, especially with regard to nitric oxide depletion, may occur [139].

With specific regard to HDL, recent work has demonstrated that attack by the aforementioned ROS, generated in large part by actions of the NADPH oxidase system and MPO as noted above, result in nitration and chlorination of specific tyrosine residues and other residues as well on apolipoprotein A-I (apoA-I), the major apolipoprotein constituent of HDL particles and major mediator of multiple HDL functionalities [108, 140, 141]. It is now widely held that such apoA-I modifications can compromise aspects of, for example, RCT including loss of ABCA1-mediated HDL cholesterol acceptor activity and lecithin-cholesterol acyltransferase (LCAT) activation. Additionally, recent evidence suggests that important functions of HDL beyond RCT may be compromised by apoA-I oxidation including anti-apoptotic and anti-inflammatory activities [142]. Another aspect of HDL dysfunctional transformation related to oxidative stress may involve resulting modifications in the HDL particle proteome [108]. It is clearly the case that further work must be undertaken to elucidate the actual importance of these and additional processes responsible for dysfunctional transformation of HDL from antiatherogenic to proatherogenic forms.

9. Conclusion

Crucial roles for inflammation in the development of atherosclerosis are evident by an increasing number of studies. Protein SUMOylation has been suggested to regulate a number of biological processes, including inflammation. Therefore, SUMOylation is one of the potential strategies to inhibit inflammation. Because SUMOylation is also required for normal cellular function, targeting the global SUMOylation system might not be an effective strategy to control inflammation. The effect of SUMOylation on inflammation undoubtedly depends on individual proteins that are modified. Therefore, targeting specific SUMOylated proteins that are involved in inflammatory events might be a rational and effective way. We have identified several SUMOylation pathways such as ERK5 SUMOylation, p53 SUMOylation, and MK2 SUMOylation that influence EC inflammation and EC apoptosis, and these pathways have potential relevance to early events of atherosclerosis. These identified SUMOylation pathways could serve as potential targets in reducing EC inflammation.

In addition to chronic inflammatory disorders, atherosclerosis is recognized as a diffuse, multisystemic disease involving the vasculature, metabolic disorder, and immune systems with various local and systemic manifestations. Thus, merely based on the recognition of a single unstable atherosclerotic plaque to predict the vulnerability of patients to atherosclerosis and CVD is insufficient. Rather, parameters that include a total burden of the atherosclerotic and vulnerable plaques in the aorta, coronary, carotid, and femoral artery as well as blood vulnerability factors is considered important. Currently, attentions are focused on the interaction between inflammation and traditional lipoprotein risk factors. In our study using a subgroup of patients at high risk for recurrent coronary events, we identified high HDL-C as a significant and independent predictor of risk, which also can be employed to evaluate the vulnerability of patients to atherosclerosis and CVD.

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Clinical Study

Serum Levels of Gelatinase Associated Lipocalin as Indicator of the Inflammatory Status in Coronary Artery Disease

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Background. Atherosclerosis is a chronic inflammatory disease and the acute clinical manifestations represent acute on chronic inflammation. Neutrophil gelatinase-associated lipocalin (NGAL) is found in the granules of human neutrophils, with many diverse functions. The aim of this study was to evaluate the hypothesis that levels NGAL in blood may reflect the inflammatory process in various stages of coronary artery disease. *Methods*. We studied 140 patients, with SA 40, UA 35, NSTEMI 40, and STEMI 25, and 20 healthy controls. Serum NGAL was measured upon admission and before coronary angiography. *Results*. Significant differences were observed in median serum-NGAL(ng/mL) between patients with SA (79.23 (IQR, 37.50–100.32)), when compared with UA (108.00 (68.34–177.59)), NSTEMI (166.49 (109.24–247.20)), and STEMI (178.63 (111.18–305.92)) patients and controls (50.31 (44.30–69.78)) with significant incremental value from SA to STEMI. We observed a positive and significant correlation between serum-NGAL and hs-CRP (spearman coefficient rho = 0.685, $P < 0.0001$) as well as with neutrophil counts ($r = 0.511$, $P < 0.0001$). *Conclusions*. In patients with coronary artery disease serum levels of NGAL increase and reflect the degree of inflammatory process. In patients with acute coronary syndromes, serum levels of NGAL have high negative predictive value and reflecting the inflammatory status could show the severity of coronary clinical syndrome.

1. Introduction

Systemic inflammation participates in atherosclerosis evolution from the early development of endothelial dysfunction, to formation of mature atheromatic plaques, to the ultimate endpoint, rupture, and thrombotic complications [1]. Plaque rupture with the formation of an occlusive thrombus is the cause of acute coronary syndromes (ACS) [2]. Inflammatory cells, involving activated neutrophils, are more frequently found in plaques vulnerable to rupture [3]. Neutrophil activation has been reported in unstable angina (UA) and acute myocardial infarction (AMI) but not in patients with stable angina (SA) [4–10]. This activation seems to precede myocardial injury in patients with AMI [11]. Therefore biomarkers of neutrophil activation could be of prognostic and even diagnostic importance.

Recent studies have shown that gelatinase B also known as matrix metalloproteinase-9 (MMP-9), an endopeptidase capable of degrading the extracellular matrix, is thought to be associated with atherosclerosis, and plaque rupture [12, 13]. Therefore, MMP-9 is considered to be an important mediator of vascular remodeling and plaque instability. The MMP-9 action is enhanced b neutrophil gelatinase-associated lipocalin (NGAL), also known as lipocalin-2, a 25 kDa glycoprotein, that is, found in the granules of human neutrophils, with many diverse functions, such as scavenger of bacterial products, modulator of inflammation, iron trafficking, and apoptosis [14].

The formation of a complex with NGAL and MMP-9 is crucial for atherosclerotic plaque erosion and thrombus formation [15]. NGAL is also produced by kidney tubular cells in response to various ischemic or toxic insults and has

been proposed as an early biomarker for the diagnosis of acute kidney injury [16, 17].

In this study, we hypothesized that levels NGAL in blood may reflect the extent of neutrophil activation in various stages of ACS and could discriminate various types of ACS (UA, NSTEMI, and STEMI) and stable from unstable coronary syndromes.

2. Methods

2.1. Study Design and Population. One hundred and seventy consecutive patients programmed for coronary angiography to the Invasive Cardiology Department of the KAT General Hospital Athens, Greece, were recruited for this study, from June 2010 to October 2010.

The study was performed according to the principles of the Declaration of Helsinki and was approved by the hospital's ethics committee. Written informed consent was obtained from all participating patients.

Thirty patients were excluded from the study. Exclusion criteria included a negative coronary angiography in patients with a typical chest pain which was considered as angina or had a false positive single photon emission computed tomography (SPECT), any surgery in the previous six months, liver disease, end stage renal disease, renal cardiac or liver transplantation, neoplasia, and infection since all these can affect serum-NGAL levels.

The 140 patients who fulfilled the study criteria after the clinical assessment and final diagnosis were divided into the following 4 groups: SA ($n = 40$), UA ($n = 35$), NSTEMI ($n = 40$), and STEMI ($n = 25$). Twenty (20) healthy amateur athletes without risk factors served as control group (Figure 1). The demographics and clinical characteristics of patients and controls are shown on Table 1.

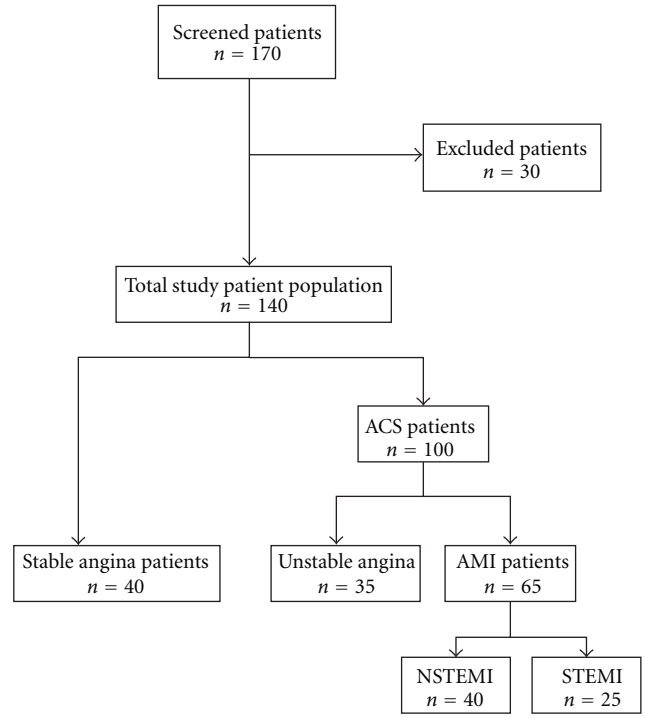

Figure 1: Flow diagram of subject recruitment. Ten patients were excluded for the following reasons: malignant diseases ($n = 1$), active infections ($n = 3$), and end stage renal disease ($n = 3$) recent surgery ($n = 3$). Also 20 patients with negative coronary angiography were excluded.

2.2. Clinical Assessment. All patients, upon presentation in emergency room, underwent an initial clinical assessment that included clinical history, physical examination, 12-lead ECG, continuous ECG monitoring, and standard blood tests (including white blood cell, polymorphonuclear neutrophil counts, and troponin-I). These tests were repeated at 6 at 12 and 24 hours as long as clinically indicated. To determine the final diagnosis for each patient 2 cardiologists blinded to NGAL results reviewed all patients available records (including patient history, laboratory results, radiologic testing, ECG, echocardiography, and coronary angiography) at the completion of their hospital stay.

The SA group consisted of patients with angiographically documented organic coronary stenosis >70% by quantitative coronary angiography in major arteries who had chronic symptoms of angina or a positive SPECT test. UA was diagnosed in patients with typical angina at rest, or a sudden increase in episodes of a previously stable angina.

AMI was diagnosed when there was evidence of myocardial necrosis in a clinical setting consistent with myocardial ischemia. Necrosis was diagnosed by a rising and/or falling pattern of troponin-I with at least one value above the cutoff value (defined as the 99th percentile of a normal population where the assay shows an imprecision <10%). Our troponin-I assay fulfills the imprecision criteria for concentration >0.2 ng/mL.

2.3. Sample Collection. Serum and K_2EDTA-plasma samples were collected from all SA patients in the morning before the coronary angiography. In all ACS patients PCI was performed within 24 hours from admission and the blood samples were collected on admission. From all healthy subjects, samples were collected in the morning and before training. Serum samples were kept frozen at -80°C until tested.

2.4. Laboratory Tests. Total white blood cell count (WBC), and peripheral polymorphonuclear neutrophil count (PMN) were assessed using the Cell-Dyn Sapphire haematology analyzer (Abbott, Chicago, Il, USA). Serum creatinine was measured with a modified jaffe method on Architect ci16200 analyzer (Abbott, Chicago, Il, USA). High-sensitivity CRP (hs-CRP) was measured with a turbidimetric assay on the same analyzer. Troponin-I was measured with a chemiluminescent immunoassay on the same analyzer. Serum-NGAL was measured with an ELISA (Bioporto, Gentofte, Denmark).

2.5. Statistical Analysis. This was performed with NCSS statistical program. Normality of distributions for quantitative data was tested with the Shapiro-Wilk test. For normally

Table 1: Patient demographics and clinical characteristics.

Nr	Stable angina	Unstable angina	NSTEMI	STEMI	Control group
	40	35	40	25	20
Sex (male/female)	(31/9)	(27/8)	(33/7)	(20/5)	(16/4)
Age mean (SD)	63.8 (9.85)	64.6 (8.17)	64.5 (9.96)	64.2 (11.19)	41.5 (7.96)
BMI mean (SD)	29.76 (4.89)	28.26 (4.81)	28.65 (3.56)	26.26 (2.12)	23.58 (1.80)
Diabetes *N* (%)	16 (40.00)	14 (40.00)	17 (42.50)	12 (48.00)	0
Hypertension *N* (%)	27 (65.71)	25 (71.43)	20 (70.00)	19 (76.00)	0
Dyslipidemia *N* (%)	27 (67.50)	19 (57.15)	25 (62.50)	17 (68.00)	0
Smoking	12 active 6 quit	17 active 1quit	18 active	14 active 4 quit	0

distributed parameters data were presented as means + standard deviation. For comparisons of means among patients groups and healthy controls a standard one-way anova test was used. Because serum-NGAL did not distribute normally, nonparametric tests were used: the Mann–Whitney test and the Kruskal-Wallis test with multiple-comparison procedures (Dunn's method) for comparisons between groups, and the Friedman test and the Wilcoxon test with the Bonferroni correction for comparisons within groups. Correlations were determined with use of Spearman's rank-correlation coefficient. Chi-square statistics were used for categorical variables. A P value of less than 0.05 (two-tailed) was considered to indicate statistical significance. Data are reported as medians and interquartile ranges. Receiver-operating characteristic (ROC) analysis was performed to calculate the area under the curve (AUC) and define cutoff points. Cutoff values were chosen after cost-benefit analysis. Differences between AUCs were investigated with the non-parametric approach of DeLong, DeLong and Clarke-Pearson. Statistics were performed with the NCSS 2004 Statistical and Power Analysis Software (NCSS Inc, Kaysville, UT, USA).

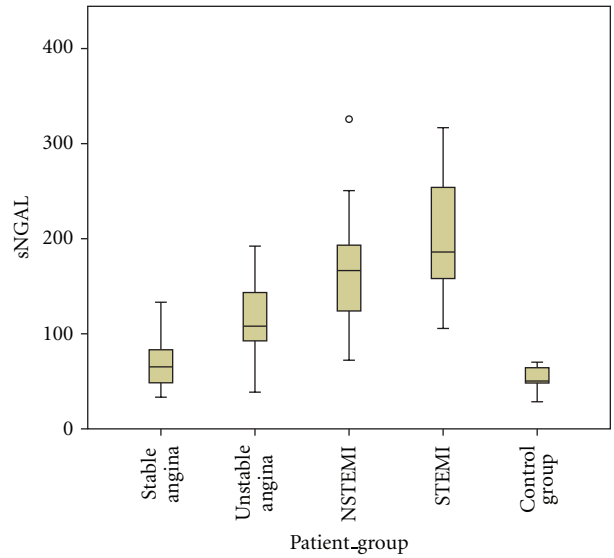

Figure 2: Box plots of median serum-NGAL values (ng/mL) among the 4 patients groups and healthy controls (y axis is in linear scale).

3. Results

The mean age and the mean BMI of the patients did not differ significantly among the four groups whereas the controls were significantly younger and their BMI was significantly lower (ANOVA-test). The proportion of diabetic patients did not differ significantly among four patient groups (chi-square = 1.69, $P = 0.639$) as well as the proportion of patients with hypertension and dyslipidemia (chi-square = 1.63, $P = 0.652$). Finally smoking habits did not differ significantly in the first three patient groups while it was significantly higher in group 4. Those risk factors were absent from our controls.

3.1. Levels of Serum-NGAL in Patients with CAD and Controls. The differences we observed in the median serum-NGAL values among the 4 patient groups and the healthy controls were significant ($P < 0.001$, ANOVA test). Further statistical analysis using multiple-comparisons between groups revealed that the median serum-NGAL levels in UA (108.00 ng/mL), NSTEMI (166.49 ng/mL), and STEMI (178.63 ng/mL) patients were significantly higher than in patients with SA (79.23 ng/mL) and healthy controls (50.31 ng/mL) (Table 2 and Figure 2). Also significant were the differences observed between healthy controls and SA patients, between UA patients and patients with AMI (NSTEMI or STEMI). Patients with STEMI had higher levels of NGAL than patients with NSTEMI but the difference was nonsignificant (Table 2).

3.2. Correlation between Levels of NGAL and Inflammatory Markers. The median plasma levels of hs-CRP were similar in patients with SA (0.40 mg/dL) and those with UA (0.69 mg/dL) and were significantly higher than the levels in the control group (0.12 mg/dL). Hs-CRP levels were significantly increased in patients with NSTEMI (1.20 mg/dL) and STEMI (6.76 mg/dL).

In order to further investigate the relationship between serum-NGAL and hs-CRP, we performed regression analysis between serum-NGAL and hs-CRP (Figure 3). This analysis revealed that there is a linear and positive correlation between hs-CRP and serum NGAL (spearman rank correlation coefficient rho = 0.685, $P < 0.0001$).

Table 2: Median and range of serum, urine NGAL, hs-CRP serum creatinine, and eGFR(MDRD) among patient groups and healthy controls.

Nr	Units	Stable angina 40	Unstable Angina 35	NSTEMI 40	STEMI 25	Control group 20
hs-CRP median (*quartiles*)	mg/dL	0.40 (0.05–0.87)	0.69 (0.11–3.69)	1.17 (0.24–13.21)	3.91 (0.31–13.62)	0.12 (0.02–0.25)
WBC count, mean (SD)	($\times 10^3$)	7.67 (1.99)	9.38 (2.32)	11.50 (2.65)	14.67 (4.92)	nd
Neutrophil count, mean (SD)	($\times 10^3$)	4.90 (1.72)	6.44 (1.96)	8.27 (2.44)	11.08 (4.15)	nd
s-NGAL ($*$) median (*quartiles*)	ng/mL	79.23 (37.50–100.32)	108.00 (68.34–177.59)	166.49 (109.24–247.20)	178.63 (111.18–305.92)	50.31 (44.30–69.78)
s-Creatinine, mean (SD)	μmol/L	79.72 (16.94)	79.86 (17.78)	86.04 (21.74)	88.65 (34.46)	81.26 (7.97)
eGFR(MDRD), mean (SD)	mL/min	89.71 (19.85)	89.63 (22.96)	85.79 (22.20)	81.75 (21.06)	97.65 (19.55)

($*$) $P < 0.05$ for the comparison between patients with SA and controls, $P < 0.005$ for the comparison between patients with SA with patients with UA, and $P < 0.001$ for the comparisons of rest groups, and P = NS for the comparison of patients with STEMI with NSTEMI.

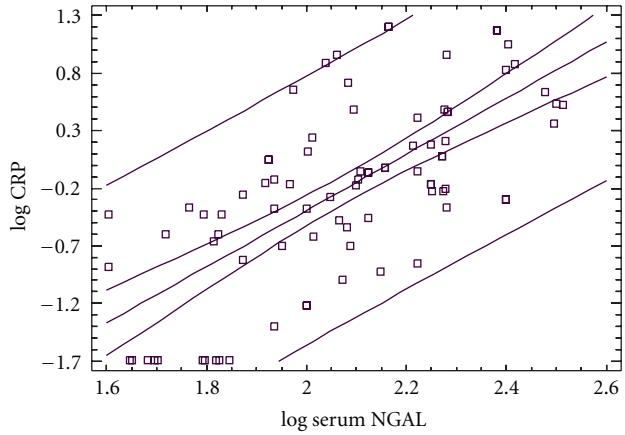

Figure 3: Regression analysis between serum-NGAL and hs-CRP.

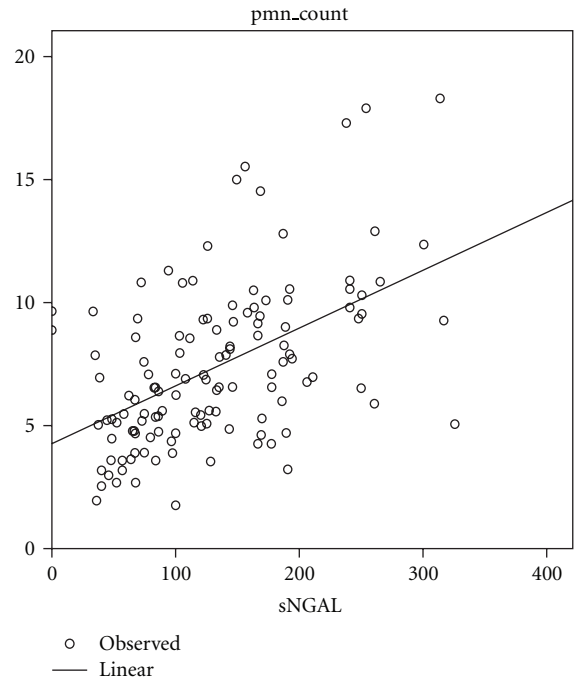

Figure 4: Scater plots of PMN values versus serum NGAL.

The differences that were observed among the four patient groups in WBC and PMN counts were statistically significant ($P < 0.001$, ANOVA-test). There was a positive and significant correlation between serum-NGAL and WBC ($r = 0.510$, $P < 0.0001$) and PMN ($r = 0.511$, $P < 0.0001$) counts (Figure 4).

In a multivariate regression analysis model entering as independent parameters age, serum creatinine, hs-CRP, and PMN count, we identified only hs-CRP ($P < 0.005$) and PMN count ($P < 0.0001$) as independent predictors of serum-NGAL levels.

3.3. The Diagnostic Value of NGAL in Discriminating Stable from Unstable Patients. ROC curves were generated for sensitivity and specificity with the respective areas under the curve for serum-NGAL hs-CRP and PMN counts (Figures 5 and 6).

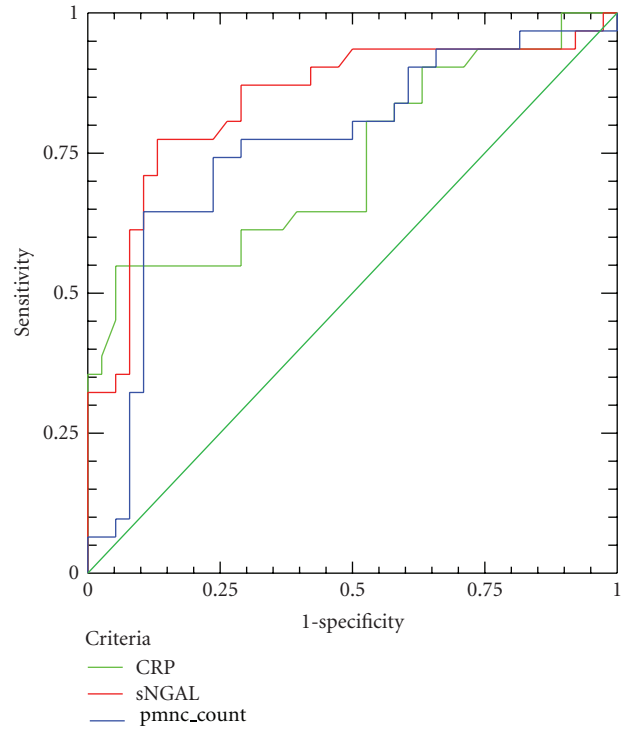

Figure 5: ROC curve analysis for CRP (green line), serum-NGAL (red line), and PMN count (blue line) for the discrimination of UA patients from patients with SA.

The diagnostic value for serum-NGAL in discriminating patients with UA, from those with SA is high (AUC = 0.852) and better than of hs-CRP (AUC = 0.735) or PMN count (AUC = 0.761). If we use as cutoff for serum-NGAL 83.74 ng/mL, we can predict an UA event with sensitivity and specificity, 82.8% and 75%, respectively. The negative predictive value of this cutoff is high (97.28%).

The diagnostic value for serum-NGAL in discriminating ACS patients, from patients with SA is high (AUC = 0.929) and better than of hs-CRP (AUC = 0.794) and PMN count (AUC = 0.830). If we use as cut-off for serum-NGAL 89.29 ng/mL, we can discriminate an ACS patient from a stable patient with sensitivity and specificity, 89.3% and 81.6%, respectively. The negative predictive value of this cutoff is high (98.65%).

4. Discussion

In this study, we demonstrated that serum levels of NGAL are higher in patients with CAD than in healthy controls patients. Among ACS patients, these levels are gradually elevated according to the severity of the coronary clinical syndrome (UA, NSTEMI, and STEMI). Also serum levels of NGAL are higher in patients with ACS than in patients with SA and could be used, with high negative value, to discriminate patients with stable or unstable coronary syndromes.

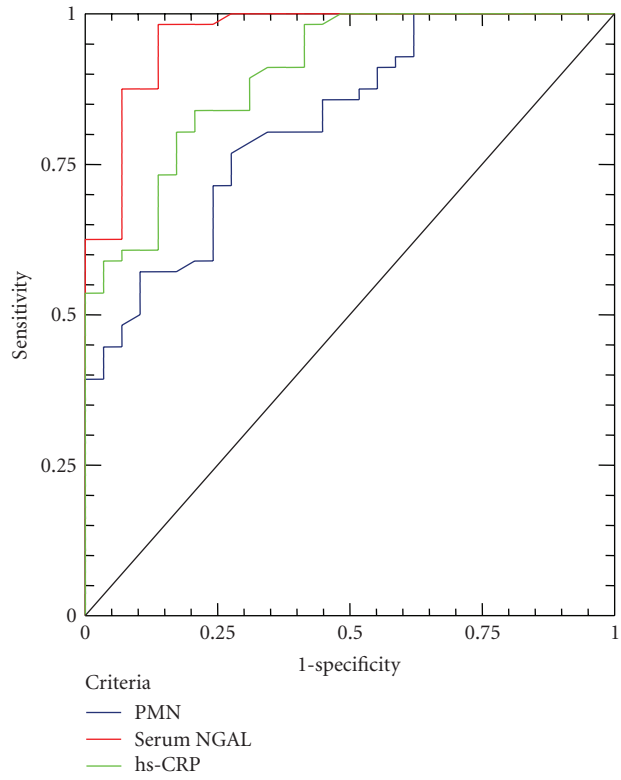

Figure 6: ROC curve analysis for CRP (green line), serum-NGAL (red line), and PMN (blue line) for the discrimination of ACS patients from patients with SA.

The relevance of NGAL to cardiovascular disease (CVD) remains primarily unknown. Elevated plasma NGAL levels were associated with atherosclerosis and were implicated as a predictor for cardiovascular mortality after cerebrovascular ischemia, possibly because of activation of blood leukocytes [18–20]. Although in recent reports has been shown that NGAL is present in atherosclerotic plaques and in human abdominal aortic aneurisms, raising the possibility that expression of NGAL can be induced in vascular cells during atherogenesis, the underlying mechanism for the induction of NGAL in vascular cells remains unknown [15, 21]. In further analysis the main source of NGAL was found to be neutrophils, probably recruited in the vascular wall by platelet activation [21].

NGAL is considered to have a protective effect on MMP-9 and enhancing its proteolytic activity, could be considered as an important factor indirectly contributing to the progression of aneurism as well as involved in the physiologic and pathologic remodeling of vessel walls. This view is further supported by the observation that similar neutrophil NGAL/MMP-9 overexpression can be found in atherosclerotic plaques, particularly those with intramural haemorrhagic debris and central necrosis [15, 22]. The above evidence supports the clinical observations that high-circulating leucocyte (particularly neutrophil) counts are independent predictors of recurrent ischaemic attacks. This may be explained by their presence in the necrotic core of unstable plaques and by their proteolytic activity towards atherosclerotic tissue and secondary mobilization of thromboembolic fragments [23]. The evidence derived from these experimental studies, showing the close link between neutrophils, their products and the natural history of atherosclerosis, and its complications, generated clinical studies that investigated the clinical utility of serum-NGAL measurements.

In two recent studies it was found that serum levels of NGAL were significantly elevated in patients with angiographically confirmed CAD compared to those with normal arteries or controls [24, 25]. Our data agree with these reports since we found that levels of serum-NGAL are significantly higher in patients with all clinical syndromes of CAD than in healthy controls, reinforcing the utility of NGAL as biomarker of detection and the extent of CAD. The expression of NGAL from vascular cells during atherogenesis can also explain the differences between patients with SA and control subjects with no risk factors observed in our study. In addition to its induction in the vessels after mechanistic injury, previous studies suggest that NGAL is strongly upregulated in atherosclerotic lesions and also in the heart after ischemic injury [15]. It is possible that NGAL produced by vascular cells could also be secreted into the systemic circulation.

Inflammation plays a critical role not only in development and progression of atherosclerosis but also in pathogenesis of the destabilization of atherosclerotic plaque that leads to ACS [1, 26]. Activation and degranulation of polymorphonuclear neutrophils and probably an underestimated critical components of an acute coronary inflammation event. Infiltrating macrophages and neutrophils participate in the transformation of stable coronary artery plaques to unstable lesions with a thin fibrous cap [27]. It has been repeatedly reported that thrombosed plaques were densely infiltrated by neutrophils and macrophages [28, 29]. Macrophages and neutrophils and some other types of leukocytes produce various proteolytic enzymes which facilitate the rupture of plaques by thinning and weakening their normally thick and firm cap [30, 31]. NGAL is one protein, that is, produced not only by the distressed kidney but also by activated neutrophils and by the vascular wall cells. Recent studies have shown that neutrophils are the main source of NGAL in blood [32, 33].

Increase in serum NGAL resulting from activation of neutrophils may reflect an acute systemic inflammatory response to events such as stroke, renal failure, or infection [18, 34–36] but are also linked with the presence of chronic inflammatory diseases such as atherosclerosis [18] whose acute clinical manifestations represent acute on chronic inflammation. Besides neutrophils, NGAL is also expressed by epithelial cells, renal tubular cells, and hepatocytes during inflammation or injury [37–39]. Our data agree with the above studies since we found a positive correlation between levels of serum-NGAL and systemic inflammation (expressed by the serum hs-CRP levels and neutrophil count), and also serum levels of NGAL were higher in patients with ACS than with SA. The higher levels of serum-NGAL observed in patients with ACS compared to SA could be explained by

the fact that neutrophil activation is present only in patients with acute coronary events (10,11). Also, our results, as far as patients with SA and AMI, are similar with the findings of a recent published study which showed that the plasma level of NGAL is higher in patients with AMI compared with the patients with stable CAD [40].

In clinical practice, levels of serum-NGAL have a high negative predictive value, 97.28% and 98.65% for patients with UA and ACS, respectively. So, serum-NGAL could be used in discriminating of patients with ACS or especially UA from whom with SA or without CAD, giving the possibility to exclude patients with symptoms similar to angina but not having true ACS. As far as the gradual increase of serum-NGAL, according to the seriousness of unstable coronary clinical syndrome, this could reflect the intensity of the inflammatory reaction, as it is expressed by the incremental increase of hs-CRP and neutrophil count and their combination with serum NGAL. Especially between serum-NGAL and hs-CRP, the correlation is linear and positive.

In conclusion, our study shows that serum levels of NGAL increase in patients with CAD with every coronary clinical syndrome and reflect the inflammatory status in the same population. Having high negative predictive value could be used as a marker for the discrimination of SA or chest pain without CAD from those with ACS. Also in patients with ACS, serum levels of NGAL reflecting the inflammatory status could show the severity of coronary clinical syndrome (UA, NSTEMI, and STEMI).

Abbreviations and Acronyms

ACS:	Acute coronary syndrome
CAD:	Coronary artery disease
SA:	Stable angina
UA:	Unstable angina
AMI:	Acute myocardial infarction
NGAL:	Neutrophil gelatinase associated lipocalin
NSTEMI:	Non-ST-elevation myocardial infarction
STEMI:	ST-elevation myocardial infarction
PCI:	Percutaneous coronary intervention.

Conflict of Interests

The authors declare that they have no conflict of interests.

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Review Article

Anti-Inflammatory Effects of Interleukin-19 in Vascular Disease

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Despite aggressive dietary modification, lipid-lowering medications, and other interventional medical therapy, vascular disease continues to be a leading cause of mortality in the western world. It is a significant medical and socioeconomic problem contributing to mortality of multiple diseases including myocardial infarction, stroke, renal failure, and peripheral vascular disease. Morbidity and mortality of vascular disease are expected to worsen with the increasing number of patients with comorbid conditions such as obesity, metabolic syndrome, and diabetes mellitus type 2. Vascular diseases such as atherosclerosis, restenosis, and allograft vasculopathy are recognized to be driven by inflammation, and as such, cytokines which mediate inflammation not only represent important targets of rational therapy, but also can be considered as possible therapeutic modalities themselves. In this paper, we will examine the role of inflammatory cytokines and lymphocyte T_h1/T_h2 polarity in vascular inflammation, with a focus on atherosclerotic vascular disease. We will then introduce a recently described T_h2 interleukin, interleukin-19 (IL-19), as a previously unrecognized mediator of vascular inflammatory disorders. We will review our current understanding of this interleukin in health and disease and present the possibility that IL-19 could represent a potential therapeutic to combat vascular inflammatory disease.

1. Inflammation, Cytokines, and Vascular Disease

Inflammation is a ubiquitous pathological process which is central to the development of multiple cardiovascular diseases. Many vascular diseases such as atherosclerosis, restenosis, and transplant vasculopathy are chronic, progressive processes initiated and propagated by local inflammation of large- and medium-sized arteries [1]. This inflammation is mediated by a variety of cell types including macrophage, lymphocyte, endothelial cell (EC), and vascular smooth muscle cell (VSMC). The multiple cell types which participate in vascular inflammation have evolved to produce common cytokines and specific membrane receptors allowing them to transmit their effects into the cell, permitting these diverse cell types to communicate by expression and recognition of multiple pro- and anti-inflammatory cytokines. As such, cytokines and their receptors are the currency of inflammation, and represent attractive targets for therapeutic modalities in numerous vascular inflammatory disorders.

Synthesis and recognition of cytokines and receptors by both vascular and inflammatory cells allows bidirectional communication between these two systems and demonstrates that, under particular conditions, we can consider vascular cells as an extended participant in the adaptive immune response. Cytokines often act in synergy with other cytokines and frequently share receptor subunits which combine into homodimers or heterodimers with receptors of other cytokines. Cytokines can drive multiple, often simultaneous cellular processes including mitogenesis, development, gene expression, fibrosis, and chemotaxis [2]. This communication initiates a series of receptor-mediated signal transduction cascades including activation of mitogen-activated protein kinases (MAPKs), protein kinase C (PKC), and transcription factors, often including the signal transducer and activator of transcription (STAT) family [3]. Proinflammatory cytokines most often lead to activation of nuclear factor-(NF-κB)

which acts as a "master switch" for transcription of numerous genes, the expression of which may be appropriate, as in host defense, or maladaptive, as in chronic vascular disease [4, 5].

Atherosclerosis is a chronic vascular inflammatory condition mediated by interactions between lymphocytes, macrophage, endothelial, and vascular smooth muscle cells which results in local inflammation of the arterial wall. While an excess of low-density lipoprotein (LDL) is an established risk factor for atherosclerosis, inflammatory mechanisms play an acknowledged role in initiation and propagation of atherogenesis. The inflammatory nature of atherosclerosis has prompted broad investigation into vascular inflammatory processes, and consequently, proinflammatory signaling mechanisms in the vascular wall have been well characterized [6–9]. Less, albeit increasing, interest has been placed on understanding the potentially protective role of anti-inflammatory cell signaling in the vascular wall [10, 11]. Such studies that do exist place a strong emphasis on the role of anti-inflammatory cytokines, for example, interleukin-10 (IL-10), in the immune cells of the plaque. Even less investigation has been carried out on the direct effect of anti-inflammatory cytokines on vascular smooth muscle cells and endothelial cells. Consequently, direct effects of anti-inflammatory cytokines constitute an emerging and promising area of study.

2. T_h1 and T_h2 Interleukins in Atherosclerosis

Interleukins are often classified according to their effects on lymphocyte function or maturation, as T_h1 (proinflammatory, cytotoxic) which promotes inflammation, and T_h2 (anti-inflammatory, antibody responses), which generally dampens inflammation [12]. Approximately 10% of the cellular content of human atherosclerotic plaque consists of CD3+ T cells [4]. The overwhelming majority of these are CD4+ helper T cells (T_h), which recognize epitopes on oxidized LDL [13]. Because atherosclerosis is primarily an inflammatory condition, it is not surprising that T_h1 interleukins are much more prevalent in human atherosclerotic lesions than the T_h2 cytokines [9, 12]. T_h1 cells drive cell-mediated immunity and are characterized by abundant expression of interferon (IFN)-γ, IL-12, tumor necrosis factor-α, and other proinflammatory cytokines, which are also highly expressed in atherosclerotic lesions. In contrast, T_h2 cytokines such as IL-4 and IL-10 are far less abundant in human and mouse atherosclerotic lesions. T_h2 cytokines dampen the inflammatory response through inhibition of proinflammatory genes (including T_h1 genes) and, conversely, T_h1 cytokines reduce expression of T_h2 cytokines. Thus, the very low levels of T_h2 cytokines detected in atherosclerotic lesions are likely due to the elevated levels of T_h1 cytokines present. The prevailing hypothesis that atherosclerosis is a T_h1 disease is supported by studies in which mice lacking IFN-γ or the IFN-γ receptor, TNF-α, or the T_h1 transcription factor T-bet have reduced atherosclerosis [14–16]. Further supporting this hypothesis, mice lacking STAT6, which is essential for T_h2 cell differentiation, have increased atherosclerosis [17]. Since so many more proatherogenic cytokines and receptors have been identified and characterized, much greater effort has gone into understanding these cytokines and the potential for inhibition of their expression or activity. Far fewer studies have pursued characterization of anti-inflammatory cytokines in vascular disease. Interleukin-10 is the archetypical T_h2 interleukin, a potent immune modulator, and the most studied in terms of vascular disease. Several studies have suggested that IL-10 is atheroprotective by several mechanisms including T_h2 T-cell polarization and attenuation of inflammatory gene expression in inflammatory cells. For example, IL-10 atherosclerotic plaque burden is reduced in IL-10 transgenic mice, and transfer of bone marrow from these mice into $LDLR^{-/-}$ mice reduced atherosclerosis [18]. As expected, atherosclerosis is increased in IL-$10^{-/-}$ mice and IL-$10^{-/-}$/ApoE$^{-/-}$ double knock-out mice. The mechanism is most likely mediated by inflammatory cells, as transfer of IL-$10^{-/-}$ bone marrow to $LDLR^{-/-}$ polarizes the T lymphocyte T_h2/T_h1 ratio toward a more anti-inflammatory phenotype [19]. Although considered to be a T_h2 interleukin, IL-4 does not appear to be antiatherosclerotic, as IL-$4^{-/-}$ mice do not have increased atherosclerosis, and administration of IL-4 into ApoE$^{-/-}$ mice does not reduce development of atherosclerotic lesions [20]. Thus, most studies consider T_h2 interleukins to be indirectly antiatherogenic by dampening the host immune response, reducing inflammation and lesion formation. Though the number of studies investigating the role of T_h2 cytokines is dwarfed by those investigating T_h1, our current understanding suggests that the balance of these two opposing "forces" can dictate outcome of the atherosclerotic lesion. Consequently, investigation of the role of T_h2 cytokines as potential therapeutics in vascular inflammatory disorders, though understudied as it is, may be considered potentially promising as anti-inflammatory therapy for vascular disorders.

3. Discovery and Characterization of Interleukin-19

Interleukin-19 (IL-19) was first identified and cloned by searching Expressed Sequence Tag (EST) databases for IL-10 homologues [21]. The IL-19 gene is located in chromosome 1q32, in an "IL-10 cluster," which includes genes for several other IL-10 family members. IL-19 is a member of a subfamily of the IL-10 family of interleukins and is more broadly classified as a class II cytokine, a class which includes the IL-10 family members and the interferons (types I, II, and III) [22–24]. More recently, IL-19 has been classified in a subfamily including IL-19, IL-20, and IL-24, though these subfamily members are recognized by and signal through different combinations of shared receptor chain complexes.

In its secreted form, human IL-19 is a compact α-helical protein composed of 159 amino acids. IL-19 has amino acid identity with IL-10 at 30 residues including 4 cysteines known to be required for correct folding of IL-10 and 41 of the 50 amino acids required for formation of the IL-10

hydrophobic core [21]. The overall IL-19 amino acid sequence shares 20% identity with IL-10, and X-ray crystallography confirms that IL-19 is structurally similar to IL-10 but with key differences [25]. While IL-10 has 6 α-helices (A–F), the last of which (F) contributes to the formation of a stable IL-10 homodimer in solution through its insertion into the core of its paired protein, IL-19 has 7 α-helices (A–G), the last of which (G) is able to fold back and stabilize IL-19 as a soluble monomer. Further, the homologous region of IL-10 that interacts with the IL-10 receptor chain 1 is far less conserved in IL-19. Together, these properties may explain why IL-19, despite its amino acid identity with IL-10, is not recognized by and cannot signal through the IL-10 receptor complex.

IL-19 shares relatively greater structural similarity with fellow subfamily members IL-20 and IL-24, each of which also forms a stable monomer in solution. The genes for these three proteins are found in a gene cluster with IL-10 on chromosome 1 and have been alternately referred to as the "IL-19 subfamily" [22] or the "IL-20 subfamily" [26]. In addition to their structural similarity, interleukins 19, 20, and 24 all signal through receptor complexes containing the IL-20 receptor β chain (IL-20Rβ) [27]. All three proteins can signal through the heterodimer formed by IL-20Rα and IL-20Rβ. IL-20 and IL-24, but not IL-19, can also signal through the receptor formed by IL-22Rα and IL-20Rβ.

4. Expression and Function of IL-19

Expression of IL-19 was first reported by Gallagher et al. [21] in LPS- and GM-CSF-stimulated primary human monocytes, and subsequent early reports on IL-19 focused on its role as a product of immune cells [28]. Among immune cells, IL-19 is primarily expressed by monocytes and, to a lesser extent, by B cells, but some investigators have questioned its role in regulating these cells due to the lack of detectable expression of the IL-20Rα chain in lymphocytes [28–31]. Notwithstanding the lack of this receptor, effects of IL-19 have been reported in lymphocytes [32, 33], including the notable observation that IL-19 treatment is able to polarize the maturation of human T cells away from the proinflammatory T_h1 phenotype to the anti-inflammatory T_h2 phenotype [33, 34]. While expression of IL-20Rα and IL-20Rβ chains is reported to be cytokineregulated, detailed studies on expression of these peptides in vascular cells or myocytes are lacking. Since its discovery and early characterization, IL-19 expression has been detected in a wide variety of nonimmune human peripheral cell types, including keratinocytes [29], bronchial epithelial cells [35, 36], synovial tissue [37, 38], fetal membranes [39], and vascular endothelial [40] and smooth muscle cells [41] (Table 1). This suggests a functional role for IL-19 distinct from T_h1/T_h2 polarization. Paradoxically, IL-19 seems to exert both proinflammatory and anti-inflammatory properties in a manner contextually governed by tissue-specific and disease-specific factors. The myriad roles of IL-19 in noncardiovascular tissues and diseases are of qualified interest to the scope of this paper as other roles of IL-19 could affect its efficacy and desirability as a therapeutic modality in vascular disease. The multiple effects of IL-19 have been well reviewed in the past [22] and will be briefly presented here.

A putative role for IL-19 has been put forth in the development of psoriasis, a chronic inflammatory skin condition characterized by increased proliferation of keratinocytes leading to the development of plaque-like epidermal lesions. Expression of IL-19, IL20Rα, and IL20Rβ can be detected in psoriatic lesions [29, 44, 47, 56, 57], and treatment of psoriasis reduces expression of IL-19 [47, 56]. Current findings suggest a possible feedback loop whereby IL-19 promotes expression of keratinocyte growth factor (KGF) in CD8+ T cells which, in turn, induces increased expression of IL-19 from keratinocytes [44]. However, while IL-20 transgenic mice are reported to have a psoriatic phenotype, IL-19 mice exhibit no such pathology. A causal role for IL-19 in psoriasis has not been well established, though the data support a stronger implication for the IL-19 family member IL-20 in this disease.

T_h2 cytokines are involved in the pathogenesis of a number of diseases, most notably asthma [58–60], a chronic inflammatory airway disease resulting in bronchospasm and consequent reversible airway obstruction. As expected, given the T_h2 nature of the disease, IL-19 has a demonstrable, though yet unclear, role in the development of asthma. IL-19 expression is increased in the lungs of mice exposed to allergens [42]. Serum IL-19 levels are increased in children with asthma when compared with normal children [42], and airway epithelial cells of asthma patients exhibit increased IL-19 expression [36]. IL-19 expression in airway cells can be modulated by adenosine receptors [48], which play a role in asthma-related cell signaling.

Recent scientific interest in IL-19 has prompted investigators to pursue exploration of IL-19 involvement in various other diseases and tissue types. IL-19 has been indicated as potentially protective against gut inflammation [50, 61], representing the potential for therapeutic use in inflammatory bowel disease. IL-19 has a suggested role in promoting the development of endotoxic ("septic") shock [49] as well as rheumatoid arthritis [38, 54]. Recent work demonstrated IL-19 expression in numerous neoplastic cell types, including cells of squamous cell carcinoma of the oral cavity, in which IL-19 promoted proliferation [52]. IL-19 also promoted proliferation and migration of breast cancer cells, and high IL-19 expression was associated with poor outcomes in breast cancer patients [53].

5. Expression of Interleukin-19 in Vascular Disease

Vascular expression of IL-19 was first identified in 2005 through cDNA microarray analysis of cultured human vascular smooth muscle cells treated with inflammatory stimuli [62]. This was unexpected as IL-19 expression had previously been thought to be restricted to leukocytes [21, 22, 34, 63]. Induction of IL-19 expression in vascular cells was further characterized, and western blot analysis of cultured human VSMC demonstrated that IL-19, while not expressed

Table 1: Effects of IL-19 in nonvascular cell types.

Tissue type	Effect	Species	Source
Immune cells	T_h2 response in T cells	h, m	[33, 34, 42]
	Inhibits IFN-γ production in T cells	h	[34]
	Induces IL-4 and IL-13 production in T cells	h	[34]
	Induces IL-10 production in monocytes	h	[43]
	Autoinduces IL-19 expression in PBMC; dendritic cells	h	[43]
	Induce KGF expression in CD8+ T cells	h	[44]
	Suppress cell-mediated immunity in postbypass patients	h	[45]
	Induced production of IL-6; TNF-α in monocytes	m	[46]
	Induced ROS production and apoptosis in monocytes	m	[46]
Skin cells	Expressed in keratinocytes in psoriatic skin	h	[29, 47]
	STAT3 phosphorylation in HaCat keratinocyte cell line	h	[33]
Airway epithelium	HBEC-produced IL-19 induces TNF-α production in THP-1 monocyte line	h	[48]
	Induced apoptosis in lung epithelium cells	h	[49]
Colon epithelium	IL-19 expression is protective against dextran sucrose sodium-induced colitis	m	[50]
Cancer cells	Inhibit proliferation in NIH:OVCAR3 (ovarian carcinoma) cells	h	[51]
	Increase proliferation in oral squamous cell carcinoma cells	h	[52]
	Increase proliferation in breast cancer cells	h, m	[53]
	Induced IL-1β, IL-6, TGF-β, MMP-2, -9, and CXCR4 in 4T1 breast cancer cells *in vitro*	m	[53]
	Induced fibronectin expression in 4T1 breast cancer cells *in vitro*	m	[53]
Fetal membranes	Induce IL-6 production	h	[39]
	Inhibit LPS-induced TNF-α production	h	[39]
Liver	Induced ROS production in Huh-7 cell line	h	[49]
Synovial fluid	Inhibits apoptosis in RASC	h	[37]
	Activates STAT3 and induces IL-6 production in RASC	h	[37]
	Induces TNF-α, IL-1β, IL-6, and RANKL in collagen-induced arthritis synovial fibroblasts	r	[54]
Nasal fibroblast	Inhibits IL-4-induced eotaxin expression by SOCS1-dependent mechanism	h	[55]

Abbreviations: HBEC: human bronchial epithelial cell, RASC: rat arthritis synovial cell, h: human, m: mouse, r: rat.

in quiescent (unstimulated) controls, can be induced in VSMC treated with inflammatory stimuli including fetal bovine serum (FBS), T-cell-conditioned media (TCM), IFN-γ, platelet-derived growth factor (PDGF), and TNF-α [41]. Analysis of endothelial cells produced similar results, showing that microvascular EC (mEC), coronary artery EC (CaEC), and human vascular EC (HVEC) can all be stimulated to express IL-19 by FBS, basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF), and oxidized LDL (ox-LDL) [40]. In contrast, IL-10 expression could not be detected in VSMC at the mRNA or protein level [41]. In histological analysis of human coronary arteries, IL-19 expression was undetectable in sections from normal arteries, but was highly expressed in EC [40], neointimal and medial VSMC [41], and CD45+ leukocytes in coronary arteries with allograft vasculopathy, a chronic vascular inflammatory syndrome. Similarly, both IL-20 and its receptor subunits are expressed in macrophage and EC in atherosclerotic plaque and are induced in these cultured cells when stimulated with inflammatory factors. IL-20 is not expressed in VSMC. In contrast, IL-19 is detected in VSMC [41], EC [40], and CD45+ leukocytes [41] in atherosclerotic plaque in aortic arch of ApoE$^{-/-}$ mice, but not aortic arch of wild-type mice, further suggesting that IL-19 is only expressed in response to vascular injury.

In another connection to vascular disease, two reports indicate that serum concentrations of IL-19 are increased in patients undergoing coronary artery bypass graft (CABG) surgery with cardiopulmonary bypass [45, 64], and that the increased IL-19 levels contribute to the cell-mediated immune suppression frequently observed in these patients [45].

6. Pleiotropic Effects of IL-19 on Vascular Cells

In vascular disease, where the participating cell types are primarily immune cells and vascular cells, IL-19 exerts a pronounced anti-inflammatory effect (Table 2). IL-19 has antiproliferative effects on the NIH:OVCAR-3 ovarian carcinoma cell line [51], and IL-10 has antiproliferative effects in

Table 2: Effects of IL-19 on resident vascular cells.

Tissue type	Effect	Species	Source
EC	Autoinduces IL-19 expression	h	[40]
	Activates STAT3, Rac1, and MAPK p44/42	h	[40]
	Increases EC proliferation	h	[40]
	Increases EC spreading, and migration	h	[40]
	Proangiogenic (increase tube, microvessel formation)	m	[40]
	Inhibits HuR nucleocytoplasmic translocation	h	[67]
VSMC	Inhibits proliferation, hyperplasia	h, r	[41]
	Autoinduces IL-19 expression	h	[41]
	STAT3 phosphorylation, translocation	h	[41]
	Increases SOCS5 expression	h	[41]
	Inhibits MAPKs (p44/42, p38)	h	[41]
	Decreases inflammatory, proliferative proteins, and mRNAs	h	[68]
	Decreases HuR protein abundance	h	[68]
	Inhibits HuR nucleocytoplasmic translocation	h	[68]
	Decreases ARE-bearing mRNA stability	h	[68]
	Inhibits PKCα activation	h	[68]
	Inhibits migration, spreading	h	[69]
	Inhibits activation of MLC, cofilin, Hsp70, Rac1, and RhoA	h	[69]
	STAT3-dependent increase heme oxygenase-1 expression	h	[70]
	Decreases ROS *in vitro* and *in vivo*	h, m	[70]
	Inhibits apoptosis	h	[70]

Abbreviations: h: human, m: mouse, r: rat.

vascular cells [65], suggesting that vascular expression of IL-19 in response to injury might represent a novel autocrine or paracrine mechanism for attenuation and regulation of VSMC proliferation. Several experiments were carried out to test this hypothesis and have uncovered multiple potential mechanisms for these effects (summarized in Figure 1). Treatment of cultured VSMC with recombinant IL-19 or with adenoviral expression of IL-19 decreased VSMC proliferation compared to controls in a concentration-dependent manner [41]. *In vivo* experiments in rats recapitulated this antiproliferative effect, demonstrating that adenoviral delivery of IL-19 to balloon angioplasty-injured rat carotid arteries decreased neointima formation and number of proliferating (Ki-67-positive) VSMCs in this tissue. IL-19 treatment of VSMCs evoked a rapid and transient activation of STAT3 as measured by both phosphorylation and nuclear translocation. IL-19 was shown to rapidly increase expression of suppressor of cytokine signaling 5 (SOCS5), an STAT-responsive gene, at both the mRNA and protein levels. IL-19-induced SOCS5 expression was dependent on STAT3 [41]. There are six SOCS family members which function to suppress cytokine signaling by binding to phosphorylated tyrosine residues on cytokine receptors and cytoplasmic signaling intermediates and targeting them for E3-ubiquitin ligase-mediated degradation. SOCS-mediated signaling inhibition is a strategy employed by numerous cytokines [66]. In VSMC, IL-19 can reduce fetal bovine serum-induced activation of the p44/42 and p38 MAPKs, both mediators of inflammation. IL-19-induced SOCS5 binds the p44/42 and p38 MAPKs, providing at least one probable mechanism for these effects [41]. This indicates that IL-19 can reduce VSMC activation by inhibition of signal transduction. In addition to IL-19, this work also implicates SOCS5 as an important mediator of anti-inflammatory signal transduction.

IL-19 can decrease FBS-mediated induction of protein and mRNA abundance of proliferative and proinflammatory genes in VSMC, including Cyclin D1, cyclooxygenase-2 (COX-2), IL-1β, and IL-8 [68]. Interestingly, this inhibition was selective, and other important regulatory proteins such as proliferating cell nuclear antigen (PCNA), Rac1, and others were not sensitive to IL-19. Other T_h2 interleukins also reduce inflammatory cytokine expression, and IL-10 in particular reduces inflammatory responses in varied cell types by inhibition of activation of the transcription factor NF-κB. In contrast to IL-10, IL-19 did not inhibit NF-κB activation, as determined by IκB degradation and p65 subunit phosphorylation in both VSMC [68] and EC [67]. This surprising finding suggested that IL-19 decreases abundance of inflammatory and proliferative genes in an NF-κB-independent mechanism and prompted a search for other possible mechanisms whereby IL-19 could decrease inflammatory gene abundance without affecting their transcription. Notably, many proinflammatory genes, including the genes affected by IL-19, are targeted for preferential degradation by cis-acting AU-rich elements (AREs) in their 3′ untranslated regions [71]. Two proteins, human R antigen (HuR) and AU-rich RNA-binding factor-1 (AUF-1), have been shown to regulate ARE-bearing transcripts by binding to ARE and modifying their mRNA stability [72], with HuR promoting increased mRNA stability and AUF-1

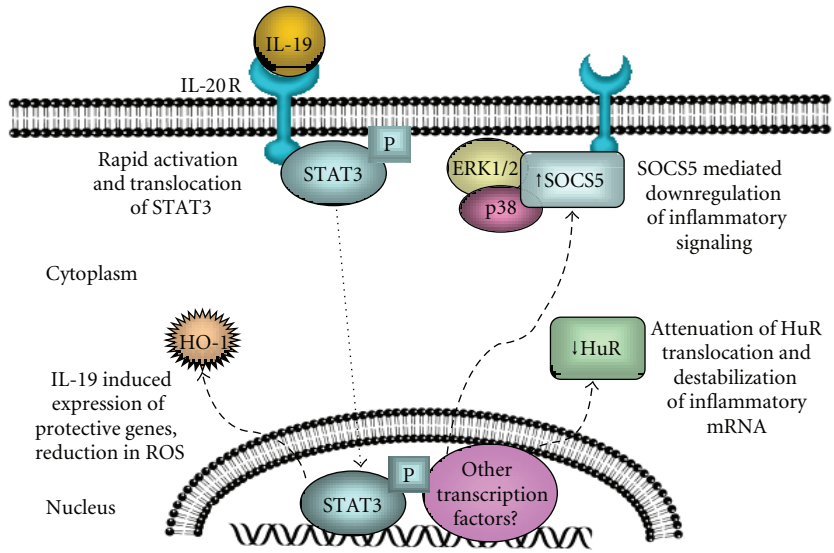

Figure 1: Proposed pleiotropic mechanisms of IL-19 effects in vascular smooth muscle cells. Several mechanisms occurring independently or concurrently may be responsible for IL-19 anti-inflammatory effects on VSMC. These include STAT3-mediated SOCS5 expression, which dampens early activation events such as signal transduction. The second is a down-regulation of HuR abundance and cytoplasmic translocation, resulting in attenuation of proliferative and inflammatory gene expression. The third is expression of HO-1, with subsequent protective effects attributed to HO-1 such as reduction of intracellular ROS. These mechanisms are not mutually exclusive, so varying degrees or combinations of each are likely.

promoting decreased stability. The half-lives of ARE-bearing transcripts may be regulated in a "yin-yang" fashion through competitive binding of HuR and AUF-1. The ability of HuR to stabilize mRNA corresponds with its translocation from a predominately nuclear location into the cytoplasm [72]. Prompted by the observation that IL-19-inhibited transcripts bear AREs, and that transcripts lacking AREs (e.g., PCNA) are unaffected by IL-19, the effects of IL-19 on HuR, AUF-1, and mRNA stability were explored. It was found that IL-19 reduces HuR translocation in both FBS-stimulated VSMC [68] and TNF-α-stimulated human coronary artery EC [67]. AUF-1 abundance and translocation were not affected by IL-19. As expected, IL-19 reduced stability of inflammatory and proliferative mRNA transcripts which contained ARE in both VSMC and EC, as measured using qRT-PCR and with the transcription inhibitor actinomycin D, but failed to affect stability of mRNA lacking AREs. This effect on stability was able to be recapitulated using HuR siRNA [67, 68]. Taken together, these observations suggested that IL-19 signaling in vascular cells is permissive of NF-κB-mediated increases in inflammatory and proliferative gene *transcription*; however, IL-19 produces a *posttranscriptional* decrease in the abundance of these transcripts through inhibition of HuR translocation, thereby mediating a decrease in transcript stability. This represents a second mechanism, in addition to IL-19-induced SOCS5 expression, through which IL-19 may exert its anti-inflammatory effects on vascular cells.

IL-19 has been shown to have a direct effect on VSMC motility. IL-19 inhibited cultured VSMC remigration into a scratch wound and also inhibited PDGF-induced migration in a Boyden chamber [69]. Molecular analysis revealed that IL-19 inhibits activation of cellular motility proteins, including myosin light chain (MLC), cofilin, Hsp70, and the monomeric G proteins Rac1 and RhoA. The precise molecular mechanism(s) of IL-19 decrease in activation of these important proteins remains to be elucidated.

In contrast to its documented antiproliferative effects in VSMC, IL-19 exhibits proliferative, promigratory, and proangiogenic effects in vascular EC. Recombinant IL-19 treatment of EC *in vitro* results in activation of STAT3, Rac1, and MAPK p44/42 with consequent increases in EC proliferation, spreading, and migration. Confirming its proangiogenic potential, IL-19 promotes formation of endothelial cell tubes in isolated cultured mouse aortic rings and promotes formation of nascent blood vessels in subcutaneous gel plugs in mice [40]. These functions are independent of bFGF and VEGF expression and are IL-19-specific, as specific antibody to IL-20 receptor significantly reduces IL-19-driven EC migration [37]. The molecular basis of these intriguing and unexpected observations should uncover interesting distinctions between EC and VSMC processing of anti-inflammatory signals.

Heme oxygenase-1 (HO-1) has powerful anti-inflammatory and antiapoptotic effects and protects against vascular inflammation through multiple mechanisms including decreasing monocyte arterial transmigration, decreasing VSMC proliferation, and acting as a potent antioxidant [73, 74]. HO-1 is induced primarily at the transcriptional level by many proinflammatory mediators including cytokines, oxidative stress, and some growth factors [75]. IL-19 can induce expression of HO-1 mRNA and protein in cultured VSMC, but not EC [70], again, another interesting distinction in IL-19 cell-specific effects. Consistent with this finding, IL-19 can reduce peroxide-induced apoptosis and

growth-factor-induced reactive oxygen species (ROS) accumulation in VSMC. This reduction in ROS was abrogated when VSMCs were transfected with HO-1-specific siRNA prior to IL-19 treatment. *In vivo*, IL-19 can reduce TNF-α-induced ROS accumulation in murine coronary arteries [70]. While it has been shown that IL-10 can induce HO-1 in monocyte/macrophages [76], induction of HO-1 in vascular cells by any anti-inflammatory cytokine or T_h2 interleukin had not been reported. This provides a third potential molecular mechanism whereby IL-19 can reduce vascular inflammation and implicates IL-19 as a potential link between two powerful and protective systems, anti-inflammation and reduction of ROS.

In unpublished experiments, $LDLR^{-/-}$ mice fed an atherogenic diet and injected with as little as 1.0 ng/g/day of recombinant IL-19 demonstrated significantly less atherosclerotic plaque lesion area in the aortic arch compared with PBS-injected control mice [77]. These mice have decreased macrophage infiltrate into the atherosclerotic lesion. Interestingly, based on serum cytokine analysis, preliminary data suggest that these mice do not have an altered T_h1 /T_h2 balance. This is in contrast to IL-20, which is proatherosclerotic [78]. Further, weight gain as well as serum cholesterol and triglyceride levels is identical in IL-19-treated mice compared with PBS controls. This is an important distinction for IL-19, as several studies have shown an association between T_h1 /T_h2 balance with hypercholesterolemia [79, 80]. Together, these preliminary, but provocative, data suggest that IL-19 can decrease atherosclerosis in susceptible mice while neither affecting T_h1 /T_h2 balance nor suppressing serum lipid levels and place emphasis on vascular cells as the primary targets for IL-19. Future studies are necessary to determine the precise molecular and cellular mechanisms for IL-19-mediated decreases in vascular disease.

7. Summary, Conclusions, and Future Perspectives

The roles of cytokines in development of vascular inflammatory diseases such as atherosclerosis, restenosis, and coronary artery transplant vasculopathy are very complex. It is clear however that the functions of putative anti-inflammatory cytokines in these disease processes hold potential as therapeutics and require further study to characterize their precise mechanism(s) of action. Interleukin-19 is rather unique among interleukins, and its expression by resident vascular cells may represent an autoregulatory, autocrine, or paracrine mechanism to promote resolution of the vascular response to inflammatory insult. IL-19 is not detectible in naïve artery, but is induced in response to vascular injury and inflammation. Similarly, IL-19 is expressed in EC and VSMC when stimulated with inflammatory stimuli, and its addition to these cell types imparts anti-inflammatory effects, with decrease in ROS abundance, migration, proliferation, and expression of inflammatory genes. Function of IL-19 outside of the immune system implies that resident vascular cells may take on a T_h2 phenotype, and the pleiotropic mechanisms of IL-19 in vascular cells suggest that IL-19 may be a valuable anti-inflammatory therapeutic modality in acute vascular injury such as balloon angioplasty as well as more chronic vascular inflammatory diseases such as allograft vasculopathy and atherosclerosis.

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Review Article

Proinflammatory Effects of C-Peptide in Different Tissues

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Atherosclerosis is well known as an inflammatory disease that can lead to clinical complications such as heart attack or stroke. C-peptide as a cleavage product of proinsulin is in the last few decades known as an active peptide with a number of different effects on microvascular and macrovascular complications in type 2 diabetic patients. Patients with insulin resistance and early type 2 diabetes show elevated levels of C-peptide in blood. Several last findings demonstrated deposition of C-peptide in the vessel wall in ApoE-deficient mice and induction of local inflammation. Besides that, C-peptide has proliferative effects on human mesangial cells. This review discusses recently published proinflammatory effects of C-peptide in different tissues.

1. Structure of C-Peptide

C-peptide is a small peptide of 31 amino acids and short half-life of approximately 30 minutes. It has been identified by Steiner 1967 as a by-product of proinsulin and its main role was in assisting in the arrangement of the correct structure of insulin [1]. Proinsulin consists of an A chain, connecting peptide (C-peptide), and B chain. C-peptide has a central glycine-rich region which allows a correct positioning of A and B chains for insulin to achieve its tertiary structure [1]. It is secreted into the bloodstream in equimolar amounts together with insulin in response to glucose stimulation. C-peptide has been since a long time considered as an inactive peptide. However, over the last two decades, numerous studies revealed that C-peptide displays a physiological role in different cell types [2, 3]. C-terminal pentapeptide of C-peptide obtains the full activity of intact C-peptide in stimulating Na^+/K^+-ATPase [4]. Amino acid sequence of C-peptide is in different species relatively variable, although it has several conserved sequence like N-terminal acidic region, glycine-rich central segment, and C-terminal pentapeptide [5]. Binding of C-peptide was investigated by fluorescence correlation spectroscopy. The authors find C-peptide binding to the cell membranes of intact fibroblasts with the saturation at the physiological levels of C-peptide [6]. Although C-peptide receptor remains unknown, it has already been shown that C-peptide activates signaling pathways in different cell types. For example, it binds to pertussis-toxin-sensitive G-protein-coupled receptor on Swiss 3T3 fibroblasts [7] and activates p38 protein kinase pathway in mouse lung capillary endothelial cells [8, 9]. Effects of C-peptide have a positive influence on long-term complications in type 1 diabetic patients. C-peptide has an impact on diabetic neuropathy via improvements of endoneural blood flow and axonal swelling [10] or improves decreased blood flow in extremities. [11]. Several studies proposed direct role of endogenous insulin and C-peptide in improvement of endothelial dysfunction [12]. Moreover, C-peptide increases nitric oxide (NO) production through ERK1/2 MAP kinase-dependent up-regulation of endothelial nitric oxide synthase (eNOS) gene transcription [13].

The effects of C-peptide in type 2 diabetes and cell proliferation are controversial. The metabolic syndrome, prediabetes, and type 2 diabetes mellitus accelerate vascular disease and increase development of the disease [14].

2. Proinflammatory Effects of C-Peptide in the Vasculature

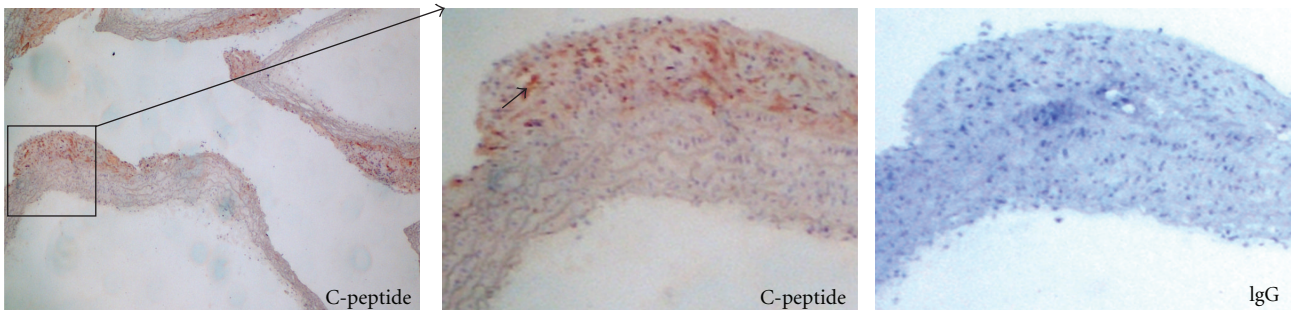
First reports about the C-peptide deposition in the vessel wall came from Marx et al., when they demonstrated deposition of C-peptide in the subendothelial space in

thoracic aorta in diabetic subjects [15]. In this study, it was found the C-peptide deposition in intima of the vessel wall in the thoracic aorta of diabetic subjects. From 21 subjects with deposition of C-peptide, 77% showed infiltration of monocytes/macrophages and 57% infiltration of $CD4^+$ lymphocytes [15]. In further studies, *in vitro* migration assays reported that C-peptide induces migration of $CD4^+$ lymphocytes and monocytes/macrophages in a concentration-dependent manner. These effects were similar to those induced by monocyte chemokine MCP-1 or T-lymphocyte chemokine RANTES. Checkerboard analysis in the same study shows that C-peptide induces chemotaxis rather than chemokinesis with maximal effect that correspond to physiological concentrations of C-peptide (1 nmol/L) [15, 16]. C-peptide mediates its chemotactic activity in $CD4^+$ lymphocytes and in monocytes through an as of yet unidentified pertussis toxin-sensitive G-protein coupled receptor and stimulates specific intracellular signaling pathways in these cells [17]. C-peptide stimulates similar signaling pathways in different cell types. For example, Na^+/K^+ATPase [4, 18], ERK1/2 MAP kinase, and PI-3 kinase [9, 16, 19, 20]. Aleksic et al. revealed that activation of PI-3 kinaseγ induced by supraphysiological concentrations (10 nmol/L) of C-peptide leads to an activation of Rho GTPases. Rho, Rac1, and Cdc42 are small GTP-binding proteins with GTPase activity. Activation of Src-kinase and RhoA, Rac-1, and Cdc42 GTPases act via PAKs (p21 activated kinase) and stimulate LIMK (LIM domain-containing protein kinase), which phosphorylates and inhibits cofilin. This leads to increased accumulation of polymerized actin at the leading edge of cells. RhoA stimulates MLC (myosin light chain) phosphorylation via ROCK (Rho kinase) activation which is important for cell body contraction and migration [17]. C-peptide positively controls the expression of the PPARγ-regulated CD36 scavenger receptor in human THP-1 monocytes. Its stimulates PPARγ activity in a ligand-independent fashion and this effect is mediated by PI-3 kinase [21].

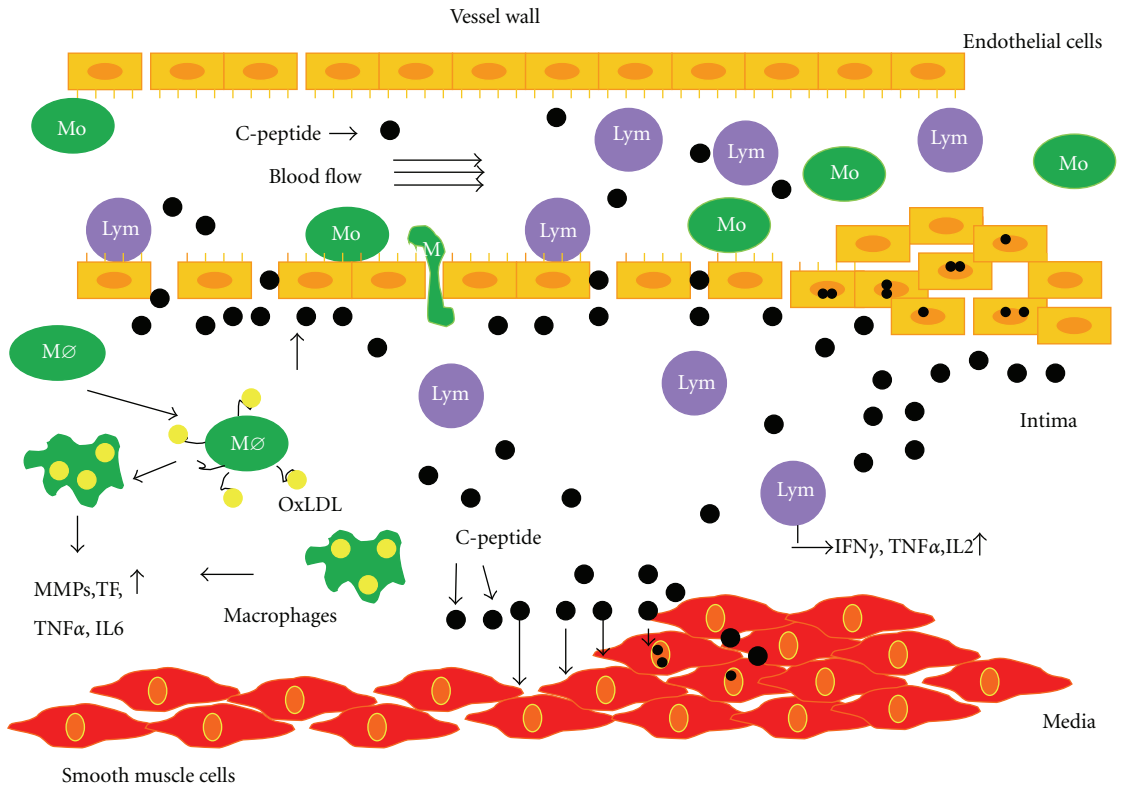
Further, effects of C-peptide on smooth muscle cell proliferation have been investigated. Walcher et al. showed that high levels (10 nmol/L) of C-peptide induces proliferation of human and rat smooth muscle cells in concentration-dependent manner assessed by Ki-67 assay and 3[H] Thymidin assay. Extent of proliferation was similar to those induced by platelet-derived growth factor (PDGF) [19]. In addition, C-peptide induces phosphorylation of protein tyrosine kinase (Src) and PI-3 kinase. Further, it induces activation of specific ERK1/2 MAP kinase [19]. VSMC proliferation by extracellular stimuli takes place in mid-to-late G_1 phase of the cell cycle, where D-type cyclins promote G_1- to S-phase transition by leading to Rb phosphorylation [22]. Walcher et al. showed that C-peptide increases cyclin D1 expression and Rb phosphorylation that suggests that C-peptide acts via similar signaling pathways [19]. In another study, Insulin cannot alter endothelial cell (EC) proliferation or migration, where 10 nmol/L C-peptide stimulates EC proliferation by 40% [23]. Proliferation effects of C-peptide have been shown in different cell types, for example, like endothelial cells, HEK293 cells, and chondrocytes. Lindhal et al. found that C-peptide stimulates rRNA synthesis and induces expression of 47S in HCS-2/8 chondrocytes derived from a human chondrosarcoma. This regulation of ribosomal RNA provides amechanism by which C-peptide can apply its transcriptional effects and its growth factor activity [24].

Summarizing these results our group tested initial hypothesis show on in the Figure 1(b). Patients with early diabetes type 2 and insulin resistance show increased levels of C-peptide in blood. Together with increased endothelial dysfunction, this leads to deposition of C-peptide in the intima of the vessel wall. According to the *in vitro* results, C-peptide may have chemotactic effect on the inflammatory cells involved in the onset of the atherosclerosis, like monocytes/macrophages and $CD4^+$ lymphocytes. Further, C-peptide has an effect on the proliferation of smooth muscle cells in the media. These cells migrate into developing atheroma and together with inflammatory cell recruitment represent initial step in the developing of atherosclerosis.

To test the hypothesis in an animal model, we used ApoE deficient mice. The animals were divided into two groups. C-peptide group numbered 18, and placebo 17 mice per group [25]. Subcutaneous injections (200 nmol/injection) of dissolved peptide increased blood C-peptide levels 5 to 6 folds compared to basal levels (12.9 ± 1.8 nmol/L compared with 2.7 ± 0.8 nmol/L; C-peptide versus placebo; $P < 0.05$). At the same time, mice were put on the Western type diet to trigger atherosclerosis. C-peptide deposition was found in the vessel wall of aortic arch and in early atherosclerotic lesions (Figure 1(a)). Computer-assisted image quantification revealed significantly higher deposition of C-peptide in treated mice, compared to placebo one (2.1 ± 0.4 versus 0.8 ± 0.1% positive area; $P < 0.01$) treated with water. Similar results were obtained in the aortic root (data not shown). This deposition of C-peptide was followed with increased local inflammation in aortic arch. After immunohistochemical staining, computer-assisted image quantification showed increased infiltration of monocytes/macrophages in the vessel wall. Further, we know that diabetes accelerates smooth muscle cell proliferation in atherosclerotic lesions and that it correlates with insulin levels [26]. Smooth muscle cells and their secreted products are the main components of advanced atherosclerotic lesions [27]. Staining of aortic arch in ApoE–deficient mice for α-actin showed increased content of smooth muscle cells in C-peptide-treated group (C-peptide versus placebo; 4.8 ± 0.6 versus 2.4 ± 0.7% positive area; $P < 0.01$) as well as a trend towards more Ki-67 proliferated cells in C-peptide treated group [25]. Analysis of lipid deposition in placebo and C-peptide treated mice revealed increased deposition of lipids stained with Oil-red-O in C-peptide-treated mice compared to placebo. Lipid deposition in *en face* preparations of the abdominal and thoracic aorta in C-peptide-treated mice did not reach statistical significance compared to placebo mice (C-peptide versus placebo; 5.64 ± 0.69% versus 3.98 ± 0.5% $P = 0.07$) [25]. Proinflammatory effects of C-peptide were obtained in the ApoE-deficient animals on top of a high cholesterol diet. Effects of high cholesterol diet can



(a)



(b)

Figure 1: C-peptide deposits in mouse aortic arch. Red areas represent C-peptide deposition indicated by arrow on the high power view. Adjacent sections represent negative control and show no immunoreactive C-peptide areas (Figure 1(a)). Lower panel (Figure 1(b)) illustrates potential hypothesis about C-peptide effects in vessel wall. Patients with insulin resistance and type 2 diabetes show increased levels of C-peptide in the blood. Together with endothelial dysfunction and increased endothelial permeability C-peptide deposits in the intima of the vessel wall and from there induces recruitment of inflammatory cells and their migration into the subendothelial layer. C-peptide deposits also in the media and has an effect on the proliferation of smooth muscle cells.

partly cover the proinflammatory effects of C-peptide in this model.

Our study revealed no differences in E-selectin and ICAM-1 levels as well as levels of the inflammatory markers such as TNFα and soluble IL-6, that is, in contrast to several findings where C-peptide has anti-inflammatory effects and reduces upregulation of cell adhesion molecules under inflammatory conditions [28, 29]. C-peptide is nowadays recognized as an active peptide with various effects. Further work is needed to identify C-peptide receptor and elucidate mechanisms by which it modulates cell signaling in different cell types. Different effects in type 1 and 2 diabetes seem to be tissue and cell specific.

3. Proinflammatory Effects of C-peptide in Kidneys

We already know that C-peptide administration in replacement dose given to diabetic rats limits or prevents glomerular hypertrophy and mesangial matrix expression [30]. In several further studies, C-peptide reduces glomerular hyperfiltration, hypertrophy, and proteinuria [31–33]. Lower

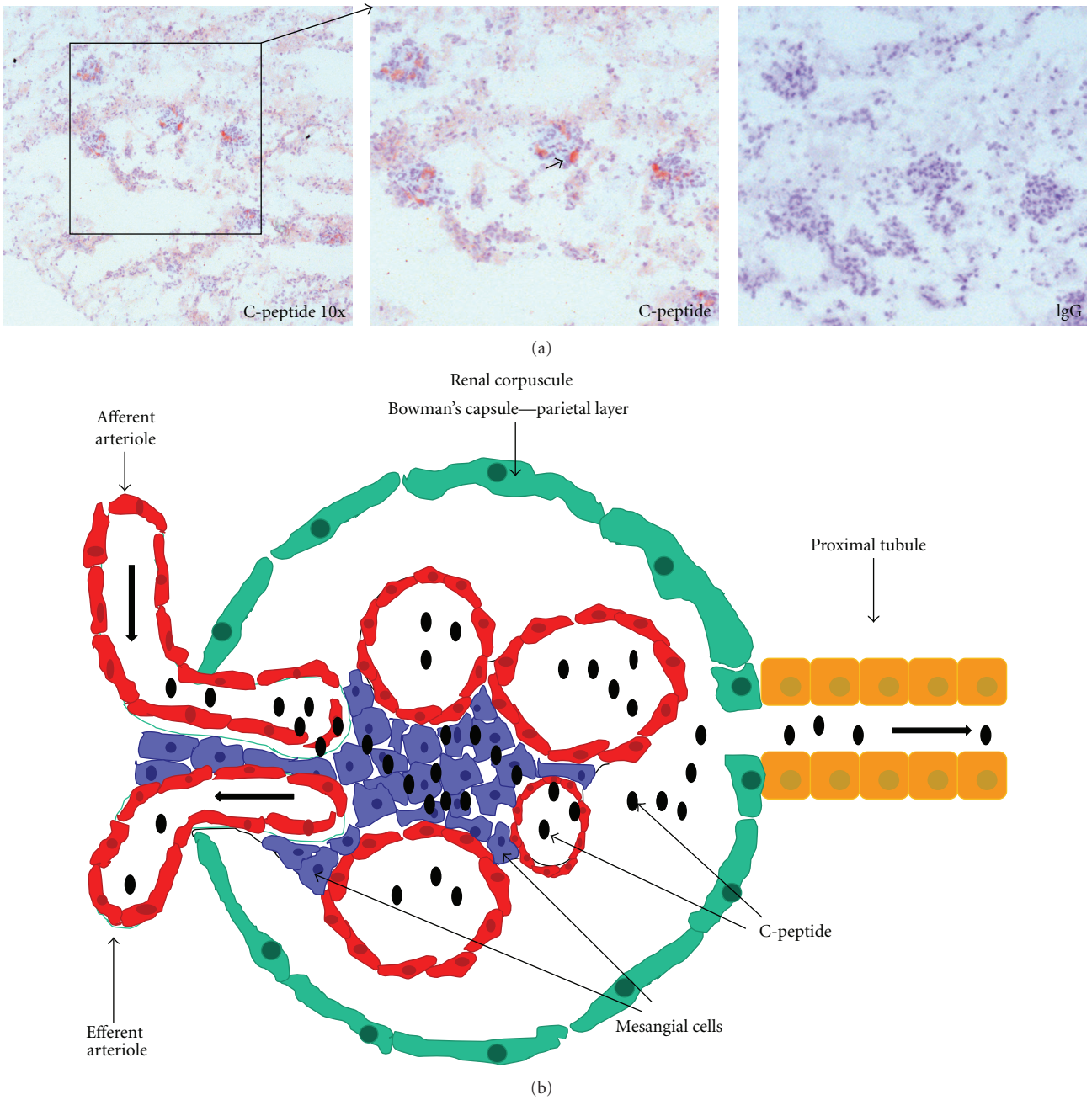


Figure 2: C-peptide deposition in mouse juxtaglomerular apparatus. Upper panel: Representative photograph of mouse kidney sections with C-peptide deposition in the glomeruli in C-peptide treated mice (Figure 2(a)). Red areas represented C-peptide deposition indicated by arrow. On the lower panel is schematic explained the way C-peptide induces proliferation of mesangial cells suggesting a possible role of C-peptide in glomerular hyperproliferation in patients with diabetic nephropathy (Figure 2(b)).

C-peptide levels are connected with increased albuminuria, retinopathy, and nephropathy [34] whereas other studies did not show relation between C-peptide levels and microangiopathic diabetic complications [20, 35]. In our previous work, we demonstrate that C-peptide exhibits mitogenic activity on human mesangial cells (MCs). High levels of C-peptide (10 nmol/L) induce proliferation of kidney human mesangial cells in a concentration-dependent manner assessed by Ki-67 assay with maximal induction of 2.6 ± 0.4 folds. Further, pretreatment of cells with inhibitors PP2 (Src kinase inhibitor) or PD98059 (MEK 1 inhibitor) decreases C-peptide-induced human mesangial cell (MC) proliferation. As well, pretreatment of cells with PI-3 kinase inhibitor wortmannin also reduces human MC proliferation. These results suggest the involvement of Src-kinase, ERK1/2 MAP kinase, and PI-3 kinase as downstream elements of the signaling pathway. We further investigated the activation of signaling pathways involved in C-peptide-induced proliferation of mesangial cells. C-peptide activates phosphorylation of Src that leads to activation of PI-3 kinase and involvement of ERK1/2 MAP

kinase. High-concentration C-peptide (10 nmol/L) increases phosphorylation of ERK1/2 MAP kinase in human MCs in a time-dependent manner with a maximal effect after 10 minutes. Cyclin D activates cyclin-dependent kinase 4 (CDK4) during G1 phase that leads to phosphorylation of retinoblastoma tumor suppressor protein (pRb) [36]. C-peptide stimulation increases activation of cyclin D1 and phosphorylation of Rb suggesting that C-peptide-induced proliferation may use similar signaling pathways. These results are in agreement with *in vitro* data of swiss 3T3 fibroblasts, where C-peptide has been shown to activate PI-3 kinase [7]. Serum levels of C-peptide are associated with the metabolic syndrome in patients with type 2 diabetes and in diabetic patients with nephropathy and vascular disease [37]. C-peptide is eliminated from the body by kidneys [38]. In the period of insulin resistance and early type 2 diabetes increased levels of C-peptide are circulating through glomeruli and could deposit in juxtaglomerular apparatus and from there could demonstrate its mitogenetic effect on mesangial cells.

The ApoE-deficient mouse model is a conventional model for investigating atherosclerosis. These mice have greatly increased plasma lipid levels [39]. Appearance of atherosclerosis is similar to those in humans induced by ApoE deficiency called type III hyperlipoproteinemia [40]. It is known that ApoE-deficient mice with increased hyperlipidemia demonstrates glomerular injury characterized by glomerular endothelial cell activation and macrophage recruitment [41]. Elevated levels of albumin in urine serve as clinical predictors of diabetic nephropathy [42]. Apolipoprotein E modulates human mesangial cell proliferation depending on the length of stimulation and cell conditions [43]. It has been shown that mice with increased hyperlipidemia in plasma have an increased progression of renal disease [44].

Assuming *in vitro* effects of C-peptide on human MCs, we further investigated deposition of C-peptide in mouse juxtaglomerular apparatus. Longitudinal sections of mouse kidneys were stained for C-peptide. Red areas in mouse glomeruli demonstrate C-peptide deposition (Figure 2). Quantitative analysis of C-peptide deposition in mouse glomeruli of ApoE-deficient mice determined increased deposition of C-peptide in glomeruli in C-peptide treated mice compared with placebo (unpublished data). Our previous work illustrated that C-peptide induces proliferation of mesangial cells, deposition in the intima and media of the vessel wall in diabetic patients, and C-peptide-induced proinflammatory effects in vascular cells. This resulted in increased C-peptide deposition in juxtaglomerular apparatus in C-peptide-treated mice. Still, the relevance of these results to human atherosclerosis or diabetic nephropathy remains to be determined.

4. Conclusion

In this review, we explained several proinflammatory effects of C-peptide on the inflammatory cells in the vessel wall and its mitogenic effects on the smooth muscle cells. Based on the previous results, we demonstrated that C-peptide deposits in the vessel wall in ApoE-deficient mice and induces local inflammation that leads to increased lipid deposition in aortic arch and increased proliferation of smooth muscle cells, crucial processes in the onset of atherosclerosis. Further, we explained an effect of C-peptide on the mesangial cell proliferation that involves Src kinase, PI-3 kinase, and ERK1/2 MAP kinase, and for the first time the deposition of C-peptide in mouse kidney juxtaglomerular apparatus. These results raise the hypothesis that C-peptide may have a role in glomerular hyperproliferation in patients with diabetic nephropathy.

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Review Article

Biomarkers of Chronic Inflammatory State in Uremia and Cardiovascular Disease

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Cardiovascular disease is the leading cause of death in the general population; traditional risk factors seem inadequate to explain completely the remarkable prevalence of cardiovascular mortality and morbidity observed in the uremic population. A role for chronic inflammation has been well established in the development of atherosclerotic disease, and, on the basis of these observations, atherosclerosis might be considered an inflammatory disease. Inflammation has been implicated in the etiology of coronary artery disease in the general population, and traditional inflammatory biomarkers such as C-reactive protein (CRP) and interleukin-6 (IL-6) have been shown to predict cardiovascular events in both symptomatic and asymptomatic individuals as well as those in the uremic population. Later on, new nontraditional markers were related to the risk of cardiovascular morbidity and mortality in general and in uremic population. As a consequence of the expanding research base and availability of assays, the number of inflammatory marker tests ordered by clinicians for cardiovascular disease (CVD) risk prediction has grown rapidly and several commercial assays have become available. So, up to now we can consider that several new nontraditional markers as CD40-CD40 ligand system and pentraxin-3 seem to be significant features of cardiovascular disease in general and in ESRD population.

1. Introduction

Patients with end-stage kidney disease undergoing chronic hemodialysis (HD) present higher mortality rates compared with the general population. Once patients are on HD, the risk of cardiovascular death is approximately 30 times higher than that in the general population and remains 10–20 times higher after stratification for age, gender, and the presence of diabetes. About half of the deaths of patients on dialysis are attributed to cardiovascular causes including coronary heart disease, cerebrovascular disease, peripheral vascular disease, and heart failure.

End-stage renal disease (ESRD) patients suffer from a state of chronic inflammation leading to cardiovascular complications, progressive malnutrition, and death [1, 2]. Inflammation is subclinical, and chronic disorders of the cytokine system or acute-phase proteins may be observed as the sole evidence of a proinflammatory disorder.

According to this hypothesis traditional inflammatory biomarkers such as tumor necrosis factor-alfa (TNF-alfa), C-reactive protein (CRP), and interleukin-6 (IL-6) have been shown to predict cardiovascular events in both symptomatic and asymptomatic individuals as well as those in the uremic population. More recently, several new nontraditional biomarkers have been introduced in the clinical practice.

2. Traditional Biomarkers of Chronic Inflammation

Low-grade chronic inflammation, as indicated by levels of high-sensitivity C-reactive protein (hs-CRP), prospectively defines the risk of atherosclerotic complications, adding to the prognostic information provided by traditional risk factors. The study of Ridker et al. [3] provides convincing evidence that, in apparently healthy subjects, baseline

serum levels of hs-CRP are predictive of future myocardial infarction and ischemic stroke. Subsequent meta-analysis of prospective population-based studies has compared patients in the lower tertile of hs-CRP with those in the upper tertile [4, 5]. With a good consistency between studies, a higher risk for major coronary events was observed for the upper tertile with the lowest tertile used as a reference. In general population most studies showed a dose-response relationship between the level of hs-CRP and risk of incident coronary disease. Recent papers also suggest association with incidence of sudden death [6, 7] and peripheral arterial disease [8]. Through stratification or multivariable statistical adjustment, hs-CRP retains an independent association with incident coronary events after adjusting for age, total cholesterol, HDL cholesterol, smoking, body mass index, diabetes, history of hypertension, exercise level, and family history of coronary disease [9, 10]. In terms of prediction of recurrent CVD events and death, the strongest association with prognosis has been with hs-CRP; hs-CRP consistently predicts new coronary events in patients with unstable angina and acute myocardial infarction [11–20].

As elevated serum levels of hs-CRP have been shown to be such a strong predictor of cardiovascular mortality in the general population, available data suggest that the association between inflammation and atherosclerosis is particularly strong in uremic patients [21, 22]. Zimmermann et al. [2] reported that chronic inflammation enhances cardiovascular risk and mortality; a few years later Ikizler et al. [23] in a prospective study assessed the importance of hs-CRP values as independent determination of hospitalization in chronic hemodialysis (HD) patients.

Recently, it has been shown that proinflammatory cytokines such as IL-6 may exert a direct inflammatory effect on the heart and peripheral circulation [24]. In a previous published paper, we investigated the joint predictive power of CRP and IL-6, in order to ascertain what is the prognostic information that each index carries independently of the other. To this aim, IL-6 and CRP plasma levels were measured in a cohort of 218 ESRD patients from different centres over a 4-year followup. Main outcomes were cardiovascular and total mortality. This study showed that plasma IL-6 rather than CRP better predicts outcome in ERSD patients. Various possible explanations may underline the advantage of IL-6 over CRP as an outcome predictor. One possibility is that, being located upstream in the cascade of events which lead to the synthesis of many acute-phase reactants, IL-6 is a better marker of the inflammatory burden affecting the development of cardiovascular disease. Another possibility is that levels of IL-6 vary less than those of CRP, leading to a more accurate classification of patients at risk when one single sample is taken. Finally, the toxic effects of IL-6 on the heart and peripheral vasculature might be stronger than those of CRP [24]. This study provides some important implications. First, it gives further support to the hypothesis about the role of inflammatory mediators in the genesis of cardiovascular disease in dialysis patients [25–27]. Secondly, it provides evidence suggesting the use of IL-6 in addition to, or even in place of, CRP for the identification of patients at risk.

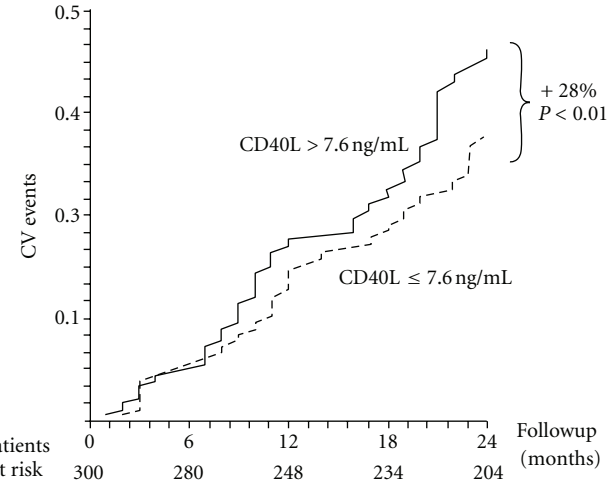

Figure 1: Prognostic value of CD40L in the RISCAVID population.

Zhang et al. [28] reported that there was no association between CRP haplotypes and cardiovascular outcome in dialysis patients; this study argues against CRP as a cardiovascular risk factor. On the other hand, because variations within the IL-6 gene were shown to affect the risk for CVD in a multiethnic dialysis cohort [29], this suggests that IL-6 should be the target for interventional studies.

According to these data, we suggest that all traditional risk factors for death should be measured accurately in uremic patients. Clinical events should be identified prospectively, and, whenever possible, IL-6 levels should be measured repeatedly during the course of followup.

TNF-alfa, a proinflammatory cytokine (17 kDa) originally associated with killing of tumor cells, has a pivotal role in regulating both pro- and anti-inflammatory mediators. TNF-alfa has been regarded a "master regulator" of the cytokine cascade that provides a rapid form of host defense against infection but is fatal in excess. TNF-alfa is highly multifunctional with effects on lipid metabolism, coagulation, insulin resistance, and endothelial dysfunction. The major cellular origin of TNF-alfa, previously known as cachectin, is activated macrophages. It should be noted that, whereas IL-6 is strongly associated with CRP and other inflammatory biomarkers, the association between TNF-alfa and CRP is rather weak. This suggests that circulating levels may be influenced by a number of different factors and that circulating TNF-alfa levels may not reflect biologic activity at the tissue levels. Although available evidence suggests upregulated TNF-alfa system activity in ESRD patients [30], data linking elevated circulating TNF-alfa levels to CVD and mortality have not been as clear as for IL-6.

3. Nontraditional Biomarkers of Chronic Inflammation

It is now generally accepted that CD40-CD40 ligand interaction is a main determinant of the proatherogenic phenotype [31]. Originally identified in B and T lymphocytes as being

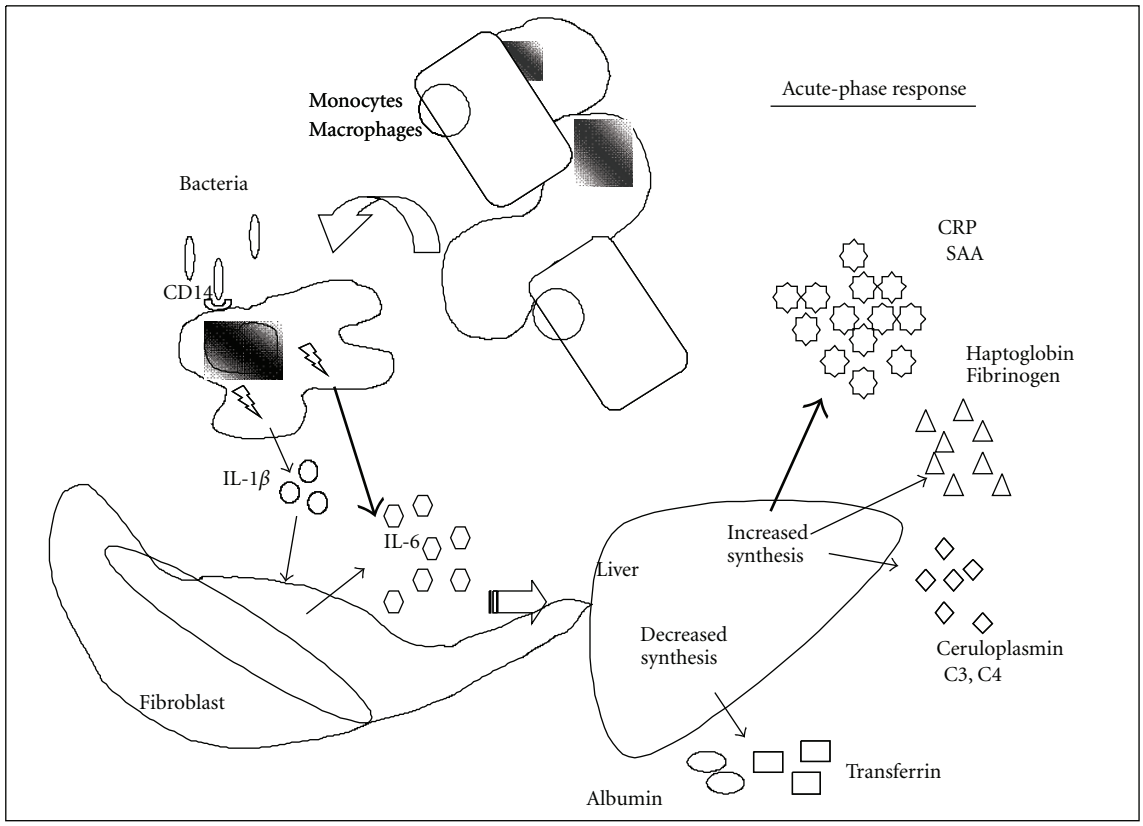

Figure 2: Acute phase of inflammation.

involved in T-cell-dependent B-cell activation and differentiation, the CD40-CD40 ligand system has been implicated in the pathophysiology of several chronic inflammatory diseases including risk factor-related vascular damage [32]. CD40, a 50 kDa integral membrane protein of the tumor necrosis factor receptor family, and its cognate agonist CD40 ligand also known as CD154, a transmembrane 39-kDalton protein structurally related to tumor necrosis factor-alpha, are coexpressed by several cells of the vasculature, including endothelial cells, smooth muscle cells, and macrophages [31]. CD40 ligand also occurs in a soluble form (sCD40L) that is considered to possess a full biological activity [33]. Increased sCD40L levels have been described in obesity [34], hypercholesterolemia [35], diabetes [36, 37], and unstable angina [38]. Furthermore, it has been recently reported that circulating sCD40L has a strong independent prognostic value among apparently healthy individuals [39] and patients with acute coronary syndromes [40] and represents an independent predictor of restenosis after percutaneous transluminal angioplasty [41]. Thus, the clinical association between soluble CD40L and cardiovascular events suggests that soluble CD40L function spans the time interval from early atherogenesis to late thrombotic complications.

According to this, Hocher et al. [42] recently demonstrated during a follow-up period of 52 months that sCD40L is an independent predictor of atherothrombotic events in patients on HD. More recently we expanded on this topic demonstrating that the prognostic value of sCD40L is evident also in over 200 chronic HD patients from the RISCAVID population at 24-month followup [43] (RISCAVID, "risk cardiovascular in dialysis" is a prospective observational study performed on a large HD population in the northwestern region of Tuscany, Italy) (Figure 1).

In this paper we were able to demonstrate that this prognostic value of sCD40L is already evident at 24 months followup thus reinforcing the strong link between sCD40L and clinical outcomes in patients in HD and suggesting a possible clinical use of this new promising biomarker to better define cardiovascular prognosis in these patients. The striking prognostic impact of sCD40L on the clinical course in patients in HD raises questions about the origin of this biomarker. Platelets represent the main source of circulating sCD40L in patients with acute coronary syndrome [38] and in hypercholesterolemia [35]. Accordingly, plasma levels of sCD40L correlate closely with markers of platelet activation in these patient populations [35, 38]. Thus, increased circulating levels of sCD40L might reflect an enhanced platelet activation in HD. According to this, it has been demonstrated that circulating activated platelets (P-selectin/CD63-positive platelets) are higher in HD patients than in controls and further increase during HD sessions [44]. Potential causes of such activation include possible stimulation of platelets by proinflammatory cytokines that have been reported to be increased in patients with end-stage renal disease [45]. Furthermore, the increased lipid peroxidation that has been found in patients with chronic renal failure might also

participate in activating platelets [46]. On the other hand, the lack of any correlation between circulating levels of sCD40L and CRP seems to exclude a role for this platelet-activating inflammatory biomarker [47] in the enhanced sCD40L signaling observed in our study population.

Pentraxin is a family of proteins considered to be markers of the acute-phase inflammation [48, 49] (Figure 2). Currently, the pentraxin protein family is divided into two subfamilies based on size: the classical "short" pentraxin (25 kDa) and the "long" pentraxin (40–50 kDa). Pentraxin 3 (PTX3) is a "long" pentraxin that is highly expressed in the heart, whereas C-reactive protein (CRP) is a "short" pentraxin and is produced from the liver [50]. PTX-3 expression occurs in a variety of cell types, including endothelial cells, mononuclear phagocytes, dendritic cells, smooth muscle cells, fibroblasts, adipocytes, and epithelial cells in response to inflammatory cytokines and Toll-like receptor engagement [51–53]. In several recent studies [54, 55] PTX3 appeared to be not only an early indicator of irreversible myocyte injury but also a prognostic marker in patients with acute myocardial infarction. Latini et al. [56] reported the acute-phase protein PTX3 as a predictor of 3-month mortality after adjustment for major risk factors and other acute-phase prognostic markers. In a recently published paper of Barbui et al. [57], the role of PTX3 as a prognostic biomarker was shown by an increased serum PTX3 that was closely related to death due to MI, in-hospital or to 6 months, in ACS patients, including STEMI, NSTEMI, and UAP groups. More recently, Suliman et al. [58] analyzed plasma PTX-3 concentrations in relation to comorbidities (Davies score), protein-energy wasting (PEW), and inflammation markers in 200 prevalent HD patients, aged 64 ± 14 years, who had been on HD treatment for a median period of 36 months. Survival (42 months) was analyzed in relation to PTX-3 levels (high PTX-3 tertile versus two lower tertiles). This study shows that high levels of PTX-3 were found in prevalent HD patients with CVD and PEW; furthermore, a powerful association of PTX-3 with comorbidities was founded. As PTX-3 predicts mortality independent of age and comorbidities in prevalent HD patients, further designed studies addressing the clinical implication and pathogenic mechanisms of this long pentraxin are warranted.

4. Conclusions

Although the successful introduction of dialysis in the 1960s has increased life expectancy in patients with ESRD, the mortality rate is still unacceptably high, due primary to a process of inflammation-associated accelerated atherosclerosis. The accelerated atherosclerotic process of ESRD may involve several interrelated processes, such as oxidative stress, endothelial dysfunction, vascular calcification, and inflammation. The explosion of new knowledge on the central role of a dysregulated cytokine and Th system activity has opened new and exciting opportunities for nephrologists to manage and prevent CVD and wasting in this diseased patient group. The use of several traditional and new biomarkers of inflammatory and cardiovascular risk is of great utility in this high-risk population.

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