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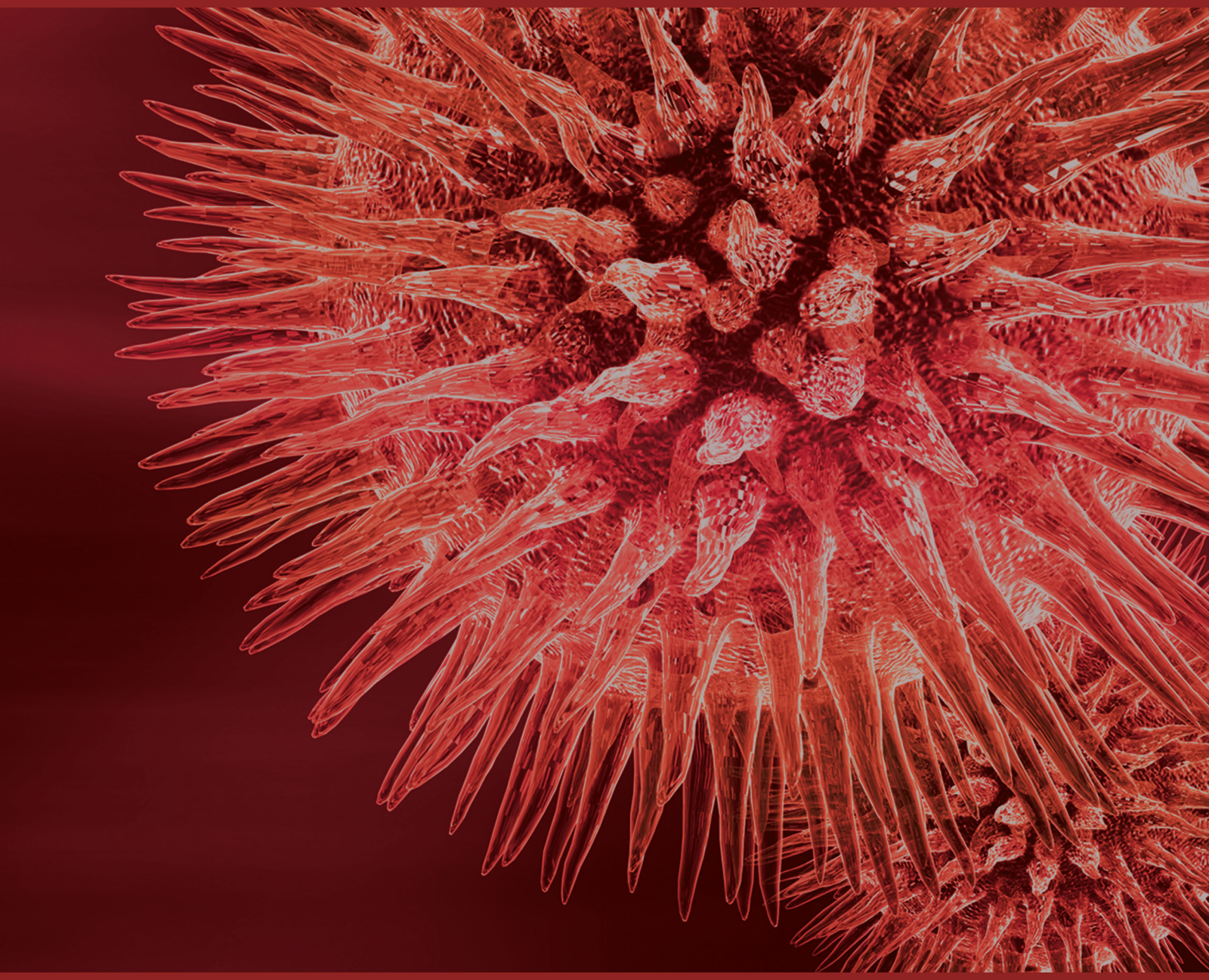
Proteoglycans/Glycosaminoglycans: From Basic Research to Clinical Practice

Guest Editors: George Tzanakakis, Ilona Kovalszky, Paraskevi Heldin, and Dragana Nikitovic



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Editorial

Proteoglycans/Glycosaminoglycans: From Basic Research to Clinical Practice

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Extracellular matrices (ECM) represent a complex network of proteins and glycosaminoglycans (GAGs) constituting the cell microenvironment including elastin, laminins, fibronectin, and GAG decorated proteoglycans (PGs). PGs are composed of independent structural domains, the sequences and arrangements of which are highly conserved and discretely glycosylated, thus determining a varying degree of matrices organization. On the other hand, both bound GAG chains and free GAGs such as hyaluronan (HA) bestow voluminosity to the ECM, due to negative charges they carry and their subsequent water binding ability. Therefore these molecules participate in maintaining the bulk, shape, and strength of tissues in vivo. However, PGs/GAGs provide much more than just mechanical and structural support but are critically important for cell growth, survival, and differentiation [1, 2]. Moreover, these molecules are key participants of various disease processes including inflammation, atherosclerosis, autoimmune diseases, and cancer [3–5]. Importantly, GAG/PG effects are clearly dependent on the specific correlation among their abundance, distribution, and disease type/stage. The evidence that PGs/GAGs have a key role in various pathological conditions has led to the conclusion that understanding the changes in PG/GAG expression and fine structure that occur in disease may lead to opportunities to develop innovative and selective therapies. We invited authors to submit original research and review articles that seek to designate discrete roles for GAGs/PGs as diagnostic markers and/or therapy targets for specific disease types and grades. Therefore, the submissions for this special issue focused on the utilization of pluripotent characteristics of PGs/GAGs and their synthesizing and/or degrading enzymes in the ongoing battle against disease.

The topics discussed in depth by experts in the field and published in this special issue include the following: mechanisms of GAG/PG action and their potential application, development of novel disease markers, roles of PGs/GAGs in wound healing (therapeutical aspects), development of immunotherapeutic strategies involving GAGs/PGs, roles of PGs/GAGs in tissue engineering, potential application of PGs/GAGs in axon regeneration, roles of PGs/GAGs in cartilage regeneration, synthesis of reliable carriers specifically designed to deliver discrete GAGs/PGs to target disease tissues, utilization of GAGs/PGs as carriers for targeted therapy delivery.

We hope that readers will find that this special issue highlights the important advances that are presently being accomplished in the preclinical and clinical applications in the rapidly growing PG/GAG field. Hopefully, the highlighted advances will encourage the application of novel drug development for elevating disease states.

George Tzanakakis
Ilona Kovalszky
Paraskevi Heldin
Dragana Nikitovic

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Review Article

Cysteine Cathepsin Activity Regulation by Glycosaminoglycans

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Cysteine cathepsins are a group of enzymes normally found in the endolysosomes where they are primarily involved in intracellular protein turnover but also have a critical role in MHC II-mediated antigen processing and presentation. However, in a number of pathologies cysteine cathepsins were found to be heavily upregulated and secreted into extracellular milieu, where they were found to degrade a number of extracellular proteins. A major role in modulating cathepsin activities play glycosaminoglycans, which were found not only to facilitate their autocatalytic activation including at neutral pH, but also to critically modulate their activities such as in the case of the collagenolytic activity of cathepsin K. The interaction between cathepsins and glycosaminoglycans will be discussed in more detail.

1. Introduction

Cysteine cathepsins are members of the papain-like cysteine peptidase family [1]. Despite the fact that the eleven cysteine cathepsins found in man [2] represent only a small fraction of the human proteolytic repertoire, these enzymes have been attracting a lot of attention for their diverse roles in physiological and pathological processes that range from nonspecific protein turnover within the endolysosomal pathway to highly specialized functions in tissue homeostasis. A number of excellent reviews have been published recently, summarizing the structural and functional characteristics of cysteine cathepsins in health and disease [3–5].

All the cathepsins share the same structural scaffold, also called the papain-like fold. The structure consists of two subdomains which have been termed the L- and R-domains referring to their position when the molecule is shown in the standard orientation (Figure 1). The active site cleft is at the top of the molecule between the L- and R-domains and contains the conserved catalytic dyad Cys-His (marked by yellow and blue spheres in Figure 1, resp.). In general, papain-like peptidases can act as endo- or exopeptidases. In a typical endopeptidase the primary specificity determinant is the S2 site [6] and well-determined sites on the enzyme interact with residues P3 through P2′ of the substrate [7]. Five of the eleven human members of the family (cathepsins F, K, L, S, and V) are exclusively endopeptidases, cathepsin B is also a peptidyl dipeptidase, cathepsin X is a carboxypeptidase, cathepsin H is an aminopeptidase, and cathepsin C is a dipeptidyl peptidase. The proteolytic activity of the remaining two members, cathepsins O and W, remains to be determined [4]. Most cysteine cathepsins are ubiquitously expressed in the human body, while some (cathepsins K, S, V, and W) are expressed in more restricted patterns [3]. Cathepsin K is abundantly expressed in osteoclasts and synovial fibroblasts [8, 9] but is also found in other cells of the hematopoietic, epithelial, and fibroblast lineages [10]. Highest expression levels of cathepsin S are found in antigen-presenting cells [11], cathepsin V is expressed predominantly in thymus and testis [12], and the expression of cathepsin W is restricted to CD8+ lymphocytes and natural killer cells [13].

2. Regulation of Cysteine Cathepsins Activity

Zymogen activation is one of the major means of regulations of cathepsin activity. All the cathepsins are namely

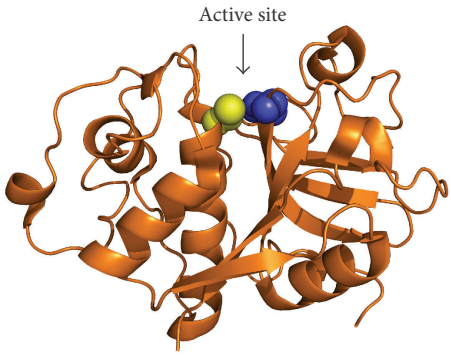

Figure 1: The papain-like peptidase fold illustrated on the crystal structure of papain. The protein is shown in cartoon representation and the position of the active site cleft is marked by an arrow. Catalytic residues Cys and His are shown as yellow and blue spheres, respectively. Coordinates were obtained from the Protein Data Bank under accession code 1PPN. The image was created with PyMOL (Schrödinger, LLC, Portland, OR, USA).

synthesized as inactive zymogens and activated in the acidic milieu of the endolysosomal vesicles. The molecular mechanism of their activation was puzzling for a long time. The critical information came from the combination of structural studies of procathepsins B, K, and L, which showed that the propeptide runs through the active site of cathepsins in the opposite direction of the substrate, thus excluding the cleavage of the propeptide in the molecule without enormous and energetically unfavorable structural movements of the propeptide [15–19], thereby eliminating the unimolecular mechanism initially suggested, and detailed kinetic studies, which clearly demonstrated that the activation of cathepsin B is a bimolecular process [20]. The current model, which is mostly based on the cathepsin B studies, suggests that the propeptide in the cathepsin zymogen switches between two conformations, the so-called "closed" and "open." In the "closed" conformation, favored at neutral to slightly acidic pH, the propeptide blocks the active site and prevents substrate hydrolysis, whereas, in the "open" form, favored at acidic pH below pH 5.0, the propeptide is removed from the active side cleft, resulting in a low catalytic activity of the zymogen. This activity is sufficient to activate another cathepsin zymogen in one or several steps, thereby starting the chain reaction, where such fully active mature cathepsin B processes the majority of zymogen molecules [21].

The other major regulators of cysteine cathepsins are macromolecular inhibitors that bind into the active site and thereby prevent association of the peptidase with its substrate. They belong to several distinct families including the cystatins, the thyropins, and the serpins that can, in addition to serine proteases, also inhibit several cathepsins [3, 22–25].

3. Glycosaminoglycans as Major Regulators of Cysteine Cathepsin Activity

Glycosaminoglycans (GAGs) are heteropolysaccharides composed of repeating disaccharide units with a high negative charge. This is a result of the presence of multiple carboxyl groups and sulfate substitutions. Most of GAGs are sulfated, including chondroitin sulfates (CS), keratan sulfate (KS), dermatan sulfate (DS), heparan sulfate (HS), and heparin, whereas hyaluronan (HA) is the only nonsulfated GAG. In recent years GAGs have been emerging as important regulators of cysteine cathepsins with diverse effects on their targets. Traditionally, cysteine cathepsins had been viewed as lysosomal proteases and, like other lysosomal enzymes, had been known to be inhibited by intralysosomal GAGs in the resting lysosome [26]. Today, however, cysteine cathepsins are established as major players in extracellular proteolysis [27]. Their action in the glycosaminoglycan-rich extracellular environment raised questions about the interplay between cysteine cathepsins and GAGs outside of the lysosome. The two groups of endogenous human cysteine cathepsins most commonly associated with extracellular proteolysis are cathepsin L-like proteases (cathepsins K, L, S, and V in humans) and cathepsin B [27]. Data accumulated over the past two decades shows that the interplay between these peptidases and GAGs goes both ways; cysteine cathepsins are capable of cleaving proteoglycan core proteins and thereby release GAGs from their support, whereas GAGs in turn affect both the activity and stability of cysteine cathepsins in the extracellular space.

The regulation of papain-like cysteine peptidases by GAGs was first described for cathepsin L [28, 29]. In these early works it was found that GAGs and various negatively charged surfaces substantially accelerate activation of the cathepsin L zymogen into the mature form, including at pH close to the neutral, such as also found in the extracellular milieu in various disease conditions. This has been confirmed for several other cathepsins, the most important being cathepsins B and S [30, 31], and even for a *T. congolense* parasite homologue congopain [32], suggesting that GAGs and other negatively charged surfaces may play a major role in extracellular cathepsin activation in disease. However, recent findings with cathepsin S at high GAG concentration suggest that this enzyme may behave somewhat differently under such conditions with chondroitin-4-sulfate (C4S) even exhibiting a decelerating effect [33]. Nevertheless, facilitating autocatalytic activation of cathepsins by the negatively charged polysaccharide dextran sulfate also became a routine method in preparation of recombinant cathepsins [20, 34–36].

The majority of information about the molecular mechanism of GAG-assisted cathepsin activation came from a study by Caglič et al. using human cathepsin B as a model [31]. As shown, GAGs seem to contribute to the processing in two ways. First, upon binding they seem to convert the cathepsin zymogen into a better substrate. Second, binding of GAGs apparently favors the open conformation of the zymogen, thereby promoting activation not only at acidic pH but also at pH values closer to neutral. This seems to be the case for most GAGs and does not depend critically on the charge density of GAGs, as also HA was able to accelerate the activation, although to a lower extent, which is unusual for a protein-GAG interaction. Moreover, already a tetrasaccharide was sufficient for a prominent acceleration

of cathepsin B autoactivation, which is substantially smaller than found for a number of other GAG-mediated reactions [37, 38]. The interaction is mediated by ionic interactions; however, there seems to be no conserved GAG-binding surface on the zymogens as completely unrelated residues in procathepsins L and B, which were largely located on the prodomains, were found to govern the interaction [31, 39].

The other important role of GAGs in the regulation of cathepsin activity came from the studies on papain, the archetypal representative of the family [40]. This mode of regulation quickly received more attention with the discovery that chondroitin-sulfate from cartilage prominently increased the collagenolytic activity of cathepsin K [41]. This particular peptidase had been discovered a few years prior as the sole protease responsible for collagen degradation in bone remodeling and immediately recognized as a potential drug candidate for the treatment of metabolic bone diseases, such as osteoporosis [42]. The interaction of cathepsin K with chondroitin-sulfate and other glycosaminoglycans was later examined in detail from both the structural and functional perspectives and glycosaminoglycans have been recognized as the first known allosteric regulators of a cysteine cathepsin peptidase [43, 44], as described in detail in the following sections. In parallel, functionally relevant interactions with glycosaminoglycans have also been documented for other members of the cysteine cathepsin family. Taken together, glycosaminoglycans have been found to affect both the activity and the stability of cysteine cathepsins. Kinetic profiles are usually consistent with hyperbolic mechanisms, indicating interactions with enzymes outside of the active site, possibly via allosteric mechanisms. The stabilizing effect is important especially because of the relative instability of cysteine cathepsins at neutral pH found in the extracellular matrix.

4. Regulation of Cathepsin K Activity and Stability

Of all papain-like peptidases, cathepsin K has been established as the cathepsin most tightly linked to glycosaminoglycans. It was originally identified as a protease expressed predominantly in osteoclasts [8] and its impaired activity was shown to result in severe bone disorders [42, 45]. Cathepsin K is a collagenase with unique activity among mammalian peptidases [46–48], which is specifically modulated by glycosaminoglycans via allosteric mechanisms [41, 44]. Due to its central role in bone turnover, cathepsin K is currently considered one of the most promising targets for the treatment of osteoporosis [49]. Apart from bone remodeling, cathepsin K is involved in diverse physiological and pathological processes (for a recent review see [10]). It can cleave a number of extracellular substrates, including proteoglycans, to release active glycosaminoglycans [50], which in turn modulate its activity. In cartilage, cathepsin K degrades both type I and type II collagens and thereby contributes to the development of various inflammatory joint diseases [9, 51, 52]. Moreover, cathepsin K has been associated with cardiovascular diseases, obesity, schizophrenia, and cancer [10].

The interaction of cathepsin K with different glycosaminoglycans has been the subject of in-depth investigation. Though several aspects of these interactions remain elusive, accumulated data suggest that the interactions are heterogeneous and diverse and probably involve multiple binding sites on the enzyme. Chondroitin-4-sulfate (C4S) was initially identified as the GAG with the most dramatic effect on cathepsin K [41] while the effects of chondroitin-6-sulfate (C6S), dermatan sulfate (DS), and hyaluronan (HA) were weaker. All tested GAGs increased the stability of cathepsin K over a broad pH range. C4S had the most prominent effect on the degradation of types I and II collagens by cathepsin K [41], whereas its effect on the hydrolysis of a synthetic substrate was virtually identical to that of C6S and DS and resulted in a twofold increase in the values of the specificity constant (k_{cat}/K_m). In a later study, keratan sulfate (KS) and C6S were found to have a stimulatory effect on cathepsin K similar to that of C4S, whereas heparin and HS had a limited effect on the collagenolytic activity of cathepsin K [53]. Early experiments have also suggested that complex formation with CS is necessary for the collagenolytic activity of cathepsin K [54]; however, recent findings have shown that type I collagen can also be efficiently degraded in the absence of glycosaminoglycans [55]. Nonetheless, bone-resident GAGs have been shown to potentiate the collagenolytic activity of cathepsin K and endogenous GAG concentrations in bone were sufficient for a maximal effect on cathepsin K activity [55].

The kinetic mechanism of the effect of CS, DS, and heparin (HP) on cathepsin K was also investigated in detail at physiological plasma pH of 7.4. Under these conditions, CS and DS were characterized as nonessential activators with a predominant effect on the affinity for the substrate K_m [44]. DS was more effective than CS, which was attributed to its greater flexibility due to fewer intramolecular hydrogen bonds [56]. Intrinsic fluorescence indicated that binding of GAGs affects the conformation of cathepsin K. In contrast to experiments performed at pH 5.5, CS and DS acted as inhibitors of collagen degradation at physiological plasma pH. In contrast, the kinetic mechanism of heparin was biphasic, indicating interaction with two distinct sites on the enzyme. Altogether, heparin was a strong activator of cathepsin K at physiological plasma pH, increasing both its collagenolytic and elastinolytic activities. Moreover, heparin had a strong stabilizing effect on cathepsin K under these conditions, resulting in a more than 5-fold increase in the half-life of the enzyme [44].

5. Structural Basis for the Interaction between Cathepsin K and GAGs

The crystal structure of cathepsin K and C4S revealed the structural basis for the interaction [43]. The binding site is located on the back of cathepsin K and interacts with three disaccharide units of CS in the crystal structure (Figure 2(a)). As usual, the glycosaminoglycan/protein interaction is mediated mostly by electrostatic interactions between the negatively charged GAG chain and positively charged residues on the enzyme. Binding of chondroitin-sulfate does not cause

significant conformational changes in cathepsin K compared to the CS-free cathepsin K. Conversely, the CS chain is bent upon binding to cathepsin K (Figure 2(b)). The bulk of the conformational change can be attributed to the interaction with a short helical region Arg8-Lys9-Lys10 which interacts with four negatively charged groups on CS (Figure 2(c)). Further close contacts include Asp6, Ile171, Gln172, Asn190, Lys191, and Leu195 and a few additional water-mediated contacts [43].

The kinetic behavior of DS was analogous to that of CS: it was hence proposed that it interacts with cathepsin K in the same manner as CS [44]. Heparin, on the other hand, was proposed to bind to two sites on cathepsin K according to its kinetic profile. While the first binding site was proposed to be identical with that for CS/DS, the second binding site was predicted on the bottom of the molecule by chemical cross-linking experiments and computational modeling [44]. From a structural perspective, the predicted binding site is a continuation of the CD/DS-binding site and consists of several basic residues (Lys10, Lys40, Lys41, Arg108, Arg111, Arg127, and Lys214) organized in a ring-shaped structure (Figure 2(d)). Kinetic experiments have confirmed that heparin can be bound to both sites at the same time [44]. However, it remains to be determined whether this requires one HP chain that interacts with both sites at the same time or two separate HP chains. This is neither clear for the interaction of other GAGs with the second HP-binding site.

6. Interactions of GAGs with Cathepsins S and B

Apart from cathepsin K, two other human papain-like peptidases, cathepsins S and B, have been shown to be regulated by glycosaminoglycans in their mature forms [33, 57]. Cathepsin S, the closest relative of cathepsin K, is unusual among cysteine cathepsins for being stable at neutral pH [58]. Cathepsin S has major physiological roles in antigen-presenting cells as the most important protease in antigen processing [59–61] and was recently found to be regulated by C4S [33]. In contrast to the activation effect observed with cathepsin K, C4S acted as an inhibitor of type IV collagen degradation by cathepsin S. Inhibition was also observed with HS, whereas HP, DS, C6S, and HA slightly increased the proteolytic activity of cathepsin S using type IV collagen as the substrate. C4S, C6S, and HS also inhibited the hydrolysis of Z-Phe-Arg-AMC via a partial, mixed mechanism. Similar to cathepsin K, subtle conformational changes in cathepsin S were observed upon C4S binding by intrinsic fluorescence spectroscopy. Three binding sites for C4S were predicted on cathepsin S by molecular docking (Figure 3(a)). One of the proposed sites is the active site, which, however, is not in agreement with the observed mixed inhibition profile of C4S; the second is located on the bottom right side of the molecule and corresponds to the recently identified allosteric site in cathepsin K [62], while the third is located at the bottom of the molecule and roughly corresponds to the secondary heparin-binding site identified in cathepsin K [44].

Cathepsin B is unique among cysteine cathepsins for being both an endopeptidase and a peptidyl dipeptidase, depending on the conformation of the occluding loop, a cathepsin B-specific structure that provides a pH-specific switch between both activities [63]. In the lysosome, the low pH restricts the protease to a closed, exopeptidase conformation, whereas the near-neutral pH of the extracellular environment promotes endopeptidase activity of cathepsin B [63]. Extracellular cathepsin B is most commonly associated with cancer and various types of arthritis [64, 65]. The protease localized to the cell surface in several studies and was found to be involved in cell migration under both physiological and pathological conditions [66, 67]. At the molecular level, it was shown to cleave a number of extracellular substrates, including laminin, type IV collagen, and fibronectin [68, 69]. Recently, cathepsin B has also been suggested to be a β-secretase that produces amyloid β peptides in secretory vesicles of neuronal chromaffin cells [70]. However, it has also been shown to degrade amyloid deposits in an animal model [71] and the overall outcome has been suggested to be determined by the balance between cathepsin B and its endogenous inhibitor cystatin C [72].

Binding of HP or HS has been shown to increase the stability of the otherwise unstable enzyme at alkaline pH (8.0), while slightly reducing the activity of the enzyme along the entire pH profile of the enzyme [57]. Computational simulations have predicted that heparin stabilizes the conformation of the molecule under these conditions and have predicted two putative GAG-binding sites (Figure 3(b)), one on each side of the enzyme [73]. The putative binding site in the L domain consists of five basic residues (Arg85, Lys86, Lys130, Lys141, and Lys144), while the one in the R domain contains only two (Lys158 and Arg235). The authors have suggested that the binding site in the R domain has higher affinity for shorter GAG fragments, such as the heparin disaccharide used in their docking simulations, whereas the one in the L domain is likely more relevant for the binding of longer GAG fragments [73].

7. Interactions of GAGs with Other Papain-Like Peptidases

Interestingly, papain has also been shown to interact with GAGs. Despite bearing no physiological relevance, these interactions point to evolutionarily conserved mechanisms of regulation within the family. HP inhibited papain by a hyperbolic mixed mechanism and affected its conformation [40]. A classical heparin-binding consensus sequence was identified in papain, in the form of the sequence 187-Ile-Arg-Ile-Lys-Arg-Gly-192. Structurally, this sequence is located on the right side of the molecule (Figure 3(c)) in a region that lies between both allosteric sites known in cathepsin K.

In addition, a few examples of proteases from protozoan parasites have been described that interact with GAGs, suggesting the possibility of their influence on host-parasite interactions. The cathepsin L homolog brucipain, a crucial virulence factor of the protozoan parasite *Trypanosoma brucei*, has been found to be allosterically modulated by HS. The effect of HS in this study was subtle and it had the ability to reverse substrate inhibition by a small dipeptide substrate (Z-Phe-Arg-AMC) [74]. A stronger effect of HS was observed for

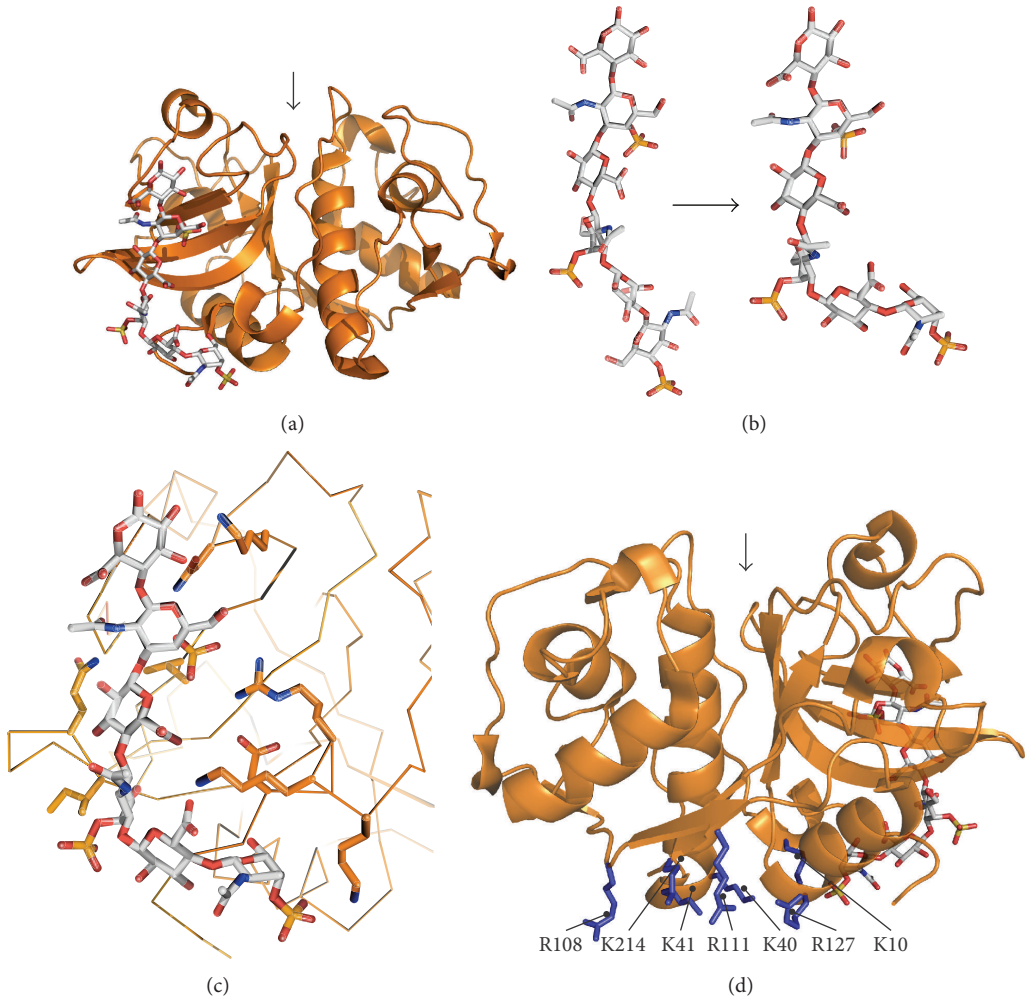

Figure 2: Interactions between human cathepsin K and GAGs. (a) Crystal structure of the cathepsin K/chondroitin-4-sulfate (C4S) complex. The protein is shown in cartoon representation and C4S is shown as sticks. (b) Conformational change in C4S upon binding to cathepsin K. (c) Detailed representation of the interaction in panel (a). C4S is shown as sticks. The backbone of cathepsin K is shown as ribbons and residues that interact with C4S are shown as sticks. (d) Location of the predicted second heparin-binding site in cathepsin K. Positively charged residues proposed to interact with heparin are shown as blue sticks. For orientation, C4S bound at the first binding site is shown as sticks. The position of the active site cleft is marked by an arrow. Coordinates of the cathepsin K/C4S complex were retrieved from the Protein Data Bank under accession code 3C9E. The solution structure of C4S was modeled using data from [14]. All images were created with PyMOL.

cruzipain from the related parasite *Trypanosoma cruzi*. In this case, HS was an activator of the peptidase that caused a significant (up to 6-fold) increase in the activity of the peptidase measured with a synthetic substrate. Moreover, HS increased the release of kinin from high molecular weight kininogen by cruzipain *in vitro* as well as by living trypomastigotes and reduced the inhibitory properties of kininogen towards cruzipain [75]. Similarly, HP was recently shown to modulate the activity of the cathepsin L-like peptidase rCPB2.8 from *Leishmania mexicana* [76]. In this case, HP and HS, but not CS or DS, inhibited the hydrolysis of Z-Phe-Arg-AMC by a hyperbolic mixed mechanism and affected the conformation of the protein [76]. Altogether these examples show that interactions with GAGs are not restricted to endogenous cysteine cathepsins but can also play diverse roles in host-pathogen interactions and can act either as part of the body's defense against invading pathogens or as factors contributing to the invasive mechanisms of the pathogen.

8. Pharmacological Targeting

Cathepsin K currently represents the most attractive drug target among the cathepsins, although cathepsin S is also a relevant target in diseases associated with elevated immune response, such as bronchial asthma and psoriasis [5, 27]. Several cathepsin K inhibitors are currently under development, which target the active site of the enzyme (collected in [10, 27]). At the moment, the most promising inhibitor is odanacatib (Merck & Co., Inc., Whitehouse Station, NJ, USA), a nitrile warhead-containing inhibitor, highly selective for cathepsin K [77]. Phase III clinical trials for odanacatib have been successfully concluded and applications for approval are expected to be filed soon. If approved,

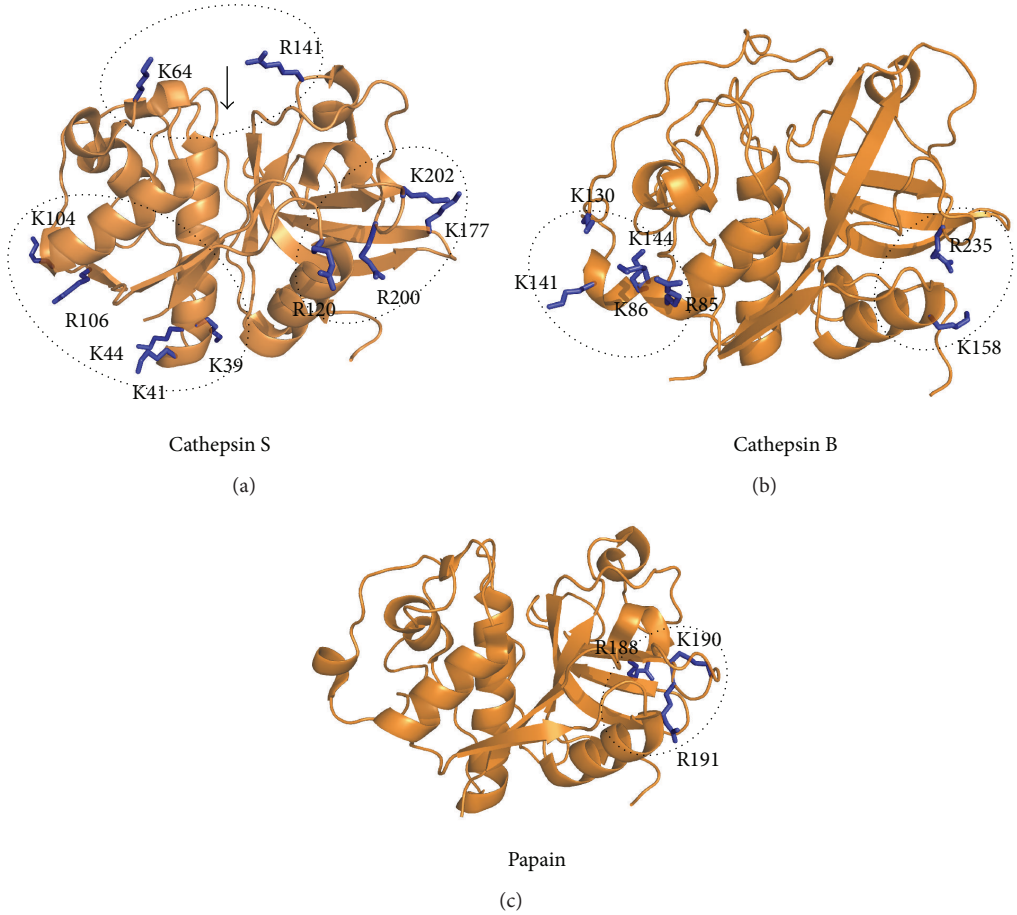

Figure 3: Predicted GAG-binding sites in papain-like peptidases. (a) Three predicted CS-binding sites in cathepsin S. (b) Two predicted HS/HP-binding sites in cathepsin B. (c) The conserved GAG-binding motif in papain. Predicted sites are shown in circles and positively charged residues at each site are shown as blue sticks and labeled. The position of the active site cleft is marked by an arrow. All coordinates were obtained from the Protein Data Bank (accession codes: 1NQC for cathepsin S, 3AI8 for cathepsin B, and 1PPN for papain, resp.). All images were created with PyMOL.

the drug will position itself in the market against other new generation drugs, such as the anti-RANK-ligand antibody denosumab (Amgen, Inc., Thousand Oaks, CA, USA) and the teriparatide, a recombinant form of parathyroid hormone (Eli Lilly and Company, Indianapolis, IN, USA), as well as the well-established bisphosphonates [78]. Targeting the cathepsin K/chondroitin-sulfate interaction would represent an alternative to these treatments. Endogenous chondroitin-sulfate is sufficient to exhibit a maximum activation effect on cathepsin K and its digestion reduces the activity of cathepsin K by 40% [55]. A reduction in bone turnover of this magnitude would likely suffice for the treatment of patients with less severe bone density reduction. An added benefit would be that cathepsin K activity *per se* as well as the viability and cell count of osteoclasts and osteoblasts would remain undisturbed.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Review Article

Chondroitin Sulfate Proteoglycans in the Nervous System: Inhibitors to Repair

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Chondroitin sulfate proteoglycans (CSPGs) are widely expressed in the normal central nervous system, serving as guidance cues during development and modulating synaptic connections in the adult. With injury or disease, an increase in CSPG expression is commonly observed close to lesioned areas. However, these CSPG deposits form a substantial barrier to regeneration and are largely responsible for the inability to repair damage in the brain and spinal cord. This review discusses the role of CSPGs as inhibitors, the role of inflammation in stimulating CSPG expression near site of injury, and therapeutic strategies for overcoming the inhibitory effects of CSPGs and creating an environment conducive to nerve regeneration.

1. Introduction

The limited ability of the human central nervous system (CNS) to repair itself following injuries has been known since the days of Ancient Egypt (3,000–2,5000 BCE), as documented in the Edwin Smith papyrus [1]. It had long been thought that neurons in the CNS were incapable of mounting a regenerative response, until the studies of Aguayo and colleagues in the early 1980's [2, 3] which demonstrated that certain classes of neurons within the CNS, particularly those neurons which sustained an axonal injury in close proximity to their cell body, were able to regenerate their axons within a permissive environment, such as a peripheral nerve graft. Aguayo's work and more recent studies [4–6] have all demonstrated that supraspinal neurons (neurons arising in the cerebral cortex or brainstem and which project their axons caudally into the spinal cord) are actually capable of mounting a regenerative, albeit brief, and response following injury, when provided with the proper environment. While advances in science have not solved the problem of this short and often abortive nature of CNS neuron regeneration, many of the studies point to the same general theme: CNS neurons attempt to regenerate, but the post-injury environment is highly inhibitory to this process due to many molecules expressed after damage to the nervous system. One family of molecules, the chondroitin sulfate proteoglycans (CSPGs), are of particular importance and have significant roles in limiting the reparative response in almost every case of CNS damage.

Injuries to the CNS can generally be classified into two overarching categories: traumatic and neurodegenerative. Traumatic lesions to the brain or spinal cord are largely contusive in nature and often result from falls, sharp blows, or sudden deceleration style injuries, rather than penetrating wounds [7, 8]. Unlike sharp lacerating wounds that sever tissue, contusion lesions occur when a physical force (compression, shearing, or tensile) is rapidly applied to neural tissue without cutting [7, 9–11]. These sudden forces cause rapid and focal compression and displacement of neural tissue, resulting in the disruption of multiple afferent and efferent neuronal fiber tracts. Nontraumatic injuries to the CNS are often caused by degenerative pathologies, such as multiple sclerosis, Alzheimer's disease, and Parkinson's disease. While research is progressing in all arenas of traumatic and degenerative CNS lesions, one common attribute is observed: the expression of CSPGs in and around the areas

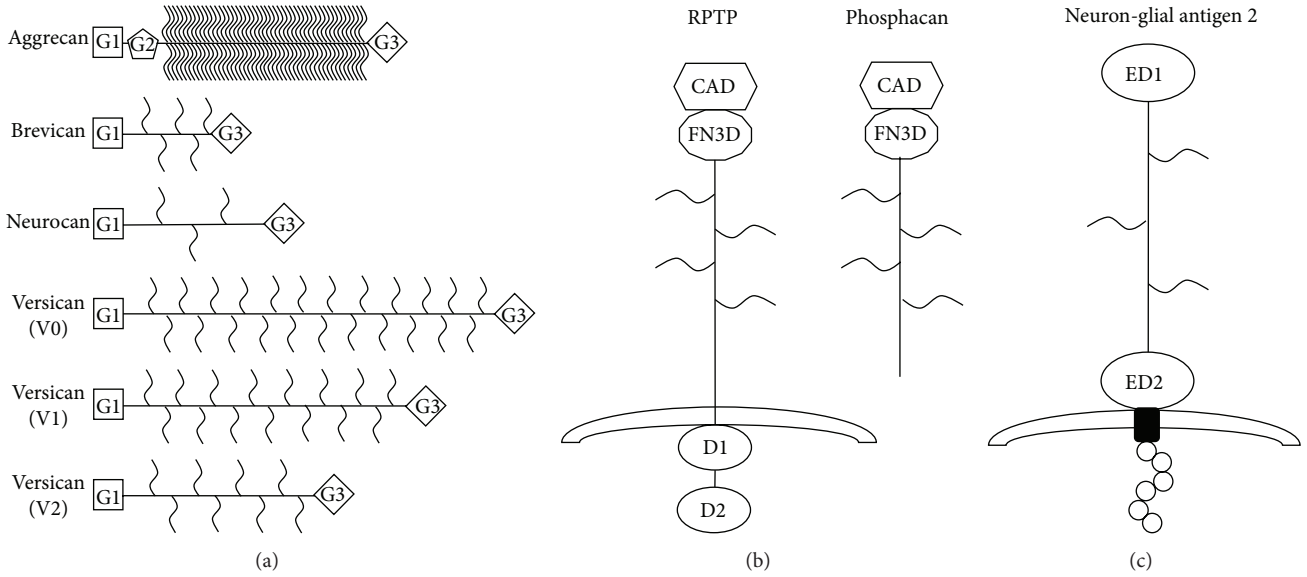

Figure 1: Schematic representation of individual proteoglycan molecules. (a) Members of the lectican family: aggrecan, brevican neurocan, and the three isotypes of versican, all share a similar homology, with a G1 domain at the N-terminus and a G3 domain at the C-terminus. The GAG side chain varies in number among the different lectican family members but is attached to the central core of the protein. (b) Phosphacan is a splice variant of the RPTP molecule, lacking the transmembrane and two intracellular domains, found in the RPTP molecule. (c) NG2 is a transmembrane proteoglycan that lacks homology to any of the other CSPGs. NG2 has two large extracellular domains separated by an extended region, where the GAGs are attached, a transmembrane domain and short cytoplasmic tail. NG2 can be cleaved by enzymes at the cell surface and released into the extracellular matrix (adapted and modified from [16, 18]).

of CNS tissue damage. It is important to understand that upregulation of CSPG expression in response to an insult is thought to be a protective mechanism, an attempt to wall off the area of damage and limit its spread [12–15]. However, this creates a cellular microenvironment that inhibits regeneration and repair. It follows then that one therapeutic approach to enhance CNS repair involves modulation of CSPG expression, which can change the cellular environment to allow for neural regeneration.

2. Chondroitin Sulfate Proteoglycans

Among the many CSPG molecules expressed in the CNS are the lectican group, which include aggrecan, three forms of versican (V0, V1, and V2), neurocan, and brevican (Figure 1). All members of the lectican family consist of a central core protein that has an N-terminal G1 domain and a C-terminal G3 domain. The central domain binds the chondroitin sulfate glycosaminoglycan side chains (CS-GAG) [16–18]. The aggrecan proteoglycan is the only member of the lectican group that has an additional globular (G2) near the G1 domain. Individual lectican molecules differ in the number of CS-GAG chains attached to their core proteins, with over one hundred GAG side chains being found in aggrecan and as little as zero to five GAG chains being found in brevican and neurocan [18] (Figure 1). The lectican family of CSPGs is largely produced by two major cell groups in the CNS: neurons and astrocytes (Table 1).

Other proteoglycans that have major roles in the pathology of CNS injury are phosphacan and NG2 (Figure 1). Phosphacan is a splice variant of receptor-type protein tyrosine phosphatase (RPTP) and lacks the two intracellular tyrosine phosphatase domains that are found in RPTP [18]. This RPTP splice variant is secreted into the extracellular environment and contains attachment regions for CS-GAG chains. Neuron-glial antigen 2 (NG2) is a unique CSPG that lacks sequence homology to other known CSPGs. NG2 proteoglycan is a transmembrane proteoglycan, composed of a large extracellular domain (two large globular domains separated by an extended region to which the GAGs attach), transmembrane domain, and a short cytoplasmic tail (Figure 1) [18, 19]. Cells that express the NG2 proteoglycan include oligodendrocyte progenitor cells, polydendrocytes, activated microglia, and activated macrophages (Table 1).

The variations in the length of the core protein and the varying number of GAG side chains attached to the central domain are major determinants of the biological activity of an individual proteoglycan. However, the biological effects of a proteoglycan are also affected by the sulfation patterns of the *N*-acetylgalactosamine and glucuronate disaccharide, the basic unit that composes the CS-GAG [16, 18]. There are four different sulfation patterns that can occur, based on either monosulfation or disulfation of the *N*-acetylgalactosamine (Gal*N*Ac) and glucuronate disaccharide (GlcA) of the GAG disaccharide, leading to the synthesis of the CS-A, CS-C, CS-D, and CS-E CS-GAGs (Figure 2) [16–18, 20]. Sulfation of the chondroitin sulfate disaccharide can occur in the 4 and 6 positions of the Gal*N*Ac and the 2 position of the GlcA [16, 18, 20]. It has been demonstrated that a CSPG molecule can be either inhibitory or permissive for axonal growth,

Table 1

Cell	Proteoglycan	CNS specific	Location	Inhibitory to axonal growth	References
Neurons	Aggrecan	No	ECM	YES	[166, 167]
	Brevican	Yes	ECM	YES	[22, 105]
	Neurocan	Yes	ECM	YES	[16, 22, 105, 168, 169]
	Phosphacan	Yes	ECM	YES	[16, 22, 105, 168, 169]
Astrocytes	Brevican	Yes	ECM	YES	[22, 105, 116]
	Neurocan	Yes	ECM	YES	[16, 22, 105, 116, 168, 169]
	Phosphacan	Yes	ECM	YES	[16, 22, 105, 116, 121, 122, 168, 169]
Activated microglial cells	KSPGs	No	TM & ECM	YES	[92, 93, 170]
	NG2	No	TM & ECM	?	[92, 108, 109, 121, 122, 171–174]
Oligodendrocyte progenitor cells	KSPGs	No	TM & ECM	YES	[92, 93, 170]
	NG2	No	TM & ECM	?	[92, 108, 109, 121, 122, 171]
	Versican (V2)	Yes	ECM	NO?	[106, 108]
Polydendrocytes	NG2	No	TM & ECM	?	[92, 108, 109, 121, 122]
Activated vascular macrophages	KSPGs	No	TM & ECM	YES	[92, 93, 170]
	NG2	No	TM & ECM	?	[92, 108, 109, 121, 122, 173, 174]

Abbreviations: TM: transmembrane; ECM: extracellular matrix.

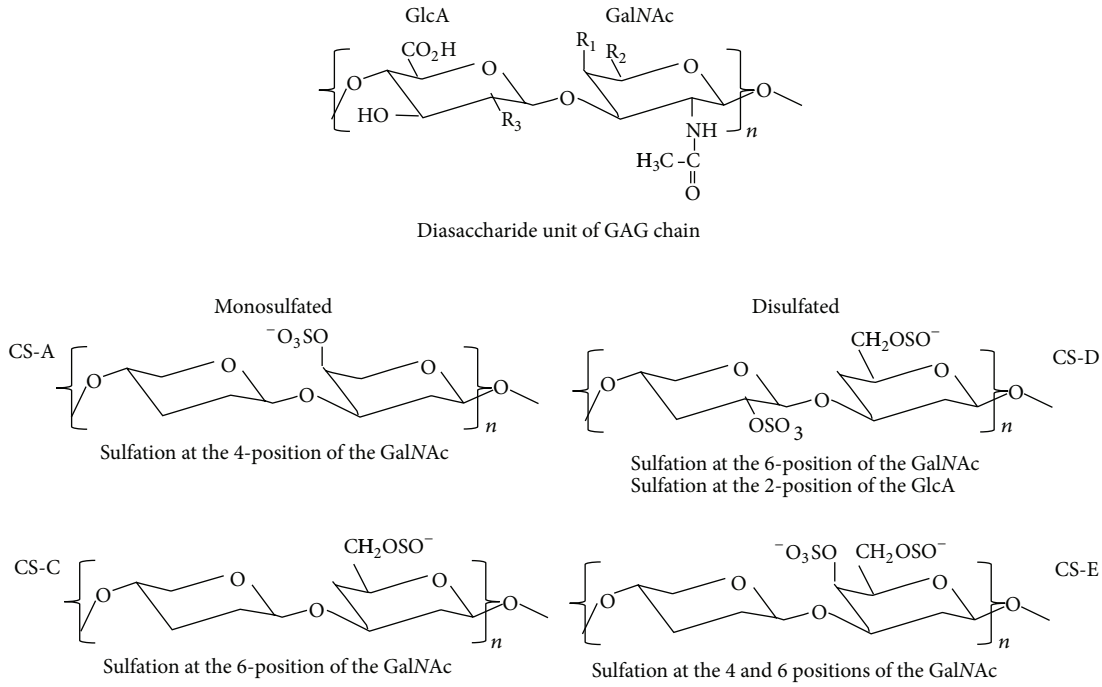

Figure 2: Diagram of the sulfation patterns of the disaccharide unit of the GAG chain. Sulfation at different carbon atom positions in the GlcA and/or Gal*N*Ac saccharide unit is one of the major factors that influence the effects of the proteoglycan. Monosulfation can occur at position 4 of the Gal*N*Ac resulting in synthesis of CS-A GAG or position 6 of the Gal*N*Ac resulting in the synthesis of the CS-C GAG. Disulfation can also occur with sulfation of position 6 of the Gal*N*Ac and position 2 of the GlcA, resulting in synthesis of the CS-D GAG, or sulfation of positions 4 and 6 on the Gal*N*Ac saccharide unit resulting in formation of the CS-E GAG (adapted and modified from [16, 18]).

based on the molecular position of the sulfation which occurs in the disaccharide [20–22]. For example, when the sulfation occurs at the 4-position (CS-A), the proteoglycan tends to be highly inhibitory to axonal outgrowth [21, 22] whereas sulfation in the 6-position (CS-C) is more controversial and has been found to be inhibitory to axonal outgrowth by some labs [23] and permissive to axonal growth in others [20]. *In vitro* experiments show that the disulphated GAGs (CS-D and CS-E) promote the growth of embryonic axons [24, 25]. Taken together, these observations reveal many aspects of

proteoglycan structure which can modulate their biological effects on cells within the CNS.

The majority of CSPGs can interact with growth factors, cell adhesion molecules, and other ECM molecules in the local environment and may regulate their biological activity. CSPGs are widely distributed throughout the normal CNS, both during development and in the adult. CSPGs expression is especially rich in the embryonic brain and can direct cell migration and axonal outgrowth by providing guidance cues [17, 18]. Phosphacan is concentrated in the regions of cell proliferation such as the ventricular zone of the embryonic brains, suggesting it may modulate cell division. In the healthy adult nervous system, the soma and proximal dendrites of certain neurons are surrounded by a CSPG rich structure known as a perineuronal net. This perineuronal net stabilizes existing synapses and inhibits the formation of aberrant synaptic connections [17, 26]. In terms of general CSPG expression patterns, white matter is rich in versican and neurocan, while brevican can be found throughout the CNS, and NG2 is found expressed among meningeal cells, blood vessels, and OPCs [27].

3. Traumatic Lesions

Vast strides have been made in characterizing and understanding the complex orchestration of biological events that occurs following a lesion to the CNS. Unfortunately, it is now widely known and accepted that the events occurring in brain or spinal cord tissue post-injury create an environment hostile to a regenerative response, resulting in the abortive nature of the reparative process [9, 28, 29]. This is due in part to a significant upregulation of CSPGs.

The extreme forces applied to the CNS during traumatic injuries result in disruption of axonal tracts, blood vessels, and glial cells located at the epicenter of the lesion (reviewed by [10, 12]). Immediately following injury, there is a marked swelling of CNS tissue caused by damage to the local vasculature, allowing for leakage of blood plasma fluid into the surrounding extracellular space [30]. This vascular damage also creates an anoxic environment that is accompanied by necrosis of tissue damaged during the injury. Dying cells release their contents directly into the extracellular environment, resulting in a massive infiltration of blood borne macrophages and microglia, the resident CNS immune cell [31–34].

In healthy CNS tissue, microglial cells exist in a nonactive or resting state, courtesy of microglia-neuronal communications [35–37]. However, with an infection or trauma, microglial cells are activated, taking on the role of a phagocytic macrophage [38]. Activated macrophages/microglia play an important role in the CNS response to injury and infection because of the various products they secrete [37], including cytotoxic molecules (free radicals, i.e., superoxides), neurotrophic molecules (nerve growth factor, e.g.), and a variety of pro- and anti-inflammatory cytokines and chemokines [37]. These cytokines can stimulate the expression of CSPGs in a variety of cells in and around the lesion. Complicit in this process is the recruitment of blood borne macrophages which invade the lesion site. As these cells perform their biological function of phagocytizing necrotic cellular debris and any foreign pathogens, they further exacerbate the inflammatory response by releasing inflammatory cytokines, which can also stimulate CSPGs synthesis from neighboring cells [38]. Most important are the astrocytes located at the border of the injury, which undergo a process known as reactive astrogliosis. In response to various cytokines, astrocytes become hypertrophic and begin secreting CSPGs, leading to the formation of a glial scar. The glial scar serves as a chemophysical barrier to axonal regeneration [32, 33, 39, 40].

4. Degenerative Lesions

While the etiologies of traumatic and degenerative lesions are different, during degeneration the CNS responds in a highly similar manner to that described for traumatic lesions, complete with reactive astrogliosis and the deposition of CSPGs. Hallmarks of Alzheimer's disease (AD) pathology include the formation of neurofibrillary tangles and extracellular accumulation of amyloid-beta ($A\beta$) fibrils [41]. This results in a loss of axonal integrity and a decline in synaptic connectivity that is believed to contribute to dementia [42]. Dystrophic neurites, activated astrocytes, and $A\beta$ fibrils form senile plaques, which are the key diagnostic criteria of AD [43, 44]. These senile plaques appear to be loci for inflammatory processes, containing a variety of molecules such as cytokines, acute-phase proteins, and complement proteins, which are secreted by reactive microglia and astrocytes around the lesion [45–47]. Microglia have been identified in close association with AD lesions, and while their function is to phagocytize the lesions, they are unable to do so. With plaque deposition, activated microglia are present on a continuous basis and thus provide a constant source of neurotoxic molecules [48]. This mechanism has been proposed to explain the toxicity of amyloid peptides (reviewed by [49–53]).

Parkinson's disease (PD) is a neurodegenerative movement disorder of unknown etiology, characterized by the selective loss of dopaminergic nigrostriatal neurons [54]. PD patients present symptoms such as resting tremor, postural instability, muscle stiffness, and slowness of voluntary movements (reviewed by [55–57]). The selective vulnerability of dopaminergic neurons observed in PD is thought to be due to various interacting factors, one of which is the microglia-mediated inflammatory response [57, 58]. A role for activated microglia in neurodegeneration is supported by studies using PD animal models. Dopaminergic neuronal degeneration can be elicited by injecting the neurotoxin bacterial lipopolysaccharide into the rat substantia nigra. These experiments revealed a significant increase in microglia activation throughout this subcortical area preceding marked neuronal death [59]. Furthermore, lipopolysaccharide-induced neuronal death was blocked by inhibition of microglial activation in this model. Administration of the neurotoxins 1-Methyl-4-phenyl-1,2,5,6-tetrahydropyridine (MPTP) or 6-hydroxydopamine (6-OHDA) also mimics PD symptoms in mice and induces activation and proliferation of microglia, as well as increased expression of inducible nitric oxide synthase

(iNOS) and MHC class-I and II molecules [57, 60–64]. The observed increase in activated microglia is highly localized and anatomically discrete, limited to the substantia nigra. Furthermore, it is directly correlated with the neuronal death (reviewed by [57]).

Multiple sclerosis (MS) is a clinically heterogeneous demyelinating disease of the CNS [65], characterized by inflammation, axonal degeneration, and gliosis [66]. The etiology of this chronic inflammatory disease is unknown, although an autoimmune response is thought to be involved. MS lesions are characterized by the presence of large regions of demyelination, commonly referred to as plaques. These plaques contain reactive glial scar formation, with infiltration of activated T cells and macrophages [67]. The blood brain barrier (BBB) is disrupted, and upregulation of various adhesion molecules has been reported on capillary endothelial cells during the early stages of disease [68]. Increased BBB permeability allows for inflammatory infiltrates like activated macrophages, T cells, and antibodies, to invade the CNS parenchyma. Tumor necrosis factor alpha (TNF-α) is a key cytokine that may influence the progression of MS. TNF-α promotes the proliferation of bovine astrocytes and human astroglioma cell lines, which leads to reactive gliosis, as seen in active MS plaques [69]. TNF-α also stimulates production of colony-stimulating factors from neighboring astrocytes, which act as chemoattractants for reactive cells. This results in increased migration of activated cells to sites of inflammation and increases in the proliferation and activation of microglia [70–72].

Amyotrophic lateral sclerosis (ALS) is a rapidly progressing, adult-onset disease that usually results in death within 5 years of its initiation. Clinical manifestations of the disease include initial muscle weakness and atrophy that progress to a spastic paralysis resulting from motor neuron degeneration [73]. About 25% of ALS cases appear to be caused by a gain-of-function mutation in the antioxidant enzyme Cu, Zn superoxide dismutase-1 (SOD-1), which catalyzes the conversion of superoxide to oxygen and hydrogen peroxide [74]. This disease shares some of the characteristic neuroinflammatory changes observed in other neurodegenerative diseases like AD and MS [75]. Neuroinflammation is a key mediator of the pathology observed in ALS [76]. Reactive microglia and macrophages have been detected in spinal cord and motor cortex of ALS patients using conventional immunohistochemistry for activated monocyte-lineage-cell antigens (reviewed by [75]). MHC-I and MHC-II molecules, β2-integrins, and leukocyte common antigen are upregulated, indicating the presence of reactive microglia in these tissues [75, 77, 78]. Activated astrocytes and leukocytes are also observed, as demonstrated by glial fibrillary acidic protein (GFAP) and leukocyte functional antigen (LFA)-1 staining, respectively. Furthermore, the majority of LFA-1$^+$ leukocytes were CD8$^+$ [79], suggesting a role for cytotoxic T lymphocytes in disease progression. This T cell presence is not as prominent as that seen in T cell-mediated diseases like MS. In contrast, it has been proposed that neuroinflammation in ALS and its animal models is driven mainly by reactive macrophages and microglia and the resulting dysregulation in cytokine expression [76].

5. Role of Inflammation in Stimulating CSPG Expression

Trauma-induced CNS injury and autoimmune/neurodegenerative CNS disorders have several important similarities and differences in terms of the immune/inflammatory response. This observation was noted by Popovich and colleagues [80] comparing MS and SCI, and it could easily be further extended to include conditions like ALS, AD, and PD. For example, one key feature observed in CNS diseases is the disruption of the BBB, which allows for an influx of inflammatory cells [81–84]. In AD, PD, MS, and ALS, the mechanisms for increased BBB permeability remain unknown. Inflammatory mediators released from microglia and T lymphocytes are related to this process, but what triggers this release is still a matter of controversy. On the other hand, in cases of traumatic CNS injuries, BBB disruption is a direct consequence of traumatic insult. Nonetheless, the chronic endothelial permeability is maintained and explained by a perpetuated intraparenchymal inflammatory response.

Both traumatic and autoimmune/neurodegenerative CNS injuries show microglial activation and immune cell infiltration. Even the temporal sequence of events is similar in these scenarios, where microglia are recruited first, releasing inflammatory cytokines and reactive oxygen species (ROS), as well as upregulating their antigen presenting cell capabilities. Following increased BBB permeability, hematogenous macrophages, neutrophils, and lymphocytes follow, mediating myelin vesiculation, lipid peroxidation and further release of proinflammatory agents and free radicals. Demyelination ensues, resulting from oligodendrocyte injury and edema. However, in SCI this process is restricted to CNS myelin, whereas in conditions like MS, myelin destruction and ROS are found in the CNS as well as in the periphery [80].

Increases in pro- and anti-inflammatory cytokine levels and ROS production are a common occurrence in traumatic and autoimmune or neurodegenerative CNS disorders. In terms of therapeutic approaches, this has been targeted repeatedly using a variety of strategies that include antibody treatments, administration of cytokine receptor antagonists, or even proinflammatory cytokines themselves [85–90]. Glucocorticoids like methylprednisolone have been used extensively in light of their ability to suppress proinflammatory cytokine synthesis [91]. Because activation of various immune cells during the inflammatory response is directly associated with secretion of ROS and tyrosine nitration, approaches such as antioxidant administration, inhibition of inducible nitric oxide synthase iNOS, and targeted depletion of hematogenous macrophages are all under investigation [92].

One of the main differences between SCI and autoimmune/neurodegenerative disorders is the induction time for cytokine expression. Trauma-induced CNS injury is characterized by a transient increase in proinflammatory cytokine levels that is followed by a relatively rapid restoration of baseline levels. In this instance, cytokines have a key role in the acute-phase response. Trauma related degeneration can be attributed to the initial trauma itself, since cell death and

tissue necrosis occur as early as one hour after injury. On the other hand, neurodegenerative conditions like MS and ALS owe their prolonged progression to a slow and persistent increase in proinflammatory cytokines. Degenerative processes seen in these cases are more likely to be the result of direct effects of cytokines [80].

As previously noted, following any type of insult to the central nervous system, an immune response is elicited and a combination of vascular macrophages and active microglia infiltrate the lesion site. It is well documented that vascular macrophages and activated microglia synthesize and secrete many different proinflammatory cytokine and chemokine molecules [93–99]. Activated microglial cells are known to secrete a minimum of at least 20 different cytokine and chemokine molecules [100]. Some of these molecules, such as Interleukin-1 (IL-1), IL-2, IL-6, IL-15, IL-18, Interferon gamma (IFN-γ), and TNF-α are all known and documented to be proinflammatory and found to be upregulated in cases of CNS tissue damage [98, 100]. Interestingly, when these proinflammatory cytokines and chemokines, specifically IL-1, IL-2, IL-6, TNF-α, IFN-γ, were injected into normal brain tissue, a significant amount of reactive astrogliosis was observed around the injection sites [93, 94, 96]. Another study demonstrated that transforming growth factor alpha (TGF-α; a cytokine expressed by macrophages) and transforming growth factor beta (TGF-β; a cytokine expressed by both microglia and macrophages) resulted in the upregulation and synthesis of chondroitin 6-sulphate proteoglycans in brain tissue and *in vitro* cell culture experiments [99]. Recent work has identified a link between the activation of microglia and the activation of astrocytes, painting a picture of crosstalk and coregulation, where astrocytes and microglia signal to each other modulating each other's activation and post-injury activities [95, 97]. Interestingly, macrophages themselves can synthesize and either degrade or secrete CSPGs and may be a potentially significant source of CSPGs in the post-injury environment [101].

6. CSPG Deposition at CNS Lesions

CSPGs are upregulated rapidly in the tissue surrounding a lesion site, due to the induction of reactive gliosis (see Figure 3). There are many members of the CSPG family and individual CSPGs are synthesized by different cell types and at different time points following injury. Reactive astrocytes synthesize brevican, neurocan, and phosphacan, while vascular macrophages, activated microglia, and endogenous OPCs account for the increased expression of NG2 and versican [17, 102, 103].

In traumatic lesions, CSPGs can be observed at the lesion very quickly, within the first 24 hours. However, the temporal pattern in which individual CSPG molecules are produced is different. Neurocan is the first to appear, with brevican and versican following. Their expression levels are maximal two weeks after injury, in a spinal cord injury model [103]. Peak expression of the NG2 proteoglycan occurs one week after injury, because it is expressed by infiltrating macrophages and OPCs. Keratan sulfate proteoglycans, which are also inhibitory to regeneration, are also produced by infiltrating

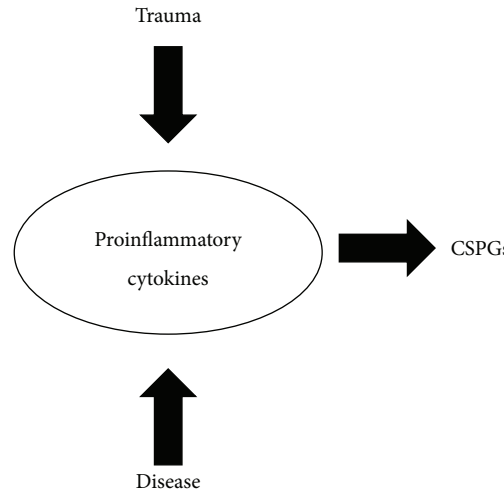

Figure 3: Damage to the central nervous system, either by trauma or disease processes, initiates an increase in proinflammatory cytokines, which stimulates the upregulation of CSPGs expression.

macrophages, microglia, and OPCs as early as 3 days post-injury [103, 104]. Interestingly, expression of phosphacan is initially downregulated during the first 72 hours after lesion but slowly increases over time, reaching peak levels approximately eight weeks after injury [103]. While all members of the CSPG family are expressed following a traumatic insult to the CNS, their location in the lesion area is also variable. Neurocan is expressed close to the lesion center, and around the immediate border, in a zone from 100 μm to 500 μm around a spinal cord lesion. Brevican lies close to the immediate injury site as well, deposited within 300 μm of the lesion border. Phosphacan and versican are expressed in a more broad range deposition, extending in a diffuse pattern as far as 600 μm from the impact site [103]. Additionally, while changes in CSPG expression in tissue adjacent to the damaged tissue are well described, alterations in CSPGs expression in areas distal to lesions are also observed. In a study conducted by Andrews and colleagues [105] following a spinal contusion at the T8 level, CSPG expression was detected in both the lumbar and cervical enlargements, far away from the injury site. They discovered a strong upregulation of neurocan at the lesion epicenter and at both the lumbar and cervical enlargements. Aggrecan and brevican were initially downregulated in the lesion and was unchanged in both spinal enlargements [105]. While the total amount of NG2 protein did not change in the tissue around the lesion, there was an accumulation of NG2 in the lumbar enlargement but not the cervical. The continual and differential expression of CSPGs changes over acute and chronic times following trauma, and the changes that occur far from the site of insult maintain a broad environment inhibitory to regenerative response for many months post-injury.

In neurodegenerative lesions, the timeline of CSPG expression is not well understood, as all histopathology in humans occurs post-mortem. However, several studies have documented the presence of CSPGs in human tissue and upregulation in animal models of disease. In Alzheimer's

disease, when the formation of the β-amyloid plaques and neurofibrillary tangles was examined, it was found that these lesions were surrounded in a shell of GFAP positive reactive astrocytes, which indicate that reactive gliosis had occurred [106]. Further examination of AD brains revealed the expression of CSPGs, heparan sulfate proteoglycans (HSPGs), and dermatan sulfate proteoglycans (DSPGs). The upregulation of CSPG expression has also been discovered in other neurodegenerative diseases such as Huntington's disease, MS, around the inclusions of Parkinson's disease, in Pick's disease, and progressive supranuclear palsy [106, 107]. The edges of active MS demyelinating lesions are rich in the CSPGs aggrecan, neurocan, and versican [108]. Further, an increase in the levels of bone morphogenic protein (BMP) has been detected in areas of demyelination. Activation of BMP stimulates increased synthesis of CSPGs from astrocytes surrounding the lesion [109]. In other forms of nontraumatic CNS injuries, such as stroke and amyotrophic lateral sclerosis, reactive astrogliosis occurs, with CSPG deposition as a result of the pathology [110, 111].

Regardless of the etiology, most CNS lesions involve an immune response, including the recruitment of vascular macrophages and activated microglial cells [10, 29, 100, 112, 113]. The cytokine and chemokine molecules expressed by these immune cell infiltrates elicit a process of reactive astrogliosis, which in turn is responsible for the upregulation of CSPG expression. While the overexpression of proteoglycans that occurs following injury is highly inhibitory to the regenerative response, it is also important to note that this process is also necessary. The upregulation of CSPGs occurs quickly to contain the tissue damage. By interfering with or preventing the formation of the glial scar, not only tissue degeneration is significantly worse, but the spread of damage into areas not initially damaged can be up to 60% greater [12–15]. This poses an interesting challenge to researchers, how to bypass or neutralize the well-known and documented inhibitory effects of CSPGs on regeneration, while not totally or permanently ablating or eliminating the formation and function of the glial scar.

7. Inhibitory Effect of CSPGs on Neuronal and Oligodendroglial Cells

At the molecular level, *in vitro* studies have demonstrated that CSPGs can interact with adhesion molecules expressed on various cell types [114]. When axonal growth cones come into contact with CSPGs, they collapse and retract. This is likely the reason for the abortive regenerative sprouting observed in spinal cord lesions [10, 33]. The interaction of CSPGs and neurons activates the Rho-ROCK and/or protein kinase C (PKC) intracellular signaling cascades, which inhibit process extension. It was also noted that by blocking activation of the Rho-Rock and/or PKC signalling pathways, the inhibitory effects of CSPGs could be reversed [26]. It has also been demonstrated *in vitro* that CSPGs can influence the activity of the axonal growth cone. In dorsal root ganglion cultures, the presence of CSPGs induced changes in local protein synthesis in the growth cone, with an increase in RhoA transcripts (a cytoskeletal regulator) being found locally within the growth cone after CSPG contact [115].

While CSPGs are widely accepted to be inhibitory to axonal regeneration, the inhibitory nature of individual CSPG molecules varies among the proteoglycans. *In vitro*, purified brevican has been shown to be inhibitory to both axonal attachment and growth [29, 116], while both neurocan and phosphacan have been shown to interact with neural cell adhesion molecules (N-CAM) similarly inhibiting axonal growth [29, 116]. Conversely, versican is not thought to be inhibitory to either axonal regrowth or adhesion. Axons are able to grow through deposits of versican *in vitro* and are not inhibited by the presence of purified versican [117, 118]. Certain *in vitro* studies have demonstrated that NG2 can be inhibitory to the process of axonal outgrowth, while other studies have demonstrated that NG2 is not only permissive to axonal growth but can stabilize the axon post-injury [103, 119–122]. A novel brain-derived proteoglycan Te38 is highly inhibitory to axon outgrowth [123], and it is present within the lesion site of a spinal cord injury [123, 124]. The Te38 proteoglycan could be detected for up to 4 weeks post-injury; however, the exact expression pattern for Te38 has yet to be determined.

While the inhibitory effects of CSPGs on axons have been known for some time, the effects they exert on other cell populations, such as oligodendrocytes, have only been recently considered. CSPGs exert a highly inhibitory influence on oligodendrocytes [125–127]. Studies utilizing an *in vitro* model of the glial scar with isolated OPCs revealed a significant inhibition of process outgrowth and differentiation [125]. When the OPCs processes came into contact with CSPGs, the cellular process retracted and avoided contact with the CSPG rich surface, similar to what was observed with neurons. Additional studies by other laboratories have confirmed these findings, suggesting CSPGs exert an inhibitory effect on oligodendrocytes [126, 127].

In vivo, CSPG expression can modulate the migration and differentiation of endogenous OPCs that are attracted to CNS lesions. Accumulation of OPCs at the edge of a lesion can be observed after spinal cord injury, in the regions distal to the lesion site [128–130]. There is somewhat conflicting evidence as to whether they can differentiate into myelinating oligodendrocytes in the area around the lesion. They may differentiate into mature cells but may not survive for longer times after injury [131, 132]. In addition to the CS-GAGs, the presence of other glycosaminoglycans like hyaluronan needs to be taken into consideration, as hyaluronan is known to modulate the behavior of oligodendrocytes. The presence of hyaluronan in active demyelinating lesions in MS and other white matter diseases can inhibit the differentiation of endogenous OPCs located near the lesions [133, 134]. Thus, the expression of glycosaminoglycans at a lesion site has effects not only on neuronal regeneration but possibly on remyelination as well.

The cellular receptors which interact with proteoglycans have only recently been identified. Potential surface receptors for proteoglycans on neurons and glial cells include the protein tyrosine phosphatase sigma (PTPσ). Loss of the PTPσ receptor by gene knockdown or inhibition of receptor

activation renders neuronal process outgrowth insensitive to CSPG deposits [134]. Studies have also recently shown that the PTPσ receptor may also be involved with the observed inhibitory effects CSPGs exert on oligodendrocytes [127, 135]. While PTPσ has been observed in both neurons and oligodendrocytes, other receptors for CSPGs have been identified in neurons only, including Nogo 66 Receptor 1 (NgR1), Nogo 66 Receptor 3 (NgR3), and leukocyte common antigen receptor (LAR) [136, 137]. To date, there are no definitive reports that NgR1, NgR3, or LAR function as CSPG receptors in oligodendrocytes.

8. Therapeutic Modulation of CSPGs: Chondroitinase ABC

Given that CSPGs are a major barrier to repair and regeneration, a key experimental strategy for enhancing axonal regeneration and plasticity has been to modify the inhibitory extracellular environment. The bacterial enzyme chondroitinase ABC (cABC) can neutralize the inhibitory nature of the CSPG molecules and thus was tested in many injury models. cABC is an enzyme produced by the bacteria *Proteus vulgaris*, which catalyzes the removal of the glycosaminoglycan side chains from the central core protein [138]. Numerous *in vivo* studies have shown that by treating a CNS lesion site with the enzyme cABC, axonal sprouting, growth, and plasticity are significantly increased [139–147]. This is often accompanied by a significant increase in recovery of motor function, which suggests that cABC is an attractive candidate for therapeutic applications. However, to date, there is little anatomical evidence to suggest that administration of cABC has allowed for axonal regrowth over long distances. While substantial axonal growth can be observed into and even through a SCI lesion, there are no studies that demonstrate axonal regrowth to its original target. This is likely due to the slow growth rates of regenerating axons, and the short time points examined in the reported experiments. It could also be due to the fact that measured improvements in motor function result from other mechanisms, such as formation of alternative neuronal circuits, improved survival of motor neurons, as well as remyelination. The underlying explanation for the motor improvements observed after cABC administration are currently not well understood.

One critical issue with the use of cABC as a therapeutic agent is the thermal instability of the enzyme. The biological activity of cABC decreases quickly when in solution and is sensitive to temperature. A study by Tester et al. [148] demonstrated that if cABC in solution was incubated at 37°C, its enzymatic activity was significantly reduced after 3 days and totally lost by 5 days. These findings reveal that while the therapeutic ability of cABC is promising, experimental stabilization of the enzyme is likely to be needed for it to be a more effective therapeutic agent [148]. Currently, methods for administering cABC range from a constant infusion, soaking a piece of gelfoam in cABC and applying it directly to the injury site [142], to directly infusing the enzyme into the ventricles of the brain [149]. There have been several experimental approaches to stabilize the enzyme or to provide a continual supply of active enzyme at a lesion. Modification of the protein structure by amino acid substitutions has generated forms of cABC that show more stable enzymatic activity [150]. Alternatively, the gene for cABC can be inserted into a viral vector, which can be either directly introduced into a CNS lesion or introduced into cells which can then be transplanted into a lesion [151–155]. Both methods can produce sustained levels of enzyme *in vivo*, with digestion of CSPGs. To avoid the use of viral vectors, we have incorporated cABC into biodegradable nanospheres, which protects the enzymatic activity for many months [156]. When tested in an *in vivo* model of spinal cord injury, the slow release of active cABC from nanospheres resulted in a significant increase in the level of CSPG digestion at 2 weeks and 1 month after injury when compared to direct injections, as well as a significant increase in the level of axonal sprouting throughout the lesion site [157].

While cABC treatment digests CSPGs, there is evidence that following deglycanation, intact CSPGs are eventually reconstituted in the tissue. This turnover occurs approximately two-weeks following deglycanation and was demonstrated in a study by Crespo et al. [138]. In this study, a CNS lesion was inflicted to the nigrostriatal tract, followed by cABC treatment. Prepared lesion site extracts were analyzed with the 1B5 antibody to identify digested CSPGs. At 1, 4, and 7 days after lesion 1B5 labeling was clearly visible, but by 14 days after lesion 1B5 immunoreactivity was no longer present [138]. This suggests that by 2 weeks postlesion the digested CSPGs have been either reformed or cleared from the lesion site. Therefore, a continual supply of cABC within the lesion site is presumably needed until axonal outgrowth and repair of neural connections is complete. Should CSPG turnover occur before axonal outgrowth or remyelination is complete, the newly deposited CSPGs would once again create a highly inhibitory influence and halt the regeneration. However, there is clearly a need for a balance of CSPG degradation and reconstitution. While cABC treatment offers a temporary breakdown of CSPGs within the glial scar to foster axonal outgrowth, total ablation of the glial scar results in more severe tissue damage [12]. Therefore, at least the core proteins of CSPGs and perhaps the intact CSPG molecule may be needed to stabilize the spinal cord environment after CNS injury.

9. Postulated Mechanisms for cABC Improvements in CNS Function after Injury

While many studies claim functional recovery is a result of axonal regeneration, they fail to rule out other adaptive mechanisms that may account for the recovery of function. Such mechanisms include possible spontaneous remyelination of spared axons or the formation of alternative neural circuits [158–160]. It is essential in the field of neural regeneration research, especially when agents like cABC are tested, that the motor behavior data be correlated with neuroanatomic data to determine the cellular mechanisms underlying motor recovery. Introducing cABC into a post-injury environment rich in CSPGs, which have well-known inhibitory effects on neurons, OPCs, and possibly other cells will change their behavior in the dynamic lesion environment. Therefore, the

observed recovery of function could be the collective results of multiple events not related to axonal regeneration.

Most studies using cABC have focused on the effects on neurons, documenting significant axonal sprouting and growth around a lesion after cABC treatment [139–144]. However, the extent of axonal regeneration is widely variable between studies, and no long distance regeneration is observed. While experimental treatment with cABC is frequent in the field of spinal cord injury, it has also been tested as a potential therapeutic agent in stroke and TBI research. Studies demonstrate that while the functional/behavioral recovery is mixed, in all cases of cABC administration following TBI or stroke, there are signs of anatomical reorganization, with degradation of the CSPG matrix, perineuronal nets, and evidence of axonal sprouting and growth [145–147].

It is also possible that what is sometimes characterized as axonal regeneration may actually be the sprouting of spared axons [161]. When the total percentage of CNS axons found to regenerate is compared to the results found after PNS injury, the results are striking. Under optimal conditions, on average 90% of axons are found to regenerate within the PNS [161]. However in regeneration studies examining CST axons, only 2–10% of CST axons are reported to regenerate and for relatively short distances [161]. Likewise ~7% of rubrospinal axons regenerate, at best, following experimental intervention [161]. And finally, the distance CNS axons actually grow is rather meager. As summarized by Bradbury and McMahon [161], the proportion of axons (CST axons) induced to grow longer than two spinal segments is less than 10%.

CSPGs have a strong inhibitory influence on OPC process outgrowth and differentiation, both *in vitro* and *in vivo*, and this may affect remyelination near a CNS lesion. There is significant OPC infiltration to a SCI lesion site after cABC treatment, which occurs quickly after injury [121, 124, 130, 162]. The inhibitory effects of individual CSPGs were identified using an *in vitro* assay; the strongest inhibition was observed with the mixes of CSPGs containing high levels of both neurocan and phosphacan. This is highly homologous to the CSPGs composition of the glial scar [124]. Cellular effects included stunting of cytoplasmic process outgrowth and myelin sheet formation, and impeding the migratory ability of the OPCs. It is well known that following an injury to the CNS, OPCs begin to migrate towards the site of oligodendrocyte loss and some spontaneous remyelination does occur; however, sustained remyelination of spared axons has never been well documented [22, 128].

Using a spinal cord injury model, treatment with cABC showed a significant increase in the number of OPCs found inside and around the lesion site [130]. This occurs quite quickly after injury and is independent of axonal sprouting. In the absence of cABC, OPCs migrate towards the distal edge of the lesion over the two week period. This finding mirrors that of previous studies, which show an accumulation of OPCs in the regions distal to the lesion site [128, 129]. Moreover, this also correlates to the time when the expression of CSPGs and establishment of the glial scar in the proximal area immediately adjacent to the lesion becomes maximal [10, 29, 103]. The administration of cABC immediately after injury allowed for a significant increase in overall number of OPCs as well as access into areas proximal and within the lesion. Interestingly, a large increase in OPC number was observed deep inside the lesion site [130]. The signal attracting OPCs into the lesion cavity is unknown. However, there is speculation that demyelination and myelin breakdown may be the cue. One element common to cuprizone and SCI models is the microglial activation and clearance of myelin debris [10, 29, 163]. Thus, demyelination followed by microglial activation may be recruitment signals for OPCs.

10. Xyloside and Other Agents

In the field of MS research, it has been demonstrated that inhibiting the synthesis of CSPGs improves the outcome of the pathology [126]. Xyloside blocks the attachment of the GAGs to the CSPG's central core protein, which is the primary inhibitory element of CSPGs. Thus, the deposition of CSPGs without GAG side chains does not create an environment that retards tissue repair. When xyloside was administered in a lysolecithin-induced model of demyelination, it not only resulted in a greatly reduced area of demyelination, but there was also a significant increase in the number of mature oligodendrocytes found within the MS plaque [126]. Due to potential side effects, xylosides may not be useful to treat SCI in humans. However, the results further support the observation that CSPGs are inhibitory to repair of the injured CNS, due to the presence of GAG side chains, and that neutralizing CSPGs allows for regenerative/reparative response to progress.

Alternatively, *in vitro* experiments have successfully targeted the enzymes responsible for the polymerization of the GAG side chains [164]. When siRNA directed against chondroitin polymerizing factor (ChPF) was introduced into astrocytes, CSPG core proteins were produced, but they were not decorated with the GAG side chain and did not pose a significant barrier to axonal growth from cerebellar granule neurons [164]. In another study, it was demonstrated that the administration of xylosyltransferase-1 (XT-1), a DNA enzyme against the GAG chain initiating enzyme, greatly reduced the presence of GAG chains, which subsequently allowed for the regeneration of microtransplanted adult sensory axons past the central core of a spinal cord stab lesion [165]. Disrupting CSPGs synthesis could provide a potentially novel therapeutic treatment paradigm, targeting assembly of the GAG chains in newly synthesized proteoglycans, rather than digestion of existing GAG chains with cABC.

11. Summary and Future Directions

Significant progress has been made in understanding the post-injury tissue response after CNS injury, especially the identification and characterization of molecules at the lesion site that inhibit axonal regeneration, and identifying agents that can enhance the capacity for repair. There is overwhelming evidence that following *any* insult to the CNS, traumatic or degenerative, an inflammatory reaction occurs, and the activation of microglia, astrocytes, and invasion of vascular macrophages result in an upregulation and synthesis

of CSPGs (Figure 3). There is also strong evidence that CSPGs form an important protective barrier, preventing further secondary tissue damage [12–15]. However, it is also a primary reason axonal regeneration and remyelination fails following any type of injury to the CNS.

Modification of CS-GAG expression on CSPGs, while not completely ablating the CSPG molecule, is a reasonable approach to facilitating repair. One promising avenue is the administration of cABC at the site of a CNS lesion to remove the inhibitory GAG chains and neutralize the inhibitory nature of CSPGs. cABC has been tested in many injury studies and often results in an improvement in motor function. However, long distance axonal regeneration is rarely observed. In the SCI field, it is widely accepted that cABC will need to be used in conjunction with agents such as neurotrophins that can directly promote neuronal survival and stimulate axonal growth. An alternative strategy targets the new synthesis of CSPGs after injury by blocking the addition of GAG side chains. These agents also reduce the presence to the inhibitory GAG chains, but they have not been as well documented in injury models. Like cABC, it is probable that they need to be utilized in conjunction with factors that directly stimulate axonal sprouting and regrowth to maximize repair and recovery of function.

While modification of CSPG expression is an attractive therapeutic approach, there are still many questions which need to be answered. How long would an agent such as cABC be needed? Axonal growth is a slow process, which can take months in animals and longer in humans; however, cABC may only be needed at specific times to allow the regeneration process to initiate. Delivery methods are very important as well. cABC is a labile enzyme, losing activity quickly in solution. There are several choices for long term delivery including viral expression and biomaterial packaging. However, how would this be silenced once regeneration is accomplished and cABC is no longer needed? Excess cABC would destabilize perineuronal nets in uninjured tissue, which could potentially result in aberrant axonal sprouting and circuit formation as well as synaptic instability. Although there are no reports of adverse effects in animal studies, there is also the possibility that the long term presence of cABC could trigger an immune response in humans.

The most important consideration for repair of the damaged CNS is that controlling CSPG expression is only one aspect of the solution. Neurotrophic agents that directly promote neuronal survival and axonal growth are just as important, particularly for regeneration of specific neuronal populations, as are methods to direct these regenerating axons to their targets. CSPG regulation is but one important step in restoring CNS function after injury or disease.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Hyaluronan-Phosphatidylethanolamine Polymers Form Pericellular Coats on Keratinocytes and Promote Basal Keratinocyte Proliferation

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Aged keratinocytes have diminished proliferative capacity and hyaluronan (HA) cell coats, which are losses that contribute to atrophic skin characterized by reduced barrier and repair functions. We formulated HA-phospholipid (phosphatidylethanolamine, HA-PE) polymers that form pericellular coats around cultured dermal fibroblasts independently of CD44 or RHAMM display. We investigated the ability of these HA-PE polymers to penetrate into aged mouse skin and restore epidermal function in vivo. Topically applied Alexa^{647}-HA-PE penetrated into the epidermis and dermis, where it associated with both keratinocytes and fibroblasts. In contrast, Alexa^{647}-HA was largely retained in the outer cornified layer of the epidermis and quantification of fluorescence confirmed that significantly more Alexa^{647}-HA-PE penetrated into and was retained within the epidermis than Alexa^{647}-HA. Multiple topical applications of HA-PE to shaved mouse skin significantly stimulated basal keratinocyte proliferation and epidermal thickness compared to HA or vehicle cream alone. HA-PE had no detectable effect on keratinocyte differentiation and did not promote local or systemic inflammation. These effects of HA-PE polymers are similar to those reported for endogenous epidermal HA in youthful skin and show that topical application of HA-PE polymers can restore some of the impaired functions of aged epidermis.

1. Introduction

Hyaluronan (HA) is a ubiquitous extracellular matrix tissue polysaccharide belonging to the glycosaminoglycan family, which is characterized by repeating hexosamines and uronic acid [1–3]. Skin HA accounts for approximately 50% of total body HA and occurs in both the epidermal, and dermal layers. Here it performs a variety of functions that are related to its rheological, viscoelastic and biological properties [4–6]. For example, its rheological properties contribute to the overall quality, hydration, permeability, and immune barrier functions of skin while its unique viscoelastic properties protect skin cells from mechanical damage [7–11]. The biological properties of HA include a contribution to cell survival, proliferation, and migration, and result from its ability to activate key signaling cascades through interactions with cellular HA receptors, which in keratinocytes is primarily CD44 [12–18]. HA is also an important regulator of skin immune surveillance [19] and response to injury processes [15, 20–25]. Although the primary structure of HA is simple and is composed only of linear repeating N-acetyl-D-glucosamine and glucuronic acid disaccharides, its functions are complexly regulated and dependent upon its organization by extracellular and cellular proteins as well as by polymer size [14, 16, 21, 23].

The organization of HA in the extracellular matrix and around cells is a critical component of its skin functions [6, 26, 27]. On keratinocytes, HA is structured as compact

pericellular coats that are maintained by CD44. Dermal HA is more abundantly extracellular and linked to a variety of proteoglycans including versican [28–34]. The cellular and extracellular organization of skin HA is critical for its retention in the papillary dermal and keratinocyte layers. Quantitative loss of HA from these layers is associated with skin pathologies including poor wound healing, reduced skin elasticity/mobility, and loss of keratinocyte tight-junctions and permeability barrier functions [4, 27, 29, 35–44]. Polymer size also contributes to the skin functions of HA. For example, high molecular weight (HMW), native HA in skin protects against tumor initiation [45], provides intrinsic water binding properties of skin [26] and is required for dendritic cell functions [15, 19, 46]. It regulates the proliferation and differentiation of the basal keratinocyte layer during homeostasis and response to injury [4, 6, 47, 48] and contributes to the barrier/hydration function [26, 34, 49] and structure of the stratum corneum [50].

Most HA in homeostatic skin is high molecular weight but fragmentation occurs following injury or prolonged exposure to UV. In cooperation with the fragmentation of other extracellular matrix components, HA fragments activate signaling cascades in keratinocytes and dermal fibroblasts that control migration, survival, and redifferentiation required for repair of injured skin [51–58]. HA fragments are also key regulators of innate immunity and are required for in-trafficking and proinflammatory cytokine expression of macrophages [15, 19]. The different functional effects of native versus fragmented HA likely result from selective interactions with specific receptors and differential effects of polymer size on the clustering/signal activation through these receptors [13, 15, 59]. The effects of native HA on homeostatic keratinocyte functions are mediated through CD44 [4, 30] while repair functions of HA fragments involve coordination of signaling through CD44 : RHAMM and TLR2, 4 complexes [55, 60, 61].

Chronological skin aging results in physiological alterations of keratinocytes and epidermal functions that contribute to epidermal thinning or atrophy, and barrier dysfunction, delayed wound repair, as well as increased susceptibility to pathologies including ulceration, dermatitis, and eczema [4, 60, 62]. Although age-associated epidermal dysfunction is not well understood, it is associated with changes in HA concentration and organization, and CD44 display [4, 63]. Experimental models have established that reduction or loss of keratinocyte CD44 results in epidermal changes that are similar to aging dysfunction such as thinning of the epidermal layer, barrier dysfunction, modified HA metabolism, reduced HA production, altered keratinocyte differentiation, and decreased skin elasticity. Application of HA to aged mouse skin partially restores permeability barrier homeostasis and epidermal thickness [41, 42]. Topical or injected HMW HA products have had variable effects in restoring a sustained physiological and hydrated microenvironment of youthful skin required for optimizing tissue repair and rejuvenation [4, 62, 64, 65]. Transepidermal or dermal HA delivery modalities, although promising, have similarly failed to reliably replenish sustained high levels of native HMW HA in the epidermis or dermis of aged skin [66–68]. This failure is likely because of poor penetration of topically applied HA formulations of MW greater than 50 KDa [39, 69–71], reduced HA capture in the epidermis as a result of declining CD44 levels [4, 42, 72] and aberrant organization as well as rapid clearance of the exogenous HA formulations [40, 60].

To address this problem, we developed a cell-based screening method for identifying HA-phospholipid (phosphatidylethanolamine, HA-PE) formulations that form pericellular HA coats on fibroblasts and keratinocytes in a CD44-independent manner [73]. We show that topical administration of HA-PE to the shaved skin of aged wild type or CD44−/− mice increased HA within the epidermal layer. This HA modification promoted basal keratinocyte and hair follicle proliferation as well as increased epidermal thickness but does not detectably alter the differentiation of keratinocytes. In addition, topical application of HA-PE in vivo did not result in either a local or a systemic inflammatory response.

2. Methods

2.1. Animals. Forty-five retired breeder ten-month old female C57BL/6 mice (Jackson Laboratory) were used for the multiple cream application studies. An additional twelve retired breeder twelve-month old female C57BL/6 mice were used for the Alexa^{647} mouse experiments.

Animals were individually caged in a temperature-controlled environment with a 12 h light/dark cycle and fed a standard mouse chow diet. All experiments were approved by and compliant with the standard operating protocols of the Animal Use Subcommittee at the University of Western Ontario, Canada (2009-051).

2.2. Preparation of HA-PE and HA. For preparation of the HA-PE cream (Patent identification: WO2011140630 A1) [74], 1.35 mL of unrefined liquid soy lecithin (Soy Lecithin GT non-GM IP, Imperial-Oel-Import, Germany) was mixed thoroughly at room temperature with 1.35 mL of 1% v/w Sodium Hyaluronate Solution (500 kDa, Medical Grade, Lifecore Biomedical, Chaska, MN, USA) and 252 μL of isopropanolol. Subsequently, 3.78 mg of dry 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) was added and mixed thoroughly as a linking agent. After mixing for 10–15 minutes, 3 mL of vehicle cream was added and mixed for another 5 minutes. For preparation of unmodified HA cream, 1.35 mL of 1% v/w Sodium Hyaluronate Solution was mixed thoroughly with 3 mL of vehicle cream.

A water-based cream (Mango Face cream, Aquatech, Toronto) was used throughout the study as the vehicle for mixing with unmodified HA or HA-PE. Formulated creams were stored protected from light at 4°C.

2.3. HA Pericellular Coat Detection. HA pericellular coats were detected using particle exclusion [75]. Dermal wild type, CD44−/−, RHAMM−/−, and CD44 : RHAMM−/− embryonic fibroblasts were plated onto 35 mm tissue culture dishes in DMEM + 10% FBS for 24 hrs. Cells were then fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer, 5 mM $CaCl_2$, pH 7.2, for 30 min then washed gently in cacodylate buffer. 1 mL of either FITC-labeled 0.4 mm microspheres (Invitrogen) or

formalized sheep erythrocytes (1×10^8 erythrocytes/mL) was added to each 35 mm culture dish and swirled gently so that cells were evenly covered. Dishes were incubated for 15–30 min at 37°C to let beads or erythrocytes settle around cells. As a control, cells were incubated with 200 μg/mL bovine testicular hyaluronidase (Sigma) for 1 h at 37°C prior to performing the particle exclusion assay. Cells were then photographed with a Nikon inverted microscope equipped with epifluorescence and Hoffmann optics.

2.4. Preparation of A^{647}-HA. In the preparation of A^{647}-HA, the solution was protected from ambient light. In a laminar flow hood, 0.0028 g of EDC (Sigma Aldrich, http://www.sigmaaldrich.com/) was dissolved in a 1 mL solution of 20 mM MES and 30% ethyl alcohol (pH 4.5) in a 15 mL tube. Subsequently, 200 μL of pharmaceutical grade 1% v/w Sodium Hyaluronate Solution (500 kDa) (Lifecore Biomedical, Chaska, MN, USA) was added. After five minutes, 300 μL of Alexa Fluor 647 Hydrazide, Tris (triethylammonium) salt (Life Technologies, http://www.lifetechnologies.com/) was added. The A^{647}-HA solution was placed on a rocker at room temperature for 12 hours and then dialyzed (10,000 dalton cut off, Thermoscientific) at 4°C. The entire volume of 1X PBS buffer was exchanged at 1, 3, and 5 days. The retained A^{647}-HA solution was retrieved from the dialysis apparatus and stored in a 15 mL tube in the 4°C fridge.

2.5. Application of A^{647}-HA/HA-PE to Mice. Mice were anesthetized using Isofluorane gas for application of cream. While anesthetized, the upper dorsum of the back was shaved with an electric razor, leaving a strip of hair in midline to define the right and left sides. 0.18 g of A^{647}-HA and A^{647}-HA-PE cream was applied to the left and right sides, respectively. Two mice were euthanized using a CO_2 chamber at each of six time points: 40 min, 2 h, 4 h, 8 h, 24 h, and 72 h following cream application.

2.6. Preparation and Analysis of A^{647}-HA-PE. A full-thickness biopsy of the A^{647}-HA/HA-PE treated area was obtained and lightly fixed for 10 minutes in 1.5% paraformaldehyde containing 0.5% cetylpyridinium chloride monohydrate [75] in PBS. Each specimen was subsequently fixed in 4% paraformaldehyde (pH 7.4) for an additional 30 min then stored at −4°C. Tissue samples were paraffin processed, mounted then analyzed with a Nikon Eclipse motorized upright microscope. The penetration of A^{647}-HA/HA-PE into the epidermis was quantified using Image J, which converted fluorescent images to pixel density per a tissue area. A total of twelve C57BL/6 retired breeder female mice were used for this experiment.

2.7. Treatment of Mice with HA-PE. Three treatment arms were used for these experiments: HA-PE cream, HA cream, and vehicle cream and 15 mice were used per group. For the initial cream application, mice were anesthetized, shaved, and treated as described above. Subsequent once daily cream applications were performed without anesthesia. Five mice in each treatment arm were sacrificed after one, five, and ten applications. At each of these times, mice were euthanized, a blood sample via a cardiac puncture and two four-millimeter punch skin biopsies was obtained. The remainder of the shaven treatment area was excised and stored at −80°C. To provide a positive control for inflammation markers, an additional mouse was wounded with a 4 mm punch biopsy as described previously [76], then tissue harvested using an 8 mm punch biopsy 3 days after wounding. All tissue biopsies were fixed in 4% paraformaldehyde/PBS at 4°C for 24 h. Samples were then processed for paraffin histology sections. For immunohistochemistry, tissue sections were deparaffinized and antigen retrieval was performed by heating tissue sections in a microwave oven in 0.01 M aqueous sodium citrate buffer (pH 6.0). Tissue sections were washed then incubated with one of the following primary antibodies: rabbit Ki67 monoclonal (1 : 100 Abcam), rabbit K10 monoclonal (1 : 7000 dilution, Abcam), and rat F4/80 monoclonal (1 : 100 dilution, AbD Serotec) followed by the appropriate biotinylated secondary antibody. Sections were counterstained with hematoxylin then mounted in Richard-Allan Scientific Cytoseal 60 (ThermoScientific). Digital images of stained tissue sections were obtained using an Aperio Scanscope. Five representative areas were taken per mouse and analyzed using Image J.

2.8. Measurement of Epidermal Thickness. To evaluate epidermal thickness, the above tissue sections stained with hematoxylin and eosin (4X magnification images) were analyzed using Image J. For each mouse, fifty serial measurements at 75 um intervals per section were made to determine epidermal thickness (*stratum basale* to the *stratum granulosum*) [77].

2.9. TNF-α ELISA. Activated macrophages and proinflammatory cytokines, such as TNFα, appear to be ubiquitous players in cutaneous inflammation regardless of the inciting stimulus whether wounding or sterile inflammation [78–80]. Dissected tissue samples from each of treatment and group and wounded tissue were mixed gently RIPA lysis buffer and a protease inhibitor tablet for 10 min and then sonicated for three 20-second pulses. Samples were incubated on a rotary shaker at 4°C in lysis buffer for an additional 45 min then centrifuged at 13,000 g (4°C) for 30 min. The pellet was discarded and supernatant used for the TNFα-ELISA assays (Abcam), which were performed per the manufacturers instructions. Triplicate samples of 100 μL of each were used for these assays.

2.10. C-Reactive Protein ELISA. Serum was obtained from blood harvested via cardiac puncture. The serum samples were centrifuged at 3000 rpm at 4°C for 10 mins using an Eppendorf Centrifuge and stored at −80°C until analysis. Samples were analyzed for the presence of C-reactive protein using an ELISA kit (Abcam) and assays were performed using the manufacturers instructions. Triplicate samples (100 μL) of each sample were used for these assays.

2.11. Statistical Analysis. One Way ANOVA and Tukey test, as a post hoc analysis, were used to determine statistical significance between groups using a P value of 0.05. Data are expressed as mean ± standard error of the mean (S.E.M) of at

least five independent samples as described above. Statistical analysis was performed using GraphPad Prism 6 (GraphPad software).

3. Results

3.1. Formulating and Characterizing HA-PE Polymers That Form Pericellular Coats. Using a cell-based screen, HA-PE polymer formulations were prepared. The polymer formulations that formed nanoparticles were discarded while those that were nonparticulate and formed pericellular coats on fibroblasts were identified using particle exclusion assays (Figure 1(a)). Addition of HA-PE polymer resulted in a significant increase in the number of cells that formed pericellular coats compared to those formed when PBS alone was added. A light but significant increase in the number of cells forming coats was also stimulated by exogenous unmodified HA of the same size used for HA-PE formulations. Nevertheless, the addition of HA-PE stimulated coat formation to a significantly greater extent than HA alone and a trend to formation of larger coats was also observed (data not shown).

CD44 has been demonstrated to be critical for keratinocyte, fibroblast, and smooth muscle cell HA coat formation [32–34, 40, 42] and both CD44 and RHAMM [12, 14, 61] have recently been implicated in the binding of HA to tumor cells and fibroblasts. The role of these HA receptors in the formation of endogenous HA and HA-PE coats was examined by comparing wild type primary mouse embryonic fibroblasts (MEF) with those lacking RHAMM, CD44, or both receptors due to genetic deletion of these genes (Figure 2(a)). Loss of RHAMM resulted in highly variable numbers of MEF forming endogenous pericellular coats but these were not significantly different from wild type fibroblasts. Loss of CD44 however resulted in a 6-fold reduction in the number of MEF exhibiting HA coats. Dual loss of CD44 and RHAMM did not further reduce coat loss from that observed in CD44−/− MEF. These results show that CD44 is the major HA receptor that facilitates endogenous HA coat formation in MEF consistent with previous studies of other cell types including keratinocytes [33, 39, 42]. The effect of HA-PE on RHAMM−/−, CD44−/−, RHAMM : CD44−/−, and wild type MEF coat formation was compared next to begin to identify the mechanisms for HA-PE-promoted pericellular HA coats. As shown in Figure 2(b), the addition of HA-PE to these different fibroblast genotypes resulted in a similar number of cells with HA coats indicating that HA-PE effects were not dependent upon HA receptor display including CD44, which has previously been shown to mediate endogenous coat formation (Figures 2(b) and 2(c)). However, HA-PE stimulated coat formation in CD44−/− MEF was sensitive to hyaluronidase as were endogenous coats (Figure 2(c)). These results predict that addition of a PE group to HA promotes its direct association with cells in a CD44-independent manner.

To determine if HA-PE promotes coat formation in other cell types particularly in vivo, its effect on skin keratinocyte HA pericellular coats, which have previously been shown to require CD44 expression [30, 42] was next evaluated. Adult mouse back skin was chosen for these studies since it has been reported to produce very little endogenous HA [29], permitting more sensitive detection of exogenous applications of HA-PE using staining methods to detect accumulated HA. Consistent with previous studies [29], very little HA staining was observed in control (PBS) keratinocytes. Topical application of HA-PE to wild type mice significantly increased HA staining in the epidermis (Figure 3) but did not detectably increase HA staining in the dermis when compared to PBS-treated controls likely because the dermis produces large amounts of HA and the staining method was not sensitive enough to detect elevation above this high background. These results show that HA-PE not only enhances pericellular HA coat formation in cultured MEF but also in epidermal keratinocytes in vivo. These results also show that HA-PE crosses the outer cornified epidermal layer more efficiently than unmodified HA.

The association of HA with keratinocytes in mouse skin in vivo depends upon CD44 expression [30]. We therefore next assessed if the loss of CD44 altered HA-PE mediated increases in keratinocyte associated HA in vivo. As shown in Figure 3(b), topical application of HA-PE increased hyaluronan staining of CD44−/− epidermis compared to PBS controls suggesting that epidermal accumulation of HA-PE is CD44 independent in vivo similar to cultured MEF. To more directly follow the association of small amounts of HA-PE with the epidermal and dermal skin layers, we labeled HA-PE with Alexa-dye and analyzed its distribution after a single topical application to mouse skin.

3.2. A^{647}-HA-PE Accumulates in the Epidermis and Penetrates into the Dermal and Subdermal Skin Layers. A^{647}-HA-was prepared, linked to PE (A^{647}-HA-PE) or not (A^{647}-HA) and applied as equal amounts of HA to the shaved back skin of mice as described in methods. Skin biopsy samples were collected from 40 min to 72 h after the single application, processed for histology, and examined with a confocal microscope. Confocal images showed accumulation of A^{647}-HA-PE and particularly A^{647}-HA on the stratum corneum but A^{647}-HA-PE also penetrated into the stratum granulosum as well as the basal keratinocyte layers (Figure 4). Fluorescence was also observed in dermal fibroblasts and even within the subcutaneous muscle layer. Further analysis of the epidermis revealed that A^{647}-HA-PE was most strongly associated with subpopulations of basal keratinocytes and formed coats around these cells (Figure 4, arrows). A^{647}-HA-PE also was detected in fibroblasts of the upper dermis. In contrast, A^{647}-HA primarily accumulated in the outer stratum corneum with much smaller amounts penetrating into the epidermis and dermis. In particular, A^{647}-HA accumulation was not concentrated in the basal layer (Figure 4).

Since the boundary of the epidermal layer is clearly identified, the amount of A^{647}-HA-PE in this layer was quantified using image analysis as described in methods. For these analyses, the fluorescent intensity of A^{647}-HA, A^{647} HA-PE, and a negative control vehicle cream were compared from the stratum basale to stratum granulosum layers of the epidermis. A^{647}-HA-PE penetrated into the epidermis as early as 40 min after application and could be detected up to 72 h after application (Figure 4). Accumulation of A^{647}-HA-PE

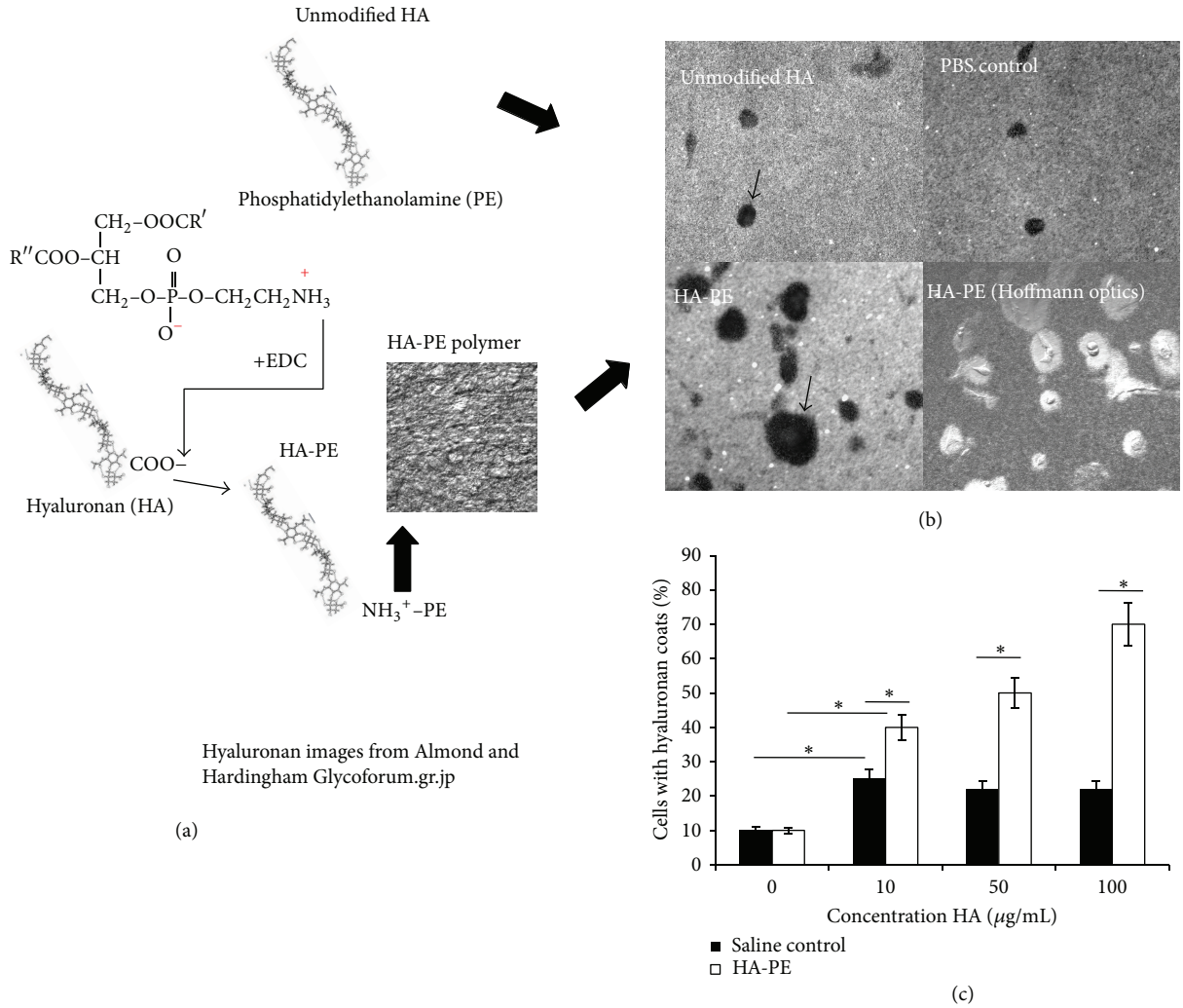

Figure 1: HA-PE simulates pericellular coat formation in cultured fibroblasts. 500 kDa HA was linked to PE using EDC to achieve a stoichiometry of 1 : 10 (HA : PE). (a) This ratio was selected because it resulted in the largest pericellular HA coats, which were detected using fluorescent particle exclusion assays. (b) The black patches on the epifluorescent images are areas of pericellular coats. A Hofmann image is included which shows cells at the center of the pericellular coats. Images were taken with a 10X objective. The percentage cells/10x field that were surrounded by HA coats were calculated using Hofmann optics. (c) Values are Mean and S.E.M $n = 3$ fields. Asterisks indicate statistical significance ($P < 0.01$).

in skin was significantly greater than A^{647}-HA at all time points but reached a maximum difference of 5 fold at 2 h. This significantly elevated accumulation of A^{647}-HA-PE versus A^{647}-HA was sustained for 24 h suggesting that HA-PE was able to establish a stable organization within the epidermis. Although A^{647}-HA-PE levels were still greater than A^{647}-HA at 72 h after application, the difference did not reach statistical significance (Figure 4).

Confocal analysis (24 h shown, Figure 4) showed that A^{647}-HA primarily accumulated in the outer stratum corneum with little penetration into the dermis. A^{647}-HA-PE was also present in the stratum corneum but unlike unmodified A^{647}-HA, accumulated around dermal fibroblasts. These results show that HA-PE readily penetrates into and is retained in the epidermal and deeper skin layers

The formation of pericellular HA coats have been linked to cellular detachment during mitotic rounding of proliferating fibroblasts and smooth muscle cells [12, 16, 81]. HA coats have also been linked to migration and differentiation of keratinocytes in organotypic cultures [4, 13, 14, 30, 82, 83]. We therefore next assessed if increasing keratinocyte HA coat formation by application of HA-PE affects the proliferation or differentiation of this skin cell type.

3.3. HA-PE Increases Epidermis Thickness and Basal Keratinocyte Proliferation but Does Not Affect Keratinocyte Differentiation or Dermal Cell Proliferation. The consequence of repeated HA-PE topical application on epidermal thickness was first quantified. As shown in Figure 5, daily topical application of HA-PE significantly enhanced epidermal

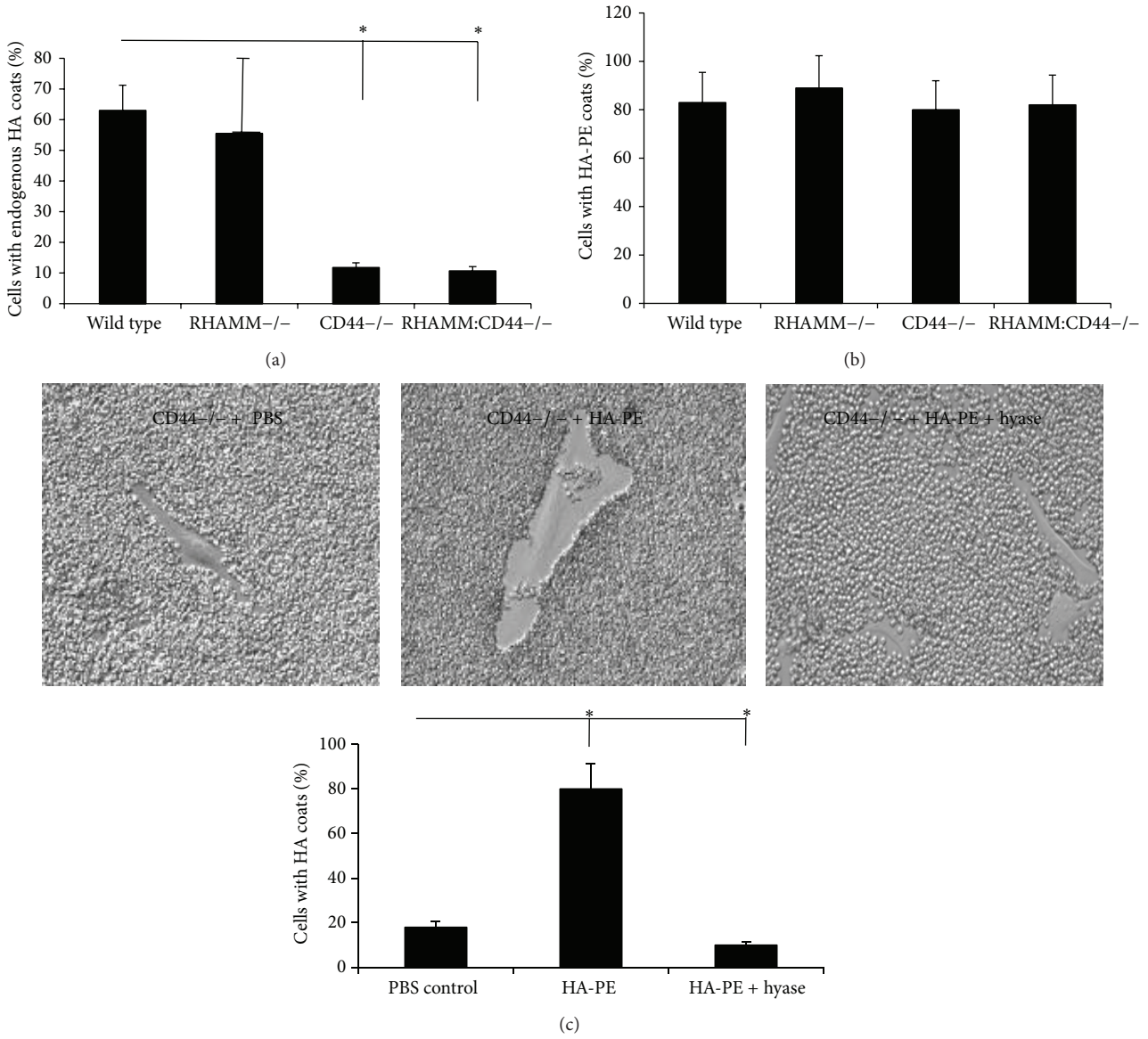

Figure 2: Endogenous HA coats require expression of CD44 while HA-PE generated coats do not. The numbers of wild type and RHAMM−/− fibroblasts that form endogenous HA coats are not significantly different. In contrast, loss of CD44 significantly reduces the numbers of fibroblasts forming coats. This is not further reduced by loss of both CD44 and RHAMM. (a) However, loss of CD44 has no effect on the number of cells forming coats as a result of HA-PE addition. (b) The pericellular coats formed by CD44−/− fibroblasts in the presence of HA-PE are destroyed by hyaluronidase. (c) Images were taken with a 20X Hoffmann objective. Values are the Mean and S.E.M $n = 75$ cells.

thickness by 24 h, 5 days, and 10 days after treatment initiation when compared to application of unmodified HA or vehicle control. Maximal epidermal thickness occurred between 24 h and 5 days and was sustained throughout the treatment period (Figure 5). The tissue samples were dehydrated prior to analysis of epidermal thickness, and therefore values do not account for any contribution of increased tissue hydration from HA.

Consistent with this thickening effect on the epidermal layer, topical application of HA-PE stimulated basal keratinocyte proliferation compared to unmodified HA or vehicle controls as detected by Ki67 staining, which is a cell proliferation marker. In addition, increased Ki67 staining of the suprabasal keratinocytes was observed in the HA-PE group. The majority of epidermal proliferation and regeneration occurs by activation of stem cells in the basal layer of the epidermis. The suprabasal levels contain transit-amplifying (TA) intermediate stem cells which are defined by a finite number of cell divisions before entering a terminal differentiation pathway (85). The HA-PE cream appears to stimulate proliferation of both the basal layer and TA stem cells. Increased keratinocyte proliferation was observed 1 day after treatment initiation and was sustained throughout the treatment period, corresponding well with the time frame observed for the increase in epidermal thickness (Figure 6). Analysis of DAPI- and Ki67 stained skin sections 24 and 72 hr

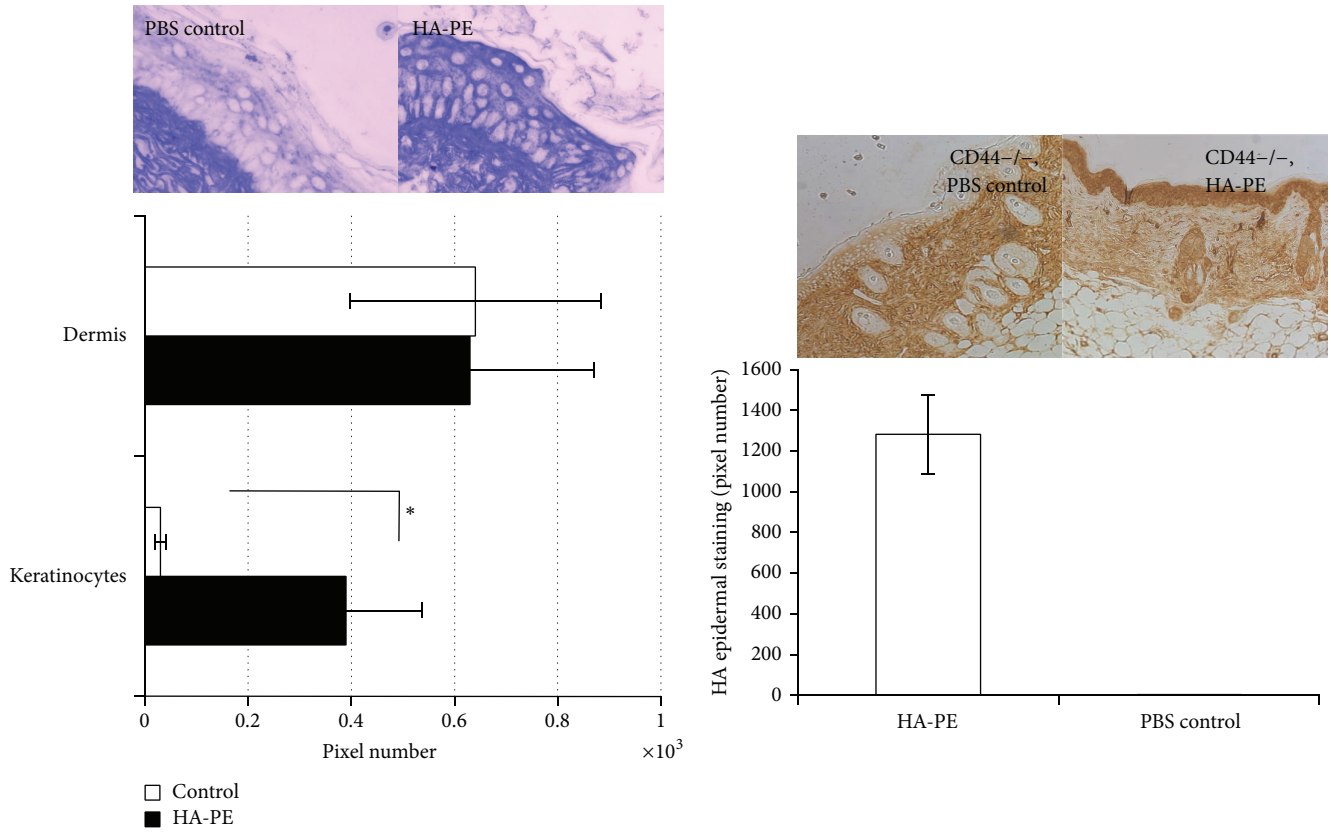

Figure 3: Topical application of HA-PE stimulates HA accumulation in wild type and CD44−/− keratinocytes in mouse skin in vivo. Topical application of HA-PE to shaved B57/BL6 wild type mice increases HA in the keratinocyte layer as detected by biotinylated HABP (blue staining indicates hyaluronan detected by HABP probe). (a) Staining 3 days after application is not detectably different in the dermis and although patchy and variable in the epidermis is significantly different in HA-PE versus vehicle controls (brown staining indicates hyaluronan detected by HABP probe) ($P < 0.05$). Images were taken with a 20X bright field objective. Similar results were obtained when the epidermal layer was analyzed for hyaluronan after HA-PE was applied to B57/BL6 CD44−/− mice. Significantly greater HA staining was observed in HA-PE treated versus vehicle controls ($P < 0.0001$). (b) Images were taken with a 10X brightfield objective. Values in (a) and (b) are the Mean and S.E.M $n = 3$ mice, 5 tissue section/mouse.

after application of HA-PE versus HA showed that dermal cell number was similar in both treatments indicating that HA-PE does not stimulate fibroblast or other dermal cell proliferation. This predicts that HA-PE effects on proliferation are limited to the epidermal layer.

Growth factors such as keratinocyte growth factor (KGF) that promote keratinocyte proliferation coincidentally inhibit keratinocyte differentiation and hyaluronan production [84, 85]. We therefore next assessed if elevating HA around keratinocytes affects their differentiation cycle. The consequence of HA-PE application on the expression of keratin-10 (K-10) was quantified using immunohistochemistry. No detectable effect of HA-PE application on K-10 staining was observed suggesting that HA-PE does not modify keratinocyte differentiation (Figure 7).

3.4. Topical Application of HA-PE Does Not Affect Local Skin or Systemic Inflammation. Since HA and its fragments are potent regulators of the immune system in particular innate immunity [15, 19, 46, 86], which could indirectly affect keratinocyte proliferation, we next determined if topical application of HA-PE affected local or systemic inflammation. F4/80 staining of skin tissue sections and TNFα was used to identify localized activation of macrophages while serum CRP levels were monitored to determine effects on systemic inflammation. As shown in Figure 9, the application of HA-PE did not increase F4/80 staining levels compared to unmodified HA or vehicle controls (Figure 8). Furthermore, the levels of macrophage activation detected by this method were very low compared to those detected in skin wounds, which are known to contain high levels of activated macrophages [87] and were used as a positive control. Similarly local TNFα and systemic CRP inflammation levels were not increased by topical application of HA-PE compared to unmodified HA or vehicle controls (Figures 9(a) and 9(b)).

4. Discussion

Our results identify a method for preparing HA-phospholipid (PE) polymers that do not require CD44 display for forming

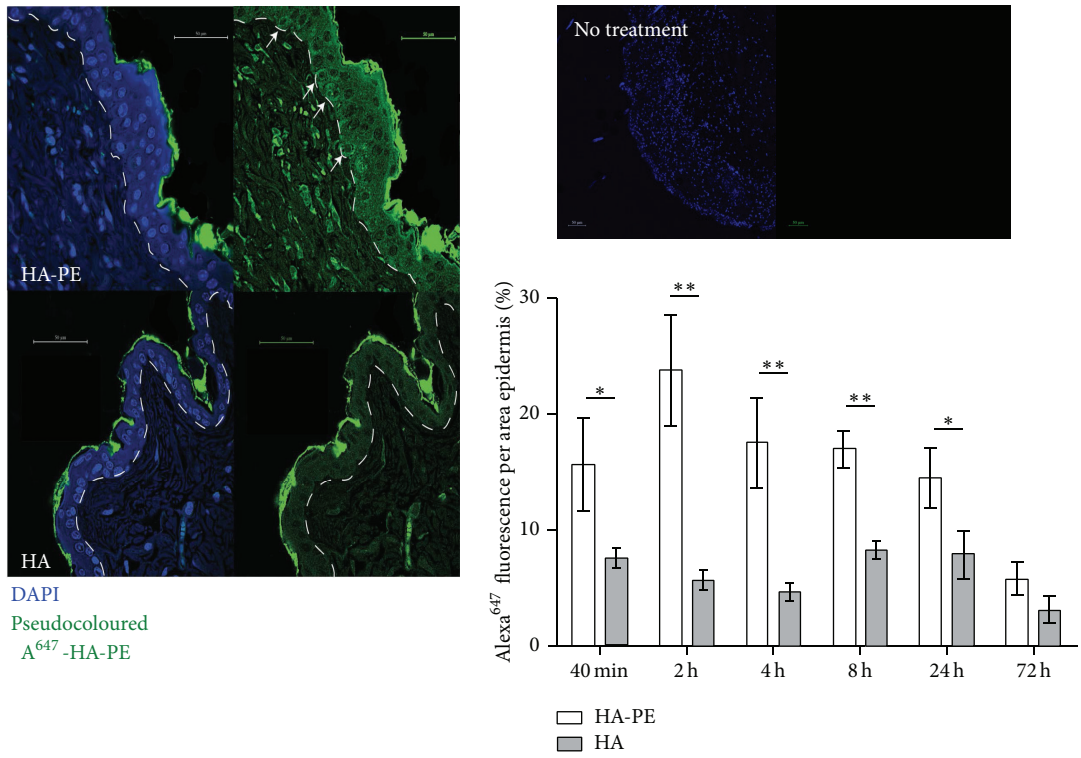

FIGURE 4: Topical A^{647}-HA-PE enters the epidermis. Significantly increased fluorescent staining of A^{647}-HA-PE is observed compared to A^{647}-HA in the epidermis (stratum basale to stratum granulosum) at all time points up to 24 h (mean ± S.E.M, 20 skin images) ($^{*}P < 0.05$, $^{**}P < 0.001$) (right panel). Representative confocal images of A^{647}-HA-PE and A^{647}-HA treated mice sectioned at 24 h are shown. Merged Dapi and $Alexa^{647}$ staining are shown to indicate cellularity in tissue sections. The dashed line indicates the junction of the epidermis and dermis. Arrowheads indicate areas of enhanced pericellular fluorescence. A^{647}-HA-PE and A^{647}-HA staining was pseudocolored green. Blue staining is DAPI. An untreated adjacent skin section (no treatment) photographed using the $Alexa^{647}$ channel is shown as a control for autofluorescence.

pericellular coats around fibroblasts in culture and aged keratinocytes in wild type and CD44−/− mice. These results are consistent with a mechanism whereby HA-PE inserts directly into the cell membrane via the phospholipid entity. The topical applications of this HA-PE polymer increased epidermal thickness of aged female mouse skin as a result of stimulating the proliferation of the basal keratinocyte layer in the absence of detectable dermal or systemic inflammation or changes in keratinocyte differentiation rates.

Topical application of unmodified HA to intact skin in situ penetrated into the epidermis and dermis as previously reported [42, 69–71] but this was limited compared to HA-PE polymers. In addition, most of the unmodified HA remained associated with the stratum corneum and was not retained in skin as long as HA-PE. The ability of unmodified HA to penetrate the skin in small amounts, which can occur in both rodent and human skin, is dependent upon molecular weight. Thus, HA polymers smaller than 50,000 daltons readily penetrate human skin ex vivo while larger HA chains do not [69]. The average MW of HA used in the present study was 500,000 Da and therefore the limited association of unmodified HA with the epidermis is likely due to the polymer size restriction. The penetration of this HA size through the outer stratum corneum is facilitated by the addition of a phospholipid moiety and this addition likely increases solubility of HA in lipids (e.g., ceramides, cholesterol, and fatty acids) [88] that are present in the stratum corneum. Retention within the epidermis and dermis is likely promoted by the ability of keratinocytes and fibroblasts to capture and retain HA-PE as demonstrated in culture. Since HA-PE is able to organize as a pericellular coat in the absence of CD44 display, HA-PE polymers are able to spontaneously organize within the epidermis and we predict that keratinocyte CD44 acts at least in part as an anchor for attaching HA to outside of cells.

Dermal and epidermal homeostasis is compromised in aging and a variety of disease processes. Aged skin is characterized as dry, atrophic, inelastic, and wrinkled. The key molecule required for water retention within skin is HA. Both the amounts and organization of HA change in skin with intrinsic (chronological) and extrinsic (photodamage) aging [4, 89, 90]. With chronological aging while HA is retained within the aged dermis, it is precipitously lost from the epidermis. HA retained within the aged dermis is however modified in its organization and this change together with epidermal loss are thought to contribute to some of the above malfunctions of aged skin [4]. Additionally, expression of skin HA receptors including CD44 are increasingly reduced with age. Even though intrinsic and extrinsic skin aging

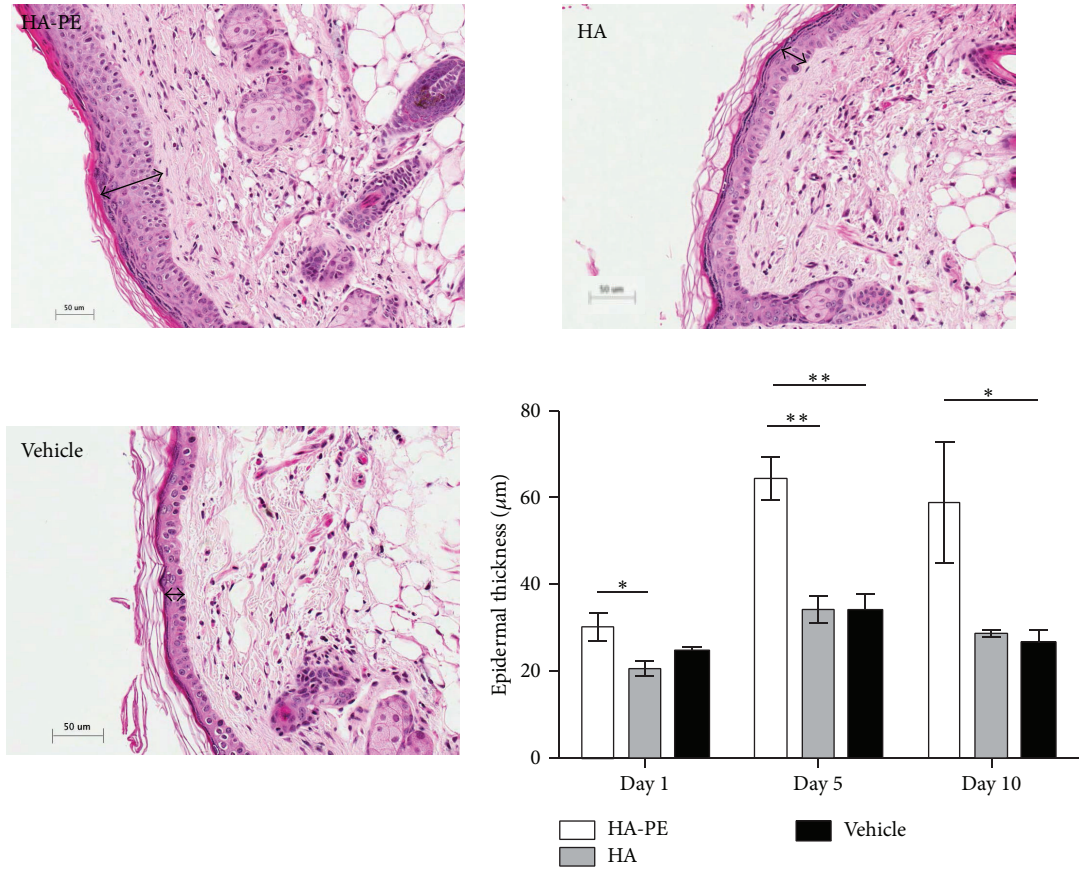

FIGURE 5: HA-PE increases epidermal thickness. There is a significant increase in epidermal thickness (μm) of mice treated daily with HA-PE for 1, 5, and 10 days compared with control groups (HA and Vehicle cream) (mean ± S.E.M, 5 mice per group) ($^{*}P < 0.05$, $^{**}P < 0.001$). A single representative H&E section from each of a day 10 HA-PE, HA, and Vehicle Cream mouse is shown, arrows indicate epidermal thickness.

are distinctive processes, they share similarities in molecular mechanisms. For example, extrinsic causes of premature aging such as chronic exposure to UV also results in loss of skin moisture, HA and HA receptor expression. Importantly, photoaging is also associated with a decrease in the size of HA that will likely affect its ability to organize into structures such as pericellular coats [72]. HA performs a variety of functions in skin and this age dependent loss is considered to impact upon skin moisture, barrier functions, epidermal thinning, and sluggish response to injury [34]. The ability of HA-PE polymers to increase rodent epidermal thickness and epidermal proliferation predict that this formulation has the potential for reversing some of the intrinsic and extrinsic age-related epidermal defects such as epidermal thinning and reduced repair response [91–93].

Our results showing that HA-PE promotes epidermal thickening and keratinocyte proliferation are similar to the ability of exogenous high molecular weight HA to affect functions of aged keratinocytes in culture and in vivo [33, 35, 39, 42] although in the present study topical HA-PE had a significantly greater effect than unmodified HA. Our data suggest that this results from increased basal keratinocyte proliferation rather than enhanced differentiation. Previous reports have similarly noted an increase in keratinocyte proliferation in response to topical HA but noted this effect was maximal for intermediate (50,000–400,000 Daltons) and no effect was noted for large HA (>400,000 Daltons) [39]. In another study, topical application of large HA promoted epidermal barrier function and keratinocyte differentiation [42]. The different effects of high versus intermediate HA fragments in these studies may have been due to the documented poor penetration of high molecular weight HA [69] that is confirmed here. The ability of 500,000 dalton HA-PE to promote keratinocyte proliferation is likely because it efficiently penetrated to and was retained in the basal keratinocyte layer to a much greater extent than unmodified HA.

A number of topical HA formulations have been developed in the past decade most of which are composed of intermediate-small HA fragments (e.g., <50,000 daltons) or nanoparticulate formulations [4, 94]. Although these have shown efficacy (e.g., [95]) in terms of treating skin disorders, we pose that HA-PE formulations will be more effective since they are designed to replace the naturally occurring HA pericellular coat that is depleted during aging and other conditions that cause epidermal atrophy [35, 39] and will also therefore be retained within the epidermis for longer times. Particulate formulations by their nature tend to stimulate endocytosis and such formulations are rapidly depleted from tissues [96–98]. Topical application of intermediate unmodified HA has the advantage of being nonparticulate and able

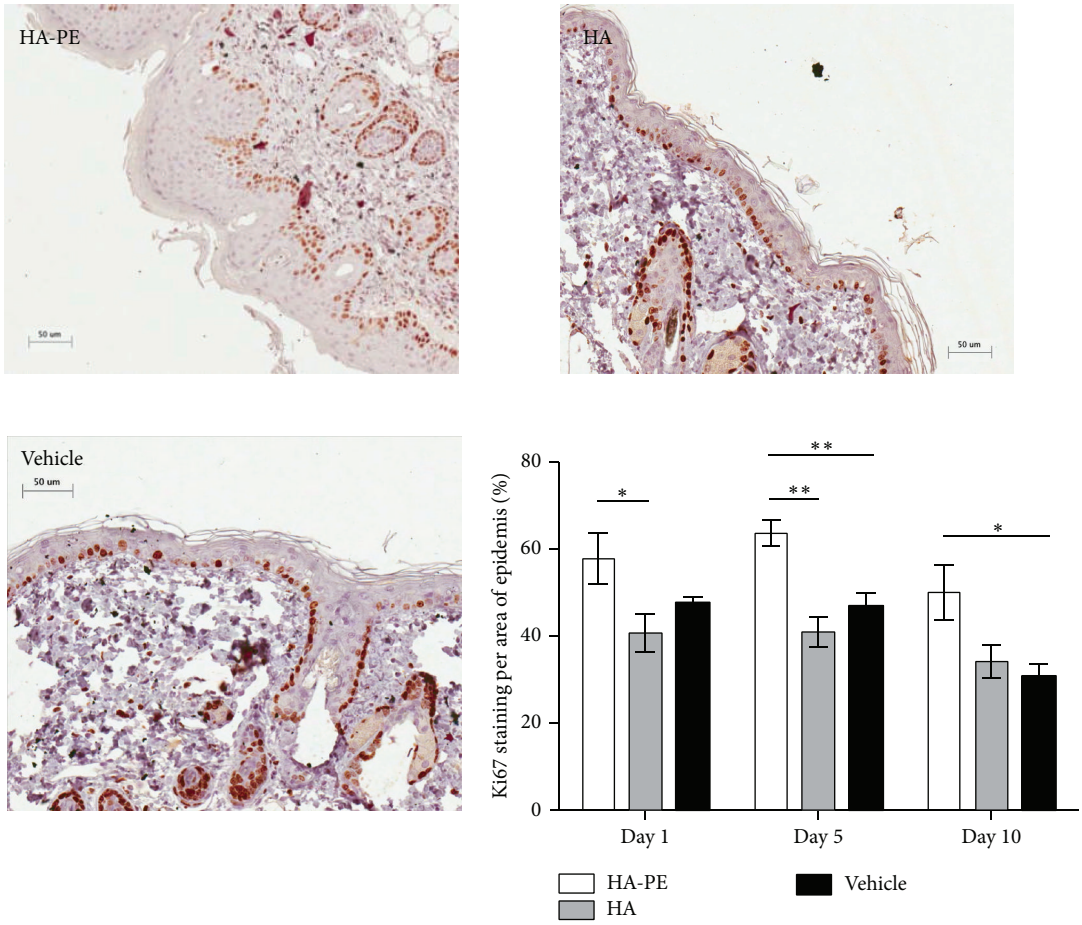

Figure 6: HA-PE stimulates keratinocyte proliferation. There is a significant increase in the percentage of positive Ki67 staining per an area of epidermis of mice treated daily with HA-PE for 1, 5, and 10 days compared with HA or Vehicle Cream (mean ± S.E.M, 5 mice per group) ($^{*}P < 0.05$, $^{**}P < 0.001$). A single representative Ki67 section from each of a day 10 HA-PE, HA, and Vehicle Cream mouse is shown.

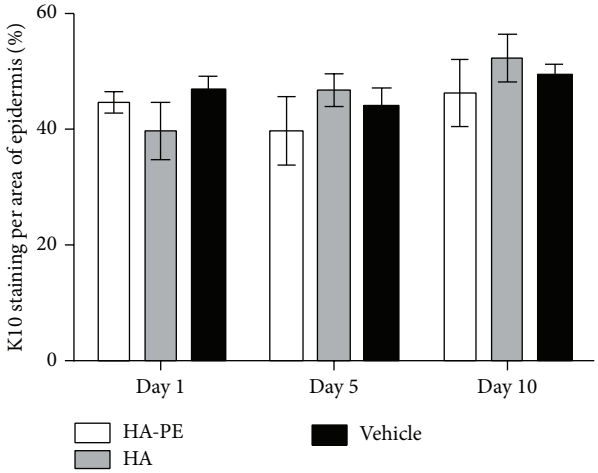

Figure 7: HA-PE cream does not affect suprabasal keratinocyte differentiation. The percentage of K10 staining per an area of epidermis for mice treated daily for 1, 5, and 10 days with either HA-PE, HA, or Vehicle Cream (mean ± S.E.M, 5 mice per group).

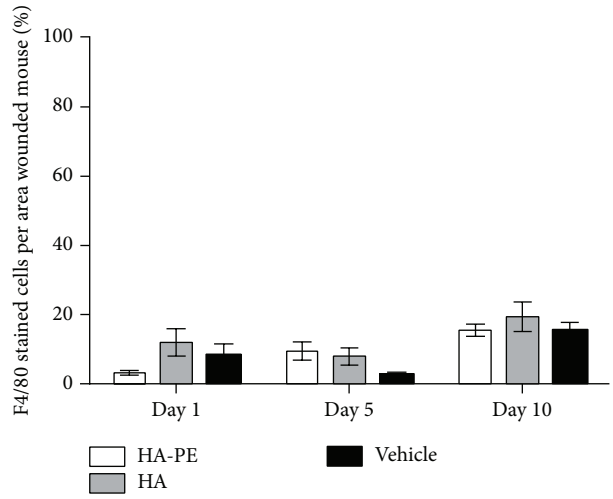

Figure 8: HA-PE cream does not elicit a local inflammatory response. The percentage of positive F4/80 staining per an area of a wounded mouse for mice treated daily for 1, 5, and 10 days with either HA-PE, HA, or Vehicle Cream (mean ± S.E.M, 5 mice per group).

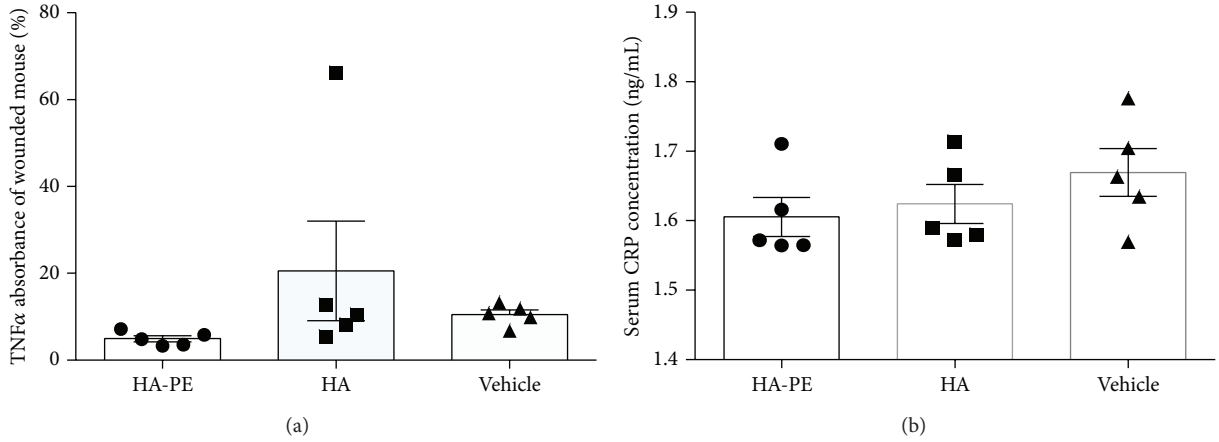

Figure 9: HA-PE cream does not elicit a local or systemic inflammatory response. The TNFα expression as a percentage of a wounded mouse control for day 10 treated mice with HA-PE, HA, or Vehicle Cream using an ELISA (mean ± S.E.M, 5 mice per group). (a) The serum CRP concentration (ng/mL) of day 10 treated mice with HA-PE, HA, and Vehicle Cream using an ELISA (mean ± S.E.M, 5 mice per group) (b).

to penetrate skin readily but is dependent upon the expression of keratinocyte CD44 for effects on keratinocyte function, which is depleted with age-related or other causes of epidermal atrophy.

The consequences of exogenous HA or inhibition of endogenous HA show that CD44 mediates the consequences of HA on keratinocyte function [4, 6, 60, 99]. For example, the effects of topical HA on keratinocyte differentiation and proliferation are ablated when CD44 function is blocked or expression is lost [35, 39, 42]. CD44 appears to regulate multiple downstream signaling pathways to control these keratinocyte processes and these include coordination of signaling through EGFR, activation of RHO GTPase and translocation of activated ERK1,2 to the cell nucleus [4, 17, 35, 99, 100]. The mechanisms by which HA-PE control basal keratinocyte proliferation and the dependence of this effect on CD44 expression are currently being investigated.

Conflict of Interests

The authors declare that there are no conflict of interests regarding the publication of this paper.

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Research Article

Heparin and Liver Heparan Sulfate Can Rescue Hepatoma Cells from Topotecan Action

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Topotecan (TpT) is a major inhibitory compound of topoisomerase (topo) I that plays important roles in gene transcription and cell division. We have previously reported that heparin and heparan sulfate (HS) might be transported to the cell nucleus and they can interact with topoisomerase I. We hypothesized that heparin and HS might interfere with the action of TpT. To test this hypothesis we isolated topoisomerase I containing cell nuclear protein fractions from normal liver, liver cancer tissues, and hepatoma cell lines. The enzymatic activity of these extracts was measured in the presence of heparin, liver HS, and liver cancer HS. In addition, topo I activity, cell viability, and apoptosis of HepG2 and Hep3B cells were investigated after heparin and TpT treatments. Liver cancer HS inhibited topo I activity in vitro. Heparin treatment abrogated topo I enzyme activity in Hep3B cells, but not in HepG2 cells, where the basal activity was higher. Heparin protected the two hepatoma cell lines from TpT actions and decreased the rate of TpT induced S phase block and cell death. These results suggest that heparin and HS might interfere with the function of TpT in liver and liver cancer.

1. Introduction

Heparin and heparan sulfate (HS) are polysulfated sugars, members of glycosaminoglycans (GAGs), present in animal and human tissue in free or protein bound forms.

Heparan sulfate glycanated proteins are found in the extracellular matrix and on the cell surface [1]. Recent studies provide ample evidence on the central role of these molecules in cell life including cellular organization, cell behavior, and cell signaling [1, 2]. Heparin und heparan sulfates bind several growth factors [3–7], hormones [8], cytokines [6, 9], and chemokines [10, 11] that are implicated in cell regulation [12] in several ways.

The cellular role of HS has been studied for years without a major breakthrough achieved [13–18]. Biochemical approaches failed to collect convincing data for intracellular proteoglycan activity. Recently tentative evidences were provided supporting the regulatory effect of HS on cell proliferation and showing that these GAGs affect DNA-transcription factor interactions [19]. Our previous experiments resulted in similar conclusions [17]. For the first time confocal microscopy evidenced the nuclear localization of GAGs and proteoglycans [20–22]. Since then the nuclear function of proteoglycans is coming to focus of interest [22]. Nevertheless, the issue is still an elusive part of proteoglycan research.

We reported that heparin and liver HS inhibit the plasmid relaxation activity of topoisomerase I enzyme in vitro [21]. Furthermore, we provided evidence for heparin and HS cellular uptake and accumulation in the nucleus [17, 22]. These observations motivated us to investigate if GAG molecules are able to interfere with topoisomerase I (topo I) activity and modify the effect of topo I inhibitory drug topotecan (TpT) [23].

2. Materials

2.1. Liver Tissue. Surgical specimens from cancer patients were sent to our department for histological diagnosis and were used with the permission of the regional ethical committee. The samples were frozen in liquid nitrogen and stored at −80°C until used.

2.2. Cells. American Tissue Type Culture Collection HepG2 and Hep3B cell lines were used after 12–15 passages. Cells were plated at a density of 2×10^5 cells/mL into six-well plates in 2 mL/well Dulbecco's modified Eagle's medium with 5% (v/v) fetal calf serum (GIBCO-BRL).

2.3. Chemicals. Unless specified otherwise, the chemicals were purchased from Merck (Darmstadt, Germany). Hind III and Klenow DNA polymerase enzymes were obtained from Promega (Madison, USA). Topotecan was a gift of SmithKline Beecham (King of Prussia, USA). Heparin was purchased from Sigma (Steinheim, Germany).

Protein concentration was determined by using the Coomassie protein assay kit of Pierce (Rockford, USA). Recombinant topo I and polyclonal human anti-topo I IgG (scl-70) from Topogen (Columbus, USA) were used for western blot.

3. Methods

3.1. Cell Numbers, Viability, and Morphology. Mitochondrial succinate dehydrogenase activity [24] was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) test, and cell numbers were counted in a hemocytometer.

Morphology of the two hepatoma cell lines was studied either by growing them onto coverslips or by preparing cytospin slides. Cells were visualized with hematoxyline-eosine staining.

3.2. Determination of Cell Cycle Parameters. HepG2 and Hep3B cells were washed twice with PBS then suspended in a buffer containing 0.1% sodium citrate, 0.1% Triton X-100, and 0.05 mg/mL ribonuclease, pH 7.7, at 10^6 cell/mL density. Before the analysis, the cells were stained with 50 μg/mL propidium-iodide (Sigma, Steinheim, Germany).

Cell cycle parameters were measured on a FACScan flow cytometer (Becton Dickinson, San Jose, USA) scanning the propidium-iodide signals and the forward and side scatter parameters. The Multicycle software of Robinovitch (Phoenics Flow San Diego, USA) was used for analyzing the results.

3.3. Protein and GAG Isolation, Quantification. Nuclei from liver specimens were isolated on saccharose gradient, according to Hogeboom [25]. The method of Duguet was used when nuclei were isolated from hepatoma cell lines [26]. Isolation was carried out in the presence of 1.0 mM phenyl-methylsulfonyl fluoride (PMSF). Nuclei were further extracted with 0.35 M NaCl buffer as described elsewhere [27]. Protein concentration of the nuclear extract was determined by the Coomassie protein kit of Pierce Biotechnology, Inc. (Rockford, USA) according to the protocol of the manufacturer. The isolation of GAG and HS, their electrophoresis, and their colorimetry were described previously [17, 21, 28].

3.4. Topoisomerase I Relaxation Assay. ATP independent relaxation of supercoiled pBR322 plasmid Stratagene (La Jolla, USA) was done in standard 30 μL reaction mixture containing various amounts of 0.35 M NaCI nuclear protein extract, 0.5 μg supercoiled plasmid DNA, 40 mM Tris-HCl, pH 7.5, 100 mM KCl, 10 mM MgCI, 2.5 mM dithiotreitol, 0.5 mM EDTA, 1 mM phenyl-methylsulfonyl fluoride, and 1 μg bovine serum albumin (as carrier protein) [29]. Samples were incubated at 37°C for 30 min with or without various GAGs as indicated. The addition of GAGs did not influence the pH of the reaction mixture. Enzymatic reactions were terminated by adding 1 μL 10% sodium dodecylsulfate and 1 μL (10 mg/mL) proteinase K. Relaxation of supercoiled DNA plasmid was determined by running the samples on analytical 1% agarose gel in Tris-boric acid buffer (50 mM Tris, 5 mM boric acid, 1 mM EDTA, pH 8.1.) for 12 h at 24 V. Gels were poststained with 0.1 μg/mL ethidium bromide and visualized under UV light [29].

3.4.1. Plasmid Cleavage Reaction for Topoisomerase I. pBR322 plasmid was linearized with EcoRI enzyme and then end-labeled with 5 μCi α^{32} dATP and 20 U Klenow DNA polymerase at 30°C for 15 min. Heating at 75°C for 10 min terminated the reaction. The labeling was removed from one end of the plasmid by Hind III digestion. Unincorporated radioactivity was separated by filtration through a Sephadex G50 (Pharmacia, Uppsala, Sweden) column. Ten microgram protein from the 0.35 M NaCI extract of liver or cancer cell nuclei was coincubated with 100 μM topotecan and 8000 cpm labeled plasmid with or without 2 μM heparin for 10 min. The reaction was terminated with SDS and proteinase as described above and the samples were run on 1% agarose gel with denaturing sample buffer (0.45 M NaOH). The gels were dried and exposed to Kodak X-omat film [29].

3.4.2. Electrophoretic Mobility Shift Assay to Study the Competition of DNA and Heparin for Topoisomerase I. pBluescript plasmid was digested with Hpa II restriction enzyme. One of the restriction fragments with 516 base pair was separated and labeled with DIG-11 dUTP and terminal deoxynucleotide transferase (Roche, Mannheim, Germany), as suggested by the manufacturer. Based on its sequence analysis the labeled fragment contained 8 potential topoisomerase I binding sequences [30]. Ten unit purified topoisomerase I enzyme was incubated with 35 ng digoxigenin labeled DNA fragment in 40 mM Tris-HCl pH 7.5, 100 mM KCl, 10 mM MgCl, 2.5 mM dithiotreitol, and 0.5 mM EDTA. 1 mM phenyl-methylsulfonyl fluoride and 1 μg bovine serum albumin (as carrier protein) in a total volume of 20 μL. Identical reaction mixtures were supplemented with 10 or 100 ng commercial heparin. Incubation was carried out at 37°C for 15 min. Subsequently, the samples were run on 1.2% agarose gel and blotted

Table 1: (a) Effect of heparin and TpT on cell cycle parameters of HepG2 and Hep3B cells (results of 3 independent experiments). Concentrations used: TpT 1 μM, heparin 100 μg/mL. TpT treatment resulted in significant alterations ($P < 0.05$) of cell cycle parameters in both cell lines. Heparin did not change the cell cycle parameters ($P > 0.05$). S phase protection of heparin at combined treatment was statistically significant only in case of Hep3B cells. (b) Induction of apoptosis after TpT and heparin treatment. Proportion of dead/apoptotic cells after TpT and heparin treatment. Concentrations: TpT 1 μM, heparin 100 μg/mL. Values corresponding to the apoptosis gate of cell cycle represent the average of three experiments. TpT induced significant increase in apoptosis compared to the control in both cell lines. Heparin raised the proportion of apoptotic cells as compared to the controls for HepG2, but not for Hep3B cells. Significant decrease of dead cell fraction was found after a combined heparin and TpT treatment at both cell lines.

(a)

% of cells	HepG2			Hep3B		
Treatment	G_1	S	G_2	G_1	S	G_2
Control	52,6 ± 4,08	35,7 ± 3,29	11,6 ± 2,19	78,7 ± 0,42	15,9 ± 0,14	5,5 ± 0,28
1 μM TpT	15,8 ± 1,3[1]	80,8 ± 15,5[1]	4,2 ± 0,07[1]	2,06 ± 0,07[1]	65,6 ± 0,99[1]	13,7 ± 1,06[1]
100 μg/mL Heparin	58,9 ± 1,39	28,3 ± 3,58	12,8 ± 0,59	81,1 ± 0,28	14 ± 0,28	5 ± 0,07
Hp + TpT	14,5 ± 0,45	70,2 ± 3,97[3]	15,2 ± 0,68	35,4 ± 0,1	56,4 ± 2,33[2]	8,1 ± 2,4

[1]Student's t-test: $P < 0.02$ for HepG2, $P < 0.005$ for Hep3B (TpT compared to control).
[2]Student's t-test: $P < 0.005$ (Hp + TpT compared to TpT alone).
[3]Student's t-test: $P > 0.1$ (Hp + TpT compared to TpT alone).

(b)

%-of cells in apoptotic gate	HepG2	Hep3B
Control	5,65 ± 0,595	**3,53 ± 0,93**
1 μM TpT	18,67 ± 3,53[1]	**16,49 ± 0,821**[1]
100 μg/mL heparin	9,06 ± 0,16[2]	**2,48 ± 0,22**
Hp + TpT	**12,69 ± 2,34**[3]	**4,44 ± 1,07**[3]

[1]Student's t-test: $P < 0{,}001$ (TpT compared to control).
[2]Student's t-test: $P < 0{,}05$ (Hp compared to control).
[3]Student's t-test: $P < 0{,}05$ (Hp + TpT compared to TpT alone).

to positively charged nylon membrane (Boehringer (Roche), Mannheim, Germany). The positions of the DNA bands were visualized by sheep alkaline phosphatase-conjugated antidigoxigenin Fab fragments (Roche, Mannheim, Germany), using NBT and BCIP (Roche Applied Science) as chromogens.

3.4.3. Analysis of the Raw Data. The assays have been run in triplicates and statistical significance has been calculated based on data distribution (normal or non-parametric) using a Student's t-test or a Mann-Whitney test using Graphpad Prism 4.03 (Graphpad Software Inc., Suite, La Jolla, CA, USA).

4. Results

4.1. Influence of Heparin on Topotecan-Induced Cell Growth Retardation. To assess the interference of heparin with TpT, the two hepatoma cell lines were treated with 1 μM TpT alone or together with 100 μg/mL heparin. The effect was evaluated measuring the growth parameters of untreated or heparin treated cells. After 48 h of plating, the serum has been withdrawn and the action of heparin and topotecan was studied under serum-free conditions. Cells were counted daily. Figures 1(a) and 1(b) show that both cell lines reached the exponential phase of cell growth around 48 h after plating. Inhibitory action of TpT occurred at 72 h and was exerted continuously thereafter ($P < 0.001$ with Student's t-test). The growth of both hepatoma cell lines was inhibited by 100 μg/mL heparin, but at a lower extent than by TpT ($P > 0.05$ with Student's t-test for HepG2, $P = 0.02$ for Hep3B). A combined treatment revealed that heparin is capable of rescuing the cells against TpT action. This effect was statistically significant in Hep3B cells ($P < 0.001$ with Student's t-test), but not in HepG2 cells ($P > 0.1$).

4.2. Changes in Cell Cycle Parameters. Although heparin inhibited the proliferation of both hepatoma lines, no changes in cell cycle parameters were discernable. TpT induced dramatic G1-S phase block and cell death in both cell lines. Both effects were reduced when TpT was administered together with heparin (Table 1(a)). After TpT exposure, the ratio of apoptotic cells increased more than three and four times in HepG2 and Hep3B cells, respectively. This level fell back to the original value after the combined heparin + TpT treatment of Hep3B cell line, while only 30% protection was achieved in case of HepG2 cells (Table 1(b)). The S phase block decreased with 13% compared to TpT treatment in both cell lines, which was statistically significant only in Hep3B cells (Table 1(a)).

4.3. Topoisomerase I Activity of Liver Specimens. The topo I enzymatic activities of surgically removed human liver and

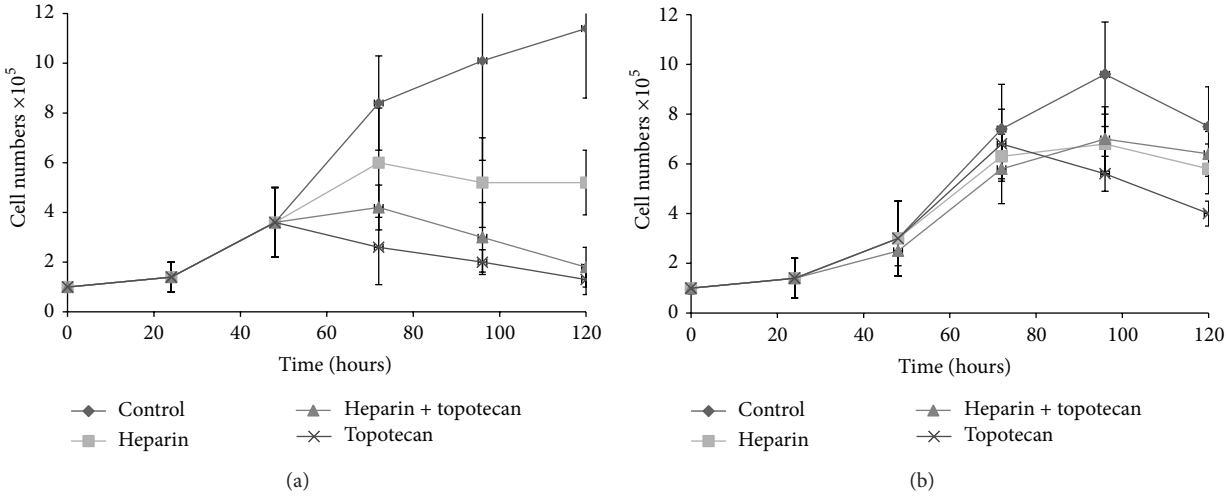

Figure 1: Effects of heparin and TpT on HepG2 (panel a) and Hep3B (panel b) cell numbers after 48–120 h incubation (results of 3 independent experiments). After 48 h of plating, serum has been withdrawn and the action of heparin and TpT was studied under serum-free conditions. Cells were grown for 72 h in the presence of 1 μM TpT, 100 μg/mL heparin alone, or in combination of the two. The curves represent the average of 6 parallels. Cells were counted daily. Both cell lines reached the exponential phase of cell growth around 48 h. Inhibitory action of TpT occurred at 72 h, which was exerted continuously thereafter (difference is significant with Student's t-test $P < 0.001$). 100 μg/mL heparin inhibited the growth of both hepatoma cell lines, but at a lower extent than TpT ($P > 0.05$ with Student's t-test for HepG2, $P = 0.02$ for Hep3B). In combined treatment heparin rescued the cells against TpT action. The protection was significant for Hep3B cells (b) ($P < 0.001$ with Student's t-test), but not for HepG2 cells (Figure 1 (a)) ($P > 0.1$).

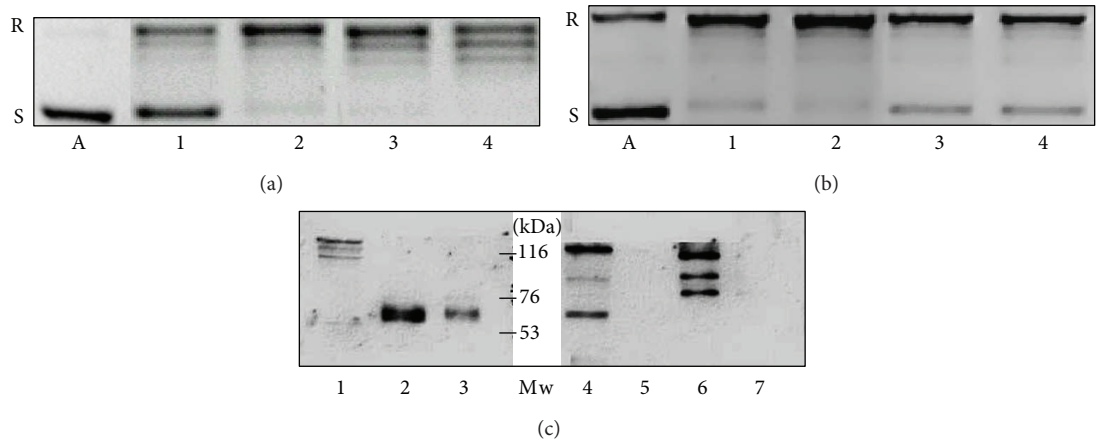

Figure 2: Differences of topoisomerase I activity in liver samples (a) and in hepatoma cells (b) (representative image of three independent experiments). Topoisomerase I activities in liver samples (a) and hepatoma cell lines (b) of 250 and 500 ng nuclear extract from peritumoral liver (a 1, a 2), hepatocellular carcinoma (a 3, a 4), HepG2 (b 1, b 2), and Hep3B cells (b 3, b 4). The activities of the specimens are related to the amounts of topoisomerase I protein in the cell nuclear extracts, as it is demonstrated on a western blot (c). Lane 1: Hep3B, lanes 2 and 3: HepG2, lanes 4 and 6: hepatocellular carcinomas, and lanes 5 and 7: peritumoral liver tissues. In addition to the 120 kDa band of the whole protein, the antibody reacts with more degradation products of the enzyme, including the 67 kDa catalytic fragment. A: plasmid control without cell nuclear extract. R: relaxed, S: supercoiled plasmid.

hepatocellular carcinomas as well as those of two hepatoma cell lines were studied. Nuclear extracts from peritumoral liver specimens showed low topo I activity. In contrast, 200 ng of nuclear protein from liver cancer resulted in total relaxation of the pBR322 plasmid. An identical amount of protein from peritumoral liver left more than half of the plasmid unattached. The activity in HepG2 cells was as high as in the primary liver cancer. Interestingly the less differentiated Hep3B hepatoma cell line retained only moderated topoisomerase I activity (Figures 2(a) and 2(b)).

Western blots loaded with 15 μg of nuclear protein extracts indicated that the measured activities were linearly

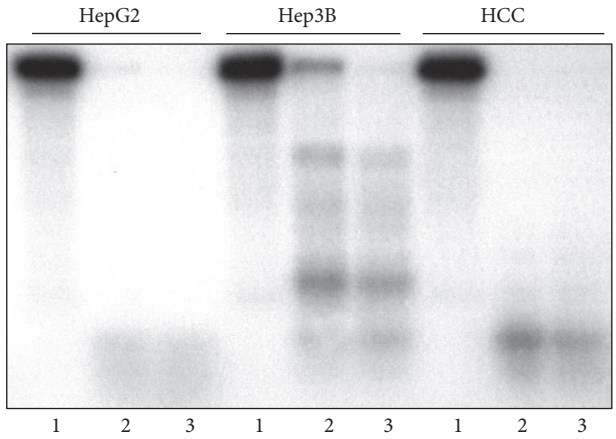

Figure 3: Differences of topoisomerase I plasmid cleavage activity trapped by TpT in liver samples and in hepatoma cells (representative image of three independent experiments). pBR322 plasmid was linearized and end-labeled with 5 μCi α^{32}P ATP by using 20 U Klenow DNA polymerase. To remove the labeling from one end, the linearized plasmid was digested with Hind III restriction endonuclease. Ten microgram 0.35 M NaCI nuclear extracts of HepG2, Hep3B, and human HCC specimens were incubated with 8000 cpm linearized plasmid in the absence or presence of 100 and 200 μM topotecan. Lanes 1: cleavage reaction without topotecan, lane 2: cleavage reaction with 100 μM topotecan, and lane 3: cleavage reaction with 200 μM topotecan. The activity of Hep3B extract was much lower than that of HepG2 and human Iiver cancer.

dependent on the amounts of topoisomerase protein in the cells. A significantly higher amount of topo I protein was detected in HepG2 cells and primary HCC than in the Hep3B cell line. The protein in peritumoral livers was below the detection level (Figure 2(c)). The cleavage reaction of TpT-trapped enzyme corroborated the activity of the liver and tumor samples. When using 10 μg HepG2 and human HCC nuclear extract, a complete fragmentation of the end-labeled plasmid was observed in the presence of 100 μM TpT. The same amount of Hep3B nuclear protein was considerably less effective (Figure 3).

4.4. Inhibitory Action of Glycosaminoglycans on Topoisomerase I Activities. Nuclear extracts with high topoisomerase I activity from HepG2 cells and a surgically removed hepatoma were used to assess the inhibitory potential of commercial heparin, normal liver HS, human hepatocellular carcinoma, and peritumoral liver tissue GAG specimens on topo I plasmid relaxation and TpT-trapped cleavage reaction. All GAG specimens but HS from liver carcinoma inhibited the plasmid relaxation assay in a dose-dependent manner. In this measure, commercial heparin was the most effective confirming an earlier report [23].

In cleavage reactions, the efficacy of heparin and normal liver HS was identical. Peritumoral liver GAG inhibited the cleavage better than the HCC GAG did (not shown). When this experiment was repeated by using isolated peritumoral and HCC HS, the result was similar (Figure 4) indicating that heparan sulfate is the active GAG component and is responsible for the inhibitory activity. The inhibitory action of GAGs depended on the topoisomerase I activity of the nuclear extract. Even liver cancer HS decreased the TpT-induced cleavage reaction of Hep3B cells (Figure 4).

We also studied if this phenomenon, observed in a cell-free system, can also be detected when cell cultures were treated with heparin. To this end, hepatoma cell lines were treated with 100 μg/mL heparin for 24 and 48 h. Thereafter, nuclear extracts were prepared and used for plasmid relaxation, as it is shown in Figure 5. Cell nuclear extracts of heparin-treated Hep3B cells did not exert topo I activity. Nevertheless, an identical amount of heparin could not inhibit the activity of the enzyme in HepG2 cells.

4.5. Competition of Heparin and DNA for Topoisomerase I. As topoisomerase I is a heparin-binding protein, we tested if heparin competed with the DNA for the enzyme. Changes in the electrophoretic mobility shift indicated that 10 μg heparin effectively inhibited the mobility shift caused by 10 unit topo I enzyme on 35 ng DNA (Figure 6).

5. Discussion

Our previous studies on various human cancer specimens revealed that the increase in the amount of proteoglycans and their sugar components is one of the most striking features of these tumors [31, 32]. As a general rule, we found about tenfold increase of chondroitin sulfate and fivefold increase of heparan sulfate in surgically removed liver and kidney cancer tissues. The biological significance of these changes awaited explanation. The pharmacological effects of heparin have been known for a long time, but contradictory observations were reported on its capacity to inhibit cell proliferation [33–36]. While heparin is mainly present in mast cells, HS that is structurally strongly related to heparin is present everywhere in the living organisms [37]. Earlier we demonstrated that cells could take up labeled heparin and liver HS and transport them into the nucleus [17, 21].

Furthermore, not only GAGs but also proteoglycans have been detected in the nucleus [38]. One possible role of nuclear HS is to shuttle the nuclear transport of heparin-binding growth factors, such as basic fibroblast growth factor (FGF-2). Once in the nucleus, these growth factors might directly modulate cellular activities [39]. A less-known way of regulation of this process is through heparanase enzyme, an endoglycosidase, implicated in cancer progression and metastasis [40, 41]. Heparanase can be localized to both the plasma membrane and the nucleus, and thus its interference with action of heparin is conceivable. Both its localization and cellular levels are finely regulated [42].

The evidences for the regulatory significance of heparin and HS justify our efforts to look for physiological or pathological cell nuclear functions where heparan sulfates could be involved [43]. We addressed the question if heparin induced inhibition of cell proliferation might be related to its ability to bind and inactivate nuclear proteins. We focused

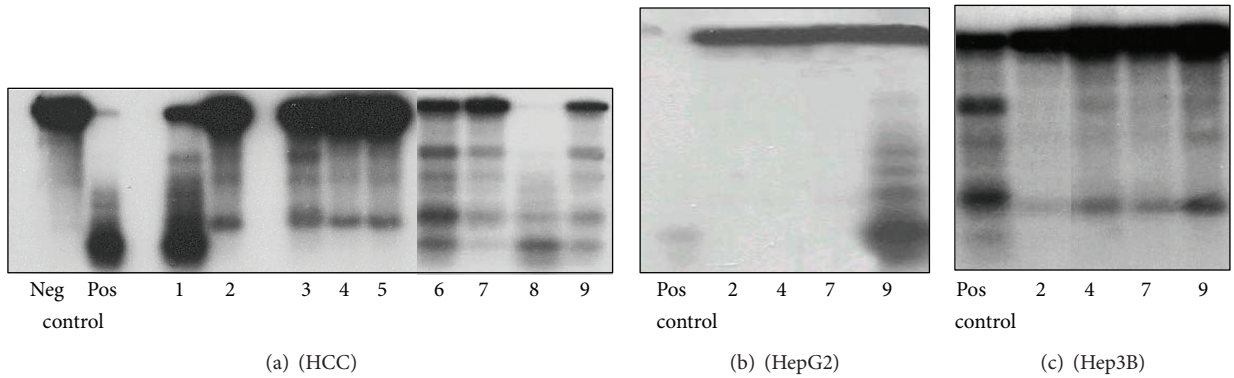

Figure 4: Inhibitory effect of heparin, normal and peritumoral liver heparane sulfate (HS), and hepatocellular carcinoma heparane sulfate on the TpT induced topo I cleavage reaction (representative image of three independent experiments). The origin of nuclear extracts: (a): hepatocellular carcinoma; (b): HepG2 cells; (c): Hep3B cells. The reaction mixture contained pBR322 plasmid DNA, nuclear extracts as described, 100 μM topotecan in all samples as well as in the positive controls. In negative control topotecan was not added. Types and concentrations of GAGs are as follows: (a1): heparin 1 μM; (a, b, c 2): heparin 2 μM; (a3): normal liver HS 1 μM; (a, b, c 4): normal liver HS 2 μM; (a5): normal liver HS 3 μM; (a6): peritumoral HS 1 μM; (a, b, c 7): peritumoral HS 2 μM; (a8): hepatocellular carcinoma HS 1 μM; (a, b, c 9): hepatocellular carcinoma HS 2 μM.

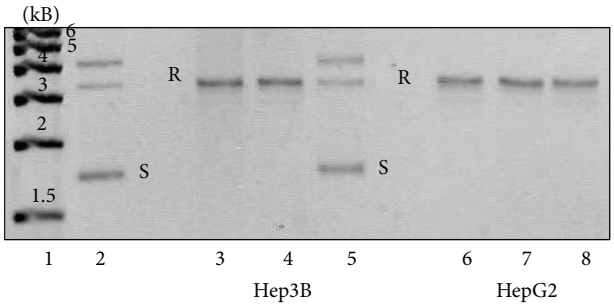

Figure 5: Effect of heparin treatment on topoisomerase I plasmid relaxation activity of HepG2 and Hep3B cells (representative image of three independent experiments). Cells were plated to 6 well plates at 2.5×10^5 cells/plate and grown for 24 h in the presence of 5% fetal calf serum. Subsequently, the serum was replaced with bovine serum albumin (BSA), and the cells were exposed to 100 μg/mL heparin for 24 h (lanes 4 and 7) and 48 h (lanes 5 and 8). Cell nuclear extracts were used for topo I relaxation activity measurements as described. Heparin exposure for 48 hours resulted in total loss of enzyme activity of Hep3B cells (lane 5). Heparin did not affect the activity of HepG2 cells (lanes 4 and 5). Lane 1: DNA size standard, 1 kiloBase (kB) ladder; lane 2: plasmid control without protein (S: superhelix, R: relaxed); lanes 3 and 6: plasmid relaxation in untreated cells.

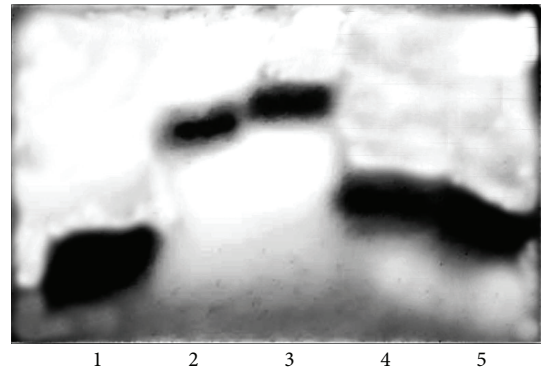

Figure 6: Competition of heparin and DNA for topoisomerase I (representative image of three independent experiments). Effect of heparin on the DNA gel retardation produced by 10 U purified topoisomerase I protein. DNA fragment of 516 base pair size with 8 potential topo I-binding sequences was end-labeled with digoxigenin-UTP. Thirty-five ng DNA was incubated with 10 U topoisomerase I alone or in the presence of 10 and 100 ng heparin. Samples were run or 1% agarose, blotted to nylon membrane, and developed with antidigoxigenin alkaline phosphatase. 1: control DNA, without protein; 2 and 3: DNA with topoisomerase I; 4 and 5: DNA and topoisomerase I, with 10 and 100 ng heparin, respectively.

on topoisomerase I. Certainly, heparin and liver HS, but not liver cancer HS, bound and inhibited topoisomerase I plasmid relaxation in vitro [21, 44]. Our present work also demonstrates that heparin and HS hinder the in vitro plasmid cleavage effect of TpT. This effect was much more obvious when cell nuclear extracts were obtained from liver tumor specimens with low or moderate topoisomerase I activity. This in vitro phenomenon seemed to be important from two points of view. First, heparin or HS could protect normal surrounding tissues with low topoisomerase I activity from the cytotoxic action of TpT. Second, however, the same mechanism could rescue tumors with low or modest topoisomerase I activity from TpT.

The HepG2 cell line with high and the Hep3B cell line with moderate enzymatic activity served as a model to test this hypothesis. In support of the results obtained in a cell-free system, heparin exposure abolished the moderate topoisomerase I activity of the Hep3B cell line, while HepG2 cells retained their enzymatic activities. As the efficacy of TpT depends on the actual activity of topoisomerase I, it was reasonable to expect that, if administered together, heparin will interfere with the action of the drug. Certainly, using the

Hep3B cell line with low topoisomerase I activity, the growth inhibitory effect of TpT decreased in the presence of heparin, while only a modest, transient heparin protection has been achieved on the HepG2 cell line. However, the mechanism of action was still a question.

Heparin alone did not affect the cell cycle. More likely, its protective effects against TpT action were related to its topo I binding capacity. In an assay mixture containing heparin, recombinant topo I and labeled DNA heparin appeared to compete with DNA for the binding of topo I. Thus, in the presence of heparin or HS a lower proportion of topoisomerase I could be bound covalently to DNA by TpT, thus preventing DNA fragmentation. The binding capacity and the amount of heparin or heparan sulfate can determine the proportion of topoisomerase I that will not interact with DNA. The decrease in dead cell fraction after a combined TpT-heparin treatment provided further support to this hypothesis.

Our results are in a good agreement with those clinical observations that aimed to treat liver tumors with TpT. Similarly to the HepG2 cell line, hepatoblastomas respond well for TpT treatment [45, 46], whereas the drug efficacy on liver cancers is modest at best [47]. In the general practice TpT is administered without knowing the topoisomerase I activity status of the tumors. Even though additional studies are warranted on the subject, our current results suggest that for maximum efficacy treatment regimens should avoid concomitant application of heparin and a topoisomerase inhibitor. Having said this, as HS of the tumor itself appears to be ineffective to inhibit topoisomerase I activity, it does not reduce the efficacy of TpT, at least not in the case of liver carcinomas.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

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Review Article

Hyaluronan and RHAMM in Wound Repair and the "Cancerization" of Stromal Tissues

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Tumors and wounds share many similarities including loss of tissue architecture, cell polarity and cell differentiation, aberrant extracellular matrix (ECM) remodeling (Ballard et al., 2006) increased inflammation, angiogenesis, and elevated cell migration and proliferation. Whereas these changes are transient in repairing wounds, tumors do not regain tissue architecture but rather their continued progression is fueled in part by loss of normal tissue structure. As a result tumors are often described as wounds that do not heal. The ECM component hyaluronan (HA) and its receptor RHAMM have both been implicated in wound repair and tumor progression. This review highlights the similarities and differences in their roles during these processes and proposes that RHAMM-regulated wound repair functions may contribute to "cancerization" of the tumor microenvironment.

1. Introduction

Tumors have often been compared to chronic wounds that do not heal. The tumor microenvironment, which is a critical but incompletely understood factor in promoting tumor progression, exhibits tissue remodeling characteristics similar to wounds. These include loss of cell polarity/tissue architecture and remodeling (degradation/resynthesis and reorganization) of the ECM [1], as well as cell dedifferentiation, migration, and proliferation [2–7]. A prolonged and episodic remodeling of adult tissue that results in loss of architecture is also associated with an increased susceptibility for tumor initiation. For example, gestation and involution in breast tissue, which are two periods of prolonged and repeated mammary tissue remodeling, are both linked to increased breast cancer (BCa) susceptibility [8–15].

Most adult wounds heal by fibrosis, which is characterized by an inflammatory response, changes in the composition of ECM, accumulation of biologically active ECM fragments, and scarring [16–20]. There are also accompanying changes in the cellular content of the wound environment that include the differentiation of myofibroblasts, which contribute to wound closure, the formation of a microvasculature, collagen I deposition, and scarring [17, 21–26]. Finally, there is an infiltration of circulating fibroblasts [27–29] and innate immune cells [30] that synthesize and ultimately contribute to repair completion and restoration of tissue architecture. Aspects of this fibrotic milieu provide a protumorigenic microenvironment that enhances both tumor initiation and expansion [31–34]. For example, the presence of high density or fibrotic regions in breast, often resulting from radiation treatment, are sites commonly associated with tumor recurrence [35, 36]. This observation and others suggest a model for tumor initiation that is associated with the chronic or frequent (e.g., episodic) loss of normal tissue architecture and wound-like ECM remodeling, which enhances rogue behavior of mutant cells by providing a "cancerized" microenvironment (Figure 1) [37, 38]. Once tumors are initiated, molecular mechanisms associated with malignant progression function in a dynamic and reciprocal manner with host cells to sustain and enhance this protumorigenic wound-like microenvironment. It should therefore be

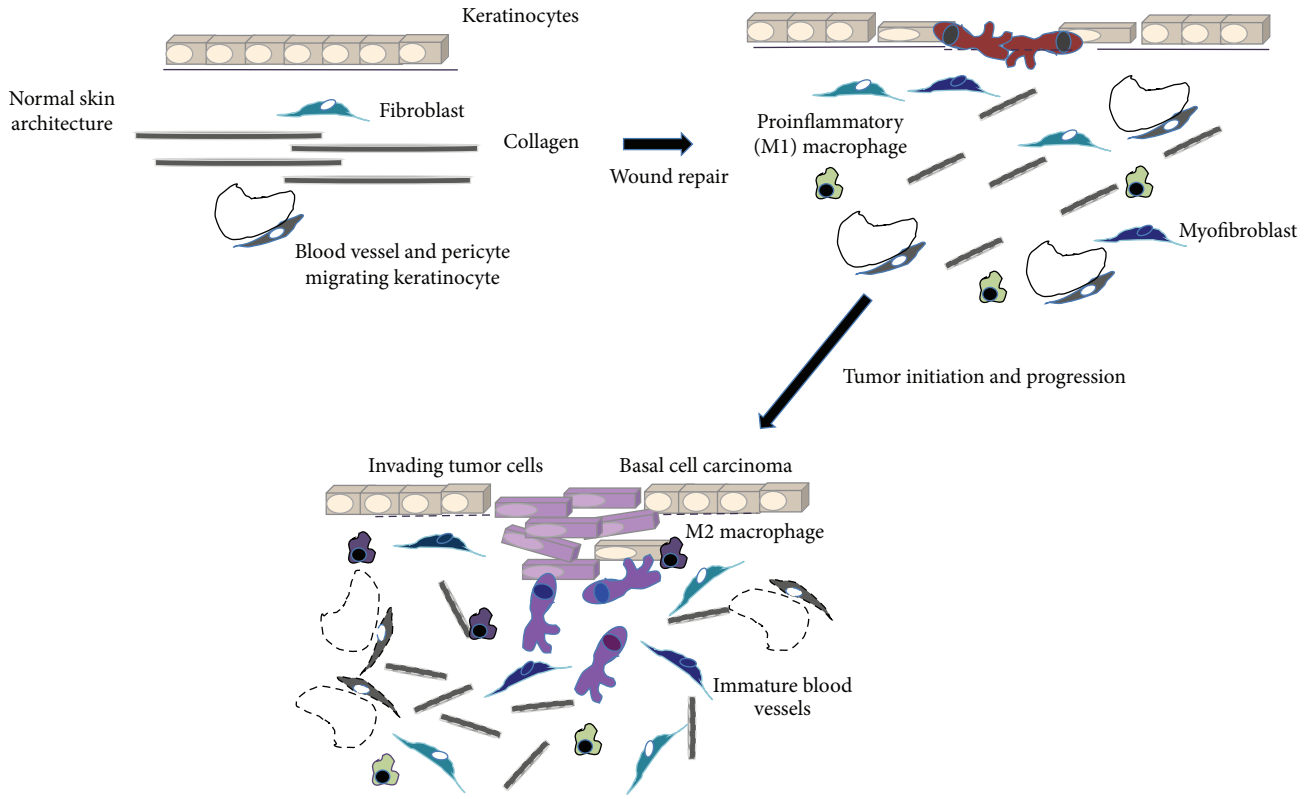

Figure 1: Schematic summarizing wound and tumor microenvironment remodeling in skin. The normal tissue architecture of skin is well-organized in both the epidermis, which consists of differentiated cohesive keratinocytes, and the dermis, which is composed of fibroblasts, blood vessels, and well-organized collagen fibrils amongst other ECM components. Tissue injury results in temporary changes in tissue architecture as keratinocytes dedifferentiate and migrate across wound gaps, proinflammatory macrophages migrate into the dermis, angiogenesis is promoted, and subpopulations of fibroblasts differentiate into myofibroblasts that organize collagen fibrils, which contribute to scar tissue. Tumor initiation also results in dedifferentiation, proliferation and migration/invasion of keratinocytes, influx of macrophages, differentiation of fibroblasts into myofibroblasts that increase deposition and scar like organization of collagen fibrils, and formation of new immature blood vessels. However, this disorganized tissue architecture is not transient as it is in wound repair but increases with tumor progression.

no surprise that gene signatures and transcriptomes of tumors are enriched in wound repair profiles and that these profiles are associated with or prognostic of poor outcome [39–44].

Quite often discussion on the importance of ECM remodeling in wound repair and protumorigenic stroma focuses upon alterations in the synthesis and fragmentation of ECM proteins [45–47]. However, a consideration of the tissue polysaccharide HA is usually not included in these discussions, despite the fact that elevated HA production is essential for tissue repair, is required for tumor progression in numerous experimental models, and is linked to poor outcome in many cancers including BCa [3, 30, 48]. Therefore the first part of this review will focus on HA metabolism as it relates to wound healing and BCa initiation/malignant progression. There is also clear and convincing evidence that HA receptors such as cluster designation 44 (CD44), receptor for hyaluronan mediated motility [49], and toll-like receptors 2,4 (TLR2,4) (to name a few) are all important contributors to malignant progression and outcome in BCa patients. There are many excellent reviews on the functions associated with these and other HA receptors in tissue homeostasis, wound repair, and tumor 4 progression [3, 30, 48, 50–53]. However, this review will focus on the multifunctional HA receptor, RHAMM (gene name HMMR), because of its clear roles in fibrotic wound repair that are apparently relevant to BCa initiation and progression. For example, the expression levels of HMMR/RHAMM are frequently increased in BCa and linked to poor clinical outcome [54] and considerable *in vivo* evidence links RHAMM expression levels to mesenchymal response-to-injury [55–60]. The disparate functions of RHAMM are related to its complex subcellular localization. RHAMM was originally described in the context of one of these functions, which is to facilitate HA mediated cell motility [48, 61–64] but was more recently shown to affect centrosomal function and mitotic spindle integrity. The purpose of this review is to highlight the roles of HA and RHAMM that converge in wound repair and BCa progression.

2. Hyaluronan Background

HA is a polysaccharide belonging to the glycosaminoglycan family of macromolecules. This biopolymer consists

of repeating disaccharides of N-acetylglucosamine and β-glucuronic acid, the latter of which confers anionic properties to HA [51, 53, 65]. There are three known hyaluronan synthases (HAS1-3) that produce HA and these are differentially expressed during wound repair and in tumors [48, 66–78]. HAS enzymes are proteins that contain multiple membrane spanning sequences and to date they are the only known glycosyltransferases imbedded in the plasma membrane. "Activated" UDP-sugars are sequentially added to the catalytic portion of the enzyme located on the inner face of the plasma membrane. The synthesized polymer is then extruded through the plasma membrane, possibly through channels created by oligomerization of the synthases [76, 79].

HA is a ubiquitous component of tissue ECM but is found in particularly high concentrations as a native homeostatic form within hydrated tissues such as the vitreous of the eye, articular cartilage, and lymphatics and skin. It is particularly enriched in the epidermis, where it is important for maintaining the hydration of this tissue so that it can form a more effective barrier to the environment [80–82]. During embryonic development HA is a crucial component of cardiac jelly and is absolutely required for heart development where it provides a migration-supporting environment for cardiac cushion cells [83]. Genetic deletion of HAS2, the HA synthase that is responsible for HA synthesis during the embryonic developmental period when heart development occurs, results in embryonic lethality as a result of defective cardiac development [84]. Genetic deletions of HAS1 or 3 do not have the same developmental consequences emphasizing that these synthases are expressed differentially. They also exhibit distinct spatial distributions in tissues [68, 85–87]. The biological significance of having 3 unique but closely related HA isoforms is not completely understood, but in addition to differential mechanisms regulating their expression, they also synthesize HA biopolymers of dissimilar average sizes. Such differences in polymer size are linked to distinct HA functions [48, 65, 88–90]. Thus, it is not surprising that there are temporal, spatial, and cell-type specific differences in HAS1-3 expression during tissue repair and in tumor progression. For example, during repair of excisional wounds, keratinocyte migration is associated with elevated HAS2 and 3 expression while peritoneal mesothelial cells upregulate only HAS2 following mechanical injury [69, 72]. There is surprisingly little information about HAS expression and HA production during tissue remodeling that is not associated with injury. For example, although branching morphogenesis in general is known to be HA dependent, to our knowledge its role in mammary gland morphogenesis has not been reported. A great deal more is known about the roles of HA and HAS isoforms in the initiation and progression of tumors from breast tissue. HAS1 and HAS2 expression are commonly upregulated in BCa. Their elevated expression and HA accumulation are linked to ominous features of malignant progression. This includes epithelial-to-mesenchymal transformation (EMT) [91–93] and increased invasion [3, 54, 94–96], providing a partial explanation for HA's relationship to poor outcome [48, 74, 93, 97].

Glycosaminoglycans such as HA bind, concentrate, present, and prevent diffusion of growth factors in tissues. HA is fundamentally important for both maintaining tissue homeostasis and orchestrating the inflammatory, fibrotic, and stem cell renewal responses in damaged tissues. High molecular weight HA is typically produced by homeostatic tissues and can reach up to 2,000 kDa. High molecular weight HA performs ECM and growth factor presenting/scaffolding functions and is critical for tissue hydration [81, 98]. By providing a relatively loose matrix, HA supports cell migration, acts as scavenger for reactive oxygen and nitrogen species, and is typically anti-inflammatory [30, 50, 65, 99]. Importantly, recent findings also suggest that high molecular weight HA exhibits tumor suppressive functions in skin [100]. By sharp contrast, the fragmentation of polymeric HA, which occurs during both normal and disease-associated tissue remodeling, drastically alters the functions of HA. HA fragments are typically proinflammatory and promigratory and promote proliferation (Figure 2). This change in function as a result of depolymerization is similar in principle to the fragmentation of certain embryonic morphogens and extracellular matrikines which have distinct biological properties compared to the intact molecules, and which occur during tissue remodeling [98]. HA fragmentation can occur as a result of hyaluronidase activity (e.g., Hyal1 and Hyal2) or the presence of reactive oxygen and nitrogen species [81, 98]. Furthermore, HA size heterogeneity within a wound can also result from altered expression levels of distinct HAS isoforms [48]. Localized changes in HA synthesis and fragmentation may therefore represent a type of "on-off switch," which is important for providing early warning signals of tissue damage, requiring host cells to respond appropriately to restore tissue function and architecture [50, 98, 101–105]. It needs to be emphasized, however, that the majority of these studies have been performed in culture with very few analyses determining the extent of HA fragmentation in intact tissues during tissue remodeling *in vivo*. Therefore aspects of these *in vitro* studies that are applicable to tissue remodeling *in vivo* have yet to be fully defined.

The development of new techniques [106, 107] that facilitate the isolation and determination of HA fragments size distributions from complex tissues will ultimately provide an important framework for understanding the biological importance of size heterogeneity of this biopolymer. While *in vivo* analysis of these fragmentation patterns is just the beginning, there is mounting evidence *in vitro* that the biological impact of HA size heterogeneity is related to the ability of different HA receptors to bind high molecular weight polymeric versus HA fragments. As an example, while higher molecular weight HA binds to CD44, smaller fragments of HA bind to RHAMM and even smaller fragments bind to TLR 2,4. There is evidence that these HA fragments can also function to inhibit the binding of higher molecular weight HA to CD44 [108, 109]. Thus, fragmented HA could impact tissues by either directly binding specific receptors or antagonizing the binding of larger HA polymers to their cognate receptors. These complex interactions control a variety of signaling pathways that regulate cell adhesion/motility, mitotic spindle formation, and transcriptomes. Clearly much work is needed to dissect the complex roles of native HA versus fragments in normal and diseased tissues, with longer term impact of

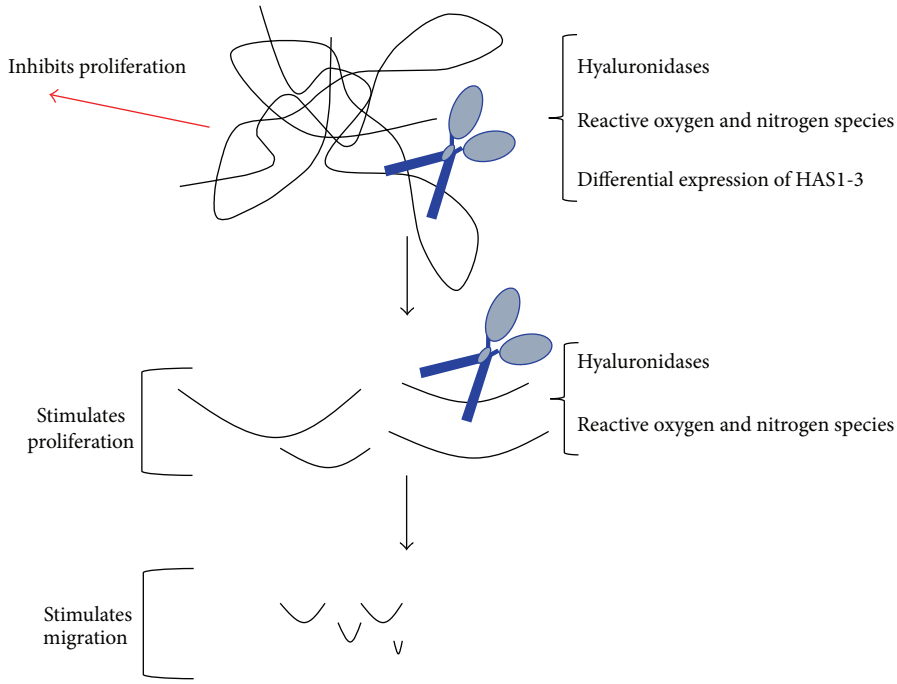

Figure 2: HA functions are molecular weight dependent. HA occurs as a large native polymer in homeostatic tissues but is degraded following tissue injury by free radicals and hyaluronidases. The resulting fragments have different bioactivity than the native polymer depending upon their size. For example, intermediate fragments can stimulate cell proliferation while smaller fragments have been reported to only promote cell migration.

providing specific targets associated with pathologies linked to altered HA metabolism.

3. Hyaluronan and Tissue Remodelling

The importance of the synthesis/fragmentation of high molecular weight HA has been most extensively studied in models of tissue repair. HA is considered a "keystone" or central molecule in regulating response to tissue stress since rapid alterations in HA production, macromolecular organization, and size within tissues are among the earliest changes that can be detected following injury including those resulting from exposure to ionizing radiation [30, 81, 98, 110, 111]. Like other ECM components (e.g., collagen), HA is fragmented during wound repair into small proinflammatory oligosaccharides by oxygen/nitrogen free radicals that accumulate in the stressed tissue [112, 113]. These wound fragments constitute an early "danger signal" that is part of the damage-associated molecular process (DAMP). DAMP stimulates the innate immune response, which, when not resolved, directly contributes to chronic inflammation and tissue fibrosis [106, 114–117]. Experimental models of tissue injury have documented the contribution of HA to DAMP and to the repair of excisional skin wounds [81, 118], vascular response to injury [119, 120], and induced lung injury [30]. The paradigm for the functions of HA in all of these injured tissues is similar, and recent reviews have summarized the literature in both vascular and lung injury models [110, 121]. Our studies on the identification and characterization of HA size heterogeneity in excisional wound healing are among the first to attempt to shed light on these issues in skin wounds *in vivo* [50, 106]. These studies have demonstrated an association between HA fragmentation and cellular infiltration into wounded tissues [56, 122, 123]. Furthermore they have shown that this cellular infiltration is defective in animals that are embryonic null for RHAMM [55, 56].

HA occurs in large amounts in skin and it is a key factor in its homeostasis since it controls both fibroblast differentiation and epidermal activation and renewal [118, 124]. As in other tissues, native, high molecular weight HA suppresses skin inflammation [30, 118, 125], myofibroblast differentiation [125], and fibrosis [118]. Similar to other injured tissues, fragmentation of HA, which results from ionizing radiation and other damage, promotes inflammation and tissue fibrosis. Homeostatic skin contains the largest depot of high molecular weight, anti-inflammatory (native) HA in the body and this is primarily organized into extracellular macromolecular complexes in the dermal ECM. It is also detected as pericellular coats, which are particularly noticeable around keratinocytes [126–128]. These HA coats, which are sustained on keratinocytes surfaces via the HA receptor CD44 [129], are required for maintenance of barrier/permeability functions of skin as well as for keratinocyte renewal, proliferation, and differentiation [81, 130–133]. Because of their exposure to the environment, a key additional homeostatic function of keratinocyte-associated coats is that they protect against ionizing radiation-induced DNA damage. For example, production of native HA protects against DNA damage resulting from either UVB or gamma radiation [81]. Native HA in skin is also linked to reduced risk of cancer (skin) and metastasis

[92, 100]. Importantly, loss of keratinocyte HA coats is linked to epidermal atrophy and other pathologic lesions including increased dermal fibrosis [124]. In addition to its effects on keratinocytes, skin HA metabolism also controls proinflammatory immune cell influx [30] and TGFβ-1 induced myofibroblast differentiation, which is largely responsible for fibrosis [118, 125]. Reepithelialization of injured skin begins early after injury and is controlled by EGF signaling, which stimulates HA production and regulates promigratory/proliferation signaling through HA and CD44 [134]. TGFβ-1 is strongly antiproliferative in these cells [135].

Reepithelialization serves as a critical function in maintaining the integrity of the dermal layers due to crosstalk between these two skin layers. Although not a great deal is known about this function, TGFβ-1/SMAD 3 signaling plays a key role. Thus, when wound repair is deregulated and reepithelialization is prevented, dermal fibrosis is enhanced [136]. The epithelium in transgenic mice, which are engineered to suppress SMAD 3, exhibits accelerated reepithelialization, reduced inflammation, and reduced dermal fibrosis [137]. Reepithelialization of wounds normally coincides with and may instruct removal of dermal myofibroblasts by apoptosis. Blocking reepithelialization prevents myofibroblast apoptosis and results in hypertrophic scars or chronic tissue fibrosis [124]. HA, which as noted above is necessary for keratinocyte proliferation and migration in response to epidermal injury, is also a key regulator of TGFβ-1 functions in fibrosis and myofibroblast differentiation [118, 125].

Fibroblast differentiation into myofibroblasts is controlled by two cooperating pathways, TGFβ-R/SMAD and HA mediated RHAMM:CD44:EGFR signaling complexes, both of which must be activated to induce myofibroblast differentiation [138]. Thus, the TGFβ-1 signaling pathway promotes HAS2 dependent HA synthesis and pericellular HA coat formation. This results in increased levels of endogenous TGFβ-1, which maintain myofibroblast differentiation via an autocrine loop consisting of HA:TGFβ-1 production [139]. TGFβ-1 and HA dependent signaling promote CD44 and EGFR interaction in lipid rafts resulting in the activation of ERK1,2 and calmodulin kinase II activation [138]. Intriguingly, increased accumulation of native extracellular HA [118] such as what occurs during repair of embryonic tissues [140, 141] or disruption of HA:RHAMM:CD44 [106] complexes in wounds can negatively regulate these signaling pathways so that myofibroblast differentiation is reduced and, conversely, reepithelialization is promoted. The role of HA in wound myofibroblast differentiation is important since these cells closely resemble cancer associated fibroblasts that are intimately involved in development of a cancerized microenvironment that facilitate the progression of BCa (e.g., [142]).

Studies implicating differential roles for high molecular weight versus fragmented HA in the repair of other tissues prompted us to further investigate the potential relationship between HA synthesis and size heterogeneity in excisional wound repair [30, 98]. As anticipated, homeostatic adult mouse skin contains mainly high molecular weight HA (>5,000 kDa) while fragmentation can be detected in wounds as rapidly as 24 h, peaking at 7 days after injury [106]. Importantly high molecular weight polymers (>5,000 kDa) as well as intermediate-to-small (30–500 kDa) and very small (<10 kDa) HA fragments coexist in these wounds. This fragmentation pattern is consistent with an important role for HA size heterogeneity in promoting excisional dermal wound repair. HA fragments signal as "on" switches to both circulating and resident cells, which promote inflammation and angiogenesis while coexisting high molecular weight polymers signal as "off" switches that limit responses to fragments. In support of this notion, it has been shown that either forced HAS1 expression or supplementing the wounds with high molecular weight HA limits the fibrotic response such that the healing of these wounds more closely resembles those observed in embryos. The effect of adding excess high molecular weight HA to these wounds leads to reduced levels of TGFβ-1, attenuated inflammation, and a reduction in biomechanical stress [22, 143, 144]. A number of studies have established that small to intermediate size HA fragments promote the influx of immune cells. Such fragments also activate the proinflammatory functions of these infiltrating cells, which include stimulating expression of chemokines such as MIF-1a and MCP and increasing the expression of profibrotic growth factors such as TGFβ-1 [30]. This size range of HA fragments also promotes branching morphogenesis of wound associated blood vessels [119] and activation of fibroblasts [56, 145].

The availability of precisely or at least restricted sizes of small HA polymers produced by recombinant technology has allowed studies that suggest a much more complex functional repertoire and interplay of high molecular weight HA versus HA fragments than previously suspected. For example, a range of HA fragment sizes (e.g., HA-12 (12 saccharides) and HA-880 (880 saccharides) and native, high molecular weight HA) all activate ERK1,2, Akt, and P38 signaling cascades and all increase expression of ECM remodeling proteinases such as MMP1,3 [122, 146–150]. However, HA-12 and native HA selectively promote expression of collagen III and TGFβ-3 and HA-12 solely promotes TIMP1 expression by dermal fibroblasts in culture [56, 149]. HA-6 (6 saccharides) but not HA-8, HA-10, 40 kDa, or native HA stimulates wound closure and increases wound macrophages and TGFβ-1 levels. In spite of stimulating TGFβ-1, HA-6 does not increase myofibroblast differentiation suggesting requirement of additional stimuli and possibly other HA fragment sizes [56]. These studies serve to emphasize the enormous amount of information that is generated by differential fragmentation of HA associated with tissue injury. The mechanism by which this information is transduced to the cell is currently not well understood. For example, are the trafficking/display patterns [54] of HA receptors that have differential binding properties for discrete sizes of HA fragments involved in signal transduction of the HA fragment pool as a whole? Furthermore, is there a temporal or spatial relationship between HA fragmentation and the response of specific cellular subpopulations expressing various HA receptors? Despite the complexity, an important first step is to document the presence and kinetics of HA fragmentation patterns in order to address these and other questions in the future. These analyses of HA functions in excisional

wounds and other models of tissue injury have uncovered an information-rich mechanism for finely regulating the key processes of inflammation, angiogenesis, and fibroblast activation that are all essential for efficient wound repair [98]. These same processes in the tumor microenvironment appear to be required for the initiation and progression of BCa.

4. Stromal Hyaluronan and Breast Cancer

In BCa it is clear that tumors progress more aggressively in a HA-rich microenvironment and that stromal HA affects both host and tumor cells to accelerate progression. Many of the functions of HA during BCa progression have been summarized in several recent excellent reviews [3, 48, 78, 97, 125]. The importance of stromal HA in mediating host responses, which support BCa progression, is the focus in this section.

Both tumor parenchyma and host cells in the tumor microenvironment express HAS isoforms and produce HA, which then accumulates in tumor parenchyma and in the peritumor stromal tissues [48, 97]. Clinical studies suggest that HA accumulating in either the tumor parenchyma or surrounding peritumor stroma is tightly linked to BCa progression and both are independent prognostic indicators of poor outcome [97]. Stromal cells, in particular cancer associated fibroblasts, express all HAS isoforms. Increased HAS expression by cancer associated fibroblasts correlates with increased HA accumulation, increased stromal CD44 expression, high relapse rate, and short overall survival [74]. Furthermore, high stromal HA accumulation is significantly associated with the appearance of a tumor reactive stroma, which associates with tumor cell positive lymph nodes, high tumor grade, and lymphatic tumor emboli [151]. The increased expression of extracellular HA binding proteins such as versican has also been reported [152]. Analyses of head and neck tumors reveal previously unappreciated stromal cancer associated fibroblast heterogeneity involving HA: a subpopulation of cancer associated fibroblasts produces high levels of HA and promotes local tissue invasion by cancer cells [153].

Bigenic expression of Neu and HAS2 in ductal epithelium using the MMTV promoter in mice results in marked changes in the peritumor stroma resembling those observed clinically in tumor reactive stroma [154]. Furthermore, these studies show that HA produced by the tumor parenchyma by itself can enhance stromal cell recruitment and the formation of a tumor reactive stroma. Notable phenotypic changes include increased formation of intratumoral HA-rich stroma, accumulation of ECM components, such as versican, collagen 1, and fibronectin, neovascularization, and the infiltration of immature mesenchymal cells. Cytokine analyses suggest that the increased accumulation of these stromal cells stimulates neoangiogenesis. Coinjection experiments with cancer associated fibroblasts that are derived from these bigenic tumors reveal that the cancer associated fibroblasts are responsible for the observed increase in tumor growth, reactive stroma formation, angiogenesis, and lymphangiogenesis [155]. Others have shown that tumor-associated HA supports tumor cell epithelial-mesenchymal transformation that also enables the growth and spread of tumor cells [154–157]. Collectively these studies indicate that production of HA by tumor cells favors recruitment of mesenchymal cells that remodel the peritumor stroma to create a tumor friendly microenvironment.

In addition, microenvironments rich in HA provide mitogenic and motogenic signals for tumor cells. For example, we have shown that human BCa lines are heterogeneous in their ability to bind to HA and that exposure to this glycosaminoglycan promotes specific subpopulations within these lines to divide rapidly while stimulating other subpopulations to invade aggressively but proliferate slowly [54]. This type of functional heterogeneity may be partly responsible for the relationship between HA-rich tumor microenvironments and relapse/poor outcome reported by a number of clinical studies. Elevated stromal HA is linked to HER2 positivity and several key clinicopathologic features including poor prognosis factors such as tumor size, lymph node positivity, hormone receptor negativity, increased relapse rate, and shortened survival [158]. The mechanisms by which stromal HA effects BCa progression and the roles of native versus fragmented HA are not yet well understood. However, considering the evidence from multiple model systems (discussed above) it is clear that these effects are mediated through HA receptors. Multiple HA receptors (e.g., CD44, RHAMM and LYVE-1, and TLR2,3 among others) are known to be involved in BCa progression [3, 48, 156, 159–161]. The role of RHAMM in progression of this disease and in wound repair will be considered here because RHAMM is unique in the ways in which it converts HA "signaling" into multiple key aspects of cellular functions that are relevant to response to injury and to tumor progression.

5. RHAMM Background

Studies using RHAMM null animals have clearly established an important role for this protein in tissue response to injury [55, 57]. Furthermore, a number of studies have linked RHAMM expression to BCa since it is frequently elevated in breast and other cancers and is associated with poor outcome [54, 61, 162]. RHAMM is a largely hydrophilic helical protein (Figure 3) that was originally isolated from conditioned medium from chicken heart explant cultures exhibiting high HA production and increased cell migration [163]. It binds to HA via positively charged amino acid clusters in the carboxyl terminus that are structurally distinct from the link module responsible for HA binding to CD44 [164]. RHAMM also binds to microtubules via sequences located in its N- and carboxyl termini. It directly binds to ERK1 via a sequence with homology to a D-box MAP kinase interaction site [95, 165, 166]. The protein contains several leucine zippers and these together with its potential for forming a coiled coil predict that it can self-associate as dimers or trimers (Figure 3).

In vivo, RHAMM expression is tightly regulated: it is poorly expressed in most homeostatic adult tissues with the exception of ovaries, testes, and ciliated epithelium of the respiratory tract in which elevated RHAMM mRNA levels are detected [167–169]. However, RHAMM mRNA and protein expression are strongly but transiently increased in response to injury. A number of mechanisms have been

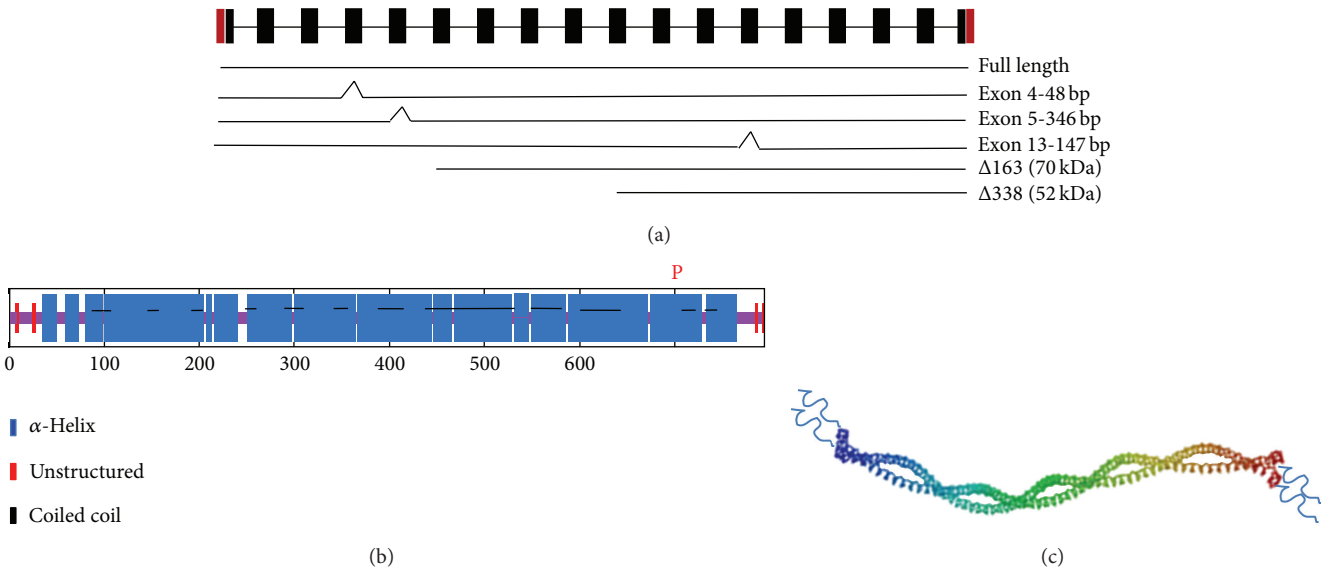

Figure 3: RHAMM isoforms, protein secondary structure, and posttranslational modification (a) RHAMM exon structure is shown as black boxes. Lines underneath this diagram show the known isoform structures. The full-length protein (85 kDa in human) is largely associated with interphase microtubules and the mitotic spindle during the cell cycle. Three isoforms are generated by alternative splicing of exon 4, 5, or 13. Loss of exon 4 sequence disrupts stable association with interphase microtubules and results in the appearance of RHAMM in the interphase cell nucleus. N-terminal truncations that may be generated by posttranslational mechanism or alternative start codon usage are very transiently expressed during early tissue injury but are constitutively present in some aggressive breast cancer cell lines and tumors. These accumulate in the nucleus and on the cell surface. (b) RHAMM protein is predicted to be largely a-helical, with unstructured sequences at the extreme N and C-termini. The orange P at the carboxyl terminus indicates an AURKA and ERK1,2 phosphoacceptor site. RHAMM also contains approximately 30 putative protein kinase C phosphoacceptor sites (not shown). This posttranslational modification is associated with the nuclear accumulation of RHAMM. Although RHAMM is phosphorylated by protein kinase C, the acceptor sites have not yet been reported. Protein kinase C modification of RHAMM is linked to interphase centrosomal placement. (c) The secondary structure predictions shown in (b) indicate that RHAMM proteins can self-associate to form random coiled coils.

identified that either promote or suppress RHAMM expression. Promoting factors include TGFβ-1, RON, and the YAP-HIPPO pathway while tumor suppressors such as p53 and BRCA1 reduce its expression [49, 61, 96, 162, 170–176]. Analyses of RHAMM knockout mice [57, 58, 177] show that it is perhaps surprisingly not required for embryogenesis or homeostatic adult functions, the latter predicted by its low or absent expression in most tissues [177]. However it is essential for a variety of tissue repair processes that like embryogenesis require cell migration, invasion, and ECM remodeling. Since elevated levels of RHAMM are associated with poor prognosis in human cancers, it would appear that tumor cells usurp these wound repair functions of RHAMM to facilitate their survival and progression. The restricted expression of RHAMM makes it a potential target for cancer and wound repair therapy with low toxicity. Indeed, RHAMM peptides that are currently being tested in phase II clinical trials for multiple myeloma and myelodysplastic syndrome show efficacy and low toxicity in patients [178, 179]. The biological functions of RHAMM are complex. It is one of the first proteins to be identified for which its extracellular and intracellular functions differ markedly.

6. RHAMM Signaling

The signaling functions of RHAMM are multifaceted and context dependent as might be expected by its complex subcellular compartmentalization. RHAMM is a cytoskeletal, centrosomal, mitotic spindle, and nuclear protein [49, 61, 166, 180], which is exported to the cell surface by unconventional mechanisms during wounding [181] by cytokines such as TGFβ-1 [173]. Cell surface RHAMM associates with several integral protein tyrosine kinase and nonprotein tyrosine kinase receptors including PDGFR [182], TGFβ Receptor-1 [170], CD44 [55, 64], CD44-EGFR complexes [183, 184], bFGFR [185], and RON [171]. RHAMM impacts upon the signaling competency of these receptors in response to their cognate ligands (Figure 4). Cell surface RHAMM:CD44 complexes, in association with one or more of the above growth factor receptors, promotes random cell motility in a protein tyrosine and ERK1,2 kinase dependent manner [186–188] (Figure 4). This random motility function does not require intracellular RHAMM proteins and immobilized recombinant cell surface RHAMM isoform (70 kDa) added to RHAMM−/−:CD44−/− fibroblasts is sufficient restore fibroblast motility speed to that of wild type or RHAMM-rescued fibroblasts [55]. Cell surface RHAMM also likely participates in functions required for wound repair such as cell division fidelity, mitotic spindle integrity, and cell cycle progression that were originally thought to be HA-independent functions of intracellular RHAMM proteins. For example, blocking cell surface RHAMM signaling reduces cell cycle progression of fibroblasts through G2M, a stage in the cell cycle where RHAMM and HAS2 mRNA are transiently elevated and

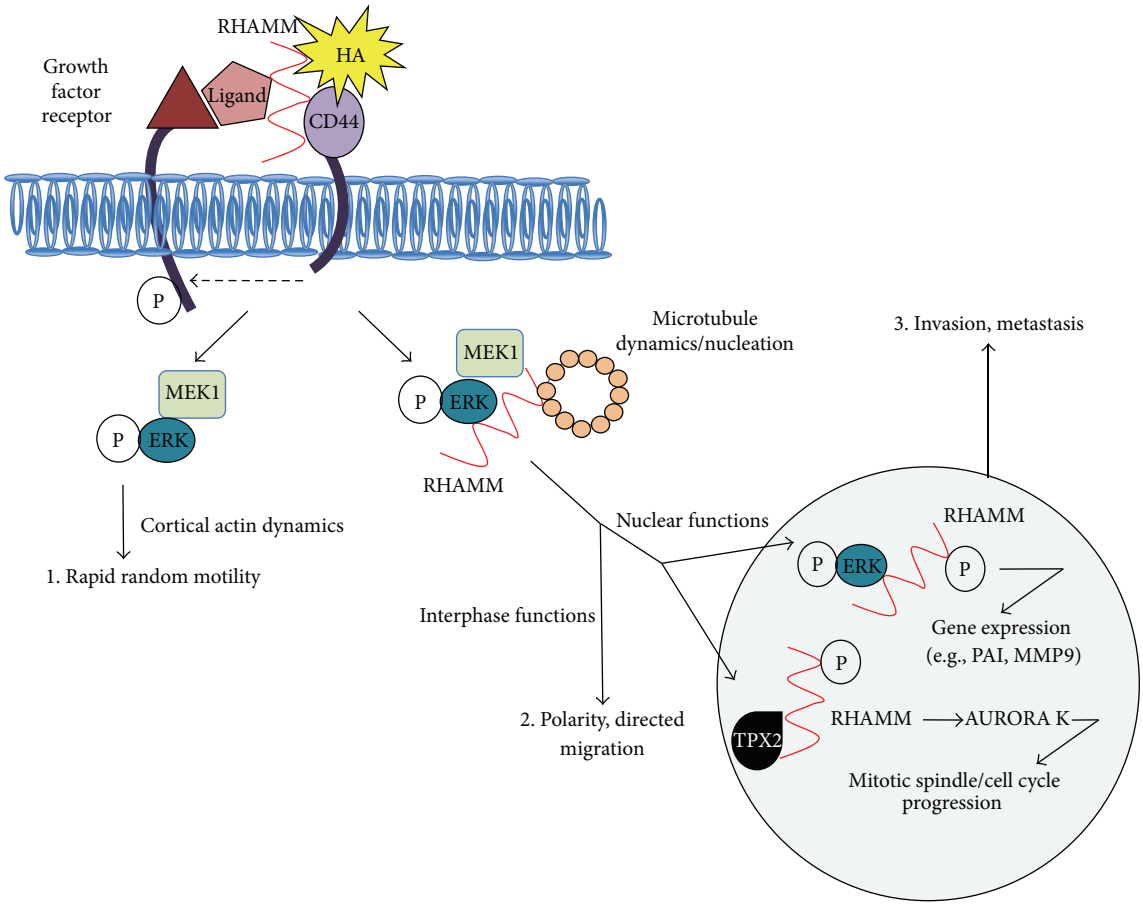

Figure 4: Model of RHAMM signaling. Model summarizes the known signaling functions of cell surface and intracellular RHAMM. Cell surface RHAMM interacts with CD44, HA, and growth factors to activate protein tyrosine kinase signaling cascades that activate the ERK1,2 MAP kinase cascade. This signaling can increase random motility in the absence of intracellular RHAMM. Intracellular RHAMM also binds to a number of protein partners that mediate its functions as a regulator of microtubule dynamics, centrosome structure/function, and gene expression. For example, during interphase, cytoplasmic RHAMM:protein partner interactions (MEK1/ERK1,2 shown) contribute to the dynamic properties of interphase microtubules and the number, placement, and structure of centrosomes, which affect cell polarity and direct cell migration. Nuclear RHAMM:MEK1:ERK1,2 complexes also control expression of genes involved in cell motility such as PAI-1 and MMP-9. During the cell cycle, RHAMM:TPX2 complexes contribute to mitotic spindle integrity and cell cycle progression through G2M while RHAMM:supervillin complexes promote cytokinesis.

for which HA production is necessary to facilitate cell rounding [189]. Exogenous HA also promotes the association of microtubule-associated protein homolog (TPX2) with nuclear RHAMM and phosphorylation of AURORA Kinase A (AURKA) to stimulate progression through the cell cycle [183]. The details of these signaling pathways as they are regulated by cell surface and intracellular RHAMM protein forms have been recently reviewed in detail [48, 61, 62]. The coordinated and separate signaling functions of intracellular and cell surface RHAMM in wound repair and in BCa remain to be resolved.

A simplified model of the proposed coordinated extracellular and intracellular RHAMM signaling functions is depicted in Figure 4. It is intriguing that both share the ability to regulate activation and subcellular localization of components of the MAP kinase (ERK1,2) cascade [48, 61, 62]. HA stimulation of cell surface RHAMM has consistently been shown to control the duration of ERK1,2 activity [55]. Intracellular RHAMM proteins form complexes with MEK1 and ERK1,2 and target these kinases to the cytoskeleton [165] and nucleus [55]. These signaling functions are required for random motility, mitotic spindle integrity, progression through the cell cycle, and gene expression (e.g., PAI-1 [170] and MMP9 [190]). RHAMM:ERK1,2 complexes are also likely to be important to centrosomal function since both RHAMM and ERK1,2 are required for microtubule nucleation [133, 191] and both are functionally linked to key centrosomal proteins such as TPX2 and AURKA [49, 183, 192]. In addition to microtubules, intracellular RHAMM partners with cortical actin proteins such as supervillin [193]. Supervillin, a membrane bound actin binding protein that participates in myosin II mediated contractility, interacts with calponin and regulates the activity of another RHAMM binding partner, ERK1,2 [194]. Supervillin coordinates processes that require dynamic cytoskeleton and membrane turnover including cell migration and cytokinesis [193, 195]. Indeed, RHAMM−/−

cells often undergo aberrant cytokinesis causing the formation of multinucleated cells [165]. To date, the signaling functions of RHAMM can therefore be roughly divided into those that require (1) intracellular RHAMM and cell surface RHAMM (e.g., to control microtubule dynamics/nucleation and gene expression), (2) only cell surface RHAMM (e.g., to control random motility speed), and (3) only intracellular RHAMM (e.g., possibly cytokinesis).

While cell surface RHAMM controls the kinetics of ERK1,2 activation, intracellular RHAMM appears to target MEK1/ERK1,2 complexes to microtubules thus contributing to the dynamic turnover of interphase microtubules [196] and mitotic spindles [165]. Mitotic spindle formation is complex and includes key proteins such as AURKA and TPX2, which is a regulator kinase of AURKA. RHAMM-regulated ERK1,2 activity is required for bipolar spindle formation and loss of RHAMM can be compensated for by mutant active MEK1 in this function [165]. Intracellular RHAMM:TPX2 interactions and additional function interactions with BRCA1/BARD1 [49] also regulate the number, structure, and placement of centrosomes, in part through regulating AURKA activity. However, Hatano and colleagues have shown that nuclear RHAMM:TPX2 colocalization only occurs during metaphase. This group further showed that addition of HA stimulates both an association of RHAMM with TPX2 and an increase in the phosphorylation of the TPX2 regulator kinase, AURKA [183]. Since endogenous HA levels are high at G2M, it is likely that RHAMM:TPX2 interactions noted in other studies [49] are also controlled by HA. This centrosomal function of intracellular RHAMM is required for cell division fidelity in vascular response to injury, mitotic spindle integrity, progression through G2M, and basal-apical polarity of breast epithelial cells [57, 59, 197–199]. Nuclear RHAMM may play a further role in sequestering TPX2 [49] to these compartments to prevent premature changes in microtubules/mitotic spindle assembly and to facilitate repair of DNA aberrations caused, for example, by ionizing radiation [200]. In addition, RHAMM together with CD44 or TGFβR1, and possibly intracellular HA, may directly affect the transcription of genes controlling cell migration and proliferation [170, 190, 201] (e.g., PAI-1, MMP-9, Figure 4).

7. RHAMM Subcellular Compartmentalization

The mechanisms responsible for the complex subcellular targeting of RHAMM are still incompletely understood but are likely to be contributed to by isoform structure and posttranslational modification. RHAMM is subject to mRNA splicing and is phosphorylated by a variety of serine threonine kinases (Figure 3). Several RHAMM mRNA splice variants have been identified in breast and other cancers including a 48 bp deletion in exon 4 (RHAMM-48), a 346 bp deletion in exon 5, and a 147 bp deletion in exon 13 although the presence of these forms in wound repair has not been reported [202–205]. RHAMM is phosphorylated by protein kinase C, AURKA, and ERK1,2 [49, 197, 206]. Additionally, smaller than full-length N-terminal truncated RHAMM proteins have been reported during wound repair and in breast cancer cell lines [95]. Expression of these RHAMM isoforms and production of phosphoprotein specific antibodies have been utilized to identify isoform-specific subcellular targeting. These studies have shown that full-length RHAMM is largely associated with the cytoskeleton in interphase cells and in particular binds to microtubules [165, 207]. Targeting the nucleus is achieved by either alternative splicing of the full-length form in exon 5 [207], truncation of N-terminal sequence [208], or phosphorylation at T703 (human, but evolutionarily conserved [49]). The phosphorylation of RHAMM by protein kinase Cα is required for rear-polarization of the microtubule organizing center (MTOC) of migrating neointimal smooth muscle cells [59, 197]. Cell surface labeling of cultured cells reveals a predominance of N-terminal truncated RHAMM proteins [209]. These small truncated proteins, which are generated by as yet unknown mechanisms, are less prominent on the cytoskeleton than the full-length protein, and FRAP studies show they are more mobile within the cell, accumulating in the nucleus and at the cell surface [61]. Since the multiple functions of RHAMM appear to be dictated by subcellular location it is likely that the various isoforms perform different functions and regulate distinct signaling pathways. Although isoform expression levels and interplay have been linked to tumor progression, their roles in response to injury and the differential manner in which they regulate specific functions are still poorly understood. The subcellular compartmentalization and signaling functions of RHAMM isoforms are critical for efficient repair of adult tissues and appear to provide some tumor cell types with growth, survival, and invasive advantages.

8. RHAMM and Tissue Remodelling

RHAMM mRNA and protein expression are coordinately and transiently upregulated following tissue injury. RHAMM protein expression is detected at the site of excisional skin wounds 24 hrs after injury, peaks at 3 days, and disappears by day 7 [55]. A similar rapid and transient increase in RHAMM expression is observed following scratch wound injury of fibroblasts and smooth muscle cells in cell culture [181]. Analyses of response to injury processes in RHAMM knockout mice or following functional blockade of RHAMM protein in wild type animals show that it regulates HA mediated ECM remodeling, polarized cell migration, cell division fidelity, and mesenchymal differentiation. These functions have been particularly well studied in vascular, lung, and excisional skin injury models [55, 57, 106, 119, 181, 197, 209, 210].

RHAMM was first shown to be required for smooth muscle cell migration into scratch wounds in the mid-1990s [181] and then later demonstrated to be required for endothelial cell signaling and migration during vessel morphogenesis in culture [123, 170, 185, 211]. More recently, the role of RHAMM was studied following vessel damage using RHAMM−/− mice [57, 119, 181, 197]. Cell culture studies comparing RHAMM−/− and wild type smooth muscle cells and blocking RHAMM function with antibodies show that RHAMM:HA interactions mediate smooth muscle cell adhesion and contraction of collagen gels. *In vivo*, loss of RHAMM

increases vessel lumen size and reduces the size of adventitia and collagen deposition within the artery wall [57]. These results suggest that cell surface RHAMM:HA interactions promote lumen constriction and blocking this function of RHAMM may be clinically useful in preventing restenosis. The role of RHAMM in smooth muscle proliferation following balloon injury of rat carotid arteries has also been reported [197]. These studies show that the rapid proliferation of neointimal smooth muscle cells is RHAMM mediated. In this injury setting, RHAMM:dynein complexes localized to the mitotic spindle are required to promote mitotic fidelity by controlling centrosome placement. Furthermore, intracellular RHAMM phosphorylated by protein kinase Cα is required for correct placement of centrosomes and directed migration of smooth muscle cells into wounds [59, 197]. In lung, RHAMM:HA fragment interactions are required for macrophage chemotaxis in surfactant-stimulated and bleomycin injured lungs [209]. RHAMM expression was first linked to skin wound fibroplasia and fibrosis *in vivo* using a transplantation model comparing incisional and excisional wounds [212]. The former heal without fibroplasia or scarring while repair of the latter is accompanied with extensive fibroplasia and scar formation. Only the excisional wounds exhibit increased CD44 and RHAMM expression. Later studies using RHAMM−/− mice, function blocking antibodies, and RHAMM mimetic peptide antagonists have established that HA:RHAMM interactions are critical for macrophage influx into the wound, as well as for fibroplasia and angiogenesis [55, 106]. Thus, blocking RHAMM function or deleting RHAMM expression results in a reduction of both M1 and M2 macrophages. Furthermore, loss of RHAMM function reduces the level of wound TGFβ-1, causes reduced fibroblast migration into wounds, and inhibits their differentiation into myofibroblasts. There is also a reduction in collagen 1 accumulation and in the number of wound blood vessels.

Evidence from these studies and others [123] suggests that RHAMM binds to fragmented HA and that these interactions may be important in stimulating a RHAMM mediated "danger signal" to cells within injured tissues. Importantly, the binding of RHAMM to HA fragments is surprisingly size specific: a mixture of 4–20 saccharides promotes endothelial cell migration through RHAMM but HA-6 present in this mixture uniquely promotes wound closure, M1 and M2 macrophage influx into wounds, and TGFβ-1 production through RHAMM and CD44 [56, 123]. In wound dermal cells, RHAMM:CD44 appears to cooperate to activate ERK1,2 and FAK. These results emphasize that HA-receptor interactions in healing wounds are complex and that multiple HA receptors can collaborate to control important aspects of wound repair. Similar RHAMM-regulated signaling appears to be at play in BCa progression.

9. RHAMM and Breast Cancer

To date, the role of RHAMM in BCa and other tumors have focused upon tumor cell parenchyma. However, it is likely that RHAMM expressed either by tumor or host cells directly or indirectly facilitates tumor progression. Blocking RHAMM in certain tumor cells inhibits tumor proliferation and migration/invasion while in others it primarily affects migration and invasion [48, 62]. RHAMM mRNA and protein expression are increased in most tumors and these high levels are positively associated with aggressive tumors. However, in a few tumor types (e.g., malignant peripheral nerve sheath tumors) knockdown of RHAMM levels actually enhances tumor aggression. Intriguingly, loss of RHAMM in these tumors is associated with increased AURKA activity and enhanced sensitivity to AURKA inhibitors [206, 213, 214]. RHAMM is also implicated in promoting the self-renewal and tumorigenic potential of tumor stem cells in cancers such as glioblastoma [215]. Despite the complexity of its functions in tumors, levels/distribution of RHAMM isoforms have diagnostic or prognostic value such as identifying which tumor types are sensitive to targeted therapy (e.g., AURKA inhibitors). Considering its involvement in many of the critical driver pathways important for malignant progression, the targeting of RHAMM may also have therapeutic value in some cancers including BCa (see below). For example, RHAMM silencing blocked the self-renewing capability of glioblastoma stem cells, and loss of RHAMM in malignant peripheral nerve sheath tumors or multiple myeloma enhances the sensitivity of tumor cells to AURKA inhibitors. RHAMM hyperexpression occurs in castration-resistant prostate cancer and is also associated with the likelihood of biochemical recurrence in prostate cancer patients with intermediate grade (Gleason grade 7) prostate tumors [206, 215–217].

Data bank indicates that hyperexpression of RHAMM mRNA expression is common in BCa and these elevated levels are often linked to poor clinical outcome [54]. Common genetic mutations at the low penetrance susceptibility RHAMM/HMMR locus enhance breast cancer risk in BRCA-1 mutation carriers [49]. Furthermore, in a large BCa patient cohort, RHAMM hyperexpression in breast tumor cell subsets predicts poor clinical outcome and is associated with elevated risk of peripheral metastases [218]. Other studies demonstrate that RHAMM expression is linked to increased BCa cell invasion and metastases [48, 62]. RHAMM transcription, which is regulated by mevalonate and HIPPO pathways, is required for ERK1,2-controlled BCa cell line migration and invasion, with relatively little impact on proliferation [96]. Similarly, RHAMM is an essential part of an autocrine motility mechanism in aggressive BCa lines for sustaining motility and invasion that requires HA production, ERK1,2 activation, and CD44 display [95]. Human BCa lines of all subtypes are heterogeneous in their ability to bind to fluorescent-HA probes. Subpopulations of tumor cells sorted according to their ability to bind to HA exhibit very different phenotypes. Cells that bind high levels of HA display both CD44 and RHAMM, proliferate slowly but are highly invasive in culture-based assay and *in vivo,* and are metastatic *in vivo*. In contrast, cell subpopulations, which bind low or no HA, express only CD44, proliferate rapidly but are poorly invasive in both culture based assays and *in vivo*, and are poorly metastatic [54]. These studies predict that major RHAMM functions in BCa are to support invasion and metastasis and that coordinated HA:cell surface RHAMM: intracellular

RHAMM signaling contributes to BCa metastases in multiple but as yet incompletely understood ways [95, 165].

10. Conclusions

Wound repair and tumor progression are two complex but similar biological processes that share many molecular mechanisms for controlling cell migration, invasion, survival, and proliferation. HA and its receptors control essential functions in these two processes and this effect appears to be controlled in part by its binding to RHAMM. RHAMM is also upregulated during both processes where it appears to be similarly involved in the control of cell migration, invasion, proliferation, and differentiation. RHAMM is a multifunctional protein that signals through the ERK1,2 and TPX2 pathways at multiple steps. Its action on these pathways appears to be coordinately important to the initiation and progression of BCa and normal response to injury. The selective expression of RHAMM during times of tissue remodeling makes it a promising marker and target for diagnosis and therapy of disease involving aberrant wound repair and cancer.

Abbreviations

AURKA:	AURORA Kinase A
BCa:	Breast cancer
CD44:	Cluster designation 44
ECM:	Extracellular matrix
ERK1,2:	Extracellular regulated kinase 1,2
HA:	Hyaluronan
HAS:	Hyaluronan synthase
Hyal:	Hyaluronidase
HMMR:	Human HA mediated motility receptor
MMP-9:	Metalloproteinase-9
PAI-1:	Plasminogen activation inhibitor 1
RHAMM:	Receptor for HA mediated motility
TLR2,4:	Toll-like Receptor 2,4
TPX2:	Microtubule-associated protein homolog.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Accumulation of Extracellular Hyaluronan by Hyaluronan Synthase 3 Promotes Tumor Growth and Modulates the Pancreatic Cancer Microenvironment

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Extensive accumulation of the glycosaminoglycan hyaluronan is found in pancreatic cancer. The role of hyaluronan synthases 2 and 3 (HAS2, 3) was investigated in pancreatic cancer growth and the tumor microenvironment. Overexpression of HAS3 increased hyaluronan synthesis in BxPC-3 pancreatic cancer cells. In vivo, overexpression of HAS3 led to faster growing xenograft tumors with abundant extracellular hyaluronan accumulation. Treatment with pegylated human recombinant hyaluronidase (PEGPH20) removed extracellular hyaluronan and dramatically decreased the growth rate of BxPC-3 HAS3 tumors compared to parental tumors. PEGPH20 had a weaker effect on HAS2-overexpressing tumors which grew more slowly and contained both extracellular and intracellular hyaluronan. Accumulation of hyaluronan was associated with loss of plasma membrane E-cadherin and accumulation of cytoplasmic β-catenin, suggesting disruption of adherens junctions. PEGPH20 decreased the amount of nuclear hypoxia-related proteins and induced translocation of E-cadherin and β-catenin to the plasma membrane. Translocation of E-cadherin was also seen in tumors from a transgenic mouse model of pancreatic cancer and in a human non-small cell lung cancer sample from a patient treated with PEGPH20. In conclusion, hyaluronan accumulation by HAS3 favors pancreatic cancer growth, at least in part by decreasing epithelial cell adhesion, and PEGPH20 inhibits these changes and suppresses tumor growth.

1. Introduction

Pancreatic ductal adenocarcinoma is the fourth-leading cause of cancer-related deaths in the United States with a median 5-year survival rate of 6% [1]. Pancreatic cancer is characterized by a desmoplastic response involving stromal fibroblasts, inflammatory cells, and pathological deposition of altered extracellular matrix [2, 3] that contains high levels of fibrous collagen, proteoglycans, and glycosaminoglycans including hyaluronan (HA, hyaluronic acid) [3]. Hyaluronan is a negatively charged linear glycosaminoglycan composed of repeating disaccharide units of N-acetylglucosamine and D-glucuronic acid. Excess hyaluronan is found in several tumor types including pancreatic, breast, and prostate cancers [4–7] and its accumulation has been shown to be a factor associated with poor prognosis for cancer patients [5, 7, 8]. Hyaluronan accumulation leads to increased tumor interstitial fluid pressure and poor perfusion [6, 9] explained by continuous tumor cell secretion of hyaluronan and its substantial absorption of water molecules (~15 per disaccharide) [10]. Elevated

tumor interstitial fluid pressure and poor perfusion can be normalized by hyaluronan depletion from the tumor [6, 9]. In addition, hypoxia, often found in advanced solid tumors and characterized by upregulation of hypoxia-inducible factor-1α (HIF-1α) [11], has been associated with increased hyaluronan accumulation [11, 12].

The hyaluronan molecule, composed of 2,000–25,000 disaccharides, with molecular weight of 1–10 million Da, is synthesized at the plasma membrane by three transmembrane synthases (HAS1-3) and is simultaneously extruded to extracellular space. Hyaluronan interacts with several binding proteins and can be incorporated into the extracellular matrix or bound to its cell surface receptors. Hyaluronan binding to its best-characterized receptor, CD44, induces activation of the PI3K-Akt and MAP kinase pathways and promotes tumor cell proliferation, invasion, and chemoresistance [13, 14]. Accumulation of intracellular hyaluronan is also found in several cell types [15–17] where it has been suggested to be a result of hyaluronan endocytosis and degradation [18] or activation of intracellular hyaluronan synthesis [17, 19]. Hyaluronan is degraded mainly by the hyaluronidase-1 (HYAL1) and hyaluronidase-2 (HYAL2) enzymes [20]. In addition to hyaluronidases, exoglycosidases and reactive oxygen species are known to participate in the degradation of high molecular weight hyaluronan to smaller fragments [21]. Recently, KIAA1199 has been suggested to be a hyaluronan binding protein and may be involved in hyaluronan degradation that is independent of CD44 and HYAL enzymes [22].

Hyaluronan synthases share 57–71% identity at the amino acid level [23] but have different expression patterns, differ in their enzymatic properties, and are differentially regulated [24]. Overexpression of any of the HAS genes in COS-1 and rat fibroblasts leads to the formation of a pericellular hyaluronan coat, where HAS1 produces smaller coats compared to HAS2 or HAS3 [25, 26]. The HAS2 isoform has been most studied and is the only isoform required for embryonic development [27]. HAS2 is believed to be the main HAS in epithelial cells and has been reported to mediate epithelial-mesenchymal transition (EMT) [28, 29]. Overexpression of HAS3 leads to the formation of long plasma membrane protrusions [30] which have recently been associated with the increased release of hyaluronan-coated and plasma membrane-derived microvesicles [31]. Moreover, overexpression of HAS3 has been reported to induce misorientation of the mitotic spindle and disturbed organization of epithelium [32], which is associated with malignancies [33]. Recently, HASs have also been suggested to form homodimers and heterodimers which may affect their function and regulate their activity [24, 34].

In human pancreatic cancer, 87% of tumors contain high levels of hyaluronan [4–6]. Similarly, extensive hyaluronan deposition is also found in a transgenic mouse model (*LSL-Kras*$^{G12D/+}$*; LSLTrp53*$^{R172H/+}$*;* and *Pdx-1-Cre* (KPC)) of pancreatic adenocarcinoma [4, 6]. The importance of hyaluronan in the pancreatic cancer microenvironment is further supported by the finding that pancreatic cancer cells encapsulated within hyaluronan gel produce faster growing tumors and are more metastatic than cancer cells with no hyaluronan in a mouse model [35]. Suppression of hyaluronan synthesis by downregulation of HASs has been previously shown to inhibit the growth of implanted breast, prostate, squamous cell carcinoma, and osteosarcoma tumors [15, 36–38]. Similarly, hyaluronan synthesis inhibitor 4-methylumbelliferone or its derivatives suppress metastasis of several tumor types [38–41].

In agreement with previous findings, enzymatic removal of hyaluronan by pegylated human recombinant hyaluronidase (PEGPH20) leads to suppression of tumor growth and metastasis and enhanced delivery of chemotherapy in hyaluronan-rich tumor models of prostate, lung, and pancreatic cancer [5, 9, 42]. In the KPC mouse model of pancreatic adenocarcinoma that closely resembles human disease, PEGPH20 suppressed tumor growth, increased drug delivery, and increased overall survival when used in combination with gemcitabine compared to gemcitabine monotherapy [4, 6]. Increased drug delivery of gemcitabine was associated with stromal remodeling, reduction of tumor interstitial fluid pressure, expansion of intratumoral blood vessels, and ultrastructural changes in tumor endothelium, characterized as formation of fenestrae in tumor endothelium [4, 6].

Over the years, HAS2 has been the focus of most research in this area and has been widely associated with malignant transformation and aggressive tumor growth [28, 29, 43]. However, elevated HAS3 protein levels have also been associated with ovarian cancer [44], and overexpression of HAS3 promotes tumor growth in a preclinical model [45]. Regulation and possible differential mechanisms of HAS2- and HAS3-mediated tumor growth are not completely understood. To date there are no reports comparing the roles of HAS2 and HAS3 in pancreatic cancer. In this study, we explored the biological consequences of HAS2 and HAS3 overexpression in BxPC-3 pancreatic cancer cells and in xenograft tumor models. HAS3 overexpression led to increased accumulation of extracellular hyaluronan that was associated with faster tumor growth and enhanced response to PEGPH20. Deposition of extracellular hyaluronan was associated with loss of adhesion proteins from the plasma membrane that was inhibited by hyaluronan depletion. These results are further supported by the finding that more plasma membrane E-cadherin was observed in KPC tumors as well as in a human non-small cell lung cancer (NSCLC) patient biopsy after PEGPH20 therapy.

2. Material and Methods

2.1. Cell Lines. BxPC-3 cells were obtained from American Type Culture Collection (Manassas, VA). BxPC-3 cells and lentiviral-transduced BxPC-3 cell lines overexpressing empty vector, HAS2, or HAS3 were cultured in RPMI medium with L-glutamine (Cat# 10-040-CV, Cellgro, Mediatech, Manassas, VA) containing 10% fetal bovine serum (Foundation B, Gemini Bio-Products, Sacramento, CA) at 37°C in a humidified incubator supplied with 5% CO_2/5% air. For experimental use, cells were thawed and maintained in culture for no longer than 10 passages. Each cell line was routinely tested negative

for mycoplasma contamination by MycoAlert Mycoplasma Detection Kit (Lonza Biologics, Hopkinton, MA).

2.2. Establishment of BxPC-3 Vector, BxPC-3 HAS2, and BxPC-3 HAS3 Cell Lines. Lentiviral vectors pLV-EF1a-MCS-IRES-Hyg (vector only), pLV-EF1a-hHAS2-IRES-Hyg (HAS2), and pLV-EF1a-hHAS3-IRES-Hyg (HAS3, Biosettia, San Diego, CA) were added to subconfluent BxPC-3 cultures, followed by centrifugation for 30 min at 1,200 rpm and incubation at 37°C for 6 h. Fresh medium was added to the cultures, and incubation was continued for 48 h, followed by selection and expansion of stable cultures using medium containing hygromycin.

2.3. Hyaluronidase-Sensitive Particle Exclusion Assay. To visualize aggrecan-mediated hyaluronan pericellular matrices in vitro, particle exclusion assays were performed as previously described [9, 42], with some modifications. Subconfluent cultures were treated with 1 mg/mL bovine nasal septum proteoglycan (Elastin Products, Owensville, MO) for 1 h at 37°C, followed by incubation with vehicle or 1,000 U/mL recombinant human PH20 (rHuPH20, Halozyme Therapeutics, San Diego, CA) as a negative control for 2 h at 37°C. Glutaraldehyde-fixed mouse red blood cells were added to the cultures, which were then imaged with a phase-contrast microscope coupled with a camera scanner and SPOT advanced imaging program (Version 4.6, Diagnostic Instruments, Sterling Heights, MI). Particle exclusion area and cell area were measured and relative hyaluronan coat area was calculated using the formula: *matrix area–cell area* (expressed as μm^2).

2.4. Hyaluronan Assay. To analyze hyaluronan secretion, tissue culture supernatants were collected from subconfluent cultures after 24 h culture, and cells were trypsinized and counted for normalization. Hyaluronan concentration in the samples was quantified using an enzyme-linked hyaluronan-binding protein sandwich assay (Cat# DY3614, R&D Systems, Minneapolis, MN) according to manufacturer's instructions [42].

2.5. Isolation of Secreted, Cell Surface, and Intracellular Hyaluronan. Isolation of secreted, cell surface, and intracellular hyaluronan was performed as previously described [16], with some modifications. The conditioned media containing secreted hyaluronan were collected from subconfluent cultures after 48 h of culture. Cells were detached and collected by centrifugation and supernatant was transferred to a clean tube. Cell pellets were rinsed with 1 × PBS and centrifuged, and supernatant was combined with the supernatant from the previous centrifugation and was designated as "cell surface hyaluronan." Combined supernatants were incubated briefly at 100°C to inactivate trypsin. The cell pellet was rinsed, the supernatant was discarded, and the cell pellet was designated as "intracellular hyaluronan." To ensure that no residual cell surface hyaluronan was present, some of the cell fractions were treated with 1,000 U/mL rHuPH20 for 20 min and rinsed with ice-cold 1 × PBS followed by centrifugation and collection of the cell pellet. All the samples were digested with Proteinase K at +55°C overnight, followed by heat inactivation at 95°C for 10 min and centrifugation at 12,000 rpm at 4°C for 10 min.

2.6. Fluorescent Staining of Intracellular Hyaluronan and CD44. For fluorescent staining, subconfluent cultures were fixed with 4% paraformaldehyde, and pericellular hyaluronan was digested with rHuPH20 (Halozyme Therapeutics, Inc., San Diego, CA). Cells were then permeabilized with 0.3% Triton-x-100 (Calbiochem, La Jolla, CA; 1% BSA) and blocked with 3% BSA. Hyaluronan and CD44 were stained with biotinylated hyaluronan-binding protein (bHABP, 2.5 μg/mL, Calbiochem/EMD Chemicals, Inc., San Diego, CA) and anti-CD44 antibody (1 μg/mL, BBA10, R&D Systems) overnight at 4°C. bHABP and anti-CD44 antibody were detected by Fluorescein-labeled streptavidin (0.5 μg/mL, Vector Laboratories, Burlingame, CA) and Texas Red labeled anti-mouse antibody (0.5 μg/mL, Vector), respectively. Nuclei were stained with Prolong Antifade with 4′,6-diamidino-2-phenylindole (DAPI; Invitrogen, Carlsbad, CA), and cultures were imaged with a spinning disk microscope (Nikon Eclipse TE 2000-U (Nikon, Eugene, OR) and BD Bioscience spinning disk unit (BD Bioscience, Rockville, MD)) using 60x objective and MetaMorph software version 7.7.6.0 (Molecular Devices, Sunnyvale, CA).

2.7. Pancreatic Cancer Xenograft Models. Six- to eight-week-old nu/nu (Ncr) athymic mice, handled in accordance with approved Institutional Animal Care and Use Committee protocols, were used for xenograft studies. Mice were inoculated with 0.05 mL of 5×10^6 BxPC-3, BxPC-3 vector, BxPC-3 HAS2, or BxPC-3 HAS3 cells (concentration 1×10^8 cells/mL) peritibially in the hind leg (adjacent to the tibia periosteum). Tumor growth was monitored by ultrasound imaging (VisualSonics Vevo 770/2100 High Resolution Imaging System (VisualSonics, Ontario, Canada)) until the average tumor size reached 200–500 mm^3. The animals were then divided into treatment groups and treated intravenously (*i.v.*) twice a week for 2-3 weeks with vehicle, 37.5 μg/kg, 1,000 μg/kg, or 4,500 μg/kg PEGPH20. At the end of the study, tumors were collected and divided into parts; one part was fixed in 10% formalin for histology, and the other parts were frozen for later biochemical analysis. Some additional tumor-bearing mice with large tumors (~2,000 mm^3) received two injections of vehicle or 4,500 μg/kg PEGPH20, and tumors were harvested in 10% formalin 6 h after treatment.

2.8. KPC Mouse Model. Mouse pancreatic cancer tissue sections from KPC mice [46] were obtained from the Jacobetz et al. 2013 study [4]. As described previously, mice were treated *i.v.* with a single injection of 4,500 μg/kg PEGPH20 once tumor volume reached 270 mm^3, and tumors were collected 1, 8, 24, and 72 h after dosing [4].

2.9. Human Patient Samples. Pretreatment and posttreatment biopsies were obtained from a patient with advanced NSCLC who was enrolled in a Phase 1 study of

PEGPH20 given *i.v.* to patients with advanced solid tumors (NCT01170897). The protocol was approved by Institutional Review Board and all patients consented to study. The patient was treated with a single dose of 5.0 μg/kg PEGPH20. Posttreatment biopsy was taken 7 days after *i.v.* treatment.

2.10. Tissue Samples. Tissues were fixed in 10% neutral buffered formalin and processed to paraffin. Five micrometer sections were used for Hematoxylin and Eosin (H&E) staining that was performed according to a standard protocol. For hyaluronan assay, fresh frozen tumor pieces were digested with Proteinase K at +55°C overnight, then heat-inactivated at 95°C for 10 min, and centrifuged at 12,000 rpm at 4°C for 10 min.

2.11. Hyaluronan Staining. Hyaluronan in tumor tissues was localized as previously described [42], with some modifications. Briefly, five micrometer sections were deparaffinized and rehydrated, and endogenous peroxidase was blocked with Peroxo-Block solution (Invitrogen). Nonspecific staining was blocked using 2% BSA (Jackson ImmunoResearch, West Grove, PA) and 2% normal goat serum (Vector) for 1 h, followed by blocking of endogenous avidin and biotin (Avidin/Biotin Blocking Kit, Invitrogen). Hyaluronan was detected by incubating sections with 2.5 μg/mL bHABP (Seikagaku, Tokyo, Japan) for 1 h at 37°C. Signal was amplified by incubation with streptavidin-horseradish peroxidase solution (HRP; BD Biosciences) and detected with 3,3′-diaminobenzidine (DAB, Dako North America, Carpinteria, CA). Sections were then counterstained in Gill's hematoxylin (Vector) and mounted in Cytoseal 60 medium (American MasterTech, Lodi, CA). Specificity of the staining was assessed by incubation of a section of each sample with rHuPH20 (12,000 U/mL) in PIPES buffer (25 mM PIPES, 70 mM NaCl, 0.1% BSA, pH 5.5) at 37°C for 2 h prior to incubation with bHABP.

2.12. Immunohistochemistry. For cleaved caspase-3 (CC3), phosphohistone 3 (PH3), E-cadherin, and β-catenin immunohistochemistry, five micrometer sections were deparaffinized, rehydrated, and pretreated in boiling citrate buffer (pH 6.0) for 10 min. For CC3 and β-catenin stainings, endogenous peroxidase was blocked with 3% H_2O_2, followed by blocking with 5% normal goat serum. Sections were incubated with CC3 (0.21 μg/mL, Cat# 9661, Cell Signaling, Danvers, MA) and β-catenin (40 μg/mL, Cat# 8480, Cell Signaling) antibodies in SignalStain Antibody Diluent (Cell Signaling) overnight at 4°C. Signal was detected with anti-rabbit SignalStain Boost IHC Detection Reagent (Cat# 8114, Cell Signaling) and DAB. For PH3 immunohistochemistry, sections were blocked with 3% H_2O_2 and 5% normal goat serum. Then PH3 antibody (65 ng/mL, Cat# 9701, Cell Signaling) was added and incubated overnight at 4°C. Primary antibody was detected with HRP-conjugated anti-rabbit immunoglobulin G (IgG) (0.17 μg/mL, Cat# 7074, Cell Signaling) and DAB. For E-cadherin immunohistochemistry, slides were blocked with 3% H_2O_2, followed by blocking of endogenous avidin and biotin. E-cadherin (0.25 μg/mL, ab76055, Abcam, Cambridge, UK) antibody was diluted in mouse-on-mouse (M.O.M.) diluent and added to the sections for 1 h (Cat# PK-2200, Vector). Subsequently, M.O.M biotinylated anti-mouse IgG was added to the slides, and signal was amplified with Vectastain ABC reagent (Vector) and DAB. Then slides were counterstained with Gill Hematoxylin and mounted with synthetic media.

2.13. Western Blot. Tumor pieces were homogenized in cold hypotonic buffer (25 mM Tris. HCl pH 7.4, 50 mM NaCl, 10% glycerol, 0.5% Triton X-100, and 5 mM EDTA with protease and phosphatase inhibitor cocktail); the homogenates were incubated at 4°C for 30 min and centrifuged at 7,500 rpm for 10 min at 4°C. The pellet was resuspended in nuclear extraction buffer (25 mM Tris-HCl pH 7.4, 250 mM NaCl, 10% glycerol, 0.5% Triton X-100, and 5 mM $MgCl_2$ with protease and phosphatase inhibitor cocktail), treated with protease-free DNAse I, briefly sonicated, and incubated with 200 μg/mL ethidium bromide for 1 h. Finally, samples were centrifuged and the supernatant was collected. Nuclear extracts were separated on SDS-PAGE, transferred into a nitrocellulose membrane, and blocked with 5% BSA in PBS with 0.1% Tween 20. The membrane was probed with HIF-1α antibody (0.5 μg/mL, ab16066, Abcam), Snail (1 μg/mL, ab130245, Abcam), β-actin (13 μg/mL, Cat# 2148, Cell Signaling), and Histone 3 (1.1 μg/mL, Cat# 9717, Cell Signaling) antibodies. HIF-1α and Snail antibodies were detected with HRP-conjugated goat anti-mouse IgG antibody (1 : 10,000, Jackson ImmunoResearch) and chemiluminescent reagent (GE Healthcare Life Sciences, Piscataway Township, NJ) and β-actin and Histone 3 with HRP-conjugated anti-rabbit IgG and chemiluminescent reagent. Images were captured using GE ImageQuant 400 (GE Healthcare) and bands were quantified using Image-Pro Analyzer 7.0 software (Media Cybernetics, Rockville, MD).

2.14. Quantification of Staining. Stained sections were scanned at Flagship Biosciences LLC (Boulder, CO) and automated quantification of CC3 staining was performed using Aperio Positive Pixel algorithm v9 (Aperio, Buffalo Grove, IL) and results were normalized to the total number of pixels. Quantification of PH3 stainings was performed by Aperio Imagescope using Nuclear v9 algorithm.

2.15. Statistical Analysis. Values are presented as mean ± S.D. or SEM. Results expressed as ratios or percent values were log transformed prior to the analysis. Statistical difference was analyzed by t test, one-way ANOVA, and Tukey's post hoc test or two-way ANOVA with Bonferroni's post hoctest using Graphpad Prism 5 software (Graphpad, La Jolla, CA). Statistical difference was set at $P < 0.05$ ($^{*}P < 0.05$; $^{**}P < 0.01$; and $^{***}P < 0.001$).

3. Results and Discussion

3.1. HAS3 Overexpression Is Associated with Increased Levels of Hyaluronan Production. Extensive hyaluronan accumulation is found in pancreatic cancer [4, 6], which is characterized by massive desmoplastic stroma, high interstitial tumor pressure, poor perfusion, and resistance to therapy [47]. Hyaluronan is synthesized by three HAS enzymes at the plasma membrane, and HAS overexpression has been associated with cancer growth [43, 45, 48]. In this study, we used BxPC-3 pancreatic cancer cells that overexpress HAS3 to assess the roles of this HAS isoform on pancreatic cell lines and tumors.

Human BxPC-3 pancreatic adenocarcinoma cells were engineered to overexpress HAS3, and functional consequences of hyaluronan production by HAS3 were analyzed by hyaluronan secretion and size of pericellular hyaluronan matrix. BxPC-3 cells secreted 181 ng hyaluronan to culture medium per 10,000 cells over 24 h, while hyaluronan secretion of BxPC-3 HAS3 cells was 20-fold higher, at 3,607 ng per 10,000 cells (Table 1). Similarly, relative hyaluronan matrix area was increased by 2.4-fold after HAS3 overexpression (Table 1). Overexpression of HAS2 and HAS3 has been reported to lead to increased hyaluronan secretion and increased size of hyaluronan coat in several cell lines [25, 26]. Thus, we generated and tested HAS2-overexpressing BxPC-3 cells and found that similar amounts of hyaluronan were secreted by BxPC-3 HAS2 and BxPC-3 HAS3 cells (Table 1), resulting in similar hyaluronan coat sizes. The size of hyaluronan coat on BxPC-3 cells transduced with an empty vector was also analyzed, and coat size did not significantly differ from that on parental cells (data not shown).

To further characterize BxPC-3 HAS3 cells, the quantity of extracellular and intracellular hyaluronan was analyzed. In line with earlier results (Table 1), the amount of extracellular hyaluronan, composed of secreted and cell surface hyaluronan, was 9.9-fold higher in BxPC-3 HAS3 cells compared to BxPC-3 cells (Figure 1(a)). Overexpression of HAS3 also induced a slight 2.8-fold increase in intracellular hyaluronan content (Figure 1(b)). HAS2-overexpressing cells contained a similar amount of extracellular hyaluronan as HAS3-overexpressing cells (Figure 1(a)), but their intracellular hyaluronan level was 35-fold higher than in BxPC-3 cells (Figure 1(b)). Intracellular hyaluronan was also visualized in cultures after fixation and digestion of cell surface hyaluronan. CD44 was stained in the cultures to visualize plasma membranes of the cells. Parental or HAS3-overexpressing cells contained very little intracellular hyaluronan (Figures 1(c) and 1(e)) while HAS2-overexpressing cells showed intensive accumulation of intracellular hyaluronan (Figure 1(d)). These data are consistent with previous reports that HAS2 expression has been associated with the presence of intracellular hyaluronan, induced by epidermal growth factor, keratinocyte growth factor, or hyperglycemia [16, 19, 49].

Table 1: Hyaluronan production of BxPC-3, BxPC-3 HAS2, and BxPC-3 HAS3 cells.

Tumor cell line	Relative hyaluronan coat size (μm^2)[1]	Hyaluronan in CM (ng/10,000 cells in 24 h)[2]
BxPC-3	502 ± 259	181 ± 54
BxPC-3 HAS2	1417 ± 614	2832 ± 643
BxPC-3 HAS3	1154 ± 439	3607 ± 349

[1]Assessed via particle exclusion assay as described in Section 2, Mean ± S.D.
[2]Mean in culture media ($n = 3$, independent cultures) ± S.D.

3.2. Amount and Localization of Hyaluronan in HAS3-Overexpressing Xenograft Tumors. In a follow-up in vivo study, mice bearing BxPC-3 and BxPC-3 HAS3 tumors were dosed twice with vehicle or 4,500 μg/kg PEGPH20, and tumors were collected 6 h after the second dosing. Histochemistry of the tumors revealed that HAS3 overexpression changed the structure of the tumor stroma (Figures 2(a)–2(c)). HAS3-overexpressing tumors contained a large amount of hyaluronan located in the extracellular space of the stroma, while hyaluronan in BxPC-3 tumors was mostly associated with cancer cells (Figures 2(g) and 2(i)). Total hyaluronan content of BxPC-3 HAS3 tumors was 1,864 ng/mg, while hyaluronan content of BxPC-3 tumors was 344 ng/mg (Figure 2(m)). In line with the in vitro results, BxPC-3 HAS2 tumors contained a similar amount of hyaluronan as BxPC-3 HAS3 tumors (Figures 2(h)-2(i) and 2(m)) but in addition to stromal hyaluronan accumulation, cancer cell-associated hyaluronan resembling intracellular hyaluronan was found (Figure 2(h)). Chronic treatment with PEGPH20 (twice a week dosing for 3 weeks) depleted 95% of hyaluronan in BxPC-3 HAS3 tumors, while in BxPC-3 and BxPC-3 HAS2 tumors hyaluronan removal was 72% (Figure 2(m)). The results are consistent with previous reports showing that PEGPH20 can efficiently remove hyaluronan in the tumors [4, 6, 9, 42]. Hyaluronan staining of the PEGPH20-treated tumors revealed that PEGPH20 removed most of the extracellular hyaluronan (Figures 2(j)–2(l)) and reduced the amount of stroma (Figures 2(d)–2(f)), although intracellular hyaluronan in cancer cells was still apparent (Figures 2(j)–2(l)). After treatment with PEGPH20, intracellular hyaluronan was most prominent in HAS2 tumors (Figure 2(k)), in agreement with in vitro observation of intracellular accumulation of hyaluronan in HAS2-overexpressing cells (Figures 1(b) and 1(d)). The significance and origin of intracellular hyaluronan accumulation in HAS2-overexpressing pancreatic cancer cells and tumors is a subject requiring further investigation. Coexpression of HAS2 with CD44 and HYAL2 has been reported and could potentially lead to the cleavage of hyaluronan at the plasma membrane and CD44-mediated internalization and accumulation of intracellular hyaluronan [36, 50, 51]. Intracellular hyaluronan synthesis has also been reported in hyperglycemia and inflammatory conditions [17, 19]. These results also confirm that PEGPH20 only degrades hyaluronan within the extracellular space, probably due to lack of a mechanism to enter the cells.

3.3. HAS3 Overexpression in BxPC-3 Cells Is Associated with Faster In Vivo Growth and Enhanced Tumor Growth Inhibition. HAS2 has been widely shown to be associated with cancer [43, 48]; however, much less is known about the role of

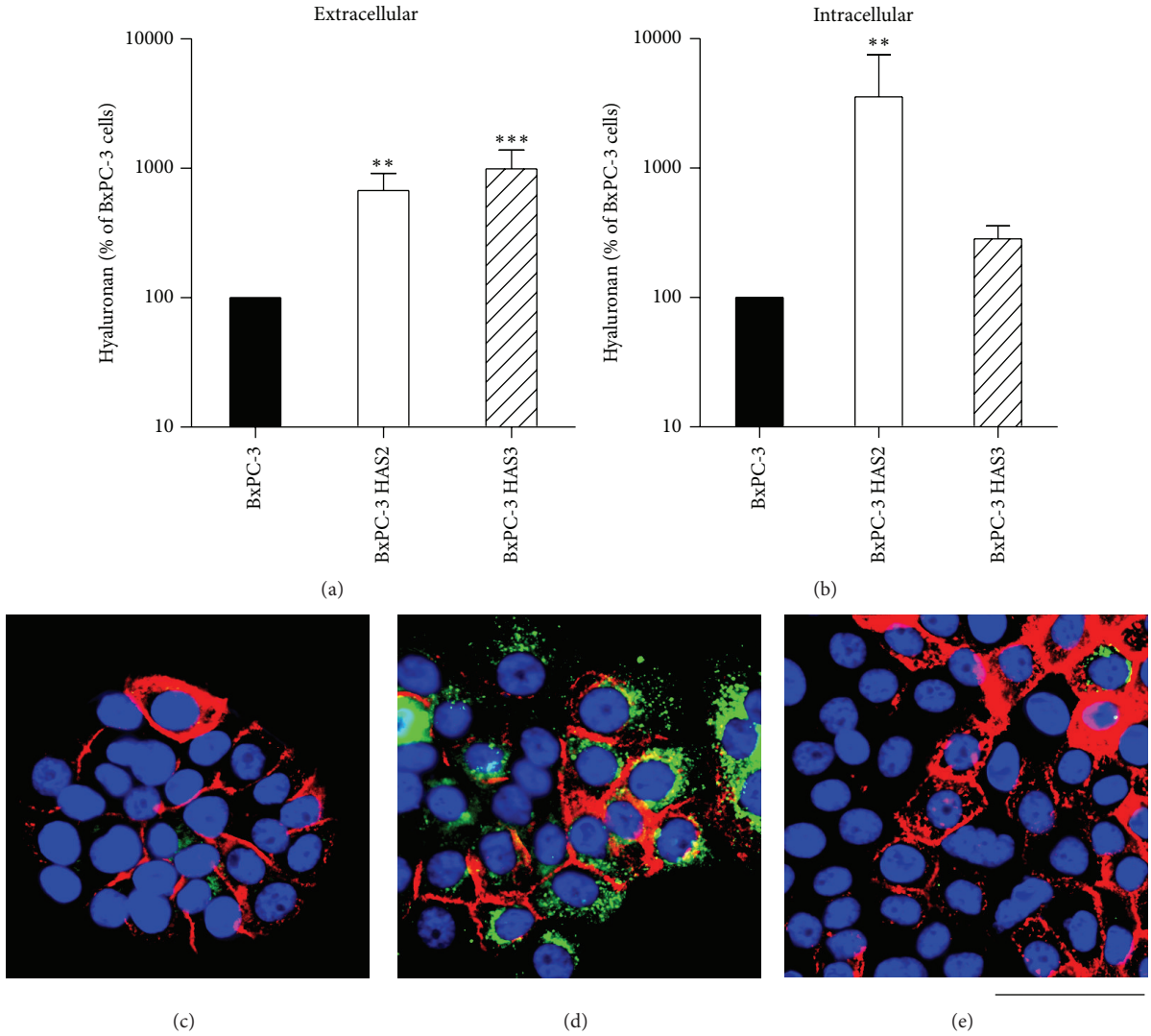

Figure 1: HAS3 overexpression is associated with high extracellular hyaluronan accumulation and HAS2 with increased extracellular and intracellular accumulation of hyaluronan. Amount of secreted, cell surface, and intracellular hyaluronan was analyzed in BxPC-3, BxPC-3 HAS2, and BxPC-3 HAS3 cells with a hyaluronan assay, and results were presented as extracellular hyaluronan showing combined amount of secreted and cell surface hyaluronan (a) and intracellular hyaluronan (b). The results were expressed as % of hyaluronan content in BxPC-3 cells, and data represent mean ± S.D. of three independent experiments. To visualize intracellular hyaluronan, subconfluent cultures of BxPC-3 (c), BxPC-3 HAS2 (d), and BxPC-3 HAS3 cells (e) were stained for intracellular hyaluronan (green) and CD44 (red) using bHABP and anti-CD44s-antibody, respectively. Nuclei were visualized with Prolong Antifade with DAPI. Scale bar in (c–e) is 50 μm. Statistical differences between the groups in (a) and (b) were tested using one-way ANOVA and Tukey's post hoc test ($^{**}P < 0.01$; $^{***}P < 0.001$).

HAS3 in tumor progression. To compare the in vivo growth rate and PEGPH20 response of BxPC-3 and BxPC-3 HAS3 cells, both cells were inoculated peritibially in the hind limb of nude mice to generate tumors, and the mice were dosed *i.v.* twice a week with vehicle or 4,500 μg/kg PEGPH20. BxPC-3 HAS3 cells generated faster growing tumors than parental cells (Figures 3(a) and 3(c)), which correlates with the amount of extracellular hyaluronan in the tumor stroma (Figures 2(g) and 2(i)). The average size of BxPC-3 HAS3 tumors was 1,905 mm^3 on day 15 after initiation of vehicle treatment while the average size of BxPC-3 tumors was 820 mm^3 (Figures 3(a) and 3(c)). This is consistent with the report that HAS3 overexpression enhances extracellular matrix deposition and tumor growth of prostate cancer cells [45] and that metastatic colon cancer cells have upregulated levels of HAS3 [52]. HAS3 protein level is higher in human ovarian cancer than in normal ovary, and the amount of HAS3-positive cancer cells correlates with hyaluronan accumulation in the stroma [44]. Overexpression of HAS2 also increased the tumor growth in a BxPC-3 model but led to less aggressive tumors than those with HAS3 overexpression (Figure 3(b)). Stromal hyaluronan has been reported to contribute to high interstitial fluid pressure, compression of tumor blood vessels, and poor drug delivery in pancreatic cancer tumors [4, 6]. To date

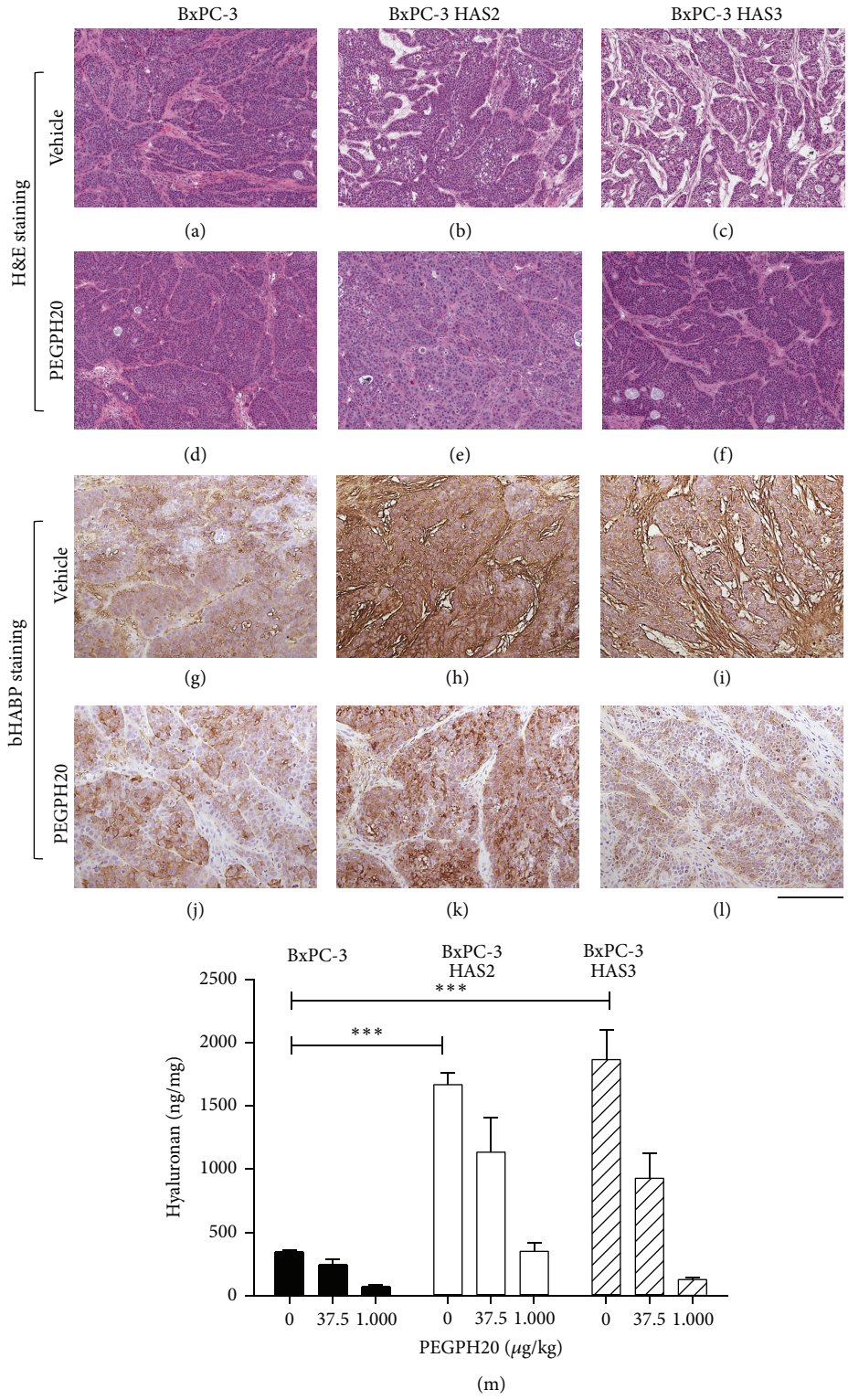

FIGURE 2: Localization and amount of hyaluronan in BxPC-3, BxPC-3 HAS2, and BxPC-3 HAS3 tumors and response to PEGPH20. Mice carrying BxPC-3, BxPC-3 HAS2, and BxPC-3 HAS3 peritibial xenograft tumors were treated twice with vehicle or 4,500 μg/kg PEGPH20, and tumors were collected 6 h after the last dose. Tumor sections were stained with H&E to visualize morphology of the tumors (a–f) and with bHABP for hyaluronan (g–l). Scale bar in (a–f) and (g–l) is 500 μm. Hyaluronan concentration in the tumors after two weekly treatments of vehicle, 37.5 μg/kg or 1,000 μg/kg of PEGPH20 ($n \geq 3$/group) for three weeks was analyzed by hyaluronan assay (m). Statistical differences between the groups shown in figure (m) were tested using two-way ANOVA and Bonferroni's post hoc test ($^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$).

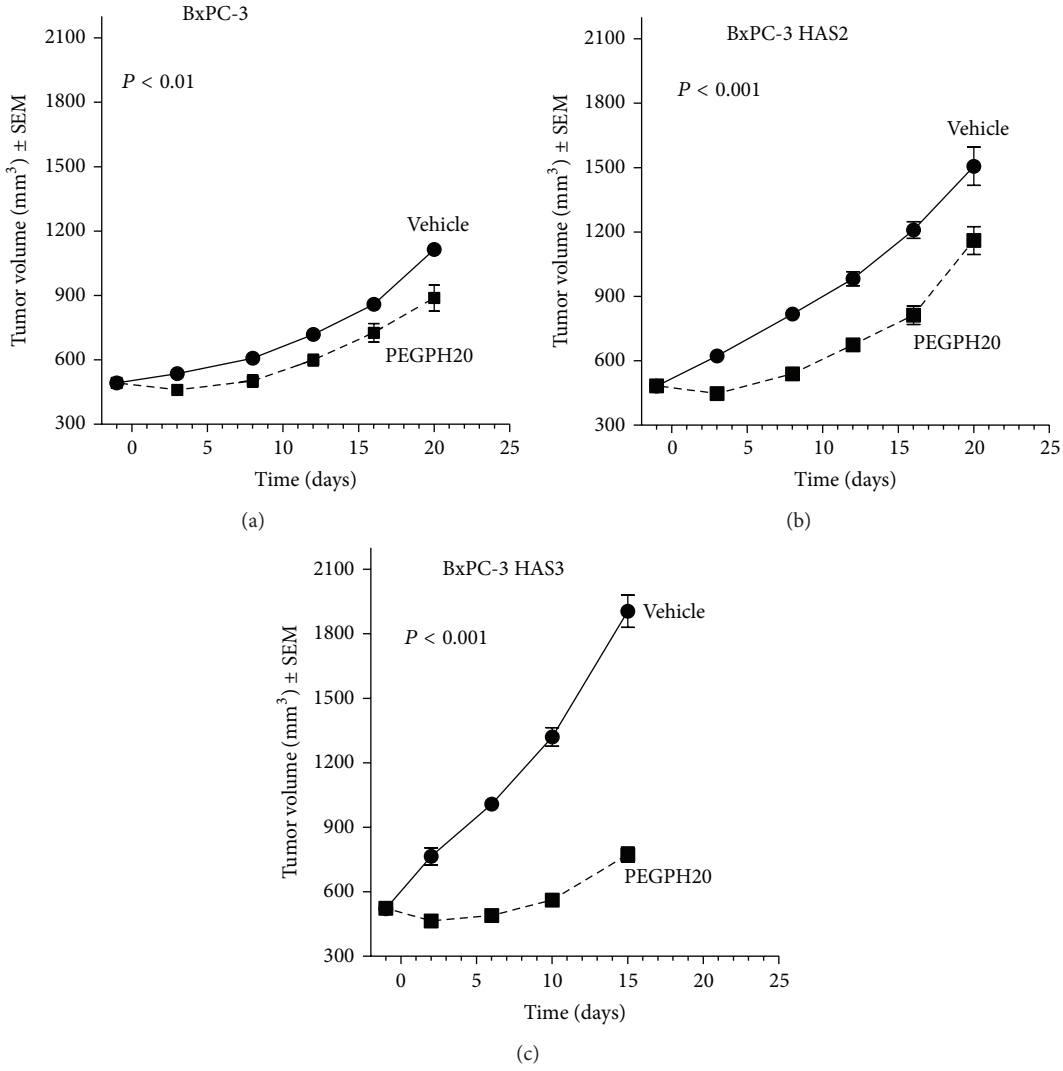

Figure 3: Pancreatic cancer xenograft tumors overexpressing HAS3 grow faster and respond better to hyaluronan removal than HAS2 or parental tumors. To compare in vivo growth, BxPC-3, BxPC-3 HAS2, and BxPC-3 HAS3 tumor cells were inoculated adjacent to the tibial periosteum in the hind limb of nu/nu mice ($n = 7$/group), and tumor growth was monitored using ultrasound imaging. Once average tumor size reached 500 mm^3, mice were treated twice a week with an *i.v.* injection of vehicle or PEGPH20 (4,500 μg/kg) (a–c). Statistical difference was tested using repeated measured two-way ANOVA and Bonferroni's post hoc test.

there have been no observed substantial differences in the amount of stromal hyaluronan between HAS2- and HAS3-overexpressing tumors. However, consequences of HAS2 and HAS3 overexpression on vasculature are not well understood and the possibility that HAS2 and HAS3 might have distinct effects on vascular function cannot be completely ruled out. Interestingly, BxPC-3 HAS3 tumors showed a strong response to PEGPH20, which caused 86% tumor growth inhibition (Figure 3(c)). In BxPC-3 and BxPC-3 HAS2 tumors, PEGPH20 induced tumor growth inhibition of 34% and 32%, respectively (Figures 3(a) and 3(b)). Removal of hyaluronan by PEGPH20 has been shown to lead to decompression of tumor blood vessels, changed ultrastructure of tumor endothelia, and increased drug delivery to the tumor [4, 6, 9]. We show that intravenous injection of PEGPH20 led to similar depletion of stromal hyaluronan in HAS2- and HAS3-overexpressing tumor models (Figures 2(k) and 2(l)) suggesting no differences in drug delivery of PEGPH20 to these tumor types. Taken together, the data show that tumors with HAS3 overexpression show more aggressive tumor growth than parental tumors or tumors with HAS2 overexpression. In HAS3-overexpressing tumors, PEGPH20 removes most of the hyaluronan and has a strong inhibitory effect on tumor growth, whereas in HAS2-overexpressing tumors PEGPH20 removes the extracellular but not the intracellular hyaluronan and causes a weaker inhibitory effect on tumor growth. Our results are in line with previous work showing that depletion of hyaluronan or suppression of its synthesis leads to inhibition of tumor growth in multiple tumor models [9, 36–38, 41, 42].

3.4. Accumulation of Extracellular Hyaluronan by HAS3 Overexpression Induces Loss of Plasma Membrane E-Cadherin. Hyaluronan is known to be involved in EMT-associated changes in cancer progression [27–29]. E-cadherin and β-catenin are essential adhesion molecules in normal epithelium, and loss of plasma membrane E-cadherin and catenins leading to disruption of cell-cell junctions is acknowledged to be an early indication of EMT [53, 54]. Because accumulation of hyaluronan by HAS3 in intercellular space has been suggested to interrupt cell-cell interactions [55], we hypothesized that hyaluronan removal would reverse changes that may have occurred in adhesion events. E-cadherin and β-catenin were visualized by immunohistochemistry in vehicle-treated and PEGPH20-treated BxPC-3 and BxPC-3 HAS3 tumors. HAS3-induced extracellular hyaluronan accumulation resulted in loss of plasma membrane E-cadherin (Figure 4(a)) and increased accumulation of cytoplasmic β-catenin in tumor cells of BxPC-3 tumors (Figure 4(b)). This result supports previous findings that increased hyaluronan production leads to disruption of adherens junctions and leads to EMT [28, 43]. Since the majority of pancreatic adenocarcinomas have high hyaluronan accumulation [4, 5]; these results are in line with the previously reported loss of membrane E-cadherin in human pancreatic adenocarcinoma compared to pancreatic intraepithelial neoplasia or normal ducts [53]. Additionally, HAS overexpression also induces loss of plasma membrane E-cadherin in breast cancer cells and spongiotic keratinocytes [28, 43, 55]. Overexpression of HAS2 led to the same changes in E-cadherin and β-catenin adhesion proteins in BxPC-3 tumors (Figures 4(a) and 4(b)). Interestingly, in mammary epithelial cells, TGF-β-induced EMT is mediated by HAS2 but not by extracellular hyaluronan [29]. This suggests that HAS2 and HAS3 may mediate EMT-associated events via different mechanisms. HAS3 seems to initiate EMT-associated events by extracellular hyaluronan, while the effect of HAS2 on EMT may be additionally mediated by intracellular hyaluronan accumulation or by HAS2 function that is not dependent on hyaluronan synthesis [29].

We then tested the effect of hyaluronan removal by PEGPH20 on EMT-related events in HAS3-overexpressing tumors. Removal of extracellular hyaluronan by PEGPH20 induced translocation of E-cadherin and β-catenin back to the plasma membrane (Figures 4(a) and 4(b)), and PEGPH20 showed a stronger effect in HAS3-overexpressing tumors than in BxPC-3 tumors. It has been previously shown that PEGPH20 removes most tumor-associated hyaluronan in the KPC mouse model [4], so we investigated translocation of E-cadherin in tumors from KPC mice treated with PEGPH20. Translocation of E-cadherin to plasma membrane was observed 8 h after treatment with PEGPH20, and it was most prominent in peripheral duct-like structures at the edges of the tumor (Figure 4(c)). However, the effect seems to be transient, since 24 to 72 h later the plasma membrane residence of E-cadherin started to disappear (Figure 4(c)). E-cadherin status was also studied in pretreatment and posttreatment biopsies of a lung cancer patient treated with PEGPH20 (Phase 1 study, NCT01170897). In the posttreatment biopsy, a decrease in extracellular hyaluronan (data not shown) and an increase in cell surface E-cadherin were found in comparison to the pretreatment biopsy (Figure 4(d)). Removal of extracellular hyaluronan is associated with translocation of epithelial markers E-cadherin and β-catenin back to the plasma membrane, suggesting a reorganization of intercellular junctions and an inhibition of early EMT-associated events, potentially contributing to growth inhibition by PEGPH20 [43].

3.5. Hyaluronan Removal Decreases Nuclear Levels of Hypoxia-Related Proteins. Since hypoxia has been reported to be associated with and able to induce EMT [56, 57], we explored the effect of extracellular hyaluronan accumulation and PEGPH20 on hypoxia-related proteins in BxPC-3 tumors. Nuclear protein levels of HIF-1α and Snail were analyzed by Western blotting (Figures 5(a)–5(f)). Overexpression of HAS3 did not cause a major change in nuclear HIF-1α level, probably because BxPC-3 tumors already contain a low amount of hyaluronan (Figures 5(a)–5(c)). However, hyaluronan depletion by PEGPH20 decreases nuclear HIF-1α levels in both BxPC-3 and BxPC-3 HAS3 tumors (Figures 5(a)–5(c)), suggesting a decrease in hypoxic conditions after hyaluronan removal. Nuclear levels of transcription factor Snail, a target of HIF-1α, were also suppressed by hyaluronan depletion in HAS3-overexpressing tumors (Figures 5(d)–5(f)). These results suggest that PEGPH20 suppresses HIF-1α-Snail signaling in tumors with high levels of hyaluronan. In agreement with our observations, previous reports have described an association of hyaluronan accumulation and tumor hypoxia [12], and that depletion of hyaluronan synthesis by 4-methylumbelliferone prevents EMT-associated changes [58]. EMT has been reported to be mediated via HIF-1α signaling [56, 57], and our data suggest that removal of hyaluronan can be one step in the inhibition of this process.

3.6. Extracellular Hyaluronan Inhibits Apoptosis of BxPC-3 Tumors. To further study the mechanism of how HAS3 overexpression favors tumor growth and leads to stronger response to PEGPH20, the amount of proliferative and apoptotic cells was analyzed in BxPC-3 and BxPC-3 HAS3 tumors. The number of apoptotic cells, assessed by CC3 positivity, was decreased in HAS3-overexpressing tumors (4-fold, $P < 0.05$), suggesting that BxPC-3 HAS3 overexpression protects cells from apoptosis (Figure 6(a)). PEGPH20 treatment showed a trend (2.3-fold) to increase CC3-positive cells in BxPC-3 HAS3 tumors but had no effect in BxPC-3 tumors (Figure 6(a)). The modest effect of PEGPH20 on promoting apoptosis in the BxPC-3 HAS3 tumor model may be due to incomplete removal of hyaluronan from the cancer cell surface (Figure 2(l)). Since HAS3 continuously produces new hyaluronan chains at the plasma membrane, binding of newly synthesized chains to hyaluronan receptors on the cancer cell surface may induce some cell survival signaling and reduce the biological effect of PEGPH20. Alternatively, overexpression of HAS3 may have other hyaluronan-independent effects on cell survival, as reported with HAS2 on TGF-β-induced EMT in mammary epithelial cells [29].

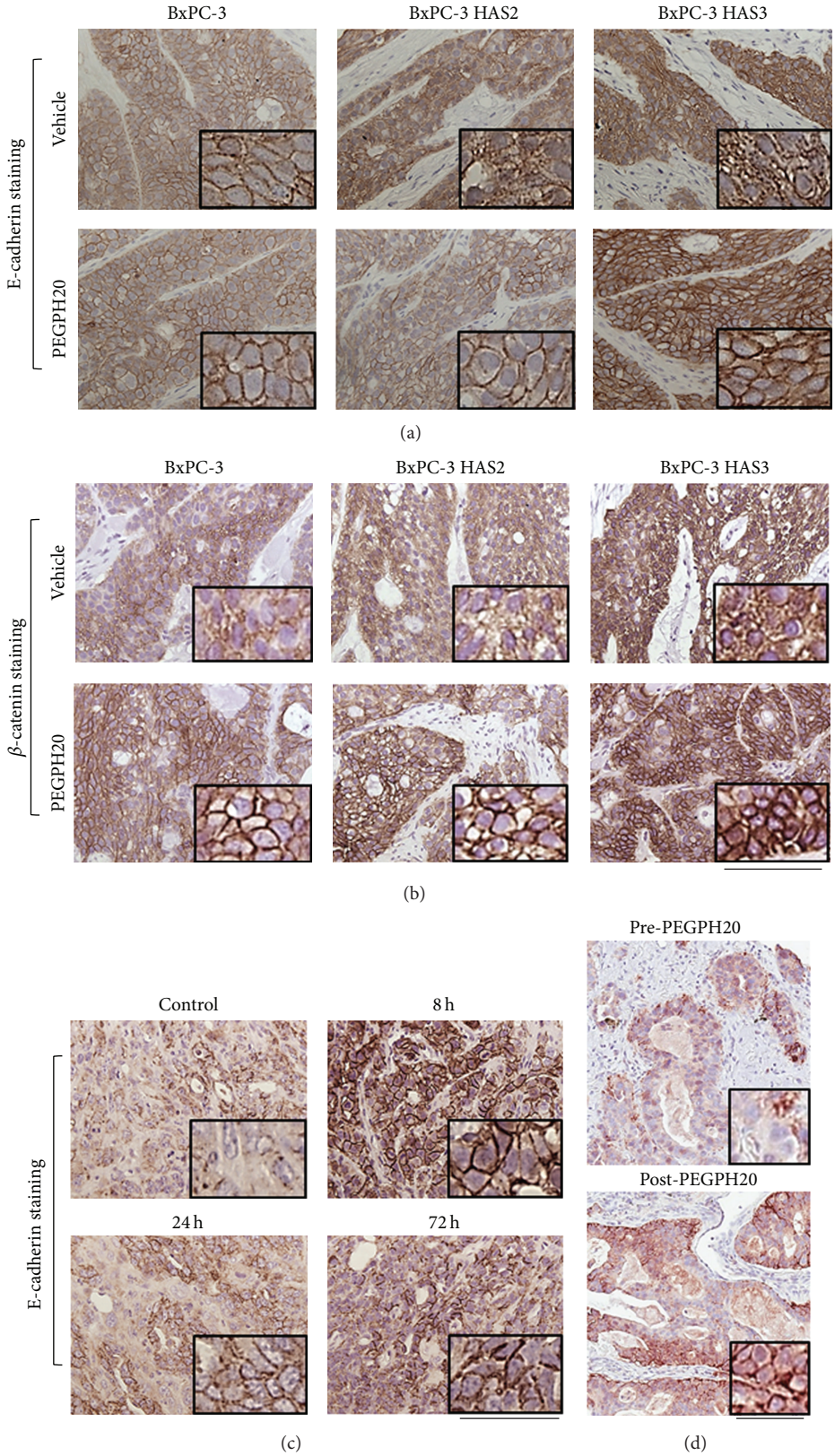

Figure 4: HAS overexpression induces loss of plasma membrane E-cadherin and accumulation of cytoplasmic β-catenin, and removal of hyaluronan translocates them to the plasma membrane. Vehicle- and PEGPH20- (2 doses; 4,500 μg/kg; n = 3/group) treated tumors were stained for E-cadherin (a) and β-catenin (b). E-cadherin was also localized in pancreatic tumors from a KPC mouse model after 0, 8, 24, and 72 h treatment with PEGPH20 (c; n = 3/group) and in human NSCLC biopsies before and after PEGPH20 therapy (d). Scale bar in (a–d) is 250 μm. Insets in (a–d) represent 3x magnification of the original micrograph.

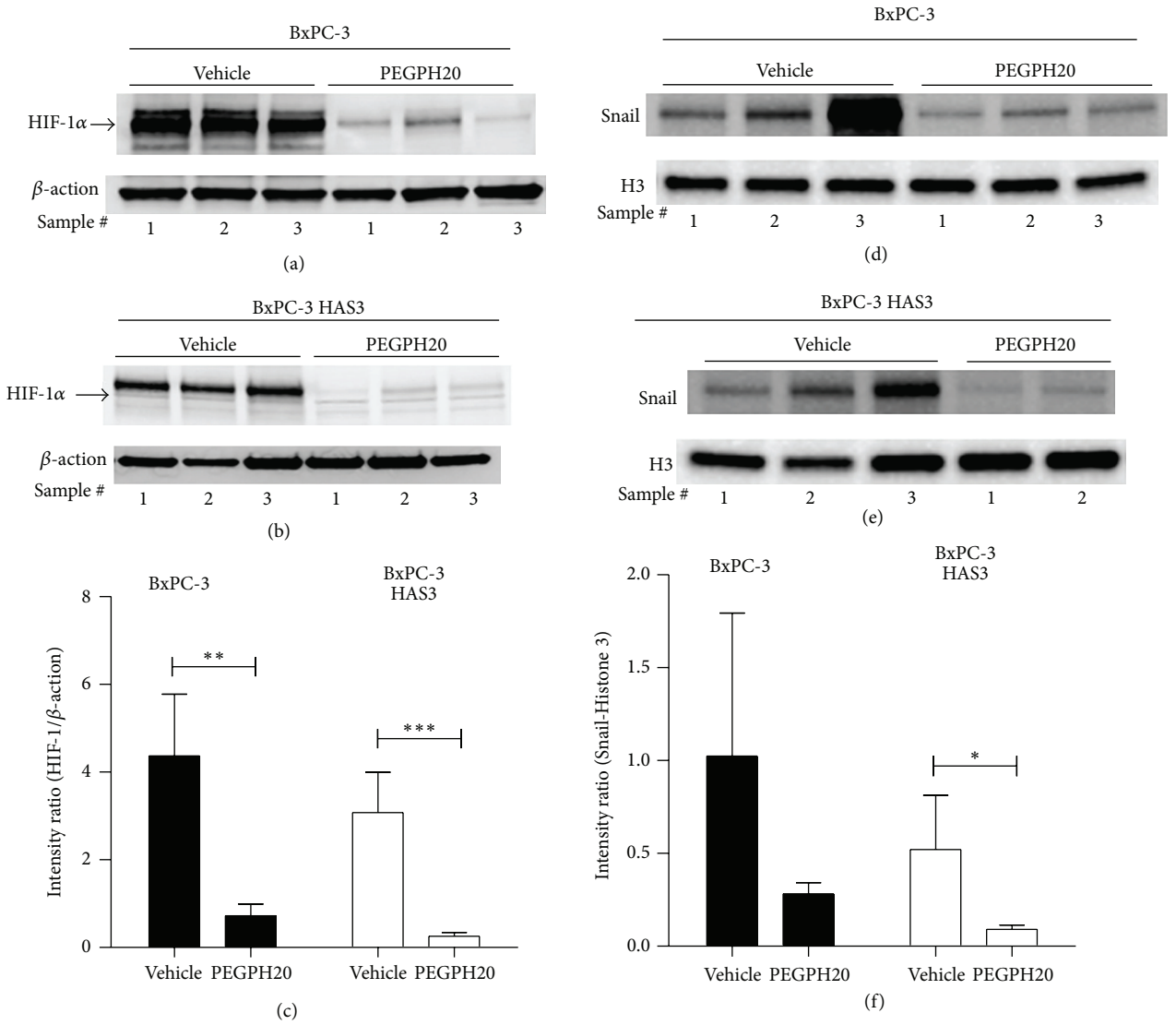

Figure 5: Removal of extracellular hyaluronan decreases HIF-1α and Snail signaling. Vehicle- and PEGPH20- (2 doses; 4,500 μg/kg) treated BxPC-3 and BxPC-3 HAS3 tumors were homogenized and nuclear extracts were analyzed with Western blot using HIF-1α (a) and (b), Snail (d) and (e), β-actin (a) and (b), and Histone 3 (c) and (d) antibodies. In (a) and (b), the blot is a representative of three experiments, and each lane represents a piece of the same vehicle-treated or PEGPH20-treated tumor. The HIF-1α band is indicated with an arrow. In (d) and (e), lysates from the same vehicle-treated or PEGPH20-treated tumor were combined and each lane represents Snail level of one tumor. Intensities of the bands in the cropped blots were quantified using Image-Pro Analyzer 7.0 software and normalized to the intensity of the housekeeping protein ((c) and (f)). Data were plotted as mean ± S.D., and statistical difference between the groups was tested with t test ($^{*}P < 0.05$; $^{**}P < 0.01$; and $^{***}P < 0.001$).

Cell proliferation, assessed by PH3, was not increased by HAS3 overexpression or with PEGPH20 treatment (Figure 6(b)). Overexpression of HAS2 and PEGPH20 treatment in HAS2-overexpressing tumors did not show a major effect on the number of CC3- and PH3-positive cells. These results suggest that, in addition to effects on stromal remodeling and early EMT-associated changes, accumulation of extracellular hyaluronan after HAS3 overexpression may protect pancreatic cancer cells from apoptosis. Hyaluronan removal alone may not be sufficient to induce substantial cell death in this model, but PEGPH20 may be effective in combination with chemotherapy as reported previously in the prostate cancer model [9] and in the transgenic mouse model of pancreatic cancer [6]. Consistent with our work, hyaluronan has also been previously associated with cell survival and protection of apoptosis in colon carcinoma cells [59]. In conditional Has2 transgenic mice that develop mammary tumors, Has2 overexpression decreases apoptosis but also increases the proliferation of neu-initiated tumors [43]. Differential results on proliferation may be explained by the fact that the net effect of HASs and hyaluronan on proliferation depends on several factors including cell type, cell density, and the final concentration of hyaluronan around the cells [15, 45, 60]. In KPC mice, PEGPH20 decreases

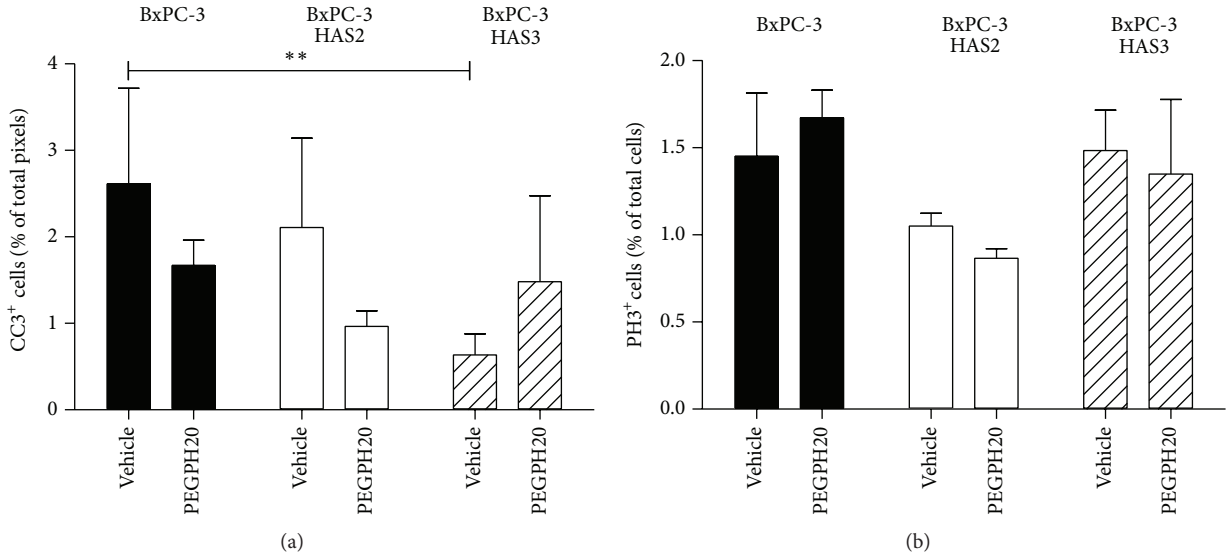

Figure 6: Effect of HAS2 and HAS3 overexpression and hyaluronan removal on apoptosis and proliferation in BxPC-3, BxPC-3 HAS2, and BxPC-3 HAS3 tumors. Vehicle-treated and PEGPH20-treated (2 doses; 4,500 μg/kg; $n = 3$/group) BxPC-3, BxPC-3 HAS2, and BxPC-3 HAS3 xenograft tumor sections were stained for CC3 and PH3 to visualize apoptotic and proliferative cells, respectively. Number of positive cells per tumor section was analyzed using Aperio Positive Pixel v9 and Nuclear staining algorithms, respectively, ((a) and (b)). Data were plotted as mean ± S.D., and statistical difference between the groups was tested with two-way ANOVA and Bonferroni's post hoc test ($^{**}P < 0.01$).

proliferation and increases apoptosis in combination with gemcitabine compared to gemcitabine alone [4, 6]. Although PEGPH20 alone might not have strong effects on proliferation and apoptosis, it causes remodeling of the tumor microenvironment and could sensitize tumors to chemotherapy. The results from this study shed a light on potential application of extracellular hyaluronan and HAS3 protein expression as an indication for stromal hyaluronan depletion by PEGPH20.

4. Conclusions

In recent years, the importance of the tumor microenvironment in cancer progression has been increasingly recognized, and stromal components including hyaluronan have become attractive targets for cancer therapy. Hyaluronan is a major component in the stroma of many solid tumors [5, 8], and its accumulation is known to promote malignant transformation and tumor growth [28, 43, 61]. The most extensive hyaluronan accumulation is found in pancreatic cancer [4, 6], and enzymatic depletion of hyaluronan in combination with chemotherapy is currently being investigated in patients with advanced pancreatic adenocarcinoma. However, there is very little information about mechanisms leading to hyaluronan accumulation in desmoplastic response of pancreatic cancer and the roles of different HASs in these dramatic changes of tumor microenvironment.

In this study, we demonstrate that overexpression of HAS3 in pancreatic cancer cells results in more aggressive tumors with extracellular hyaluronan accumulation, whereas overexpression of HAS2 is associated with moderate-growing tumors and both extracellular and intracellular accumulations of hyaluronan. An increase in extracellular hyaluronan induces loss of plasma membrane E-cadherin and accumulation of cytoplasmic β-catenin, indicating disruption of epithelial cell adhesion and an early stage of EMT. PEGPH20 depletes extracellular hyaluronan and leads to strong inhibition of tumor growth in HAS3-overexpressing tumors. Tumor growth inhibition is associated with decreased nuclear levels of hypoxia-related proteins and translocation of E-cadherin and β-catenin to the plasma membrane. The fact that removal of hyaluronan also causes E-cadherin translocation to the plasma membrane in the transgenic mouse model and in a hyaluronan-rich human NSCLC biopsy sample further justifies the relevance of the hyaluronan-rich tumor model and highlights the role of extracellular hyaluronan in the early EMT-associated events.

Further knowledge about the mechanisms and consequences of hyaluronan accumulation in pancreatic cancer and effects of hyaluronan removal by PEGPH20 will increase our understanding of the role of hyaluronan in the development of malignancies and will enable the development of new biomarkers and therapies.

Nonstandard Abbreviations Used

bHABP: Biotinylated hyaluronan binding protein
CC3: Cleaved caspase-3
DAB: 3,3′-Diaminobenzidine
DAPI: 4′,6-Diamidino-2-phenylindole
EMT: Epithelial-mesenchymal transition
H&E: Hematoxylin and Eosin
HAS: Hyaluronan synthase
HIF: Hypoxia-inducible factor
HYAL: Hyaluronidase
IgG: Immunoglobulin G

KPC: *LSL-Kras$^{G12D/+}$; LSLTrp53$^{R172H/+}$; Pdx-1-Cre*
M.O.M.: Mouse-on-mouse
NSCLC: Non-small cell lung cancer
PEGPH20: Pegylated recombinant human PH20 hyaluronidase
PH3: Phosphohistone 3.

Conflict of Interests

All authors except for Jeffrey R. Infante, Michael A. Jacobetz, and David A. Tuveson are employees, consultants, and/or shareholders of Halozyme Therapeutics. The authors declare no other conflicts of interests.

Authors' Contribution

Conception and design were performed by A. Kultti, C. Zhao, R. J. Osgood, P. Jiang, X. Li, C. B. Thompson, G. I. Frost, H. M. Shepard, and Z. Huang. Development of methodology was performed by A. Kultti, C. Zhao, N. C. Singha, R. Symons, and X. Li. Data were acquired by (and provided animal and human samples) A. Kultti, C. Zhao, N. Singha, S. Zimmerman, R. J. Osgood, R. Symons, P. Jiang, J. R. Infante, M. A. Jacobetz, and D. A. Tuveson. Analysis and interpretation of data were performed by (e.g., image analysis and statistical analysis): A. Kultti, C. Zhao, N. C. Singha, R. J. Osgood, and P. Jiang. Writing, reviewing, and/or revision of the paper were performed by A. Kultti, C. Zhao, N. Singha, R. J. Osgood, R. Symons, P. Jiang, X. Li, J. R. Infante, M. A. Jacobetz, D. A. Tuveson, H. M. Shepard, and Z. Huang. Administrative, technical, or material support was handled by A. Kultti, P. Jiang, X. Li, J. R. Infante, and D. A. Tuveson. Study was supervised by C. B. Thompson, G. I. Frost, H. M. Shepard, and Z. Huang.

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Research Article

Diagnostic and Prognostic Value of Soluble Syndecan-1 in Pleural Malignancies

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Background. The distinction between malignant and benign pleural effusions is a diagnostic challenge today and measuring soluble biomarkers could add to the diagnostic accuracy. Syndecan-1 is a proteoglycan involved in various cellular functions and is cleaved from the cell surface in a regulated manner. The shed fragment, which can be recovered in effusion supernatant and in serum, retains its binding capacities, but often with different functions and signalling properties than the cell-bound form. *Aim*. This study aimed to investigate the diagnostic and prognostic value of soluble syndecan-1 in pleural effusions and sera from patients with pleural malignancies. *Study Design*. Using two cohorts of patients, we assessed the diagnostic and prognostic value of soluble syndecan-1 in pleural effusions and sera, using enzyme-linked immunosorbent assays. *Results*. In pleural effusions, syndecan-1 distinguished malignant and benign diseases, with an odds ratio of 8.59 (95% CI 3.67 to 20.09). Furthermore, syndecan-1 in pleural effusions predicted a survival difference for patients with pleural metastatic disease and malignant mesothelioma of 11.2 and 9.2 months, respectively. However, no such effects were seen when syndecan-1 was measured in serum. *Conclusion*. Soluble syndecan-1 is a promising candidate biomarker for the cytopathological diagnosis and prognostication of malignant pleural effusions.

1. Introduction

Pleural effusion is a common symptom of cancers in the pleural cavities. Accumulation of pleural fluid is caused partly by an increase in vascular permeability and tissue leakage and can limit lung expansion and impede breathing. The effusion is withdrawn, and malignant cells from pleural effusions are often available for cytopathological diagnosis long before biopsies are obtained [1]. Pleural effusions are in these cases often the first material available for the diagnosis [2, 3]. Tumours affecting the pleural space include metastatic adenocarcinomas from the lung (36%), breast (25%), ovary and gastric cancer (5%), and malignant lymphomas (10%) [4–6]. The primary tumour of the pleura is malignant mesothelioma; however, it is overall less frequent than metastatic cancers [6, 7].

The cytological diagnosis of malignant disease in the pleura is supported by ancillary methods such as immunocytochemistry, fluorescent in situ hybridization (FISH), electron microscopy, and biomarker analyses [8, 9]. Among proposed biomarkers, the secreted phosphoprotein osteopontin is secreted by several different tumour types [10–18]. When a definite diagnosis is not reached by these methods, clinical and radiological findings may motivate a more invasive sampling of tissue [19]. However, these invasive procedures would preferably be avoided when the diagnosis can be reached by less invasive methods [6].

Syndecan-1 (CD138), a cell surface proteoglycan, has been proposed as a cellular marker for distinguishing adenocarcinoma from mesothelioma [20–22]. This proteoglycan regulates various biological processes including cell proliferation,

Table 1: Demographic data.

Pleural effusions	Number of patients	Male (%)	Female (%)	Age, median (IQR)
Benign	93	63 (68)	30 (32)	68 (54–80)
Malignant	74	23 (31)	51 (69)	68 (62–78)
Lung cancer	*43*	*14 (33)*	*29 (67)*	*70 (62–79)*
Breast cancer	*8*	*1 (12)*	*7 (88)*	*64 (47–80)*
Ovarian and fallopian cancers	*5*	*0 (0)*	*5 (100)*	*70 (66–78)*
Other malignancies	*8*	*3 (37)*	*5 (63)*	*65 (63–77)*
Cancer of unknown primary	*10*	*5 (50)*	*5 (50)*	*68 (52–80)*
Malignant mesothelioma	89	79 (89)	10 (11)	70 (63–78)
Sera	Number of patients	Male (%)	Female (%)	Age, median (IQR)
Benign	66	52 (79)	14 (21)	59 (48–71)
Benign asbestos pleuritis	*24*	*23 (96)*	*1 (4)*	*62 (54–73)*
Malignant	74	44 (59)	30 (41)	61 (54–69)
Lung cancer	*44*	*34 (77)*	*10 (23)*	*63 (57–70)*
Breast cancer	*9*	*1 (11)*	*8 (89)*	*56 (48–71)*
Ovarian and fallopian cancers	*3*	*0 (0)*	*3 (100)*	*57 (45–64)*
Other malignancies	*16*	*9 (56)*	*7 (44)*	*62 (52–72)*
Cancer of unknown primary	*2*	*1 (50)*	*1 (50)*	*56 (50–61)*
Malignant mesothelioma	91	34 (37)	57 (63)	65 (56–69)
Epithelioid	*62*	*23 (37)*	*39 (63)*	*65 (54–71)*
Biphasic	*13*	*4 (31)*	*9 (69)*	*57 (55–69)*
Sarcomatoid	*10*	*8 (80)*	*2 (20)*	*63 (61–65)*
Undetermined	*6*	*2 (33)*	*4 (66)*	*64 (55–70)*

Age (IQR: interquartile range) and patient subgrouping in the two analysed materials. The high proportion of female mesothelioma patients in the serum material is most likely due to environmental asbestos and erionite exposure, which relates to geographical distribution and has also been reported by others [36].

differentiation, invasion, migration, and angiogenesis. These processes are largely regulated by syndecan-1 through the interactions with growth factors, growth factor receptors, and several integrins [23–26]. The extracellular domain of syndecan-1 is proteolytically cleaved by metalloproteinases [27, 28] and thereby is shed to body fluids. The ratio of membrane-bound and shed syndecan-1 is altered in certain pathological conditions, including cancer and metastasis [29, 30]. However, it is noteworthy that, depending on tumour type, syndecan-1 has shown to be either a tumour suppressor or a tumour promoter [31]. The presence of syndecan-1 on mesothelioma cells is associated with favourable prognosis [32], while high levels of syndecan-1 in breast cancer indicate poor prognosis [33]. Similarly, high level of soluble syndecan-1 in serum indicates poor prognosis for patients with multiple myeloma [34] and lung cancer [35]. This dual effect of syndecan-1 seems to depend on the source and forms of syndecan-1: whether it is tumour cell-derived or synthesized by the stroma and whether it is cell surface-bound or shed.

This study aimed to evaluate the diagnostic and prognostic performance of soluble syndecan-1 in patients' serum and pleural effusions.

2. Materials and Methods

2.1. Study Participants. In this study, 256 pleural effusions (74 carcinoma, 89 malignant mesothelioma, and 93 benign effusions) and 231 serum samples (74 carcinoma, 91 malignant mesothelioma, and 66 benign conditions) were analysed for syndecan-1 and compared to their osteopontin levels. Pleural effusions were prospectively and consecutively collected in different time periods at the Department of Pathology and Cytology, Karolinska University Hospital in Huddinge, Sweden, between the years 2005 and 2011. Late time periods only included patients with lung cancer or malignant mesothelioma. Serum samples were prospectively and consecutively collected at the Chest Diseases Department of Eskisehir Osmangazi University in Eskisehir, Turkey, between the years 2002 and 2004. Serum samples were collected as part of a parallel study that evaluates the diagnostic effect of seven different biomarkers (including syndecan-1) and their combined diagnostic value for a malignant pleural mesothelioma. All samples were collected before any treatment was given. Samples were centrifuged for 1,700 g for 10 minutes without delay. Acellular supernatants were stored without additives at −20°C (effusions) or −80°C (sera). For patient demographics, see Table 1.

Inclusion criteria have been previously described [37]. In brief, all included effusions from malignant involvement of the pleura contained malignant cells. Diagnoses of malignant mesothelioma were further verified on histopathology supported by immunohistochemistry or on electron microscopy of effusion cell pellets. Metastatic pleural malignancies were diagnosed by histopathology and/or cytolopathology supported by immunocytochemistry with at least 4 antibodies. Patients diagnosed with nonmalignant disease were followed up for at least one year and excluded if they were diagnosed with any type of malignancy or were deceased, within a year from sampling. No attempt was done to distinguish exudates from transudates. Patients thought to have benign

asbestos pleurisy were monitored by thoracoscopy with biopsies showing fibrinous pleuritis.

Metastatic pleural disease correlates with an advanced stage, while information of stage for malignant mesothelioma patients was not available for this study. For this reason we measured vascular endothelial growth factor (VEGF) in a subset of pleural effusions from patients with malignant mesothelioma ($n = 16$), since VEGF has been described as a surrogate marker for stage in this disease [38].

In order to evaluate the relation between cell-bound and shed syndecan-1, a separate cohort of additional 18 pleural effusions, containing well-preserved malignant cells, was collected from 2012 to 2013 at Karolinska University Hospital in Huddinge. These samples were compared simultaneously for their pleural effusion concentrations of soluble syndecan-1 and their expression of membrane-bound syndecan-1 on tumour cells.

2.2. Enzyme-Linked Immunosorbent Assay (ELISA). Syndecan-1 and VEGF were measured using ELISA: Human CD138 (syndecan-1) from Gen-Probe Diaclone, France (cat. number 950.640.192) and Human VEGF Quantikine ELISA from R&D Systems, UK (cat. number DVE00), respectively. ELISAs were performed according to the manufacturer's instructions. Effusions were diluted 1 : 3 for syndecan-1 analysis and 1 : 5, 1 : 10, or 1 : 25 for VEGF analysis using kit-dilution buffers as blanks. Optical densities were determined using a spectrophotometer (BioTek's PowerWave HT, Winooski, VT, USA) at 450 nm. Patient samples were analysed in duplicates by investigators blind to patients' diagnoses and survival times.

2.3. Immunocytochemistry. To investigate the relationship between soluble and cell-bound syndecan-1, the latter was assessed by immunocytochemistry on tumour cells from the pleural effusion paired with their ELISA readout of shed syndecan-1 levels in the corresponding effusion supernatant. For immunocytochemistry, the pleural effusions were centrifuged for 10 min at 8,000 g and if necessary, erythrocytes were lysed using ammonium chloride (BD Pharm Lyse, BD Biosciences, CA, USA). Cells were immobilized on Super-Frost Plus Slides (Thermo Fisher Scientific Inc., Waltham, MA, USA), using cytospin preparations. Immunostaining was performed using a Lecia BOND-III automated IHC. Epitope retrieval was done by pretreating the slides in a citrate buffer, pH 6.0 (Bond Epitope Retrieval Solution 1, Leica Microsystems GmbH). Endogenous peroxidase activity was blocked with 3% H_2O_2 followed by incubation with syndecan-1 primary antibody (CD138, clone MI15, diluted 1 : 100, IgG1, DakoCytomation, CA, USA). Bound antibodies were demonstrated with the Bond Polymer Refined Detection kit (Leica DS 9800), and the cells were counterstained with haematoxylin.

Two experienced cytopathologists (KD and AH) evaluated all slides independently and were blinded to clinical diagnosis and levels of soluble syndecan-1. Cell-bound syndecan-1 expression was assessed by semiquantitative scoring which includes (i) the percentage of syndecan-1 positive tumour cells (0–100%) and (ii) the signal intensity (4-point scale). The scoring for signal intensity corresponded to 0, negative; 1, weak staining; 2, moderate positive; and 3, strong positive staining. The semiquantitative immunocytochemical (ICC) score for the cell-bound syndecan-1 expression level was provided by the multiplication of the percentage (0–100%) of syndecan-1 positive staining by the factor (1–4) corresponding to the staining intensity of the tumour cells.

2.4. Statistical Analyses

2.4.1. Analyses of Biomarker Expression in Pleural Effusions and Sera. Levels of soluble syndecan-1 and osteopontin were compared between patients with cancer and those without, using the Mann-Whitney test calculating two-tailed exact P values. Nonparametric tests were used since biomarkers were not normally distributed (D'Agostino and Pearson omnibus normality test; data not shown). Analyses of syndecan-1 levels between multiple patient subgroups were performed using the nonparametric Kruskal-Wallis one-way analysis, with Dunn's post hoc test comparing mean rank of each patient's group to the mean rank of the benign patients' group. Correlation between soluble syndecan-1 in paired effusion and serum samples was analysed using Spearman correlation. Osteopontin levels were extracted from an earlier study [37] and used as a reference biomarker for malignancy [10–18]. Correlation between soluble syndecan-1 and osteopontin levels, in either pleural effusions or sera, was analysed using Spearman correlation. Statistical analyses were performed using GraphPad Prism software (v. 6.01, GraphPad Software Inc.).

2.4.2. Logistic Regression. Logistic regression was used to create a predictive model for each biomarker, with cancer or without cancer as outcome, coded as 1 and 0, respectively. Univariate odds ratios were calculated. Model calibration is recommended by several studies [39, 40]. Model calibration was assessed by graphical inspection as well as calculation of Nagelkerke's r^2. Goodness of fit was quantified using the le Cessie-van Houwelingen-Copas-Hosmer unweighted sum of squares test [41] using the R software v. 3.0.1.

2.4.3. Receiver Operator Characteristic (ROC) Analyses. ROC plots with areas under the curves (AUC) and their 95% confidence intervals were made with the GraphPad Prism software. The calculations of sensitivity, specificity, positive predictive value, and negative predictive value were calculated with cutoffs from maximum sensitivity × specificity from ROC curves.

2.4.4. Discrimination Slopes. Discrimination slopes are recommended by several studies [39, 40]. Discrimination plots were constructed to assess the predicted probability for each biomarker of a malignant pleural effusion. Discrimination slope (DS) is the difference between predicted probabilities; in this study it is the probability that syndecan-1 or osteopontin predicts malignant effusions over a benign. Analyses were performed using the R software v. 3.0.1 and visualized using GraphPad Prism software.

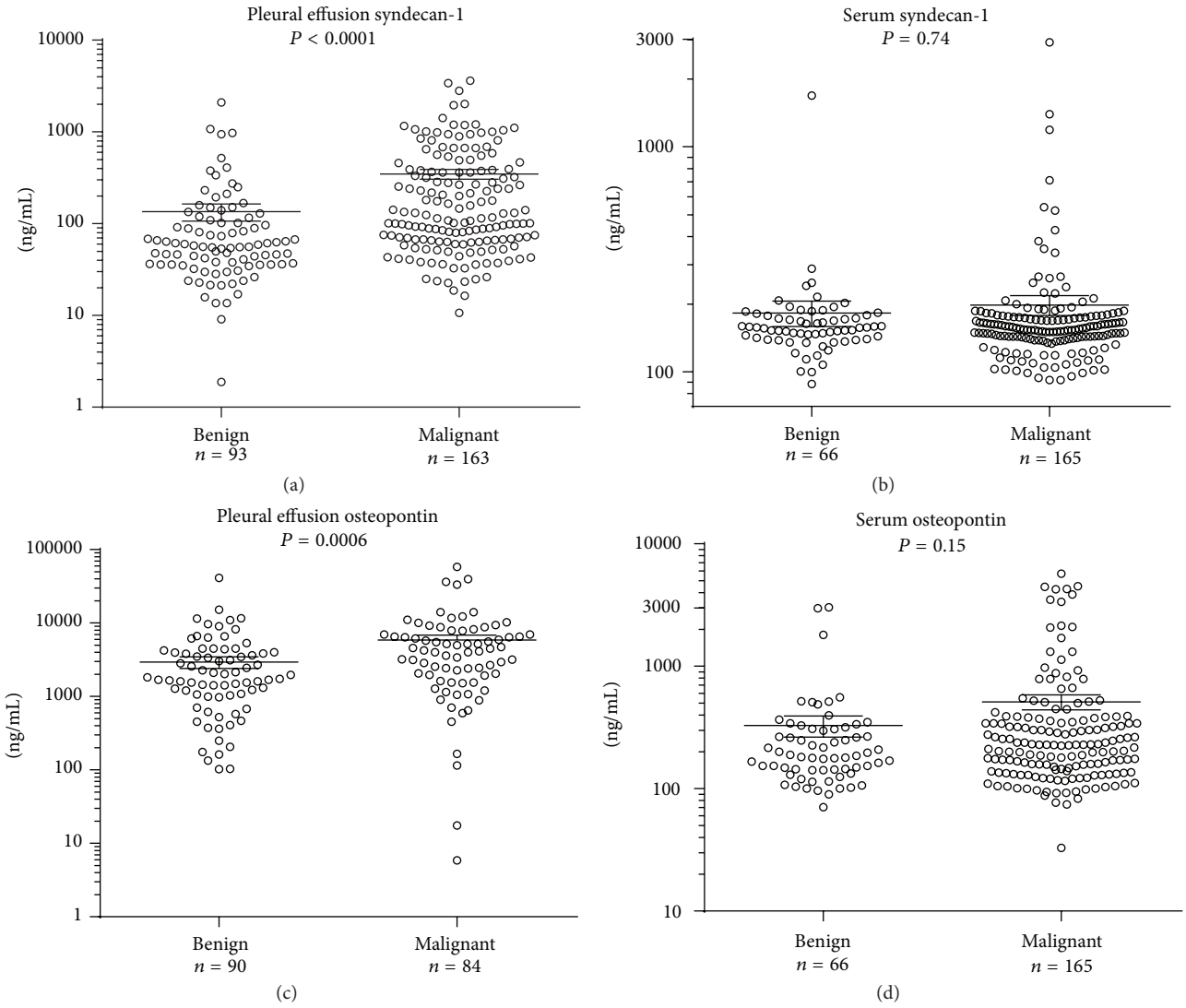

Figure 1: Levels of syndecan-1 and osteopontin are higher in pleural effusions from malignant tumours than from benign conditions. Syndecan-1 and osteopontin expression levels in benign and malignant pleural effusions, as measured by ELISA. *P* values are from Mann-Whitney tests, with Dunn's *post hoc* tests. Lines represent mean and error bars represent standard error of the mean (SEM).

2.4.5. Survival Analysis. Survival analysis was performed with cutoff values based on the highest and most significant hazard ratio [42]. Survival times were available for a subset of patients; thus, survival data for patients providing serum samples was available for 19 patients, which is too few to extract a hazard ratio based cutoffs. So, for comparisons in sera the median was instead used as a dichotomizing value. With determined cutoffs, the prognostic information was estimated using the Kaplan-Meier survival analysis. The log-rank (Mantel-Cox) test compared survival curves and estimated hazard ratios and *P* values. Survival analyses were performed and graphs were created using the GraphPad Prism software.

2.4.6. Correlation between Soluble and Cell-Bound Syndecan-1 Levels in Paired Pleural Effusion and Cytospin Samples. Spearman correlation analysis was used to assess the relationship between soluble syndecan-1 in effusions and corresponding cell-bound syndecan-1 in patients suffering from various pleura malignancies. Analyses were performed and graphs were created using the GraphPad Prism software.

2.5. Ethical Permits. This study was approved by the ethical review board of Stockholm, Sweden (2009/1138-31/3), and the ethical review board of Eskisehir University, Turkey. All patients had given informed consent.

3. Results

3.1. Expression Levels of Syndecan-1 and Osteopontin. Syndecan-1 and osteopontin levels were both significantly higher in malignant pleural effusions than in those with benign conditions, the difference being more pronounced with syndecan-1. However, neither soluble syndecan-1 nor osteopontin differentiated patient groups in sera (Figure 1). The pleural effusion levels of syndecan-1 were highest in carcinomas,

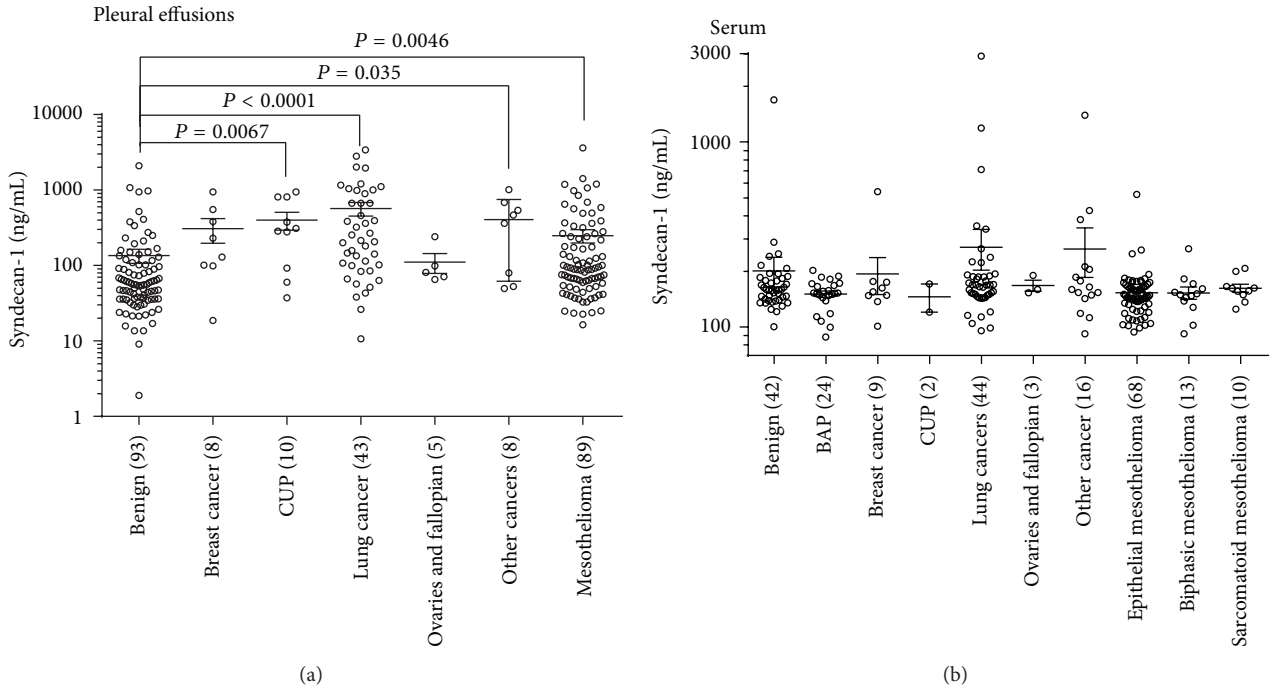

Figure 2: Soluble syndecan-1 levels in various malignant tumours. Significantly elevated soluble syndecan-1 levels were measured in several malignant tumours compared to benign disease. *P* values are derived from Kruskal-Wallis tests, with Dunn's *post hoc* tests. Benign asbestos pleuritis (BAP) and cancer of unknown primary (CUP). Where no *P* values are stated, it indicates a nonsignificant association. Lines represent mean and error bars represent standard error of the mean (SEM).

Table 2: Cut-offs were derived from the maximum sensitivity × specificity in respective receiver operator characteristic curve.

Biomarker	Fluid	Cut-off (ng/mL)	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
Syndecan-1	Pl. effs	65.7	74.9	61.3	0.77	0.57
Syndecan-1	Sera	151.6	46.1	59.1	0.70	0.27
Osteopontin	Pl. effs	2034.0	65.5	61.1	0.60	0.65
Osteopontin	Sera	217.3	52.7	60.6	0.77	0.34

Pl. effs: pleural effusions; PPV: positive predictive value; NPV: negative predictive value.

although patients diagnosed with malignant mesothelioma also had significantly elevated levels compared to benign disease (Figure 2). Paired pleural effusion and serum samples showed moderate correlation of soluble syndecan-1 ($r = 0.45$, $P = 0.05$). Furthermore, syndecan-1 and osteopontin levels correlated in both pleural effusions ($r = 0.18$, $P = 0.019$) and sera ($r = 0.25$, $P < 0.0001$; see Supplementary Figure S1 in Supplementary Material available online at http://dx.doi.org/10.1155/2014/419853). ELISA results for syndecan-1 and osteopontin of all patients can be downloaded from the Dryad online repository (doi: 10.5061/dryad.c42t7).

3.2. Diagnostic Performance of Syndecan-1 and Osteopontin. Syndecan-1 levels in pleural effusions significantly predicted malignant disease (odds ratio 8.59, 95% CI 3.67 to 20.09). Area under the ROC curve was 0.71 (95% CI 0.65, 0.78), model calibration was acceptable (Nagelkerke's $r^2 = 0.29$), and goodness of fit was good ($P = 0.99$). Discrimination slope was 0.24 (Figures 3(a), 3(c), and 3(e)). Syndecan-1 had a sensitivity of 74.9% and specificity of 61.3% at the cutoff of 65.7 ng/mL (Table 2). However, in sera, syndecan-1 showed poor prediction of a malignant disease (odds ratio 1.31, 95% CI 0.26 to 6.65; AUC 0.51, 95% CI 0.43 to 0.59 (Figure 3(a)); Nagelkerke's $r^2 < 0.01$; goodness of fit = 0.54; discrimination slope < 0.01).

Osteopontin in effusions also significantly predicted a malignant disease (odds ratio 1.39, 95% CI 1.03, 1.88). Area under the ROC curve was 0.65 (95% CI 0.57 to 0.73), the model calibration was poor (Nagelkerke's $r^2 = 0.06$), and goodness of fit was poor although not below the usual 0.05 threshold for rejection ($P = 0.09$). Discrimination slope was 0.04 (Figures 3(b), 3(d), and 3(f)). Osteopontin had a sensitivity of 65.5% and specificity of 61.1% at the cutoff of 2034.0 ng/mL. Sensitivities, specificities, positive predictive

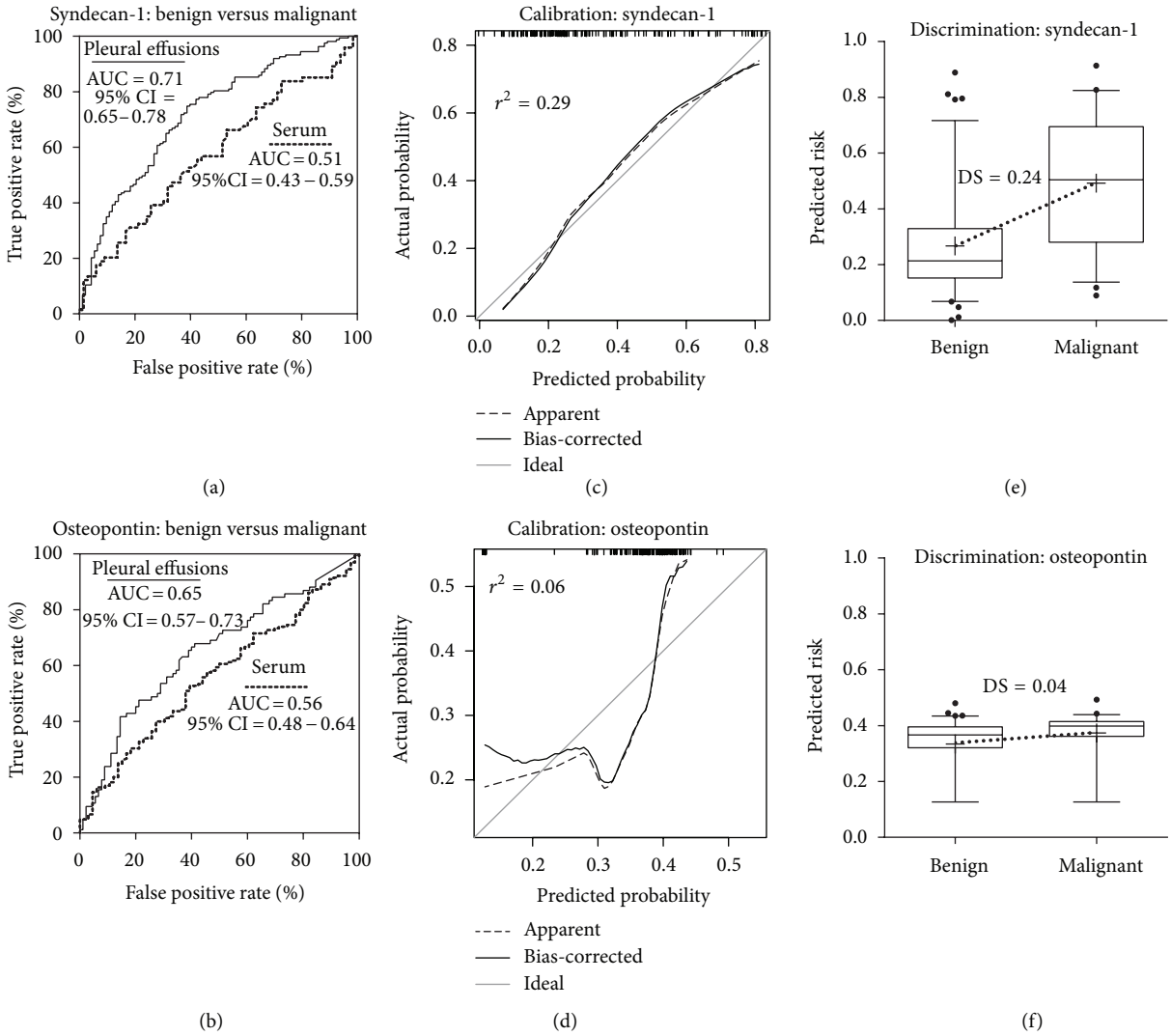

Figure 3: Soluble syndecan-1 has a diagnostic value for malignancy in pleural effusions. ROC plots for syndecan-1 in pleural effusions and serum (a) and osteopontin in pleural effusions and serum (b). Figures (c) to (f) only pertain to pleural effusions. Calibration curves evaluate the relation between the predicted and actual probabilities for soluble syndecan-1 and osteopontin, panels (c) and (d), respectively. Nagelkerke's r^2 is displayed in Figures (c) and (d). Box plots of predicted risks show a greater discrimination between malignant and benign effusions when using syndecan-1 as opposed to osteopontin (e, f). Discrimination slopes (DS; dotted lines) are calculated as the difference between mean predicted risks (+). Boxes show median and interquartile ranges, while whiskers represent the 5th to 95th percentile. Outliers are presented as black dots.

values, and negative predictive values are represented in Table 2. Serum osteopontin also showed poor prediction for a malignant disease (odds ratio 1.99, 95% CI 0.87 to 4.58; AUC 0.56, 95% CI 0.48 to 0.64 (Figure 3(b)); Nagelkerke's r^2 = 0.02; goodness of fit = 0.58; discrimination slope = 0.01).

3.3. Prognostic Value of Soluble Syndecan-1 and Osteopontin. Cutoff values derived from highest and most significant hazard ratios for pleural effusions are reported in Supplementary Figure S2. Median survival time of patients with pleural metastases and an effusion syndecan-1 level <235.1 ng/mL was 12.9 months, compared to only 1.7 months in patients with higher syndecan-1 levels (hazard ratio 2.38, 95% CI 1.56 to 5.44). Stratifying malignant mesothelioma patients in "low" and "high" syndecan-1 level using a cutoff of 100.2 ng/mL resulted in median survival times of 17.0 and 7.8 months, respectively (hazard ratio 2.77, 95% CI 1.35 to 5.68; Figure 4). Pleural effusion osteopontin levels also predicted survival time; at the cutoff level of 6.14 μg/mL, the median survival differed from 5.1 to 2.2 months for patients with metastatic pleural disease between "low" and "high" expression, respectively (hazard ratio 2.05, 95% CI 1.19 to 6.12). For patients with malignant mesothelioma, the effusion osteopontin cutoff of 1.6 μg/mL resulted in median survival times of 29.0 and 13.0 months, for "low" and "high" expression, respectively (hazard ratio 2.16, 95% CI 1.16 to 4.15; Figure 4).

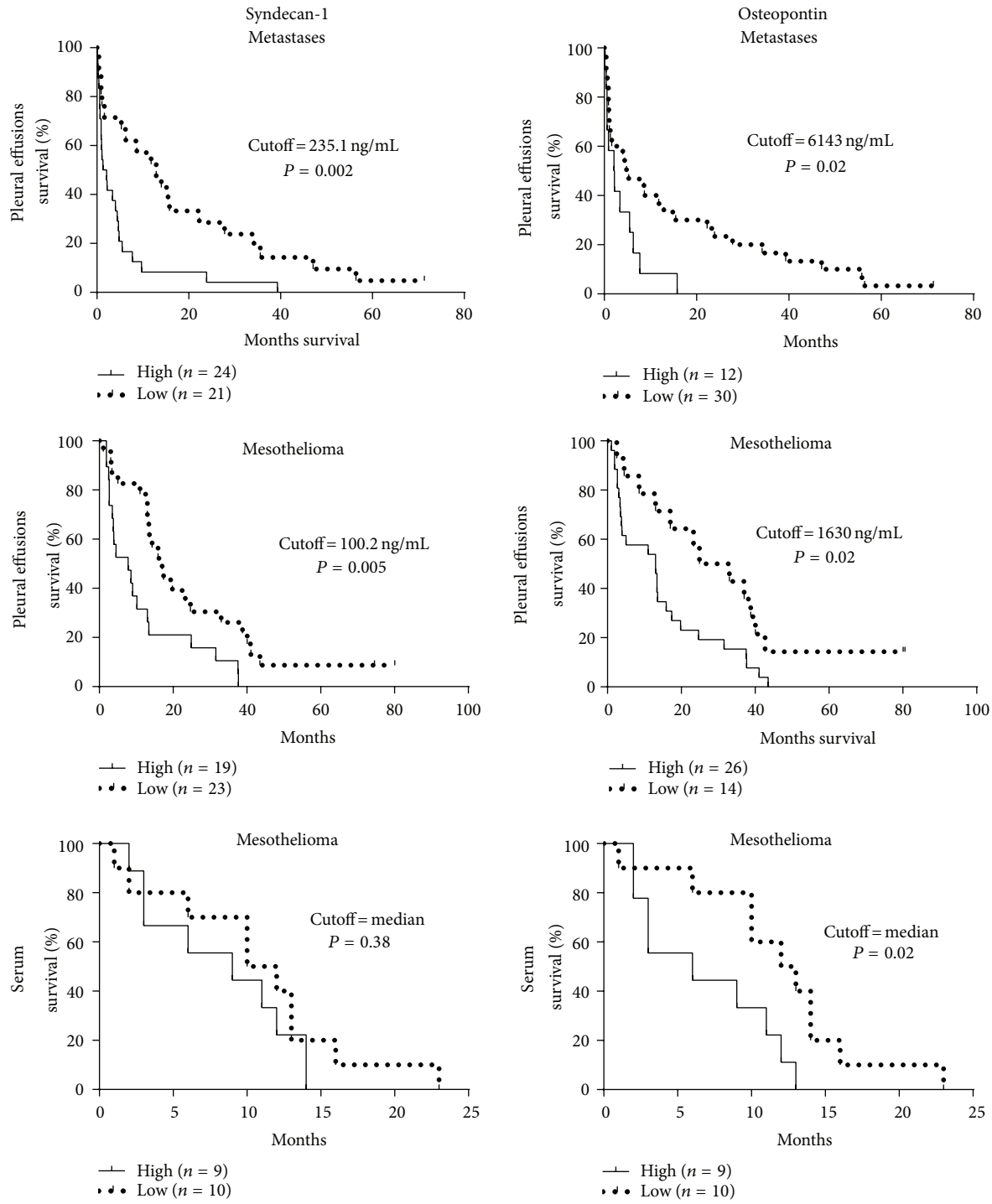

Figure 4: Both soluble syndecan-1 and osteopontin have prognostic roles for patients with malignant mesothelioma or metastatic pleural disease. Cutoffs for "high" and "low" syndecan-1 expression were identified by the online web application Cutoff Finder [42]. The effect of each marker on a time-to-event outcome is presented in corresponding Kaplan-Meier plots. *P* values are based on log-rank (Mantel-Cox) tests.

Among mesothelioma patients with serum syndecan-1 levels higher than the median (144 ng/mL), the median survival time was 9.0 months, while in those with lower serum levels, it was 11.0 months; however, this difference was not statistically significant (hazard ratio 1.43, 95% CI 0.63 to 3.98; Figure 4). The median osteopontin level in serum (185 ng/mL) separated mesothelioma patients in those with "low" and "high" expression, showing median survival times of 12.5 and 6.0 months, respectively (hazard ratio 2.45, 95% CI 1.36 to 10.32; Figure 4). There was no significant difference in

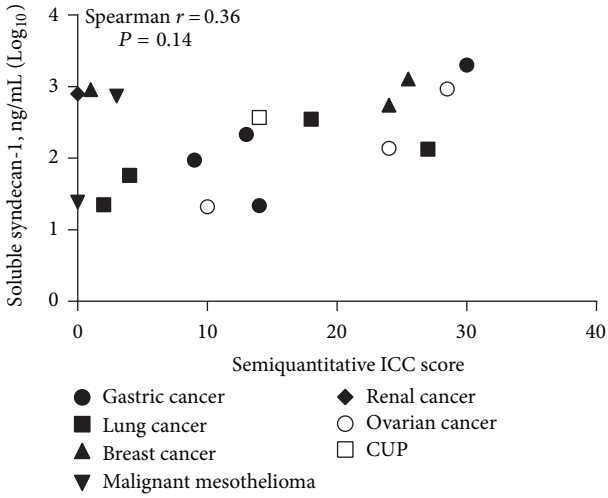

Figure 5: Strong cellular immune-reactivity for syndecan-1 on malignant cells correlates with higher levels of soluble syndecan-1 in pleural effusions. Spearman correlation was used to test the goodness of fit between levels of soluble syndecan-1 and cell bound syndecan-1 intensity times the percentage of positive tumour cells (semiquantitative ICC score; $r = 0.36$; $P = 0.14$). ICC = immunocytochemistry.

VEGF, age, or gender between any of the analysed groups of "low" and "high" expression levels (Mann-Whitney test; data not shown).

3.4. Relation between Cell-Bound and Soluble Syndecan-1. To assess the relationship between cell-bound and soluble syndecan-1, we performed immunocytochemistry on cells in pleural effusion samples paired with their ELISA readout of soluble syndecan-1 levels ($n = 18$). Linear regression of staining intensity times percentage syndecan-1 reactive tumour cells (semiquantitative ICC score) and pleural effusion levels indicate a positive correlation ($r = 0.36$, $P = 0.14$; Figure 5).

4. Discussion

The importance of the syndecan proteoglycan family has been implicated in several aspects of cancer [20–22, 25, 43–45]. Particularly in epithelial cancers, syndecan-1 has been associated with angiogenesis, invasion, proliferation, differentiation as well as diagnosis, and patient survival [23–25, 30, 46–48]. In malignant mesothelioma, Kumar-Singh et al. have shown that the membrane associated form of syndecan-1 is a good prognostic factor, where high tissue levels of syndecan-1 correlate with longer survival [32]. Furthermore, syndecan-1 has higher expression in epithelioid cell lines as compared to sarcomatoid cell lines [32], a phenotypic correlation that can explain part of the prognostic findings. Overexpression of syndecan-1 induces epithelial morphology and inhibits proliferation in mesothelioma cell lines [49], indicating a syndecan-1 dependent differentiation along the epithelial mesenchymal transition axis in this cancer. In this study we analyse the role of soluble syndecan-1 for its diagnostic and prognostic importance in pleural effusions and serum from patients with malignant diseases. The levels of shed syndecan-1 were elevated in pleural effusions from both metastatic carcinomas and malignant mesothelioma compared to patients with nonmalignant disease. As demonstrated by higher odds ratios, increased AUCs, better calibration, and better discrimination, soluble syndecan-1 had better diagnostic accuracy for a pleural malignancy compared to the reference biomarker osteopontin. The obtained AUCs indicate that effusion contents of these two compounds could be of diagnostic use if combined with other biomarkers in a battery, while their importance in serum seems to be more limited.

Stratifying the patients into disease subgroups, the highest levels were seen in effusions due to metastases from lung cancer, breast cancer, and gastric cancer, possibly reflecting their epithelial origin. Syndecan-1 levels were also higher in effusions from patients with mesothelioma. This suggests a more general pathophysiological role of syndecan-1 in cancers.

Effusion and serum levels of syndecan-1 correlated moderately, but the diagnostic value of syndecan-1 could not be demonstrated in sera. Here the differences between analysed patient groups are smaller, as is the dynamic range *within* groups. Pleural effusions have been shown to carry higher diagnostic accuracy than serum for several malignant mesothelioma biomarkers, a phenomenon that has been attributed to less interference by liver metabolism or elimination by the kidneys [50, 51]. The lack of diagnostic value in serum could also be explained, in part, by the contribution from other body fluids into this medium.

We further show that soluble syndecan-1 in pleural effusions carry strong prognostic value for patients with pleural malignancies. Low levels of shed syndecan-1 predict a more favourable prognosis, showing more than 11 months increased median survival for patients with pleural metastases and 9.2 months for malignant mesothelioma; both are diseases with average median survival times of around, or less than, one year. This trend is also seen in the two main subgroups of metastatic tumours—that is, lung and breast adenocarcinoma—although both these subgroups are too small to show statistical significance. These findings are in concordance with earlier studies on multiple myeloma [34] and lung cancer [35], in both elevated levels of syndecan-1 in sera predicted poor prognosis. There are several possible explanations for the prognostic properties of syndecan-1. In the malignant cell, syndecan-1 participates in the regulation of basic functions such as proliferation and cell migration, thereby possibly influencing the cancer progression and ultimately patient survival. One caveat to keep in mind is that although all pleural metastases represent advanced stages, it could still be that the increase in soluble syndecan-1 merely reflects tumour burden and thereby can be linked to shorter survival.

Structurally, the extracellular domain of syndecan-1 carries attachment sites for heparan sulfate and chondroitin sulfate chains. Since heparan sulfate chains act as binding sites for several ligands such as growth factors and chemokines, proteolytic shedding of the extracellular domain is crucial in regulating various signaling pathways [52–54].

In our cohorts, higher levels of soluble syndecan-1 corresponded to stronger immunocytochemical staining of syndecan-1. It seems that the soluble fraction increased proportionally to the expression of syndecan-1 on these cells. We cannot, however, exclude a concomitant increase in protease activity and syndecan-1 ectodomain shedding in the same cells. Such shedding has been associated with cancer progression, risk for recurrence, and prognosis [27, 28, 55–57]. It can, however, not be excluded that some of the released syndecan-1 is the result of cellular lysis or tumour derived exosomes containing this proteoglycan [58].

Several studies suggest elevated pretreatment soluble serum syndecan-1 level as a predictor of poor prognosis and impaired effect of chemotherapy [59, 60]. Furthermore, low serum syndecan-1 level can predict sensitivity to anticancer therapy in larynx and hypopharynx cancer, whereas high posttreatment level is an indicator of relapse [61].

In this study we show a possible diagnostic and prognostic role for soluble syndecan-1 in effusion cytology. The use of this proteoglycan as an effusion biomarker could be helpful in the evaluation of effusions. Similar to most established biomarkers—immunological or immunocytochemical—the discriminatory power of this analysis is insufficient to use as a sole diagnostic marker in the individual case. The combination of this parameter with other biomarkers in logistic models remains to be studied. This, however, should then warrant further studies on the mechanisms behind the release of syndecan-1 as well as possible predictive effects and effects on epithelial mesenchymal transition in tumours.

5. Conclusion

In summary, this study describes the clinical value of analysing soluble syndecan-1 in serum and pleural effusions. Syndecan-1 separates malignant and benign conditions when measured in pleural effusion supernatants. Furthermore, we show a striking correlation between syndecan-1 levels and patients' survival, which is of interest for future predictive translational research. Hence, the inclusion of measuring syndecan-1 could be a valuable clinical tool, whether on its own or in a panel of soluble biomarkers.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Review Article

Towards Understanding the Roles of Heparan Sulfate Proteoglycans in Alzheimer's Disease

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Alzheimer's disease (AD) is the most common form of dementia, characterized by progressive loss of memory and cognitive dysfunctions. A central pathological event of AD is accumulation and deposition of cytotoxic amyloid-β peptide (Aβ) in the brain parenchyma. Heparan sulfate proteoglycans (HSPGs) and the side chains heparan sulfate (HS) are found associated with Aβ deposits in the brains of AD patients and transgenic animal models of AD. A growing body of evidence from *in vitro* and *in vivo* studies suggests functional roles of HSPG/HS in Aβ pathogenesis. Although the question of "how and why HSPG/HS is codeposited with Aβ?" still remains, it is within reach to understand the mechanisms of the events. Recent progress by immunohistochemical examination with advanced antibodies shed light on molecular structures of HS codeposited with Aβ. Several recent reports have provided important new insights into the roles of HSPG in Aβ pathogenesis. Particularly, experiments on mouse models revealed indispensible functions of HSPG in modulating Aβ-associated neuroinflammation and clearance of Aβ from the brain. Application of molecules to interfere with the interaction between HS and Aβ peptides has demonstrated beneficial effects on AD mouse models. Elucidating the functions of HSPG/HS in Aβ deposition and toxicity is leading to further understanding of the complex pathology of AD. The progress is encouraging development of new treatments for AD by targeting HS-Aβ interactions.

1. Introduction

Structure of Heparan Sulfate Proteoglycans. Heparan sulfate proteoglycans (HSPGs) are heavily glycosylated proteins, in which several heparan sulfate (HS) glycosaminoglycan (GAG) chains are covalently attached to a core protein. HSPGs are expressed on the cell surface and in the extracellular matrix (ECM) in all tissues. Cell surface HSPGs are membrane-spanning syndecans (SDCs) and lipid-anchored glypicans (GPCs). There are four members in SDC family (SDC 1–4) and six in GPC family (GPC 1–6). Secreted HSPGs are agrin, collagen type XVIII, and perlecan [1]. HS polysaccharide chains are characterized by highly structural heterogeneity with respect to the chain length and sulfation pattern, generated by a complex biosynthetic process within the Golgi apparatus [2, 3]. Functions of HSPGs are mainly attributed to the HS side chains that interact with a spectrum of protein ligands including growth factors, cytokines, enzymes, lipase, apolipoproteins, and protein components of the ECM, exerting biological activities in development, homeostasis, and diseases [3, 4].

The diverse functions of HS in different biological settings have been extensively studied, and substantial information is obtained. One of the most studied molecular mechanisms of HS is in signal transduction process, particularly growth factor medicated signaling. For example, HS mediates high affinity binding of fibroblast growth factor-2 (FGF-2) to its receptor promoting the formation of a stable tertiary signal complex of FGF-2-HS-FGF-2 receptor [5]. Apart from mediating growth factor activities, HS also functions as coreceptors in other biological activities, for example, modulating the interaction of neuropeptide agouti-related protein with melanocortin receptors 3 and 4 (MC3R and MC4R) in the hypothalamus and regulating food consumption [6–8]. Moreover, membrane HSPGs also act as endocytic receptors

for diverse macromolecules such as lipid, growth factors, receptor ligands, and morphogens [1, 9].

Secreted HSPGs, agrin [10] and perlecan [11], constitute major structural molecules in the ECM and basement membrane (BM) along with collagens and other proteins (for review, see [12]). In the ECM, HSPG serves as storage for a number of molecules, such as growth factors and chemokines. In addition, HSPG also plays important roles in maintaining the integrity of ECM and BM [13, 14] and modulating cell mobility [15–17] (also for review, see [4]). In the BM, HSPG, along with collagen IV and laminin-entactin/nidogen complex, controls blood vessel permeability and takes a part in transportation of solutes between vessels and ECM [18, 19]. The ultrastructure of BM can be changed in disease conditions [20] and aging [21], probably due to abnormal production and breakdown of BM components including HSPGs [20].

Heparanase. Heparanase is an endo-β-glucuronidase that specifically cleaves HS side chains of HSPG, releasing oligosaccharide products at the size of 4–7 kDa (10–20 sugar units) [22]. Heparanase is normally expressed at a low level in majority of tissues including the brain [23]. Surprisingly, this unique HS-specific glycosidase is not essential for animal development and homeostasis, as demonstrated by targeted interruption of the heparanase gene in mouse [24]. The heparanase null mice produce longer HS chains in comparison to wildtype mice; however, there is no accumulation of the polysaccharide in organs, indicating that heparanase is not an indispensible enzyme for HS catabolism. In contrast, overexpression of heparanase in mice resulted in extensive modification of HS chains, producing short fragments with increased sulfation that exert higher potency for FGF-2-HS-FGF-2 receptor resembling [25]. This makes the heparanase transgenic mouse (Hpa-tg) a valuable tool for study of HS functions in different diseases [26–29]. Changes in expression of heparanase in tissues, mainly upregulation, have been reported in several diseases, particularly in cancers [30]. Increased expression of heparanase is detected in brain tumor glioma tissues from human and animal models, where heparanase is suggested to play an important role in the control of tumor cell proliferation and invasion [31]. Cerebral ischemia markedly increased heparanase levels in endothelial cells and astrocytes of mouse [32] and rat [33] brains. Available information suggests that heparanase may function as a regulatory factor in different pathological conditions, including tumor and inflammation, exerting its functions through modification of HS structure [34]. Moreover, heparanase has been shown to have nonenzymatic activities, most likely through direct interaction with cell surface receptors, which needs further investigations [35].

Aβ Pathology of Alzheimer's Disease. Alzheimer's disease (AD) is a major central nervous system disease characterized by a progressive neurodegeneration with a clinical phenotype of cognitive impairment. A histopathological hallmark of AD is extracellular Aβ deposition in brain parenchyma manifested as senile Aβ plaques [36]. The pathological Aβ peptides of 40 or 42 amino acids are products of sequential cleavage of the amyloid β precursor protein (AβPP), a transmembrane glycoprotein, by β-secretase (β-site APP cleaving enzyme 1: BACE1) [37] and γ-secretase, a multisubunit protease complex composed of at least 4 proteins including presenilin 1 and 2 [38]. Deposition of Aβ in the brain is attributed to excessive accumulation and aggregation of Aβ in the brain. Accumulation and deposition of Aβ most probably resulted from overproduction in the brain or/and impaired removal of Aβ from the brain [39]. Autosomal dominant mutations in three genes, that is, AβPP gene (*APP*) and presenilin 1 and 2 genes (*PSEN1* and *PSEN2*), can cause early onset familial AD, accounting for <10% of AD cases [40–42]. All these mutations can result in overproduction of the Aβ peptides, leading to their accumulation and aggregation in the brain [43–45]. In clinic, the most common form of AD is late-onset sporadic AD accounting for about 90% of AD cases. Sporadic AD is not associated with genetic mutations, and no overproduction of Aβ was found. In these cases, it is generally believed that overall Aβ clearance is impaired, resulting in accumulation of Aβ peptides [46, 47]. In the brains of AD patients and some aging individuals with no clear diagnosis of dementia, Aβ is found to accumulate and deposit in blood vessel walls, named cerebral amyloid angiopathy (CAA), which has been interpreted as a sign of impaired Aβ clearance from the brain [48].

There are several ways for Aβ clearance, including degradation by proteolytic enzymes [49], receptor mediated Aβ transport across the blood-brain barrier (BBB) in which the main receptor is low-density lipoprotein receptor related protein-1 (LRP-1) [50], phagocytosis by innate immune cells (macrophages) [51], and perivascular drainage along the BM of blood vessels [52].

2. Interaction of HS with Aβ

Several *in vitro* studies demonstrate interaction of Aβ with GAGs including HS and heparin (a HS analogue with higher sulfation degree) [53–56]. It has been found that the HHQK domain at the N-terminus of Aβ is a HS binding motif and this sequence has also been shown to bind microglial cells, suggesting that microglia interact with Aβ through membrane associated HS [57]. Concurrently, a HS sequence of *N*-sulfated hexasaccharide domain containing critical 2-O-sulfated iduronic acid residues binds fibrillar Aβ and was identified in human cerebral cortex. Interestingly, this HS domain also serves as a binding site for the neuroprotective growth factor FGF-2. This evidence suggests that, in AD brain, neurotoxic Aβ may compete with neuroprotective FGF-2 for a common HS binding site [58]. Affinity of HS binding to Aβ is associated with its sulfation pattern, as heparin shows a higher affinity to Aβ, while desulfated HS essentially lost binding capacity to Aβ. This interaction is also dependent on chain length of the GAGs, as heparin fragments shorter than 6-sugar units do not bind to Aβ [58]. Furthermore, it has been proposed that the Aβ-HS interaction is mutually protective, such that HS is protected from heparanase degradation [53] and Aβ is protected from protease degradation [59].

3. Codeposition of HS with Aβ in AD Brain—Updated Findings

The presence of glycosaminoglycans (GAGs) in Aβ plaques in AD brain was first identified using Congo red staining for Aβ fibrils and Alcian blue dye for sulfated GAGs in brain sections of autopsy specimens of AD patients about 30 years ago [60]. The presence of HSPGs in Aβ plaques and CAA was later revealed by immunostaining with specific antibodies recognizing the core proteins of HSPGs [61–63]. With these antibodies, subtypes of HSPGs including SDC 1–3, GPC 1, and agrin have been immunolocalized in Aβ plaques and CAA of AD brains [64, 65]. Development of antibodies recognizing different Aβ fragments further promoted characterization of interaction between Aβ and HS.

Recent studies employed advanced type of anti-HS antibodies that differentially recognizes certain structures of HS polysaccharide chains [66, 67]. For example, phage display antibodies EV3C3 and HS4C3 recognize fully N-sulfated motifs in HS chain, while RB4EA12 and HS4E4 recognize partially N-sulfated and N-acetylated HS motifs [66, 68, 69]. Availability of these unique antibodies allowed us to analyze the molecular structure of HS codeposited with Aβ in the brain. By costaining the AD brain sections with an anti-HS phage display antibody HS4E4 and antibodies specific for Aβ species, we found that HS is differentially deposited with Aβ40 or Aβ42 in neuritic and diffuse plaques [70]. In sporadic AD cases, HS4E4 immunosignals are preferentially colocalized with Aβ40 in the cores of senile plaques; however, the HS4E4 signals are absent from Aβ42-rich diffuse deposits. In a recent study, antibodies (EV3C3 and HS4C3) recognizing highly N-sulfated HS detected strongest immunosignals in both fibrillar and nonfibrillar Aβ plaques, while antibodies (RB4EA12 and HS4E4) recognizing HS regions with lower degree of N-sulfation only stained fibrillar Aβ plaques [68], indicating a distinct property of HS structures in interaction with different Aβ aggregates *in vivo*. These reports are in agreement with our findings, confirming that only fibrillar Aβ plaques of Aβ40 deposits are colocalized with lower sulfated HS motifs. We have identified the membrane bound HSPGs, GPC 1, and SDC 3 in glial cells associated with Aβ deposits in dense core plaques, proximal to sites of HS accumulation, and suggested that HS codeposited with Aβ40 in neuritic plaques is mainly derived from glial cells [70]. RB4CD12 is another phage display antibody that recognizes highly sulfated domains of HS [71]. This antibody strongly stained both diffuse and neuritic Aβ plaques in the brains of AD and several transgenic AD mouse models. Interestingly, the RB4CD12 epitope accumulated in Aβ plaques can be demolished by extracellular sulfatases (Sulf-1 and Sulf-2) *ex vivo* [72], suggesting that 6-O-sulfated glucosamine residues are within the HS sequence interacting with Aβ.

These recent findings of selective deposition of HS with different species and forms of Aβ strongly suggest distinct roles of HS in Aβ aggregation and deposition. These studies point that HS/HSPG constitutes a part of Aβ plaques and the findings support the notion that HS plays a role in Aβ plaque formation and persistence.

4. HS Mediated Aβ Uptake—Implications in Aβ Cytotoxicity and Clearance

In the brain, Aβ are present in both extracellular and intracellular pools and extracellular Aβ contributes to intracellular Aβ through internalization mechanisms [73]. Cell types in the brain are known to engulf Aβ including neurons, endothelial cells [74], smooth muscle cells [75], and glial cells (microglia and astrocytes) [76, 77]. Internalization of Aβ into cells has been shown to be associated with Aβ cytotoxicity [78, 79]. Several cell surface macromolecules of microglia/macrophages are reported to play roles in Aβ uptake, including toll-like receptor [80], complement receptors [81], scavenger receptors [76, 82], LRP-1 [83], and transmembrane protein CD33, a member of the sialic acid-binding immunoglobulin-like lectins [84] (also for review, see [85]). HSPG functions as a cell surface receptor for entry of diverse macromolecules into cells; in this context, both the core protein and the HS side chains of HSPG are attributed to regulation of endocytosis (for review, see [9]). Having this in mind, we studied Aβ40 uptake and associated toxicity in Chinese hamster ovary (CHO) cell lines. After exposure to Aβ40, the CHO wildtype cells (CHO-WT) survived poorly, whereas the HS-deficient CHO pgsD-677 cells were resistant to the treatment. In correlation with Aβ cytotoxicity, the added Aβ40 was substantially uptaken by CHO-WT but barely by CHO pgsD-677 cells [86]. Likewise, Aβ40 cytotoxicity was attenuated in human embryonic kidney cells (HEK293) overexpressing heparanase due to extensive degradation of HS chains [86]. These findings suggest that cell surface HS mediates Aβ internalization and toxicity.

According to "amyloid hypothesis," the cause of the majority form of AD, that is, late-onset sporadic, is due to impaired clearance of Aβ from the brain [47, 87]. Transport of Aβ across the BBB from brain to blood is an important route for Aβ clearance, where transcytosis requires Aβ to attach to cell surface after which it is internalized and subsequently released at the luminal side of the endothelium. LRP-1 at the surface of blood vessel endothelial and smooth muscle cells has been reported to function as Aβ cargo in this process [50, 75]. It has been recently reported that LRP-1 and HSPGs mediate Aβ internalization in a seemingly cooperative manner, in which HSPG is more important for Aβ binding to cell surface than LRP-1 [88]. Another important player in this context is apolipoprotein E (ApoE). ApoE and HS are consistently codetected in Aβ deposits and have been ascribed various roles in the pathogenesis of AD [89, 90]. ApoE can bind to HSPG forming functional complex of ApoE/HSPG; alternatively, it joins HSPG/LRP-1 uptake pathway in which ApoE first binds to HSPG and then presents to LRP-1 for uptake (for review, see [91]). The finding of codistribution of ApoE, HS, and LRP1 in Aβ40-positive microvasculature in the hippocampus of individuals with Down's syndrome (DS), diagnosed with AD, encouraged us to investigate correlation of these molecules in Aβ uptake and clearance [92]. We investigated the functional relationship between Aβ and ApoE and their interactions with cell surface HS and LRP-1 [92]. Coincubation of Aβ with CHO cells either deficient in HS (CHO pgsD677) or in LRP-1 (CHO

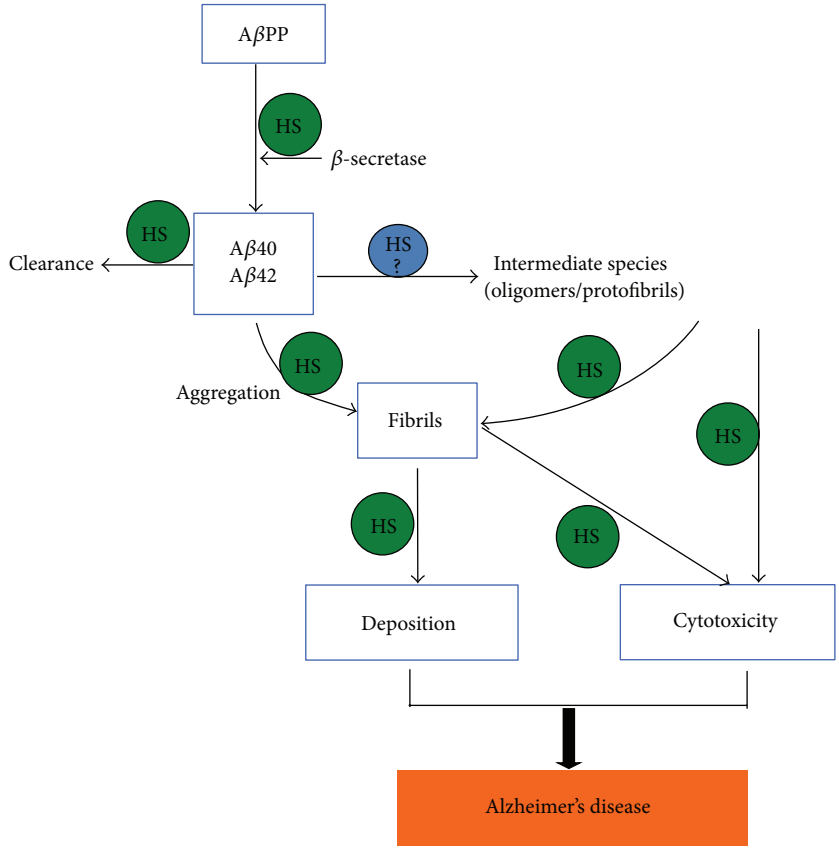

Figure 1: Heparan sulfate (HS) is involved in essentially each step of amyloid-β (Aβ) pathological development in Alzheimer's disease. HS modulates β-secretase (BACE) activity and accelerates Aβ aggregation and fibrillization. It is unclear whether HS is involved in formation of the toxic oligomers/protofibrils; however, HS mediates toxic effect of different types of Aβ fibrils. HS in the basement membrane participates in clearance of Aβ.

13-5-1) along with CHO-WT revealed that addition of ApoE in the cell culture increased Aβ association to the cells, which is dependent on presence of HSPG and LRP-1 on the cell surface. ApoE uptake by the cells does not require presence of both HSPG and LRP1; however, lack of HS in the CHO pgsD677 cells resulted in aberrant intracellular ApoE processing. These data propose that the complex interactions of ApoE, LRP-1, and HSPG facilitate Aβ internalization, which may represent one of major routes for Aβ clearance through transportation of ECM Aβ across BBB into the vessel lumen [92].

5. Heparanase in Aging and AD—Implications in Transmigration of Blood-Borne Monocytes

Heparanase expression in the brain is at marginally detectable level [23, 29], while, in several pathological conditions of the brain, expression of heparanase has been found elevated [31, 32]. Although limited information is available regarding the impact of heparanase on AD pathogenesis, Aβ40 has been shown to protect heparanase-catalyzed degradation of HSPGs *in vitro* with predicted effect contributing to the stability and persistence of Aβ plaques [53]. Our recent study has revealed increased vasculature expression of heparanase in the brains of AD patients and a mouse model that overexpresses human AβPP (Tg2576 mice) [29]. Since HS is involved in almost every step of Aβ pathogenesis found in AD (Figure 1), it is of great importance to study expression and activity of heparanase in the brain of aging subjects, both human and animal models.

In the brain, perivascular macrophages derived from blood-borne mononuclear cells play an important role in Aβ clearance [51, 93, 94]; Aβ peptides are uptaken and subsequently degraded by proteases [95]. Several *in vivo* studies have demonstrated the multiple functions of HS and heparanase in inflammatory reactions with regard to infiltration of blood-borne immune cells into infected tissues [28, 96]. In this scenario, molecular structures of HS, for example, sulfation pattern and chain length, are pivotal in interaction between endothelial cells and leukocytes as well as with the soluble inflammatory cytokines. Accordingly, we have recently studied the potential roles of heparanase and HS in mediating blood-borne monocytes across blood vessel wall into the brain parenchyma on the transgenic mouse model overexpressing heparanase (Hpa-tg). Overexpression of heparanase resulted in shorter HS

chains in the brain of Hpa-tg mouse [29]. In the study, we applied two experimental regimens, that is, localized cerebral microinjection of aggregated Aβ42 and systemic challenge by intraperitoneal injection of lipopolysaccharide (LPS), a bacterial endotoxin. Microinjection of aggregated Aβ42 into the brain elicited an inflammatory response restricted to the injection site of the wildtype mice, characterized by massive infiltration of microglia/macrophages. This inflammatory reaction clearly showed a beneficial effect for clearance of the injected Aβ. In comparison, recruitment and activation of immune cells (microglia and blood-borne monocytes) were significantly attenuated around the injection site of Hpa-tg mouse brain, which resulted in detainment of the injected Aβ42 [29]. The LPS-treated wildtype mice also showed massive activation of resident microglia as well as recruitment of monocyte-derived macrophages in the brain parenchyma, whereas Hpa-tg mice exhibited restricted inflammation with significantly fewer infiltrated macrophages. The mechanism for the reduced recruitment of inflammatory cells into the brain of Hpa-tg mice was verified with an *in vitro* BBB model constituted with primary endothelia cells and pericytes [29].

The integrity of ECM and the capillary vascular basement membrane (VBM) scaffold is often found severely damaged in association with Aβ deposition [97, 98], which may be responsible for perturbed elimination of solutes and Aβ from parenchyma, consequently leading to development of CAA [99]. As HSPGs are major components of the ECM and VBM and heparanase activity is strongly implicated in structural remodeling of the ECM and BM through degradation of HS, heparanase expression may markedly contribute to pathological changes in the ECM and VBM in AD brain, accordingly affecting Aβ clearance. There is essentially no information with this regard and studies are needed to explore the implications of HS in Aβ transportation and clearance.

6. Conclusion and Perspectives

Principle treatments for AD with regard to Aβ pathology are to reduce production, improve clearance, and prevent aggregation of the pathological peptides. Considering that HS-Aβ interaction contributes to every stage of the Aβ pathogenesis in AD, including production, clearance and accumulation, aggregation, and toxicity of Aβ (Figure 1), it is rational to hypothesize that interfering HS-Aβ interaction may have multiple beneficial effects. Earlier studies show that treatment with low molecular weight heparin (LMWH) reduced Aβ burden in the brain of an AD mouse model overexpressing human AβPP [100]; the effect is probably that the LMWH competes with endogenous HS, blocking the HS-Aβ interaction. This assumption is supported by our findings that the fragmentation of HS by overexpressed heparanase in mouse attenuated deposition of serum A amyloid (SAA; another amyloid protein) [27]. Though it is improper to use LMWH for treatment of AD, it is possible to apply non-anticoagulant LMWH or HS mimetics for the purpose. With the progress in characterization of HS molecular structures dissected from Aβ plaques, it should be possible to design compounds mimic to the HS structures that interact with Aβ to block its aggregation as well as to neutralize its toxicity. Moreover, targeting Aβ producing enzymes, that is, BACE1 and γ-secretase, constitutes one of the potential treatments for AD. Interestingly, HSPG has been found to modulate BACE activity [101, 102], and efforts are being made to synthesize HS-oligosaccharides as inhibitors of BACE [103]. In light of experimental and clinical evidences addressing the role of HS in Aβ pathology, it is plausible to expect that novel treatments by targeting HS-Aβ interaction may contribute to AD treatment and to improve effects of other treatments. Apart from designed synthesis of HS mimetics, natural anionic oligosaccharides, such as glycosaminoglycans isolated from marine animals and natural herbs, should also be explored for the potential to be developed as drug candidates for this particular application.

Conflict of Interests

The authors declare that they have no conflict of interests regarding the publication of this paper.

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Review Article

The Motile Breast Cancer Phenotype Roles of Proteoglycans/Glycosaminoglycans

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The consecutive stages of cancer growth and dissemination are obligatorily perpetrated through specific interactions of the tumor cells with their microenvironment. Importantly, cell-associated and tumor microenvironment glycosaminoglycans (GAGs)/proteoglycan (PG) content and distribution are markedly altered during tumor pathogenesis and progression. GAGs and PGs perform multiple functions in specific stages of the metastatic cascade due to their defined structure and ability to interact with both ligands and receptors regulating cancer pathogenesis. Thus, GAGs/PGs may modulate downstream signaling of key cellular mediators including insulin growth factor receptor (IGFR), epidermal growth factor receptor (EGFR), estrogen receptors (ERs), or Wnt members. In the present review we will focus on breast cancer motility in correlation with their GAG/PG content and critically discuss mechanisms involved. Furthermore, new approaches involving GAGs/PGs as potential prognostic/diagnostic markers or as therapeutic agents for cancer-related pathologies are being proposed.

1. Introduction

Cancer Microenvironment. It is now increasingly recognized that the microenvironment plays a critical role in the progression of tumors. The consecutive steps of tumor growth, local invasion, intravasation, extravasation, and invasion of anatomically distant sites are obligatorily perpetrated through specific interactions of the tumor cells with their microenvironment. Free glycosaminoglycans (GAGs) and proteoglycan- (PG-) containing GAGs, key effectors of cell surface, pericellular and extracellular microenvironments, perform multiple functions in cancer by virtue of their coded structure and their ability to interact with both ligands and receptors that regulate cancer growth [1–4]. Specifically, these extracellular matrix (ECM) components critically modulate the tumor cell "motile phenotype" affecting their adhesive/migratory abilities which are directly correlated to the metastatic cascade [5, 6].

Glycosaminoglycans (GAGs) comprise a class of linear, negatively charged polysaccharides composed of repeating disaccharide units of acetylated hexosamines (N-acetyl-galactosamine in the case of chondroitin sulphate and dermatan sulfate or N-acetyl-glucosamine in the case of heparin sulphate and heparin) and mainly of uronic acids (d-glucuronic acid or l-iduronic acid) being sulfated at various positions. The exception constitutes keratan sulphate whose uronic acid is substituted by galactose. Based on the epimeric form of uronic acid and the type of hexosamine in their repeating disaccharide units, GAGs are classified into four major types; hyaluronan (HA), chondroitin sulfate (CS) and dermatan sulfate (DS), heparin and heparan sulphate (HS), and keratan sulfate (KS). HA is synthesized in the absence of a protein core at the inner face of the plasma membrane and consequently found in the form of free chains whereas other GAG types are covalently bound into protein cores to form proteoglycans (PGs). With the exception of HA, all

GAG types are variably sulfated which contributes to the intricate complexity of their structures. Free GAGs chains are secreted to the extracellular space and distributed both in the pericellular matrix and extracellular matrix proper. GAGs bound into PGs are located to the extracellular matrix, basal membrane, and cell surface [7]. Cell type and tissue specific alterations in fine GAG structure, which are strictly predetermined [8–10], allow these molecules to modulate with high specificity different cellular processes [7]. Cell-associated and tumor microenvironment GAG content and distribution is markedly altered during tumor pathogenesis and progression [11, 12].

PGs, molecules which consist of a protein core that is covalently modified with GAG chains, are distributed both to the ECM "proper" associated with the cell membrane as well as located to intracellular compartment. These main PG groups are further classified into families according to their gene homology, core protein properties, size, and modular composition. Thus, secreted to the ECM PGs include large aggregating PGs, named hyalectans, small leucine-rich PGs (SLRPs), and basement membrane PGs. Cell-surface-associated PGs are distributed into two main families (syndecans and glypicans), whereas serglycin is the only intracellular PG characterized to date [13, 14]. The wide molecular diversity of PGs is derived from the multitude of possible combinations of protein cores and GAG chains. Thus, PGs are also classified, regarding their GAG content, into heparan sulfate PGs (HSPG), chondroitin/dermatan sulfate PGs, (CS/DSPGs), and keratan sulphate PGs. The specific structural characteristics of both the protein cores and GAG types provide the structural basis for the plethora of their biological functions which include acting as structural components in tissue organization or dynamic regulators of cellular behaviour [3].

2. Is the Expression of PGs/GAGs in Breast Cancer Correlated to Disease Progression?

Importantly, ECM components, including PGs and GAGs, are involved in the molecular events that are associated with tumor progression. It is well established that during malignant transformation, significant changes can be observed in the structural and mechanical properties of respective ECM components. Indeed, the alteration of cell shape and changes in the interactions with the ECM are considered as important hallmarks of cancer cells [15, 16]. Changes in the composition and organization of ECM regulate cancer progression by promoting cellular transformation and metastasis. Moreover, altered expression of ECM molecules also deregulates the behavior of stromal cells and promotes tumor-associated angiogenesis and inflammation, leading to the generation of a tumorigenic microenvironment [17–19].

HSPGs have been closely correlated to breast cancer tumorigenesis. Major HSPGs members are the transmembrane proteins syndecans (SDCs), with the SDC family consisting of four members: SDC1, SDC2, SDC3, and SDC4 [20]. A complex pattern describing SDCs' expression in tumor and stroma compartments during the progression of malignancy is emerging. Most reports have focused on the involvement of SDC1, an epithelial marker, during the progression of this insidious disease. Thus, increased expression of SDC1 was demonstrated in the stroma of invasive breast cancer [21–23]. Moreover, the expression of SDC1 in both epithelium and stroma may be a predictor of unfavorable prognosis in breast cancer, whereas loss of epithelial SDC1 was associated with a more favorable outcome [21]. Importantly, SDC1 has also been linked with the promotion of proliferation of human breast cancer cells *in vitro* [23]. The distribution of SDC1 to cell membrane has predominantly been described in breast cancer; however, shed SDC1 in other tumor types has been directly associated with increased invasion and cancer progression [24, 25]. Indeed in breast cancer, SDC1 is suggested to be a poor prognostic factor for breast cancer since its upregulation at both the mRNA and protein levels has been associated with higher histological tumor grade, as well as increased mitotic index and tumor size [26]. The expression of other SDC family members in breast cancer tissues has also been studied. Thus, in estrogen receptor-negative and highly proliferative breast carcinoma subtypes, SDC1 and SDC4 were found to be overexpressed [27]. Similarly, the overexpression of these two PGs has been demonstrated in a highly invasive breast cancer cell line (MDA-MB-231) [28]. However, another report suggests that SDC4 expression is downregulated in malignant breast tissue [29]. The data on SDC1' roles seem to be more uniform as high expression of SDC1 has been linked with increased tumor aggressiveness and poorer prognosis in breast carcinomas [30]. Functionally, this correlates well with the proposed role of SDC1 as a coreceptor which activates mitogenic growth factor signaling which in turn modulates tumor angiogenesis, cell adhesion, and motility [31]. Moreover, a study conducted in postmenopausal women with breast cancer or dense-mammographic breast tissue demonstrated that the distribution of SDC1 changes from the epithelium to the stroma [32, 33]. Interestingly, SDC1 expressing breast carcinomas show decreased response to chemotherapy [34], whereas it has also been indicated that the loss of SDC1 expression may be a potential predictive factor for response to preoperative systemic therapy [35]. These data define SDC1 as a potentially significant therapy target.

The glypicans (GPCs) are HSPGs anchored through the glycosylphosphatidylinositol (GPI) link to the outer layer of cell membranes. GPCs have been shown to regulate the binding properties of bone morphogenetic protein (BMP) and fibroblast growth factor (FGF) [36]. Most of the studies concerning the roles of GPCs in breast cancer progression focus on the role of the GPC3 member. Intriguingly, the GPC3 gene silencing has been identified in human breast cancer cells, through a mechanism which involves the hypermethylation of the GPC3 promoter. Thus, GPC3 seems to be a negative regulator of breast cancer cell proliferation, since it was shown that its ectopic expression inhibited the growth rates of 8 in a panel of 10 breast cancer cell lines [37]. Furthermore, it has been established that GPC3 guides MCF-7 breast cancer cells to apoptosis through a mechanism that involves the anchorage of the GPC3 core protein to the cell membrane [38]. The role of the other members of the GPC family in breast cancer pathogenesis has not been widely

investigated. The up to now obtained data suggest that the expression of GPC3 and GPC4 was negligibly increased in tumor as compared to normal tissues, whereas the expression of GPC5 and GPC6 was below the level of detection in both normal and cancerous breast tissues. On the contrary, in the same study GPC1 was found to be strongly expressed in human breast cancers with a low expression in normal breast tissues [39].

The family of PGs secreted to the ECM and known as hyalectans is comprised of versican, aggregan, neuroscan, and brevican [12]. Versican seems to have a prominent role in breast cancer progression due to its ability to interact with molecules determined to be regulators of key cellular processes [40]. Importantly, extracellular versican has been found to be elevated in a variety of human tumors including breast carcinoma [41–43]. The distribution of versican in tissue samples is mostly allocated to breast cancer margins. Indeed, the high expression of versican has been described in the interstitium at the invasive margins of breast carcinoma. Versican is suggested to be a prognostic marker as it has been found to be predictive of cancer relapse, negatively affecting overall survival rates of breast cancer patients [44]. On the other hand, the increased expression of versican within peritumoral stromal matrix was predictive of relapse-free disease prognosis, in women with node-negative breast cancer. These authors therefore propose that versican may be a predictor for risk and rate of relapse, independent of tumor size in patients with node negative disease [45]. Recently, various histotypes of breast *in situ* carcinomas have been examined in order to assess the immunohistochemical expression of versican in the stroma and correlate these findings to disease progression. This study provided evidence that versican is strongly expressed in the perilesional stroma of a subclass of ductal *in situ* carcinomas and that the extension of versican immunostaining is statistically related to the high grade. On the other hand, the expression of versican in the cases of classic lobular *in situ* carcinomas was confined to the anatomical structures that usually contain this PG in adult breast tissues [46]. Thus, Canavese et al. suggest that various histotypes of breast *in situ* carcinomas could follow different pathways of epithelial stromal interactions. Structure-function studies focusing on versican suggest that its G3 domain is closely correlated to breast cancer progression. Thus expression of versican G3 domain both increases breast cancer cell proliferation *in vitro* and *in vivo* and also enhances tumor cell migration *in vitro* and systemic metastasis *in vivo* [47, 48]. The exogenous expression of a versican G3 construct in breast cancer cell lines enhanced their resistance to anthracycline-dependent apoptosis when cultured in serum free medium by upregulating pERK and GSK-3b (S9P) [49]. On the other hand, versican G3 promoted cell apoptosis induced by C2-ceramide or Docetaxel by enhancing expression of pSAPK/JNK and decreasing expression of GSK-3β (S9P). Inhibition of endogenous versican expression by siRNA or reduction of versican G3's expression by linking G3 with 3′UTR prevented G3 modulated cell apoptosis. Thus, the G3 domain appears to have a dual role in modulating breast cancer cell resistance to chemotherapeutic agents [49]. The importance of versican in breast cancer pathogenesis is well illustrated in a recent study by Kischel et al. These authors demonstrate that all known versican isoforms as well as new alternatively spliced versican isoform, named V4, were significantly overexpressed in the malignant lesions [50].

The small leucine-rich proteoglycans (SLRPs) are characterized by a relatively small protein core with leucine rich-repeat (LRR) motifs into which GAG chains are covalently bound [13, 51, 52]. These secreted proteins have the ability to interact with collagen, modifying the deposition and organization of collagen fibers in the extracellular matrix. A study on SLRP expression in breast tumors showed that lumican and decorin are the most frequently expressed SLRPs, whereas biglycan and fibromodulin are rarely detected [53]. Decorin is physiologically secreted by stromal fibroblasts of normal breast tissue [54]. Indeed, the expression of decorin, which is abundant in the stroma, can be used as an indicator of tumor progression [55]. Specifically, low expression of decorin has been correlated to large tumor size, a shorter time to progression, and poorer survival [55]. In a study by Reed et al., it has been shown that the primary tumor growth was strongly diminished after treatment with decorin protein core. In the same study, the utilization of an adenoviral vector containing the decorin transgene caused the elimination of metastases [56]. Decorin has also been shown to decrease tumor growth in experiments conducted in a rat model [56]. Moreover, it has been indicated that decorin inactivates the oncogenic ErbB2 protein [57]. Another important member of the SLRP family, lumican, is specifically expressed in breast cancer tissues, but not in normal breast tissues. Furthermore, it has been proposed that lumican is differentially expressed during breast tumor progression [58]. The overexpression of lumican in breast cancer tissues is associated with a high tumor grade, a low estrogen receptor (ER) expression level, and young age of patients [58].

Hyaluronan (HA) is an anionic, nonsulfated GAG which differs from the other members of the GAG family as it neither contains sulfate groups nor is it covalently linked into a core protein [59]. This GAG is synthesized by three types of integral membrane proteins denominated hyaluronan synthases: HAS1, HAS2, and HAS3. The degradation of HA within tissues, on the other hand, is performed by enzymes known as hyaluronidases (HYAL). A significant number of studies demonstrate that HA deposition is elevated in various types of cancer tissues including breast cancer [60]. Specifically, immunochemistry revealed elevated amounts of HA in the stroma of human breast cancer, correlating with tumor invasion, metastasis, and adverse clinical outcome [61, 62]. The magnitude of the HA accumulation in the tumor stroma (breast, ovarian, and prostate cancers) strongly correlates with an unfavorable prognosis of the patient, that is, advancement of the malignancy [59]. HYAL1 and HYAL2 are found to be overexpressed in breast cancer tumors, downregulating the expression of HA [63].

Taking into consideration all the above, it can be concluded that PGs/GAGs, which are abundantly present in the stromal compartment of breast cancer cells, play a major role in several biological processes of carcinogenesis. The overexpression of many of these molecules has been

associated with the malignant phenotype and with poor prognosis. The *de facto* contribution of these molecules to tumor cells' malignant properties defines them as relevant therapeutic agents.

3. The "Motile" Phenotype

Tumors of solid organs (carcinomas, sarcomas, and central nervous system tumors) kill patients mainly by dissemination from the primary site as once the cells migrate beyond the primary site into adjacent or distant tissue, they are difficult to extirpate. This dissemination may take two forms: (i) localized invasion throughout the tissue and into the adnexa or (ii) metastatic dissemination [64]. An obligatory component of the dissemination process is the obtaining of a "motile phenotype." In order for the tumor cells to efficiently migrate, specific cytoskeleton modifications must be executed. First, actin cytoskeleton organization has a well-established role in cell migration and is regulated by a plethora of extensively studied molecular mediators. Specifically, Rho GTPases, cAMP/PKA, and integrins were found to have a central role in modulating the actin cytoskeleton alterations during migration and have been shown to be closely regulated during epithelial to mesenchymal transition (EMT) processes [65, 66]. Integrins are heterodimeric cell-surface molecules that on one side link the actin cytoskeleton to the cell membrane and on the other side mediate cell-matrix interactions [67]. In addition to their structural functions, integrins mediate signaling from the extracellular space into the cell through integrin-associated signalling and adaptor molecules such as FAK (focal adhesion kinase) [68] or ILK (integrin-linked kinase) [69]. Intermediate filaments (IFs) play a central role in maintaining cell structure, stiffness, and integrity. The IF network of epithelial cells comprises cytokeratins, while the mesenchymal IF network is primarily constituted of vimentin. During EMTs, many cytokeratins are downregulated and vimentin is upregulated [70]. Overexpression of vimentin IFs in the breast carcinoma model leads to augmentation of motility and invasiveness *in vitro*, which can be transiently downregulated by treatment with antisense oligonucleotides to vimentin. Additional experimental evidence suggests that the mechanism(s) responsible for the differential expression of metastatic properties associated with the interconverted phenotype rest(s) in the unique interaction, either direct or indirect, of IFs with specific integrins interacting with the extracellular matrix [71].

The "motile phenotype" of cancer cells is expressed only through direct interactions with the tumor environment as inevitably the tumor cells will respond to local stimuli. These stimuli include cues for motility and migration, which normally appear in tissues undergoing formation, remodeling, or healing. Carcinoma cells are likely to be sensitive to the motility cues that normally regulate epithelial morphogenetic movements such as ingression, delamination, invagination, and tube or sheet migration [72]. Understanding how such motility cues arise and act, in tumor tissue, may provide one of the key "answers" in cancer research.

4. The Role of Matrix Molecules in Breast Cancer Cell Epithelial-to-Mesenchymal Transition

The huge proliferative ability of tumor cells leads to genetic diversity which facilitates their responsiveness to microenvironmental factors resulting in an increased degree of phenotypic plasticity [73, 74]. Therefore, during primary growth, some tumor cells can acquire traits that endow them with a malignant phenotype that leads to increased tumor cell motility, invasiveness, and propensity to metastasize [75]. Importantly, during epithelial-to-mesenchymal transition (EMT), tumor cells acquire a phenotype that encompasses all these traits as EMT is characterized by a loss of cell polarity and adhesion and gain of motile characteristics. Thus, the EMT promotes the detachment of cells from the primary tumor, facilitating their migration and metastatic dissemination [76]. Moreover, a strong link between EMT and acquisition of a tumor-initiating phenotype is suggested [77]. Early studies suggested the involvement of EMT in aggressive breast cancer behaviour as cells exhibiting a mesenchymal-like phenotype (vimentin expression, lack of cell border associated uvomorulin) show dramatically increased motility, invasiveness, and metastatic potential in nude mice [78]. Moreover, using an intravital imaging approach, Giampieri et al. showed that single breast tumor motile cells that have an active TGF-β-Smad2/3 EMT promoting signaling were capable of hematogenous metastasis to distal organs, while those lacking this signaling pathway were prone to passive lymph metastasis [79]. However, EMT is not the "ultimate" event as it involves various morphological and functional alterations [80] and is not always correlated to a more aggressive phenotype [81]. In addition, an apparent contradiction to the association between EMT and metastasis comes from clinical observations that distant metastases derived from a variety of primary carcinomas resemble an epithelial phenotype.

Importantly the EMT as well as the mesenchymal to epithelial transition (MET) is partly regulated through the "crosstalk" between the tumor microenvironment and the cancer cells [82]. Growth factor stimulation appears to be a part of this "crosstalk" as epidermal growth factor (EGF) leads to epitheliomesenchymal transition-like changes in human breast cancer cells including upregulation of vimentin and downregulation of E-cadherin. EMT was associated with increased ability of these cells to adhere to ECM molecules as well as to migrate [78]. Furthermore, TGF-beta1-mediated breast cancer invasion is associated with EMT and matrix proteolysis [83]. Likewise, constitutively active type I insulin-like growth factor receptor causes transformation and xenograft growth of immortalized mammary epithelial cells and is accompanied by an epithelial-to-mesenchymal transition mediated by NF-kappaB and snail [84]. Interestingly, TGFβ-dependent hyaluronan synthase expression (HAS2) expression, but not extracellular hyaluronan, has an important regulatory role in TGFβ-induced EMT [85]. Furthermore, when breast cells were induced to exhibit EMT, there was a strong upregulation of HAS2 [86].

Indeed, the implication of matrix molecules contribution to EMT was evident even from early studies [87]. LOX is a secreted amine oxidase that catalyses collagen and elastin cross-linking in the extracellular matrix, previously shown to regulate breast cancer metastasis, and is correlated to EMT [88]. Enhanced tenascin-C expression and matrix deposition during Ras/TGF-beta-induced EMT of mammary tumor cells was reported [89]. Noteworthy, there seems to be a shift in proteoglycans expression as significant correlation was found between the loss of the HSPG, SDC1, and epithelial expression during EMT. This loss was correlated with increased SDC1 stromal expression and a high grade of malignancy ($P = 0.011$). Therefore, the authors concluded that the loss of SDC1 epithelial expression was of strong prognostic value in breast carcinomas [90]. Along the same lines, SDC1 coexpression with E-cadherin was found to be synchronously regulated during EMT in breast cancer [91].

Importantly, the mesenchymal to epithelial transition (MET) of metastatic breast cancer cells upon reaching distant metastatic sites appears also to be regulated by ECM molecules as a unique paracrine crosstalk between the microenvironment and the cancer cells has been identified [92]. Thus, versican stimulated MET of metastatic breast cancer cells by attenuating phospho-Smad2 levels, which resulted in elevated cell proliferation and accelerated metastases. Analysis of clinical specimens showed elevated versican expression within the metastatic lung of patients with breast cancer [92]. Thus, mechanisms regulating both the EMT and MET processes are dependent on PG/GAG participation highlighting their relevance in breast cancer progression.

5. The Roles of GAGs/PGs in Breast Cancer Cell Motility

Breast cancer is characterized by significant quantitative changes of extracellular network constituents. Previously, it has been well established that changes in unique ECM properties of tumor cells and their microenvironment may lead to changes in cell behavior during cancer progression [12, 93]. The PG component of the ECM has been shown to participate in and regulate key cellular events, acting either directly on cells or modulating growth factor activities [94].

Thus, HSPGs are involved in multiple cellular events and functions such as cell adhesion, ECM assembly, and growth factors storage [95]. Their HS chains have the ability to bind not only to numerous "heparin-" binding growth factors and morphogens, [31] but also to "heparin-" binding sites present in matrix ligands, including fibronectin, vitronectin, laminins, and the fibrillar collagens [31]. The SDCs are believed to have roles in cell adhesion and signaling possibly as coreceptors with integrins and cell-cell adhesion molecules [96].

Each of the four SDCs has been proposed to connect to the actin cytoskeleton, via their cytoplasmic domains [97, 98], for example, through ezrin in SDC2 and a-actinin in the case of SDC4. For SDC1, SDC2, and SDC4 at least, the external core protein can trigger integrin-mediated cell adhesion events, which may be direct or, in the case of SDC4, probably indirect [98].

Many studies indicate a strong correlation between the expression of specific HSPGs and the metastatic and invasive potential of breast cancer cells [26, 99, 100]. In fact, the expression of SDC4 and the overexpression of SDC2 are associated with the high invasive potential of MDA-MB-231 cell line [29]. Interestingly, estradiol (E2) as well as IGF and EGF signaling pathways have significant roles in regulating the expression of certain cell surface HSPGs, such as SDC2, SDC4, and GPC1, which are crucial for cell motility [101].

SDC1 participates in the generation of a proangiogenic microenvironment, supporting tumor growth and metastatic spread [11, 12, 102]. This HSPG, regulates downstream signaling pathways that are traditionally associated with the integrins [12, 103, 104], thus mediating cell migration by creating a dynamic linkage between the ECM and the cytoskeleton and by modulating Rho family members that control the activation of focal adhesion kinase (FAK). Indeed, it has been observed that in MDA-MB-231 human breast cancer cells SDC1 physically interacts with FAK [105]. Furthermore, SDC1 regulates the activation of $\alpha v\beta 3$ and/or $\alpha v\beta 5$ integrins. This activation stimulates adhesion, spreading, and migration of tumor cells, with clear consequences on tumor progression [106]. Beauvais and Rapraeger, [106] also demonstrated that SDC1 collaborates with $\alpha v\beta 3$ integrin to initiate a positive adhesion signal, which is integrin ligand independent. The activation of $\beta 1$ integrins is not required for SDC1 mediated cell spreading; consequently SDC1 is sufficient for adhesion. Actually, the inhibition of $\beta 1$ integrins activity induces cells spreading presumably by attenuating the suppression of SDC1 binding perpetrated by integrins. Additionally, SDC1 participates in the IGF-I receptor (IGF-IR) signaling pathway on adhesion. Specifically, it colocalizes with the integrin and IGF-IR and regulates activation of $\alpha v\beta 3$ and $\alpha v\beta 5$ integrins by coupling these integrins to the IGF-IR in human mammary carcinoma and endothelial cells, resulting in the activation of an inside-out signaling pathway [107]. Therefore, SDC1 is an obligatory component in the formation of this adhesion complex [107]. Additionally, SDC1 expression coordinates β-integrin dependent and interleukin-6 (IL-6) dependent cell functions, such as cell adhesion, migration, and resistance to irradiation, in MDA-MB-231 breast cancer cells [108].

SDC2 has likewise been implicated in cell adhesion and signaling [109] as well as in the progression of cancer [96]. The expression of SDC2 in breast cancer cells is regulated by estradiol (E2) through the action of estrogen receptor alpha (ERα) [110]. The increased levels of SDC2 after E2 treatment may be connected with the ability of SDC2 to modulate the tumorigenic and invasive behavior of breast cancer cells [110].

SDC3 has not been widely studied with respect to either breast or ovarian carcinoma [111], but its aberrant upregulation in vasculature associated with ovarian carcinoma has been noted [112].

SDC4 is a focal adhesion component in a range of cell types, adherent to different matrix molecules, including fibronectin [113, 114] and mediating breast cancer cell adhesion and spreading [103, 106]. The attachment of SDC4 to fibronectin triggers intracellular signaling, including protein kinase Cα and focal adhesion kinase activation, to promote

focal adhesion formation [115, 116]. SDC4 null cells are deficient in phosphorylated FAK and show impaired cell migration [116, 117]. When overexpressed, SDC4 promotes excess focal adhesion formation resulting in reduced cell migration [118]. Huang et al. [119] reported that tenascin-C, an adhesion-modulatory ECM molecule [120], binds to fibronectin (specifically to the FNIII13 of the HepII site), thereby specifically blocking cell adhesion to fibronectin through SDC4. This binding inhibits the coreceptor function of SDC4 in integrin signaling [119]. Nevertheless, the role of SDC4 on tumor progression needs more investigation, as different facets of its actions remain unclear.

An important feature of the SDC molecule necessary for signaling appears to be its ectodomain [96, 121]. Indeed, depleting epithelia of cell surface SDC1 alters cell morphology and organization, the arrangement and expression of adhesion molecules, and anchorage-dependent growth controls [121]. Therefore, Kato et al. [121] suggested a regulatory role for SDC1 ectodomain in the control of epithelial cell morphology. Soluble murine SDC4 ectodomain competes with the endogenous SDC4 for a critical cell surface interaction required for signaling during cell spreading [122, 123]. The ability of SDC4 to interact with molecules at the cell surface via its core protein as well as its GAG chains may uniquely regulate the formation of cell surface signaling complexes following engagement of this PG with its extracellular ligands [122, 123]. Moreover, shedding and membrane-associated SDC1 play distinct roles in different stages of ERαa+ breast cancer cell progression. Proteolytic conversion of SDC1 from a membrane bound into a soluble molecule marks a switch from a proliferative to an invasive phenotype, with implications for breast cancer diagnostics and potential GAG-based therapies [124].

A number of mutations related to SDCs have been recorded in breast carcinomas [111]. The mutations may influence the sequence of amino acids of the core protein and the enzymes that are involved in GAG chains synthesis. Importantly, these mutations may affect the interactions between SDCs and growth factors resulting in altered behavior of cells [125] including cell motility.

The expression of the GPC1 gene in the MDA-MB-231 may be indicative of its higher metastatic potential [29]. The expression of GPC3 is silenced in human breast cancer, but ectopic expression of GPC3 revealed that this molecule can act as a negative regulator of breast cancer cell growth [37, 39]. GPC3 may inhibit IGF and Wnt signaling, which are critical for cell motility and tumor progression, indicating that GPC3 may act as a metastasis suppressor [126, 127]. Another member of GPC family, GPC6, seems to have a key role in promoting the invasive migration of MDA-MB-231 cells through the inhibition of canonical-β-catenin and Wnt signaling and upregulation of noncanonical Wnt5a signaling through the activation of JNK (c-Jun-N-terminal kinase) and p38 MAPK (mitogen-activated protein kinase) [128]. Evidence suggests that GPCs are important in growth factor and morphogens responses, whereas roles in cell adhesion seem to be the prerogative of SDCs [111].

An important member of hyalectans, versican, is able to interact with ECM components and to bind to the cell-surface proteins CD44, integrin β1, and epidermal growth factor receptor (EGFR) [129, 130] to regulate cell processes such as adhesion, proliferation, migration, and ECM assembly [40, 130]. The expression of versican in breast carcinomas has been correlated to invasiveness [131]. Moreover, versican G3 domain enhanced breast cancer cell growth, migration, and metastasis by upregulating the EGFR mediated signaling pathways that contribute to a more metastatic phenotype [48]. Also, versican enhances breast cancer cell metastasis in mouse breast cancer cell lines, not only through facilitating cell motility and invasion but also by inhibiting preosteoblast cell growth and differentiation which supply favourable microenvironments for tumor metastases [132]. Enhanced understanding of the regulation and the involvement of versican in cancer may offer a novel approach to cancer therapy by targeting the tumor microenvironment [12].

The overexpression of decorin in the stroma of solid tumors counteracts cell growth, indicating that decorin may have a protective role in tumor progression [133]. Also, it seems to be a negative regulator for EGF signaling. Decorin's binding to EGFR initially leads to receptor's prolonged activation, followed by EGFR internalization and degradation, eliminating tumor growth and metastases [134]. Iozzo et al. [135] suggest that decorin loss may contribute to increased IGF-IR activity in the progression of breast cancer, where IGF plays a role on cell motility. Another member of SLRPs, lumican, may act as an inhibitor of migration, angiogenesis, and invasion by interfering with $\alpha 2\beta 1$ integrin activity and downregulating MMP-14 expression to induce apoptosis [136]. Moreover, winter action of lumican with growth factors affects mobility, adhesion, and cell growth [137, 138].

Serglycin is the only characterized intracellular PG found in hepatopoietic and endothelial cells [12]. It carriers either heparan sulfate or chondroitin sulfate chains depending on cell type. Korpetinou et al. [139] have shown for the first time that serglycin is highly expressed in an aggressive breast cancer cell line (MDA-MB-231). The same authors demonstrated that the overexpression of serglycin promotes breast cancer cell growth, migration, and invasion [139]. Interestingly, overexpression of serglycin lacking the GAG attachment sites failed to promote these cellular functions, suggesting that glycanation of serglycin is necessary for its oncogenic properties. This study suggests that serglycin promotes a more aggressive cancer cell phenotype and may protect breast cancer cells from complement attack supporting their survival and expansion.

HA is one of the principal ECM molecules and together with its CD44 cell surface receptor, it is implicated in cancer cell invasion and metastasis [62]. Indeed, high levels of HA are documented in malignant tumors, not only to the tumor stroma but also at the cell surface [62]. The elevated levels of the HA degrading, HYAL1 seems to regulate cell growth, adhesion, invasion, and angiogenesis of breast cancer [63]. Basal-like breast cancers (BL-BCa) have the worst prognosis of all subgroups of this disease. Indicatively, HA-induced CD44 signaling increases a diverse spectrum of protease activity including MTI-MMP and cathepsin K, to facilitate the invasion associated with BL-BCa cells, providing new insights into the molecular basis of CD44-promoted

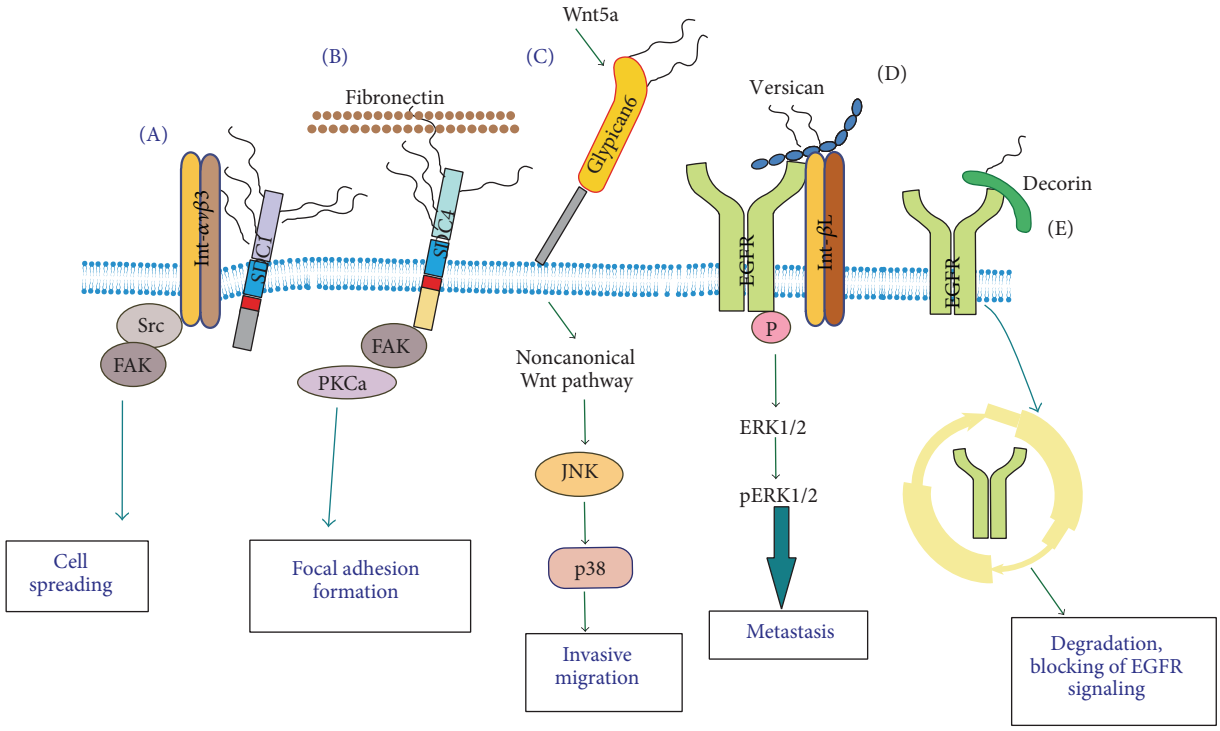

Figure 1: Roles of PGs/GAGs on the breast cancer motile phenotype. (A) Complex formation between syndecan1 (SDC1) and integrin-$\alpha v\beta 3$ activate focal adhesion kinase to facilitate breast cancer cell spreading. (B) The specific binding of SDC4, through its HS chains, to fibronectin activates FAK/protein kinase Ca (PKCa) downstream signaling to initiate focal adhesion formation. (C) Wnt5a attaches to glypican6 in order to enhance breast cancer cell invasive migration through the involvement of noncanonical Wnt pathway and JNK/p38 downstream signaling. (D) Complex formation among versican, EGFR, and Int-βL activates extracellular matrix regulated kinase (ERK1/2) signaling to promote breast cancer metastasis. (E) Binding of decorin to EGFR causes receptor internalization, degradation, and subsequent inhibition of EGFR signaling.

invasion [140]. Moreover, the cell surface HA, which is secreted by breast cancer cells, increases the adhesion ability of tumor cells, to lymphatic endothelial receptor (LYVE-1) [132]. Importantly, the molecular weight of HA seems to play a key role in the process of cell adhesion [141, 142], and particularly low molecular weight of HA promotes cell adhesion, while high molecular weight HA has no effect [143]. Indeed, LMW-HA plays an important role in CD44-TLR-associated AFAP-110-actin interaction and MyD88-NF-κB signaling required for tumor cell behaviors, which may contribute to the progression of breast cancer [143]. The roles of PGs/GAGs on breast cancer cell motile phenotype are schematically depicted in Figure 1.

6. PGs/GAGs Potential Targets in Breast Cancer

In many *in vitro* studies breast cancer cells were treated with various anticancer agents, including inhibitors of tyrosine kinase receptors and other molecules, such as small peptides, which are related to the expression of proteoglycans, in order to observe changes in cell functions [29, 144, 145]. Thus, a new generation of bisphosphonate, zoledronate (zoledronic acid, Zometa), downregulates the expression levels of SDC1, SDC2, and GPC1 and upregulates the expression of SDC4 in breast cancer cells of low and high metastatic capability [144]. Furthermore, the downregulation in the expression of HA and its receptor CD44 which is directly associated with the migration and matrix-associated invasion of breast cancer cells was also observed [144]. Imatinib, a specific tyrosine kinase inhibitor, which targets PDGFRs, had a similar effect on breast cancer cells. Imatinib resulted in an inhibition of the PDGF-BB mediated expression of HSPGs, which is associated with its inhibitory effect on the invasive and migratory potential of breast cancer cells [29]. A different approach was utilized by Rapraeger [145]. These authors used a small peptide, synstatin, to target SDC1. Thus, the site in the SDC1 ectodomain that is responsible for capture and activation of the $\alpha v\beta 3$ or $\alpha v\beta 5$ integrins by IGF1R can be mimicked by this short peptide which competitively displaces the integrin and IGF1R kinase from the syndecan and inactivates the complex. The blocking in the formation of the receptor complex attenuates breast cancer cell metastasis [145].

It has been demonstrated that degradation of HS chains by heparanase 1 (HPSE-1) reveals cryptic HS fragments that play a significant role in controlling tumor cell growth and metastasis. It is thus likely that enzymatic degradation of HS could be used as a potential treatment against carcinogenesis since HS chains are involved in fundamental biological processes of both normal and metastatic cells [146].

Synthetic proteoglycans such as neoheparin and neoCS produced by carbodiimide (EDAC) conjugation of glycosaminoglycan (GAG) chains to a protein scaffold reduce cell viability by induction of apoptosis of myeloma and breast cancer cells *in vitro*. These results demonstrate the anticancer activities of this new class of GAG-based molecules [147].

In summary, this review focused on the roles of PGs/GAGs on breast cancer motility in order to identify possible therapeutic targets. The emerging mechanisms of PG/Gas action could potentially be exploited for designating discrete therapy targets for specific breast cancer grades.

Abbreviations

(PG): Proteoglycan
(GAG): Glycosaminoglycan
(ECM): Extracellular matrix
(SLRPs): Small leucine-rich PGs
(GPC): Glypican
(SDC): Syndecan
(HYAL): Hyaluronidase
(HAS): Hyaluronan synthase
(EMT): Epithelial-to-mesenchymal transition
(MET): Mesenchymal to epithelial transition
(E2): Estradiol
(ERs): Estrogen receptors.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Obligatory Role for Endothelial Heparan Sulphate Proteoglycans and Caveolae Internalization in Catestatin-Dependent eNOS Activation

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The chromogranin-A peptide catestatin modulates a wide range of processes, such as cardiovascular functions, innate immunity, inflammation, and metabolism. We recently found that the cardiac antiadrenergic action of catestatin requires a PI3K-dependent NO release from endothelial cells, although the receptor involved is yet to be identified. In the present work, based on the cationic properties of catestatin, we tested the hypothesis of its interaction with membrane heparan sulphate proteoglycans, resulting in the activation of a caveolae-dependent endocytosis. Experiments were performed on bovine aortic endothelial cells. Endocytotic vesicles trafficking was quantified by confocal microscopy using a water-soluble membrane dye; catestatin colocalization with heparan sulphate proteoglycans and caveolin 1 internalization were studied by fluorimetric measurements in live cells. Modulation of the catestatin-dependent eNOS activation was assessed by immunofluorescence and immunoblot analysis. Our results demonstrate that catestatin (5 nM) colocalizes with heparan sulphate proteoglycans and induces a remarkable increase in the caveolae-dependent endocytosis and caveolin 1 internalization, which were significantly reduced by both heparinase and wortmannin. Moreover, catestatin was unable to induce Ser^{1179} eNOS phosphorylation after pretreatments with heparinase and methyl-β-cyclodextrin. Taken together, these results highlight the obligatory role for proteoglycans and caveolae internalization in the catestatin-dependent eNOS activation in endothelial cells.

1. Introduction

Chromogranin A (CgA) is a 48 kDa acidic glycoprotein [1–3] and the main component of granins, a family of proteins abundantly expressed in large dense core vesicles of neuroendocrine cells, neurons, and other secreting cells including cardiac cells [4]. In the heart CgA is costored and cosecreted, respectively, with catecholamines and natriuretic peptides [4]. Catestatin (CST: $hCgA_{352-372}$) is a 21 amino acid cationic and hydrophobic peptide derived from the proteolytic cleavage of CgA. It was firstly discovered as an endogenous allosteric nicotinic-cholinergic antagonist [5], but it is now established as a multifunctional peptide modulating several organs/systems, including the cardiovascular system [6, 7]. In particular, it has been shown that CST administration induces vasodilation by multiple mechanisms. One mechanism relies on the CST-dependent inhibition of catecholamine secretion, through noncompetitive binding at the nicotinic cholinergic receptor [5, 8]. Another potential mechanism for the CST-induced vasodilation is through histamine release [9, 10], which has been shown *in vitro* in mast cells mimicking the receptor-independent peptidergic pathway proposed for mastoparan and for other cationic and amphipathic peptides [10]. Finally, CST has been shown to decrease sympathetic

vascular tone by a direct excitatory effect on the GABAergic inhibitory neurons of the caudal ventrolateral medulla (CVLM), resulting in decreased sympathetic drive and a subsequent fall in arterial pressure and heart rate [11]. Therefore, CST plays crucial roles in the regulation and development of hypertension. In humans, CST plasma levels are decreased not only in hypertensive patients but also in their still-normotensive offsprings [12]. However, plasma CST level has recently been shown to be elevated in patients with heart failure [13] or with coronary heart diseases [14].

Several studies indicate that exogenous CST rescues hypertension [15] and improves baroreflex sensitivity [16] and heart rate variability [17] in CgA knockout mice.

In addition to its important role in the control of blood pressure, CST is now emerging as a peptide that has direct cardiovascular actions under both basal and stimulated conditions, suggesting that CST-induced negative inotropism and lusitropism may be important components of its hypotensive action [6, 18].

In particular in a previous study we showed a PI3K-dependent nitric oxide (NO) release induced by CST in endothelial cells [18], suggesting one of the intracellular mechanisms underlying the cardiac antiadrenergic action of this peptide.

Based on these findings, the aim of the present study was to look into the initiating step required for this intracellular cascade, as up to now, high affinity membrane receptors for CST remain unknown. We therefore tested the hypothesis of a receptor-independent cell membrane interaction.

This hypothesis was supported by several evidences: CST adopts a β-sheet structure when interacting with negatively charged membranes and may thus directly pass through cell plasma membrane [19], and this property is believed to be responsible for the antimicrobial activity against a wide array of skin pathogens, including bacteria, yeast, and fungi [20, 21]. Moreover, in endothelial cells the CgA-derived peptide vasostatin-1 (VS-1), bearing cationic and amphipathic properties, interacts with cell-surface proteoglycans and activates eNOS phosphorylation on Ser^{1179} residue through a PI3K-dependent endocytosis-coupled mechanism [22, 23]. The above findings prompted us to test whether CST, by virtue of its cationic and amphipathic properties, can work like VS-1.

2. Materials and Methods

2.1. Cell Culture, Solutions, and Drugs. Bovine Aortic Endothelial (BAE-1) cells (European Collection of Cell Cultures, Salisbury, Wiltshire, UK) were maintained in Dulbecco's Modified Eagle's medium (DMEM, Sigma, St. Louis, MO, USA) added with 10% heat-inactivated fetal calf serum (FCS, Biowhittaker, Verviers, Belgium, lot 1SB0019), 100 U/mL penicillin, 100 μg/mL streptomycin, and 2 mM glutamine, at 37°C, 5% CO_2. Cells were used at passages 2–6 and maintained in 1% FCS 24 h before the experiments. MβCD, H:ase, and Wm were purchased from Sigma.

Tyrode's standard solution used for cell washes in colocalization experiments contained (mM) 154 NaCl, 4 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 5.5 D-glucose, 5 Hepes, pH adjusted to 7.34 with NaOH.

CST was a kind gift by Professor Mahata and was synthesized by the solid-phase method, using 9-fluorenylmethoxycarbonyl protection chemistry [24]. The concentration of CST tested on BAE-1 cells was 5 nM, in the range of the circulating levels of CgA (1.36 nM) found in healthy humans [12].

Cy3-CST was purchased from Phoenix Pharmaceuticals.

2.2. Antibodies. The expressions of total eNOS and β-actin were detected with monoclonal antibodies (Invitrogen and BD Biosciences, resp.), while $P^{Ser1179}$eNOS and Caveolin 1 (Cav1) were evidenced with polyclonal antibodies that were purchased, respectively, from Invitrogen and Sigma. heparan sulphate was stained with a mouse monoclonal antibody (MAB2040, Millipore) that we labeled with Alexa Fluor 488 using the APEX Antibody Labeling Kit (Invitrogen).

The secondary antibodies employed for immunofluorescence experiments were Alexa Fluor 488 anti-mouse (Molecular Probes) for total eNOS and Cy3 anti-rabbit (Sigma) for $P^{Ser1179}$eNOS and Cav1. For Western blot experiments we used horseradish peroxidase-conjugated secondary antibodies: anti-mouse for β-actin (Invitrogen) and anti-rabbit for $P^{Ser1179}$eNOS (Amersham).

2.3. Immunofluorescence and Confocal Microscopy. Cells grown on cover slides were fixed for 20 minutes in 4% paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.3. After three washes with Dulbecco's phosphate buffer saline (PBS), cells were incubated 20 minutes with 0.3% Triton and 1% bovine serum albumin (BSA, Sigma) in PBS and stained with the primary antibody 24 h at 4°C. Cover slides were washed twice with PBS and incubated 1 h at room temperature with the secondary antibody. After two washes in PBS cover slides were mounted on standard slides with DABCO (Sigma) and observed after 24 h under confocal microscope. Fluorimetric measurements were also performed with confocal microscopy, using an Olympus Fluoview 200 laser scanning confocal system (Olympus America Inc., Melville, NY, USA) mounted on an inverted IX70 Olympus microscope, equipped with a 60X Uplan Fl (NA 1.25) and a 100X Uplan Fl (NA 1.3) oil-immersion objectives. Image processing and analysis were performed with ImageJ software (Rasband, W.S., U.S. National Institutes of Health, Bethesda, MA, http://rsb.info.nih.gov/ij/, 1997–2013).

2.4. Western Blot Analysis. BAE-1 cells were lysed with lysis buffer (100 mM Tris HCl, pH 8.0, 1 mM $MgCl_2$, plus inhibitor cocktail) and incubated at −80°C overnight. An equal volume of sucrose buffer containing 20 mM Tris Hepes pH 7.4 and 315 mM sucrose plus inhibitor cocktail was added and cell lysate was forced throughout a 25-gauge needle attached on a 1 mL syringe for several times. The inhibitor cocktail contained 2 μg/mL aprotinin, 0.1 mM PMSF, 1 mM sodium orthovanadate, and 20 mM sodium fluoride. Protein lysates

(15 μg of protein per lane) were run on 8% gradient SDS-PAGE gel, transferred to a polyvinylidene fluoride membrane (PVDF; Millipore), and blocked overnight in TBST (10 mM Tris-HCl pH 7.5, 0.1 M NaCl, and 0.1% Tween 20) plus 5% nonfat dry milk (Biorad). PVDF was incubated, with gentle agitation, 1 h at 30°C with a polyclonal anti-$P^{Ser1179}$eNOS antibody. Membranes were washed three times with TBST and were incubated 1 h at room temperature with horseradish peroxidase-conjugated secondary antibodies before being washed again three times with TBST. Protein band detection was performed by chemiluminescence using the Super Signal West Pico Kit (Pierce).

2.5. Endocytotic Vesicles Trafficking. The water-soluble membrane dye N-(3-triethylaminopropyl)-4-(p-dibutylaminostyryl) pyridinium dibromide (FM 1-43, Invitrogen) was used to label plasmalemma-derived vesicles, as previously described [25], and therefore to quantify endocytosis by confocal microscopy. BAE-1 cells grown on glass-bottom dishes (MatTek Corporation) were incubated at 37°C-5% CO_2 for 15 min in PBS containing 5 μg/mL FM 1-43, for control condition and in Hepes buffered saline solution (HBSS) plus 5 nM CST for experimental condition. Before fixation for 20 minutes in 4% paraformaldehyde, cells were washed three times in ice-cold dye-free PBS containing BSA (6 mg/mL, fraction *V*, 99% pure, endotoxin free; Sigma) to remove all unincorporated fluorescent probes from the external surface of endothelial cells. To investigate the involvement of heparan sulphate proteoglycans (HSPGs) in CST-dependent processes, we pretreated BAE-1 cells with 2 U/mL Heparinase III (H:ase) from *Flavobacterium heparinum* (Sigma) for 3 h at 37°C, 5% CO_2. After two washes with PBS, cells were incubated for 15 min with PBS plus 5 nM CST and we proceeded as described above. To investigate the role of PI3K pathway in the formation of vesicles, we pretreated BAE-1 cells with the PI3K inhibitor Wortmannin (Wm, 100 nM) for 20 min and then we added 5 nM CST. For each experiment we randomly acquired three fields/sample. Endocytosis quantification was performed with ImageJ software: briefly, after creating the *z*-axis reconstruction (i.e., average of the slices) of the stack, the quantity of intracellular stained vesicles was analyzed by evaluating the fluorescence intensity/cell/field.

2.6. Colocalization Studies with CST and HSPGs. Monoclonal anti-heparin/heparan sulfate antibody (anti-HSPGs MAB2040, Millipore) was conjugated with Alexa Fluor 488 using a commercial kit (APEX Antibody Labeling Kit, Invitrogen); briefly 10 μL of Alexa Fluor-anti-HSPGs was freshly prepared before the experiment. Fluorescent CST (Cy3-CST) was purchased from Phoenix Pharmaceuticals. BAE-1 cells grown on 35 mm glass bottom dishes were treated at 4°C for 10 min with Alexa Fluor-anti-HSPGs, followed by a rapid (2 min) exposure to Cy3-CST (5 nM), and after two washes in Tyrode's solution cells were rapidly transferred to the stage of the confocal microscope. Single fields were captured (60x objective) using separately 488 and 568 nm laser lines to avoid bleed through and ensure specific fluorescence. Images were analysed with the ImageJ/Fiji command "Colocalization Threshold."

2.7. Transfection with GFP-Cav1. Transfection of BAE-1 cells was performed with the cationic liposome Lipofectamine (Lipofectamine 2000TM, Life Technologies, Carlsbad, CA). Endothelial cells were seeded into P-10 flasks (Costar, Cambridge, MA) at 30% confluence and allowed to attach and grow to reach 90% confluence; transfection was performed using 1 μL of Lipofectamine 2000TM and 1 μg of GFP-Cav1 plasmid DNA in 120 μL of Opti-MEM (Life Technologies) for 6 h at 37°C, 5% CO_2. Before observations, transfected BAE-1 cells were incubated 24 h to allow protein expression. Confocal fluorimetric measurements were performed as described in the "Immunofluorescence and Confocal Microscopy" section.

2.8. Statistical Analysis. All values are presented as the mean ± S.E.M. Statistical comparisons were performed with ANOVA analysis followed by Bonferroni correction for post hoc tests. Significance was accepted at a P level < 0.05.

3. Results

3.1. CST Induces Endocytotic Vesicles Formation That Is Abolished by Both Heparinase and Wortmannin. The role of CST in the endocytotic process was evaluated by incubating BAE-1 cells with the styryl pyridinium membrane probe FM 1-43 (5 μg/mL), in order to visualize plasmalemma-derived endocytotic vesicles.

To quantify these results we randomly acquired three fields per sample in each experiment and then evaluated the fluorescence intensity value/n° cell/field (see Section 2), in control condition and after CST stimulation.

We observed that CST (5 nM) induced a significant increase in the FM 1-43 fluorescence (Figure 1), indicating a stimulation of the endocytotic process.

As the molecular properties of CST (little, amphipathic, and cationic peptide) resemble those of the polycationic peptides (cell penetrating peptides or CPPs), we hypothesized that the first contact of this peptide with the cell surface takes place through proteoglycans, as we previously demonstrated for VS-1 [22]; internalization mechanisms mediated by HSPGs interaction involving different routes of endocytosis have indeed been described for several CPPs [26].

In order to verify this mechanism, HSPGs were selectively removed from the cell surface by pretreatment of cells with H:ase (2 U/mL). After this step, CST strongly reduced its ability to stimulate endocytosis (Figure 1), suggesting that CST binding to HSPGs is fundamental to start its intracellular cascade.

In a recent work we showed that in BAE-1 cells CST induced a PI3K-dependent NO release [18]. To investigate the role of PI3K pathway also in CST-dependent formation of endocytotic vesicles, we performed FM 1-43 detection in the presence of the PI3K inhibitor Wm (100 nM). As shown in Figure 1, pretreatment with Wm strongly reduced the CST-activated endocytosis.

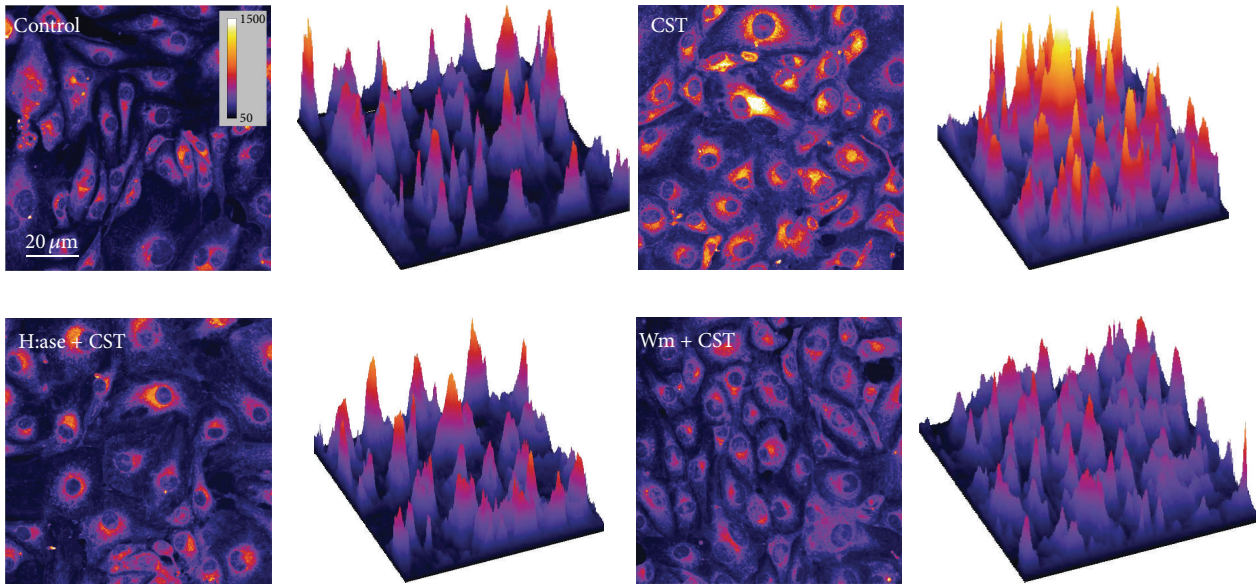

Figure 1: CST stimulates a PI3K-proteoglycan dependent endocytotic machinery in BAE-1 cells. BAE-1 cells incubated with the water-soluble styryl pyridinium membrane dye (FM 1-43). Pseudocolor images better show the fluorescence intensity increase correlated with the rise in vesicles formation consequent to stimulation with CST 5 nM. In CST + H:ase and CST + Wm samples fluorescence intensity was comparable to control levels. Surface plots close to each image display 3D graphs of pixels intensities in a pseudocolor image. The height and the color represent the pixel intensity.

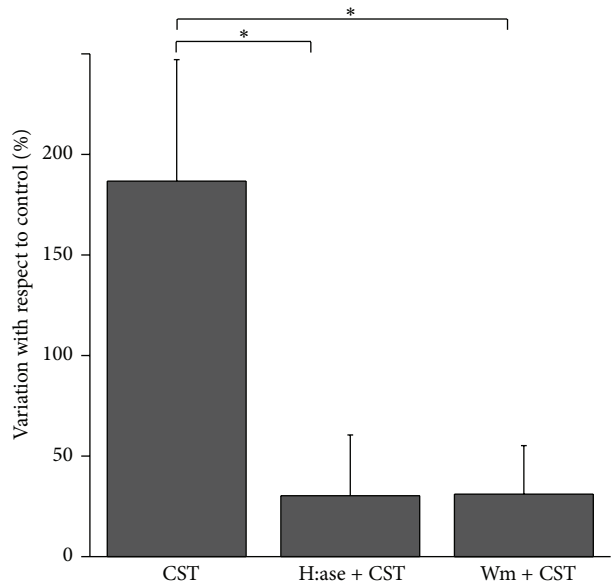

Figure 2: Endocytosis quantification. To quantify the endocytotic process stimulated by CST 5 nM, we evaluated FM 1-43 fluorescence intensity value/n° cell/field (see Section 2) in the different experimental conditions. The bar graph shows the variation of fluorescence intensity/n° cell/field with respect to control. CST = 186.8 ± 60.36%; H:ase + CST = 30.36 ± 30.24%; Wm + CST = 31.09 ± 24.11%; $n = 7$ endocytosis experiments (3 fields/sample, about 35 cells/fields); $P < 0.05$.

The bar graph in Figure 2 summarizes this set of experiments (percentage of fluorescence intensity/cell/field increase above control: CST = 186.8 ± 60.36%; H:ase + CST = 30.36 ± 30.24%; Wm + CST = 31.09 ± 24.11%; $n = 7$ endocytosis experiments: 3 fields/sample, about 35 cells/fields; $P < 0.05$).

3.2. CST Colocalizes with Heparan Sulphate Proteoglycans. To prove the interaction between CST and HSPGs we performed colocalization experiments in live cells by using Cy3-CST and Alexa Fluor 488-anti-HSPGs.

In these experiments the fluorescent antibody against heparan sulphate (1 : 200) was incubated 10 min at 4°C (to

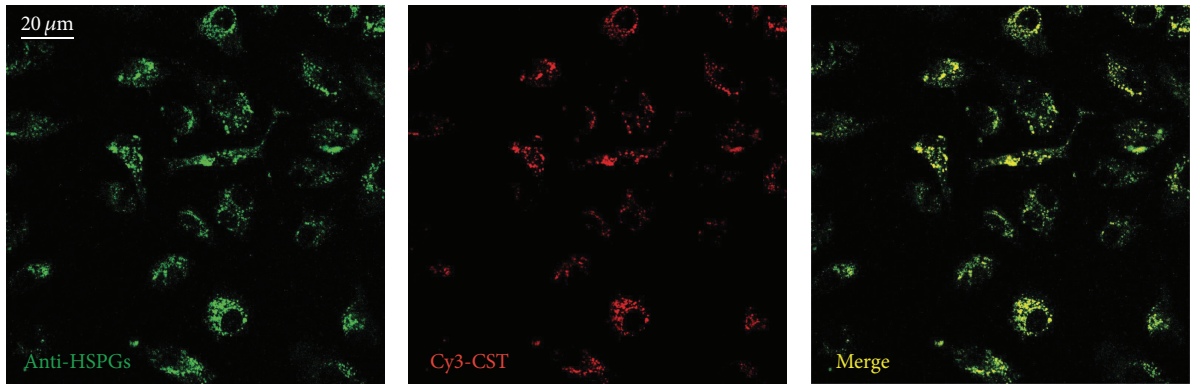

Figure 3: CST colocalizes with HSPGs. Representative confocal fluorescence images of BAE-1 cells incubated with both Alexa Fluor 488-anti-HSPGs (green signal) and Cy3-CST (red signal), showing the high levels of colocalization for CST and HSPGs (% of colocalization = 73.4 ± 0.02%, 3 separate experiments, 6 fields/sample, about 25 cells/field).

avoid endocytosis) with BAE-1 cells cultured on glass bottom dishes. The excess of antibody was removed by two washes and then cells were incubated 2 min at 4°C with 5 nM Cy3-CST. After two additional washes, cells were observed in confocal microscopy.

The results from these experiments (Figure 3) reveal that the colocalization between CST and HSPGs was very high (% of colocalization = 73.4 ± 0.02%, 3 separate experiments, 6 fields/sample, about 25 cells/field).

3.3. CST Induces Caveolin-1 Mobilization. As CST stimulates NO production [18] and several reports propose a mechanism of eNOS activation involving caveolae endocytosis [27, 28], we hypothesized that the endocytotic process triggered by CST was caveolae-dependent. To verify this hypothesis we followed the cellular localization of Cav1 by transfecting BAE-1 cells with GFP-Cav1. Cells were transfected with high efficiency (up to 95%) with GFP-Cav1 plasmid (see Section 2).

In transfected live cells GFP-Cav1 signal was confined in plasma membranes, while in the presence of CST 5 nM green fluorescence appeared clearly diffused in the cytosol, as a consequence of Cav1 internalization, thus producing a substantial increase of the overall fluorescent signal; pretreatment with H:ase strongly reduced CST ability to stimulate this process (Figures 4(a) and 4(b); percentage of fluorescence intensity variation above control: CST = 86.94 ± 25.51%; H:ase + CST = −0.07 ± 22.20%; n = 3 sets of experiments, 3 fields/sample, about 40 cells/fields; $P < 0.05$).

In order to further analyze the internalization of GFP-Cav1 in transfected BAE-1 cells, we evaluated GFP fluorescence values in specific regions localized in plasma membrane and in cytosol during CST 5 nM administration.

We found (Figure 4(c)) that during CST treatment GFP-Cav1 fluorescence intensity decreased in the plasma membrane while being increased in the cytosol (percentage of fluorescence intensity with respect to control: membrane= −19.34 ± 1.22%; cytosol = 122.60 ± 3.49%; n = 17 cells; $P < 0.05$). These results confirm that CST administration induces Cav1 displacement from plasma membrane; pretreatment of BAE-1 transfected cells with H:ase reverts CST effect (percentage of fluorescence intensity with respect to control: membrane = 54.27 ± 9.37%; cytosol = −98.46 ± 0.09%; n = 17 cells; $P < 0.05$; data not shown).

3.4. CST Breaks Caveolin-1/eNOS Colocalization. As CST stimulates endocytosis, induces Cav1 internalization, and, as shown in our previous report, enhances NO production [18] (Bassino et al., 2011), we hypothesized a mechanism for eNOS activation mediated by the displacement of the protein from Cav1 binding. Dissociation of eNOS from Cav1 has been indeed shown as a marker of eNOS activation [29].

To verify this hypothesis we followed cellular colocalization of Cav1 and eNOS by immunofluorescence experiments (Figure 5). We observed that CST strongly reduced eNOS/Cav1 colocalization at plasma membrane detected in control condition. Moreover, Wm was able to restore this colocalization, confirming the role of PI3K in mediating CST intracellular signaling.

To quantify colocalization results, Pearson correlation coefficients were calculated for each experimental condition (Figure 5(b); control = 0.84 ± 0.09; CST = −0.07 ± 0.05; Wm + CST = 0.69 ± 0.18; n = 8 sets of experiments, 3 fields/sample, about 30 cells/fields; $P < 0.05$).

3.5. Caveolae Disruption and HSPGs Removal Both Abolish CST-Induced eNOS Phosphorylation. To strongly demonstrate the proposed pathway of a CST-dependent caveolae internalization and eNOS activation triggered by HSPGs, we evaluated the level of CST-induced $P^{Ser1179}$eNOS after both caveolae disruption by methyl-β-cyclodextrin (MBCD, 5 mM) and HSPGs removal (by H:ase, 3 h). Our results from Western blot experiments (Figure 6) showed a significant reduction of the CST-dependent eNOS phosphorylation in both conditions (%$P^{Ser1179}$eNOS/β-actin: control = 3.59 ± 1; CST = 18.81 ± 1.57; MβCD + CST = 3.48 ± 1.5, H:ase + CST = 1.63 ± 0.41; n = 3; $P < 0.05$), confirming the obligatory requirement of proteoglycans and caveolae integrity to allow the CST-dependent intracellular pathway activation.

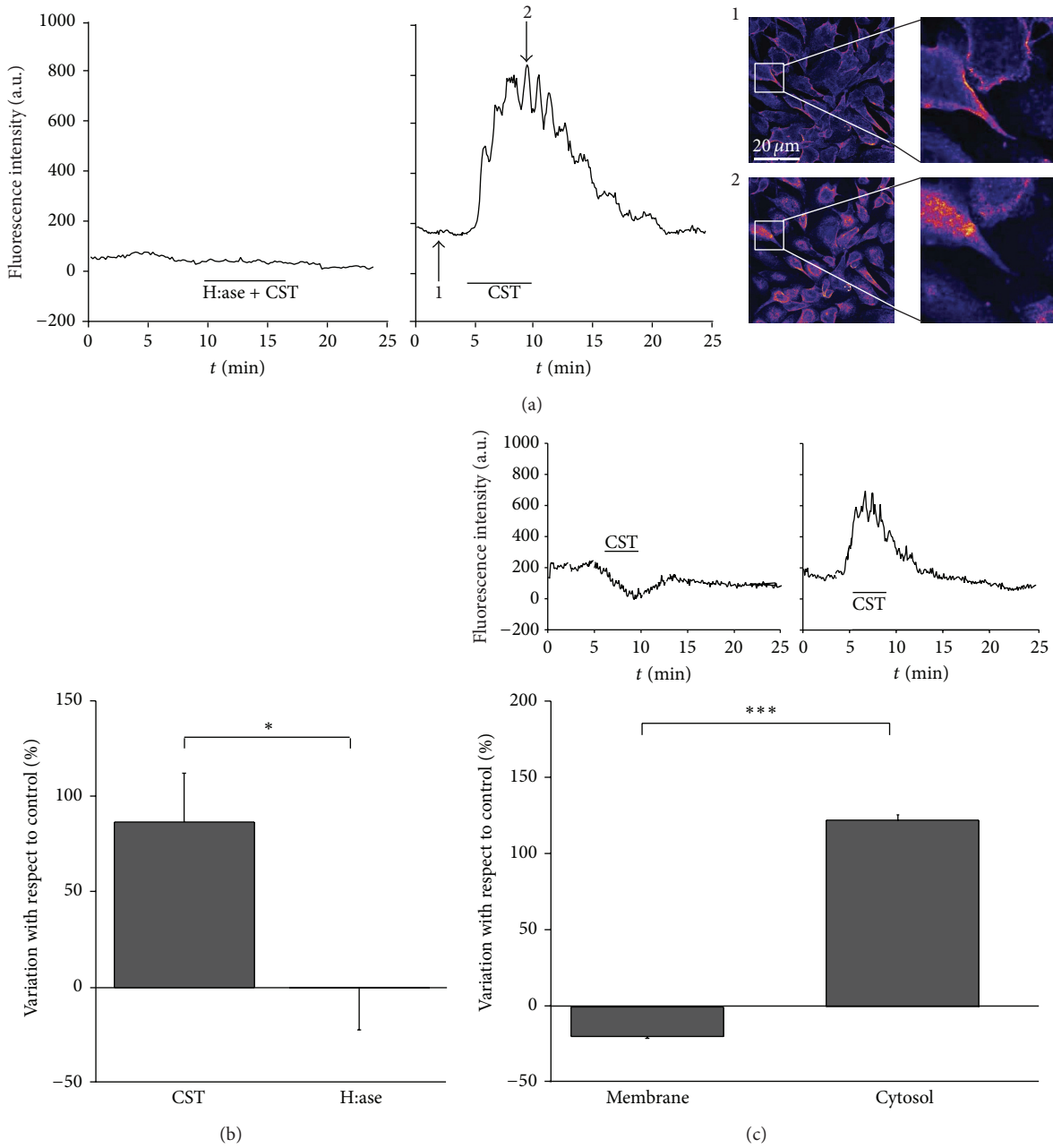

FIGURE 4: CST induces proteoglycan-dependent Cav1 internalization in GFP-Cav1 transfected BAE-1 cells. (a) Representative time course of the fluorescence intensity in single BAE-1 cells transfected with Cav-1-GFP and stimulated with H:ase + CST or with CST alone. Subpanels 1 and 2: confocal pseudocolor images of CST-treated BAE-1 cells from the correspondent time points indicated by the arrows, showing membrane (1) and cytosolic (2) localizations of GFP-Cav1 after CST treatment. (b) Bar graphs representing the percent variation with respect to control of the fluorescent GFP-Cav1 signal in the different experimental conditions (CST = 86.94 ± 25.51%; H:ase + CST = −0.07 ± 22.20%; $n = 3$ sets of experiments, 3 fields/sample, about 40 cells/fields; $P < 0.05$). (c) Bar graph representing the percent variation with respect to control of the fluorescent GFP-Cav1 signal during CST 5 nM treatment, respectively, in plasma membrane and in cytosol (membrane = −19.34 ± 1.22%; cytosol = 122.6 ± 3.49%; $n = 17$ cells; $P < 0.05$).

4. Discussion

Our results show that in endothelial cells CST activates eNOS through HSPGs and caveolae-dependent endocytotic mechanism. The findings are consistent with our previous results showing that proteoglycans/PI3K-dependent caveolae endocytosis acts as the initiating factor for the intracellular cascade activated in endothelial cells by VS-1, the major N-terminal peptide derived from CgA [22, 23]. Our previous studies identifying a PI3K-dependent NO release from endothelial cells as the intracellular mechanisms involved in the cardiac antiadrenergic action of CST [18] prompted us to

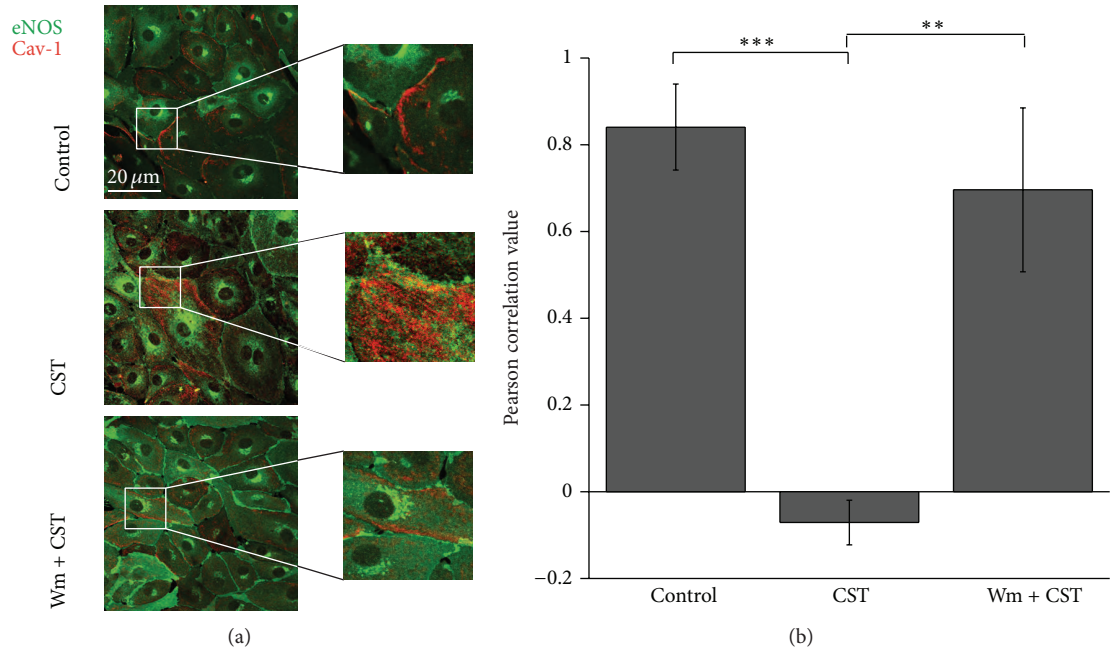

Figure 5: CST prevents Cav1/eNOS colocalization in a PI3K-dependent manner. (a) Confocal immunofluorescence images of BAE-1 cells showing the typical membrane localization of Cav1 (red staining) and eNOS (green staining). After CST administration, Cav1 dissociated from eNOS and staining diffused in the cytosol. This effect was reversed by Wm pretreatment. Immunofluorescence detection was carried out using Alexa Fluor 488 anti-mouse for total eNOS and Cy3 anti-rabbit for Cav1. Scale bar: 20 μm. (b) Bar graph representing Pearson correlation values relative to Cav1 and eNOS staining in each experimental condition (control = 0.84 ± 0.09; CST = −0.07 ± 0.05; Wm + CST = 0.69 ± 0.18; n = 8 sets of experiments, 3 fields/sample, about 30 cells/fields; $P < 0.05$).

uncover the steps upstream the intracellular cascade activated by CST in endothelial cells.

First, we found that CST stimulates endocytotic vesicles formation (Figure 1), which corresponds with the biochemical reports on CST, reporting that this peptide, like other members of the CPPs family, exhibits membrane-interaction properties because of both its amphipathic structure and extended hydrophobic region. In particular, CD and NMR data indicate that CST folds into a short helical conformation that interacts with membranes and causes considerable disordering at the level of the phospholipid head groups. Moreover, two of the five residues of the helical region of CST are arginines, an amino acid that has been proposed to form hydrogen bond interactions with phospholipids [19].

It is widely accepted that some CPPs can directly translocate across the plasma membrane of cells. CPPs-mediated toxicity, manifested as a general increase in plasma membrane permeability, could reflect some of these observed features [30]. In this context, the ability of CST to target various microorganisms such as bacteria, fungi, and parasites [21] and also host cells such as neutrophils by a direct interaction with plasma membranes [31] falls in this general property of CPPs.

However, direct translocation has been observed only at high concentrations of CPPs (>10 μM), while lower doses were shown to activate endocytotic mechanisms. For instance, studies of leukaemia cells showed that still at 2 μM extracellular concentration R8-Alexa488 labeled vesicular structures, which were shown to be endosomes and lysosomes. Upon raising the concentration to 10 μM, instead, the peptide was seen to flood into cells giving, in most cases, uniform labeling throughout the cytoplasm and nucleus [32]. In this perspective our results with CST 5 nM, higher than but comparable to the circulating concentrations of CgA found in healthy humans [12] (0.5–2 nM), are consistent with the activation of a more restricted endocytotic pathway.

Furthermore, our experiments also show that CST-activated endocytosis required the presence of HSPGs (Figures 1 and 2) on the surface of endothelial cells and that CST colocalizes with HSPGs (Figure 3). The strong anionic charge present in proteoglycans makes them favorable binding sites for cationic polymers, lipids, and polypeptides, which are used for drug and gene delivery [33, 34]. There are evidences that negatively charged carbohydrates, like HSPGs, located on the plasma membrane may serve as electrostatic traps for the cationic CPPs [30]. Interestingly, the most prominent glycosaminoglycans on the surface of endothelial cells are precisely heparan sulphates and one of the major protein core families of HSPGs is the membrane-bound glypicans, that are enriched in caveolae, where a series of molecules involved with eNOS signalling are localized [35, 36]. Furthermore, glypican-1 has been hypothesized to be the mechanosensor for eNOS phosphorylation and activation in the shear stress-induced response [37]. It could be speculated that the CST-mediated membrane perturbation through HSPGs binding and phospholipid interactions could resemble the acute membrane perturbation involved in shear stress.

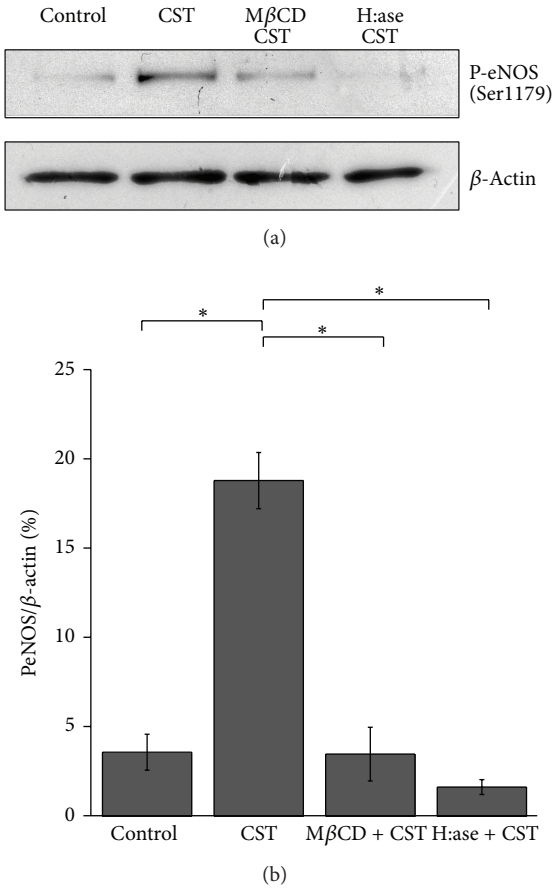

Figure 6: Both proteoglycans and caveolae are required to allow CST-dependent eNOS phosphorylation. (a) Typical Western blot experiment showing that CST-induced $P^{Ser1179}$eNOS was reduced by both MβCD (5 mM, 30 min) and H:ase (2 U/mL). (b) $P^{Ser1179}$eNOS/β-actin ratio of densitometric values from Western blots (%$P^{Ser1179}$eNOS/β-actin: control = 3.59 ± 1; CST = 18.81 ± 1.57; MβCD + CST = 3.48 ± 1.5, H:ase + CST = 1.63 ± 0.41; $n = 3$; $P < 0.05$).

This matter, together with our previous finding of CST-dependent eNOS activation [18], led us to propose a CST-induced mechanism of caveolae endocytosis and consequent eNOS activation. These assumptions are supported by our findings, from both Cav1 transfection and Cav1/eNOS immunofluorescence and colocalization experiments. Previous reports have proposed a mechanism of eNOS activation coupled with caveolae internalization [27, 28] and dissociation of eNOS from Cav1 has been shown as a marker of eNOS activation [29, 36].

In addition, we found that PI3K activity was required in both endocytosis and eNOS/Cav1 trafficking, thus representing the essential key for the CST-activated intracellular signaling. In agreement with these results, it is well known that PI3K/Akt mediated Ser^{1179} phosphorylation represents a common pathway among the multiple regulatory mechanisms affecting eNOS activity [36] and PI3K is widely reported to have an important role in membrane budding and fission in endothelial cells [38].

Finally with the last experiments (Figure 6) we confirmed our proposed pathway showing that caveolae disruption and HSPGs removal both abolished the CST-induced eNOS phosphorylation (Figure 6).

5. Conclusion

Based on our previous data on the CgA derived peptide VS-1 and the present findings, we hypothesize a novel signal transduction pathway for endogenous cationic and amphipathic peptides in endothelial cells: HSPGs interaction and caveolae endocytosis, coupled with a PI3K-dependent eNOS phosphorylation.

Moreover, giving the wide range of processes, such as innate immunity, inflammatory and autoimmune reactions, cardiovascular modulations, and several homeostatic regulations [39–45] affected by CST, the understanding of its physiological working represents a founding point for further applications.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

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Review Article

Collagen VI and Hyaluronan: The Common Role in Breast Cancer

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Collagen VI and hyaluronan are widely distributed extracellular matrix macromolecules that play a crucial role in tissue development and are highly expressed in cancers. Both hyaluronan and collagen VI are upregulated in breast cancer, generating a microenvironment that promotes tumour progression and metastasis. A growing number of studies show that these two molecules are involved in inflammation and angiogenesis by recruiting macrophages and endothelial cells, respectively. Additionally, collagen VI induces epithelial-mesenchymal transition that is correlated to increased synthesis of hyaluronan in mammary cells. Hyaluronan has also a specific role in cellular functions that depends mainly on the size of the polymer, whereas the effect of collagen VI in tumour progression may be the result of the intact molecule or the C5 peptide of α3(VI) chain, known as endotrophin. Collectively, these findings strongly support the parallel role of these molecules in tumour progression and suggest that they may be used as prognostic factors for the breast cancer treatment.

1. Introduction

The mammary gland consists of a branching ductal system that ends in terminal ducts known as ductal-lobular units (TDLUs) and an interlobular matrix consisting of fat and fibrous tissues that represent the extracellular matrix (ECM) of adipocytes and stromal fibroblasts [1]. Most breast cancers arise in the ductal-lobular units that are characterized by the presence of a basement membrane and luminal, myoepithelial, and progenitor/stem cells [1–3]. Progression of the ductal hyperproliferation involves the *in situ* and invasive carcinoma and finally the metastasis.

ECM is composed of a complex mixture of macromolecules and a variant of secreted molecules, such as growth factors, that through binding to specific ECM molecules and membrane receptors may activate signalling pathways that control or alternate cell behaviours [4]. The principal proteinaceous components of the ECM are collagens that are produced and secreted by a variety of stromal cells and provide much of the scaffold necessary for the organization of cells that constitute the tissue. Collagen VI is widely distributed in the ECM of various tissues, such as skeletal muscle and adiposal tissue, where it forms a discrete network of beaded microfilaments that interact with other ECM molecules and provide structural support for cells [5]. Furthermore, it activates signaling pathways that regulate cellular functions, such as angiogenesis and autophagy [6, 7]. A second class of molecules that play an essential role in the composition of the ECM is proteoglycans (PGs) and especially the polysaccharidic chains, known as glycosaminoglycans (GAGs), which are covalently bound to the protein core [8]. Hyaluronan is the only GAG that is not bound to a protein but has a fundamental role in tissue homeostasis, as it traps high amounts of water. The role of hyaluronan in cell functions depends on its size and the type of specialized glycoprotein receptors present on the cell membrane. The ECM is not a static structure but is constantly being remodelled by proteolytic enzymes such as the matrix metalloproteinases (MMP) or by the cells through internalization and degradation by lysosomal enzymes, as what happens in the case of hyaluronan [9]. All stages of

breast cancer are characterized by an alternate hyaluronan size and deposition in breast tissue stroma that enhances the tumour cell proliferation, invasion, and migration.

Taking into consideration that breast cancer is one of the leading causes of cancer related deaths among women, exploring the alterations of ECM of mammary gland and the role in tumour processes is of central importance in cancer biology. In fact, an increase of collagen and fat deposition in the ECM, including the collagen VI secreted by adipocyte cells, indicates a high mammographic density that is correlated to an increased risk to present breast cancer [10]. In this review, we are willing to present the role of two important ECM components in breast cancer progression: hyaluronan and collagen VI. Both molecules are implicated in the regulation of a number of cell and tissue processes, and for this reason it is worth clarifying the molecular mechanism underlying their contribution to breast tumor progression. Here, we are focused on the role of hyaluronan and collagen VI in breast cancer related angiogenesis, inflammation, and epithelial-mesenchymal transition (EMT), as well as the potential use as serum biomarkers in cancer diagnosis.

1.1. Hyaluronan Properties and Its Role in Biological Mechanisms. Hyaluronan is unique among the GAG family as it is the only polymer that is nonsulfated and not bound to a protein core. The structure of hyaluronan is characterized by repeating disaccharide units of D-glucuronic acid and N-acetyl-D-glucosamine linked by a glucuronidic $\beta(1 \rightarrow 3)$ bond. The disaccharide units are then linearly polymerized by hexosaminidic $\beta(1 \rightarrow 4)$ linkages. The number of repeating disaccharides in a completed hyaluronan molecule can reach 10,000 or more and can reach a molecular weight of 6 to 8 MDa. The average length of a disaccharide is about 1 nm. Thus, a hyaluronan molecule of 10,000 repeats could extend 10 μm if stretched from end to end, a length approximately equal to a diameter of a human erythrocyte.

Hyaluronan is ubiquitously distributed in vertebrates, whereas it has been identified in some of the lower marine organisms and in certain bacteria. It is a main component of all connective tissue and ECM in mammals and is more abundant in the rapidly growing fetal tissues, especially in the first weeks of gestation [11], than in mature adult tissues. Indeed, hyaluronan-rich environment promotes cell proliferation and migration which is important during embryogenesis for the rapid move of stem cells to the site of organ development [12]. On the other hand, the hyaluronan-rich environment actively inhibits differentiation of cells and cells must lose their hyaluronan coat in order to differentiate.

Despite the simple structure of hyaluronan, the importance that is exhibited in biological mechanisms depends on the molecular size. High molecular weight (HMW) hyaluronan forms complexes with other macromolecules of the ECM, regulating the cell adhesion, motility, and growth, whereas hyaluronan oligosaccharides regulate cell behaviours through interaction with cell surface receptors [13]. Thus, the fine regulation of synthesis or degradation to different size of hyaluronan is significant for the physiology of the tissues and is also crucial in malignancies.

Hyaluronan is closely associated with tumourigenesis as it influences tumour cell behaviour and cancer progression by modulating the hydration and osmotic balance in the tumour environment. By interaction with specific receptors, hyaluronan is capable of intracellular signal transduction that can promote the malignant phenotype [14]. Several studies have reported a relation between HA content and invasiveness, as well as a greater enrichment of hyaluronan in the stroma that surrounds tumours than in parenchymal regions. Other studies have shown that hyaluronan production by stromal cells is stimulated by interactions with tumour cells, but that synthesis is also increased in malignant tumour cells themselves. In patients with cancer, hyaluronan concentrations are usually higher in malignant tumours than in corresponding benign or normal tissues, and in some tumour types the level of hyaluronan is predictive of malignancy. However, hyaluronan levels can be increased around tumour cells themselves or within the tumour stroma. In patients with breast and ovarian carcinomas, high levels of hyaluronan in the stroma are associated with low survival rates [12].

1.2. Hyaluronan Synthesis and Catabolism: Hyaluronan Synthase (HAS). Hyaluronan is synthesized by a family of enzymes, called hyaluronan synthases (HASs). In mammals, there are three HAS isoforms (HAS1, HAS2, and HAS3) that are expressed by genes located in different chromosomes and share 55–71% sequence identity [15]. The HAS proteins have molecular masses from 42 to 64 kDa and differences in enzymatic properties particularly in their ability to form hyaluronan matrices and determine product size [16] under the control of a wide variety of cytokines and growth factors. The gene expression of HASs in mammals is tissue and cell specific and varies between normal and pathologic conditions. All HAS proteins are localized within the plasma membrane, containing multiple membrane-spanning regions and large cytoplasmic loops. The catalytic activity of HASs resides in the inner face of membrane with active sites for the two precursors, UDP-glucosamine and UDP-glucuronic acid, and the product is secreted or translocated through the HAS protein complex to the ECM [17].

The changes in hyaluronan synthesis can be related to HAS mRNA expression, to availability of the UDP-sugar precursors, or to posttranslational modifications [18]. In particular, it was demonstrated that, at low ATP/AMP ratios, HAS2 is phosphorylated by the adenosine monophosphate activated protein kinase (AMPK) at threonine 110, which resides in a cytoplasmic loop, resulting in a decreased hyaluronan synthesis [19]. Moreover, HAS2 activity is regulated by oligomerization and monoubiquitination at lysine 190 [20]. Recently, it was shown that HAS2 can also undergo regulation by O-GlcNAcylation that has an important impact on alterations in metabolism, since in hyperglycaemic conditions there is an increase of UDP-GlcNAc that, in turn, leads to an increase of O-GlcNAcylation [21].

In breast cancer, expression of HAS2 is mainly in metaplastic carcinomas of breast, which is a subtype related to the EMT, and less expressed in invasive ductal carcinomas [22]. Moreover, expression levels of stromal HAS1 and HAS2 during breast tumour are related to obesity, large tumor

size, lymph node positivity, and estrogen receptor negativity [23]. Both HAS2 and HAS3 synthesize and extrude HMW-hyaluronan, which in invasive cell lines is rapidly depolymerised into fragments ranging from 10 to 40 kDa that are involved in neovascularisation [14]. Suppression of HAS2 in MDA-MB-231 breast cancer cell line displayed decreased cellular proliferation with a transient arrest in a cell cycle, ablation of migratory phenotype, alteration in hyaluronan catabolism, and inhibition of primary and secondary tumour formation [14]. Recently, it was demonstrated that HAS2 expression in NMuMG mammary epithelial cells is required for the TGF-β-mediated EMT [24].

1.3. Hyaluronan Synthesis and Catabolism: Hyaluronidase (HYAL). Hyaluronan has a high turnover in the body, as one third is degraded each day. A major part of the circulating hyaluronan is taken up by the liver and a minor part by the kidneys [25]. In joints 20–30% of hyaluronan is catabolised by local degradation. The lymphatic tissue carries HA to the blood stream where 80–90% is degraded by receptor mediated catabolism [26]. The hyaluronan degrading enzymes, known as hyaluronidases (HYALs), hydrolyze predominantly the hexosaminidic $\beta(1 \rightarrow 4)$ linkages between N-acetyl-D-glucosamine and D-glucuronic acid residues. These enzymes also hydrolyze $\beta(1 \rightarrow 4)$ glycosidic linkages between N-acetyl-galactosamine or N-acetylgalactosamine sulfate and glucuronic acid in chondroitin and dermatan sulfates [27]. From the expressed sequence tag (EST) database, there are established six such sequences in the human genome. In the human, three genes (HYAL1, HYAL2, and HYAL3) are found tightly clustered on chromosome 3p21.3, coding for hyaluronidases 1, 2, and 3. As in synthesis, in degradation HYAL type expression is cell and tissue specific and expression levels of HYALs vary in different tumours.

Hyaluronan is mainly synthesized as a HMW-hyaluronan and then degraded in oligosaccharides by HYALs. The HMW-hyaluronan in general promotes tissue integrity and quiescence, while hyaluronan breakdown products signal that injury has occurred. The smaller fragments of hyaluronan accompanied by increased HYAL expression are involved in a variety of normal and pathological processes and can either promote or inhibit tumour progression [28]. However, HYAL has been used for therapeutic purposes in breast cancer treatment as it is relatively nontoxic, degrades the matrix hyaluronan, and enhances the penetration of cytostatic drugs [29].

1.4. The Role of Hyaluronan Receptors CD44 and RHAMM in Tumour Progression. CD44, a hyaluronan receptor, is a transmembrane glycoprotein with ability to bind HA with high specificity [30]. Importantly, this molecule participates significantly in cell-cell and cell-matrix interactions [31]. It is encoded by a single highly conserved gene but appears in multiple isoforms of different molecular size, ranging from 80 to 200 kDa. These isoforms are generated both by alternative splicing of 20 exons and through protein modifications. The isoform that is mainly expressed in normal cells is an 85 kDa protein that contains none of the variable exons and has been shown to mediate the hyaluronan-promoted motility in breast cancer cell lines [30, 32, 33]. Variant expression is linked to tumour progression and is regulated by tissue and environment-specific factors and signalling pathways involved in oncogenesis including the Ras-MAPK cascade [34]. CD44 is composed of an extracellular domain, which contains binding sites for molecules such as hyaluronan, a membrane region, a transmembrane domain, and a cytoplasmic tail. The latter domain of CD44 recruits regulatory proteins to the cell membrane activating various signalling pathways [35, 36]. It is also involved in the association of signalling complexes with the actin cytoskeleton [31, 37]. In healthy tissues, CD44 plays a fundamental role in the regulation of basic cellular processes such as cell adhesion, inflammation, and repair [38].

RHAMM is a hyaluronan-binding protein/receptor that has a diverse cellular localization including the cell surface, the cytoplasm, and the nucleus and it is also found secreted to the ECM [39]. Its expression in normal tissues is not very high but it is found to be overexpressed in many advanced cancers [33]. Different RHAMM isoforms are produced due to alternative splicing and the expression of these transcript variants is different depending on the type of the cells [40]. RHAMM protein is glycosylphosphatidylinositol-anchored to the cell membrane as it lacks a typical transmembrane domain [41]. At the cell surface, extracellular RHAMM is required for tumour cell motility and invasion, affecting hyaluronan and growth factor-induced MAPK (ERK1, 2) signalling via associations with transmembrane hyaluronan receptors such as CD44 and protein tyrosine kinase (PTK) receptors such as PDGFR and RON [42, 43]. Intracellular RHAMM, on the contrary, regulates crucial processes in cancer cells [44], through its binding to actin filaments, microtubules, podosomes, the centrosome, and the mitotic spindle [33]. Therefore, the role of RHAMM seems to be associated with its expression levels and cellular distribution.

1.5. Collagen VI Structure and Biological Role. Collagen type VI is a microfibrillar component of the ECM and has a ubiquitous distribution throughout connective tissues. It plays a key role in the maintenance of tissue integrity by providing a structural link between different components of connective tissues, basement membranes, and cells. Collagen VI is thought to participate in cell adhesion, spreading, and migration of various cell types through interaction with member of the integrin receptor family or the NG2 PGs, contributing directly by this to the regulation of cell behaviour by receptor-mediated signal transduction pathways [45]. Moreover, collagen VI monomers or individual chains can bind to collagen type I and type III, hyaluronan, heparin [46, 47], or interact with other matrix components, such as PGs, suggesting that collagen VI microfibril supramolecular assemblies act as scaffolds for the formation of the structurally critical fibrillar collagen networks [48].

Collagen VI is a heterotrimer composed of three genetically distinct α1(VI), α2(VI), and α3(VI) polypeptide chains [49] encoded by *COL6A1*, *COL6A2*, and *COL6A3* genes, respectively. The intact molecules associate laterally in an antiparallel fashion into dimmers that are stabilized by disulfide bridges [50]. The dimmers aggregate further into

tetramers that are secreted into the ECM [51, 52], where they join end to end into microfibrils. Recently, they were characterized three additional chains, the α4(VI) that forms trimers with α1(VI) and α2(VI), the α5(VI) and α6(VI), with a high degree of similarity with the α3(VI) chain, that were found accumulated intracellularly [53].

The α1(VI), α2(VI), and α3(VI) chains of collagen VI contain a short triple-helical region and globular extensions at the N and C terminus. The α1(VI) and α2(VI) chains are similar in size and structure with Mr 140 kDa, while the third chain α3(VI) is much larger with Mr ranging from 180 to 240 kDa. The α3(VI) chain has 10 vWF type A domains of approximately 200 amino acids at the N-terminus (N1–N10, N10 being the most N-terminal) compared with only one such domain in the α1(VI) and α2(VI) chains [54]. Unlike the C1 and C2 domains of all three collagen VI subunit chains, the three additional C-terminal domains of α3(VI) chain have no homology to vWF type A domains. The proline-rich C3 domain shows some similarity to salivary proteins, C4 shows homology to type III repeats found in fibronectina and tenascin, and C5 is related to apoprotein-type protease inhibitors [54]. The cleavage of C5 domain, called also endotrophin (ETP), occurs immediately after the secretion of collagen VI. Nothing is known about the protease that cleaves the ETP-peptide. However, recent studies have demonstrated that matrix metalloproteinase-11 (MMP11)/stromelysin-3, a zinc endopeptidase belonging to the MMP family, cleaves collagen VI and in particular the native α3 chain of collagen VI [55]. Moreover, it has been demonstrated in *in vivo* experiments that the absence of MMP11 in deficient mice dramatically alters the collagen VI folding and subsequently adipocytes and related ECM [55]. Additionally, the ectopic expression of MMP11 at the adipocyte-cancer cell interface leads to collagen VI alteration [55].

Missense and homozygous or heterozygous mutations of the genes expressing the three chains have been shown to cause Ullrich congenital muscular dystrophy and Bethlem myopathy, genetic diseases characterized by muscle weakness [56]. In addition, overexpression of *COL6A1* and *COL6A2* and higher synthesis of collagen VI have been observed in the umbilical cord and the ECM of the skin fibroblasts from foetuses and individuals, respectively, affected with trisomy 21 [57, 58]. As it will be discussed below, alterations in collagen VI and especially ETP peptide demonstrate a crucial role in breast cancer progression [59, 60]. Thus, abnormalities in collagen VI can lead to a wide spectrum of clinical phenotypes and pathologies.

2. Correlations between Collagen VI and Hyaluronan

Hyaluronan and collagen VI are two components of the ECM that interact with each other [46, 60]. In detail, it has been reported that the ECM of human articular cartilage comprises a network of collagen VI that interacts with PGs and hyaluronan [60]. Moreover, interaction of collagen VI and hyaluronan was also demonstrated in *in vitro* studies [46, 47, 61] and in hyaluronan-based polymer scaffolds in tissue engineered cartilage [62]. Recently, we demonstrated in our study that overexpression of collagen VI induced HAS2 gene expression and hyaluronan synthesis. Even though there are no data regarding a direct crosstalk between hyaluronan and collagen VI in breast cancer, they both promote tumour progression via common pathways. In this review, we summarize and discuss data that demonstrate the common role of collagen VI and hyaluronan in inflammation, angiogenesis, and EMT in breast cancer.

3. Collagen VI and Hyaluronan in Breast Cancer

3.1. Localization In Vivo and Synthesis In Vitro. An important factor for the breast cancer cell behaviour is the presence or absence of the estrogen receptor (ER) within the cell membrane. Clinically, ER-positive breast cancer is less aggressive than the ER-negative and is amenable to hormone therapy by ER modulators. In basic research several breast cancer cell lines have been used for the *in vitro* study of breast cancer, taking into consideration additionally the progesterone receptor (PR) and the human epidermal growth factor receptor 2 (HER2). MCF-7 and MDA-MB-231 are two representative breast cancer lines commonly used in breast cancer research. MCF-7 is an ER+/PR+/HER2-cell line, whereas MDA-MB-231 is an ER−/PR−/HER2-cell line that belongs to the triple negative breast cancer cell lines [63].

Collagen VI is found in the invasive front of the tumour that was defined as the three to six layers of tumour cells at the front edge or the scattered tumour groups between the tumour and the host tissue or organ. The ETP peptide is localized close to adipocytes within the breast tumour, which are the most representative cytotypes in breast tissues and they secrete considerable amount of collagen VI. Regarding hyaluronan, the high amount in breast cancer is observed in the immediate peritumoral stroma or associated with carcinoma cells [64]. The intensity of hyaluronan signal within the breast tumour was significantly related to poor differentiation of the tumours, axillary lymph node positivity, and short overall survival of the patients [65].

Studies performed in rodents and confirmed in human MCF-7 cell line have demonstrated that collagen VI, produced by adipocytes, promotes growth of cancer cell proliferation via NG2/chondroitin sulphate proteoglycan receptor expressed on the surface of malignant ductal epithelial cells and sequentially activates Akt and β-catenin and stabilizes cyclin D1. Interestingly, the domain that appears to be responsible for such effect seems to be the ETP peptide. Therefore, adipocytes play a vital role in defining the ECM environment for normal and tumour-derived ductal epithelial cells and contribute significantly to tumour growth at early stages through secretion and processing of collagen VI [66].

Hyaluronan is mainly produced by the stromal fibroblasts of breast tumour, even though both MDA-MB-231 and MCF-7 synthesize hyaluronan in lower amounts [67, 68]. Recent studies demonstrated that the more aggressive the breast cancer cell, the more hyaluronan produce. Indeed, the clone of MDA-MB-231 cell line that forms bone metastases (MDA-MB-231-BM) showed an abundant expression of HAS2 that

was higher with respect to wild-type and a more invasive phenotype. The increased HAS2 expression in the MDA-MB-231-BM was correlated to a suppression of TIMP-1 expression, which presumably increased MMP activity with a consequent basement membrane degradation [69].

3.2. The Role of Collagen VI and Hyaluronan in Angiogenesis. Tumour angiogenesis is the proliferation of a blood vessel network within a growing malignant tissue and is an essential process for supplying rapidly nutrients and oxygen and removing waste products. An angiogenic switch allows tumour cells to survive and grow and enhances the tumour metastasis. The neovascularisation is influenced by some ECM proteins of the tumour environment [70, 71].

Expression of collagen VI has been found within or close to the blood vessels in juvenile angiofibromas and human glioblastomas. *In vitro* studies demonstrated that endothelial cells incubated with conditioned media from ETP-overexpressing HEK-293T cells stimulated their recruitment, as well as the vessel formation [6]. Thus, ETP peptide acts as chemoattractant that recruits endothelial cells and it was found also that it stimulates angiogenesis by upregulating proangiogenic factors [6, 59].

The role of hyaluronan in angiogenesis depends on the molecular weight of the polymer. Both angiogenesis and intracellular signalling are affected by the degradation products of hyaluronan. The most expressed hyaluronidase in breast cancer cells is HYAL-2, even though recent studies showed that HYAL-1 has a pivotal role in tumour cell behaviour and angiogenesis. Indeed, knockdown of HYAL1 expression in MDA-MB-231 and MCF-7 cells results in decreased cell growth, adhesion, invasion, and angiogenesis potential. In addition, the HYAL1 knockdown showed an inhibition of breast cancer cell xenograft tumor growth and microvessel density, whereas HYAL-1 overexpression demonstrated an increase of tumour growth [72, 73]. A number of reports demonstrate that also PH-20 or SPAM1, which is thought to be a testicular hyaluronidase, is a tumour marker for breast and laryngeal cancer. In fact, PH-20 expression levels are increased in metastatic breast cancer to lymph nodes compared to ductal carcinoma *in situ* and invasive breast cancer [74]. Moreover, the upregulated PH-20 in breast cancer cells MDA-MB-231 was found to promote tumour growth when implanted into the chorioallantoic membrane (CAM) of chicken embryo to form a tumour. The growth was accompanied by an increment of neogenetic vessels and the release of FGF-2 from tumour cells indicating that upregulation of PH-20 in malignant breast tissue may degrade hyaluronan into small fragments and contribute to angiogenesis [75, 76].

The HMW native hyaluronan has been shown to be antiangiogenic by inhibiting endothelial cell proliferation and migration and capillary formation in three-dimensional matrix [77]. Administration of hyaluronan-versican aggregates showed, however, promotion of infiltration of endothelial cells within matrigel plugs containing angiogenic factors, suggesting the stimulation of HMW-hyaluronan in angiogenesis [78]. Thus, the role of the large polymer of hyaluronan in angiogenesis needs further clarification and study.

3.3. Collagen VI and Hyaluronan Induce Inflammation. Tumour-associated macrophages (TAMs) are a cell type with potent immunosuppressive functions that favour tumour angiogenesis, growth, and metastasis. TAMs are a polarized M2 macrophage population with similar functions to M2s and have an IL-10^{high} IL-12^{low} phenotype [79]. The predominant expression of these M2 macrophages is associated with more aggressive histopathological features (high tumour grade), increased microvessel density, and decreased overall survival that is the late stage of tumour progression. On the other hand, in advanced stages, TAMs release cytokines such as transforming growth factors β1 (TGF β1) and IL-10 and promote tumour development through inhibition of anticancer immune responses [80, 81].

The M2 phenotype macrophages induced by transforming growth factor (TGF-β1) and inflammatory factors, such as interleukin- (IL-) 4 and IL-10, produce high amount of collagen VI. *In vitro* studies showed also that macrophages use collagen VI to modulate their binding properties rather than to build native ECM as do other cell types, including fibroblasts and smooth muscle cells (SMC), suggesting TAMs as one of the key providers for collagen VI in tumours. [82]. Similarly to collagen VI, the ETP peptide is able to promote tumour inflammation by increasing macrophage recruitment and upregulating the production of inflammatory factors, such as IL-6 and tumour necrosis factor-alpha (TNF-α), which are abrogated by anti-ETP antibodies [6]. These outcomes suggest that the majority of chemoattractant properties exerted by collagen VI are performed by ETP [59].

The inflammatory effects of hyaluronan, pro- and anti-inflammatory, are correlated to the size of the polymer. Hyaluronan with an average molecular mass <500 kDa is considered as a fragment, although the molecular properties of a 500 kDa fragment and those of a 50 kDa are different [83]. The indicated size of hyaluronan used in a research study usually represents the average molecular weight of a polydisperse distribution. In regulatory T-cells, HMW-hyaluronan with an average molecular weight of ~10^7 Dalton binds to CD44 and promotes STAT5 signalling pathway which results in a maintenance of the cells and an inhibition of proliferation, indicating HMW-hyaluronan as an anti-inflammatory agent [84]. On the other hand, hyaluronan fragments of 200 kDa have been shown to stimulate chemokines, cytokines, growth factors, proteases, and nitric oxide by macrophages [83].

Hyaluronan degradation into fragments is performed by hyaluronidase, indicating the important role of the degrading enzyme expression in tumour environment. However, it is of high importance also to consider the role of HAS2 and the synthesis of hyaluronan by stromal fibroblasts in tumour malignancies. Indeed, hyaluronan-rich environment in tumour indicates active migration and proliferation of tumour cells. Moreover, the hyaluronan species participate in TAM trafficking into tumour masses, enhancing the neovascularisation. Recent studies on Has2 null fibroblasts showed severe impairment in recruiting macrophages when inoculated with tumour cells into nude mice, which demonstrates the contribution of stroma-derived hyaluronan in intratumoral macrophage mobilization [85]. Importantly, the upregulation of HAS2 in highly metastatic breast cancer

stem-like cells showed its critical role for the interaction of the cells with TAMs, leading to enhanced secretion of platelet-derived growth factor-BB from TAMs, which then activated stromal cells and enhanced the breast cancer stem-like cells self-renewal [86].

3.4. Correlation between Collagen VI or Hyaluronan and EMT. Another important factor for tumorigenesis and tumour progression is the epithelial-to-mesenchymal transition (EMT). EMT is characterized by the loss of cell-cell adhesions, destruction of the apicobasal polarity of epithelial cell membranes, and interactions between new plasma membrane receptors and remodelled constituents of ECM [87, 88]. Several studies have used breast cancer in model organisms in order to provide new insights regarding EMT in cancer progression. Both hyaluronan and collagen VI are among the molecules of ECM that induce EMT during cancer progression.

As described above, collagen VI and especially the ETP peptide has a critical role in various events that characterize the growth, invasive, and metastatic capacity of tumours. Recent studies demonstrated that in the ETP transgenic mice (PyMT/ETP) immune responses, cell cycle regulation, and stem cell pluripotency were significantly altered [6]. Notably, a significant decrease of E-cadherin, the loss of which is a characteristic of EMT, and an increased pulmonary metastasis of breast cancer in the PyMT/ETP with respect to the nontransgenic mice were observed [6]. The metastasis was associated with an induction of TGFβ-dependent EMT by ETP peptides [6]. Therefore, ETP may be considered as one of the ECM molecules that contribute to EMT induction.

TGFβ potently stimulates hyaluronan synthesis via upregulation of HAS2 in NMuMG [87] and MCF-10A [22]. Specifically, Porsch and colleagues [24] demonstrated that HAS2 expression plays a pivotal role in the TGFβ-induced EMT, as knockdown of HAS2 in NMuMG cells inhibited the TGFβ-induced EMT by about 50% and abolished the TGFβ-induced cell migration. Removal of extracellular hyaluronan by *Streptomyces* hyaluronidase, blocking of CD44 with antibodies, or CD44 knockdown did not inhibit TGFβ-induced EMT or cell migration, suggesting that HAS2, but not hyaluronan, has a regulatory role in EMT.

Even though nothing is known about a possible cross-linking between hyaluronan and collagen VI or ETP during the EMT process, it is evidently shown that ETP induces TGFβ-dependent EMT which in turn is regulated by HAS2 expression and results in hyaluronan increase. Therefore, both ETP and HAS2 are essential for the induction and maintenance of EMT in breast cancer and TGFβ alone is not enough for the EMT process.

3.5. Collagen VI and Hyaluronan as Serum Biomarkers. The best available approach for detection of breast cancer currently is the mammographic screening. Although resolution continues to improve, tumours smaller than 5 mm are usually not detectable and mammography effectiveness decreases in young women who physically have dense breast tissue. Several studies are focused on developed techniques for the identification of altered proteins in human serum as an additional approach for the detection, follow-up, and determination of cancer stage of breast cancer [89]. Among them, breast cancer proteins (BC) 1, 2, and 3 are used for the detection and the discrimination of the breast cancer in patients [90].

Serum hyaluronan has been used as a disease marker in pathologies such as liver fibrosis in patients affected with chronic viral hepatitis C and rheumatoid arthritis. Regarding breast cancer detection, serum hyaluronan levels were found significantly elevated in women with severe malignant breast cancer associated with high metastasis, with respect to the nonmetastatic carcinoma or with respect to those with benign breast disease [91]. On the other hand, collagen VI is not considered a serum biomarker of breast cancer, even though the α1(VI) chain was among the proteins identified by biopanning from a breast cancer cDNA T7 phage library [92]. Nevertheless, collagen VI is indicated as a diagnostic serum marker of patients with melanoma and pancreatic cancer, since the levels of this protein in human sera are significantly increased in both aggressive malignant tumours with respect to healthy donors.

4. Conclusions and Future Perspectives

As summarized in Figure 1, collagen VI and hyaluronan regulate the breast cancer progression and metastasis and seem to participate in the same biological processes in a synergistic manner. Collagen VI is produced by adipocytes during breast cancer, as well as by macrophages during inflammation. Hyaluronan is mainly produced by stromal cells, even though its increased synthesis by breast cancer cells is correlated to an increased aggressiveness of cancer. Both molecules are responsible for the recruitment of endothelial cells during angiogenesis in the breast cancer microenvironment. Moreover, macrophages produce collagen VI and secrete inflammatory factors that in turn induce hyaluronan synthesis by stromal cells. Both hyaluronan and ETP peptide regulate the trafficking and recruitment of macrophages, demonstrating their synergistic effect in breast cancer progression. Breast cancer is also characterized by EMT process. As discussed above, ETP, hyaluronan, and HAS2 are essential for the induction and maintenance of EMT progression. Thus, simultaneous targeting of these two molecules may be a promising approach for improving pharmaceutical agents and consequently breast cancer treatment.

One of the parameters to take also into consideration is the size of hyaluronan and the amount of intact collagen VI or the ETP peptide during each biological process, which means that the role of HASs, HYALs, and further studies regarding the proteases responsible for the ETP cleavage should be taken into consideration for breast cancer management and prognostication.

Abbreviations

ECM: Extracellular matrix
HMW: High molecular weight
HYAL: Hyaluronidase
HAS: Hyaluronan synthase
ETP: Endotrophin
TAM: Tumour-associated macrophages

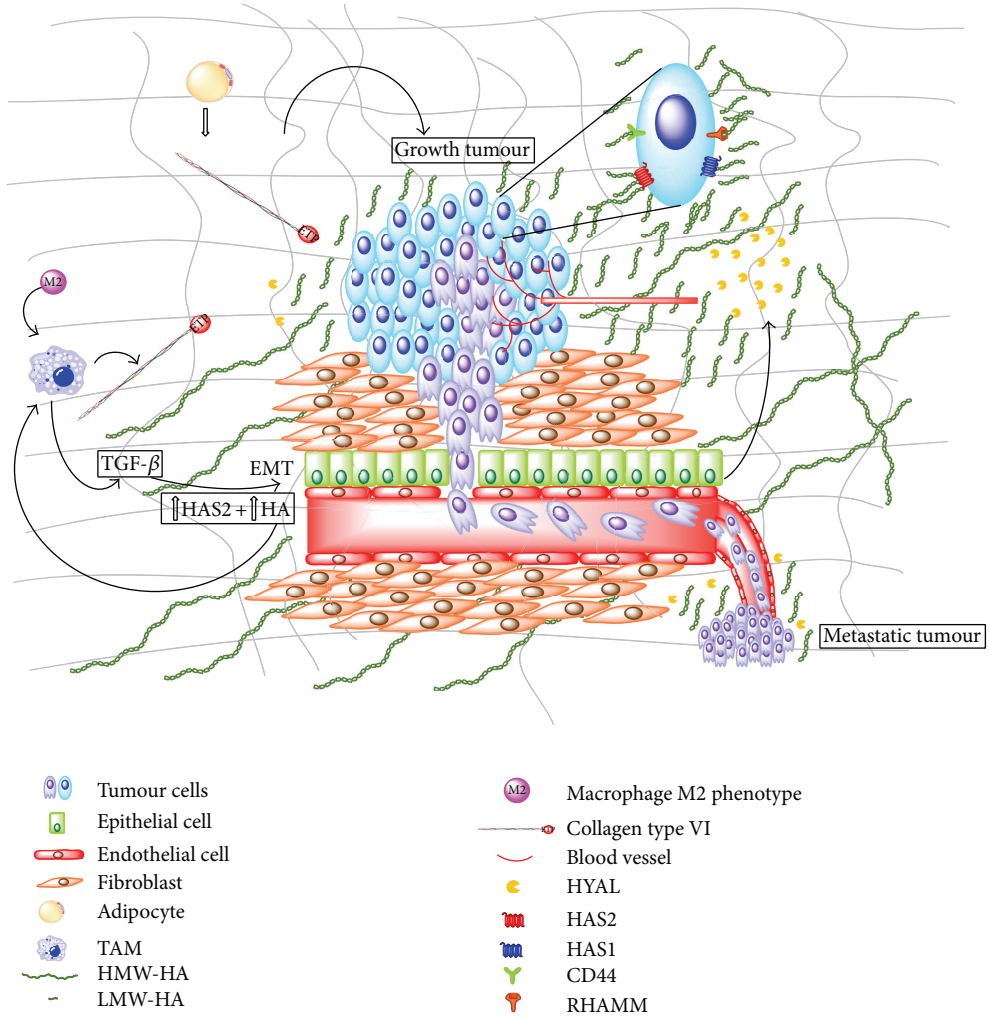

Figure 1: Schematic representation of collagen VI/ETP and hyaluronan contribution in breast cancer progression. Synthesis of collagen VI by adipocytes as well as synthesis of hyaluronan by stromal cells is increased in breast tumour. Macrophages release TGFβ and collagen VI than in turn increase HAS2 and hyaluronan in mammal cells and induce epithelial-mesenchymal transition (EMT). Increased hyaluronan and cleaved ETP induce recruitment of both macrophages and endothelial cells, resulting in neovascularisation that in turn promotes metastasis. This latter phenomenon is also induced by low molecular weight hyaluronan that is the result of HYAL activity on the HMW molecule. Both collagen VI and HMW-hyaluronan induce growth tumour. Abbreviations: ETP, endotrophin; TGFβ, transforming growth factor-beta; HMW-HA and LMW-HA: high and low molecular weight hyaluronan, respectively; HYAL: hyaluronidase; HAS: hyaluronan synthase; TAM: tumour-associated macrophages.

TGF-β: Transforming growth factor-beta
IL: Interleukin
EMT: Epithelial-mesenchymal transition
MMP: Matrix metalloproteinase.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Review Article

Human Genetic Disorders and Knockout Mice Deficient in Glycosaminoglycan

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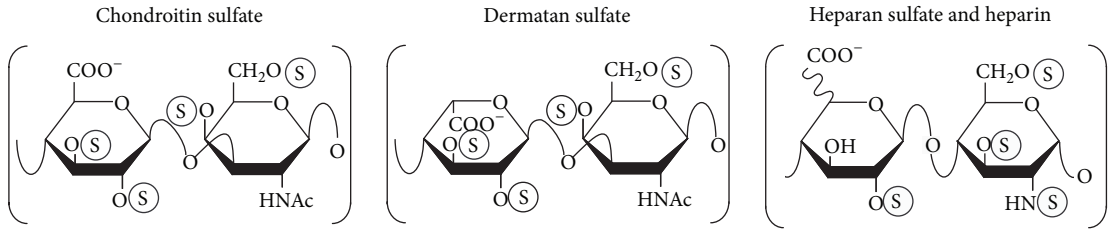
Glycosaminoglycans (GAGs) are constructed through the stepwise addition of respective monosaccharides by various glycosyltransferases and maturated by epimerases and sulfotransferases. The structural diversity of GAG polysaccharides, including their sulfation patterns and sequential arrangements, is essential for a wide range of biological activities such as cell signaling, cell proliferation, tissue morphogenesis, and interactions with various growth factors. Studies using knockout mice of enzymes responsible for the biosynthesis of the GAG side chains of proteoglycans have revealed their physiological functions. Furthermore, mutations in the human genes encoding glycosyltransferases, sulfotransferases, and related enzymes responsible for the biosynthesis of GAGs cause a number of genetic disorders including chondrodysplasia, spondyloepiphyseal dysplasia, and Ehlers-Danlos syndromes. This review focused on the increasing number of glycobiological studies on knockout mice and genetic diseases caused by disturbances in the biosynthetic enzymes for GAGs.

1. Introduction

Glycosaminoglycans (GAGs) are covalently attached to the core proteins that form proteoglycans (PGs), which are ubiquitously distributed in extracellular matrix and on the cell surface [1–7]. GAGs are linear polysaccharides that form the side chains of PGs and have been classified into chondroitin sulfate (CS), dermatan sulfate (DS), heparan sulfate (HS), and heparin based on their structural units. The backbone of CS consists of repeating disaccharide units of *N*-acetyl-D-galactosamine (GalNAc) and D-glucuronic acid (GlcUA) (Figure 1). DS is a stereoisomer of CS and composed of GalNAc and L-iduronic acid (IdoUA) instead of GlcUA (Figure 1). They are often distributed as CS-DS hybrid chains in mammalian tissues [8]. On the other hand, HS and heparin consist of *N*-acetyl-D-glucosamine (GlcNAc) and GlcUA or IdoUA (Figure 1). The glucosamine (GlcN) residues in HS and heparin are modified by not only *N*-acetylation but also *N*-sulfation. These GAG chains are modified by sulfation at various hydroxy group positions and also by the epimerization of uronic acid residues during the biosynthetic process, thereby giving rise to structural diversity, which plays an important role in a wide range of biological roles including cell proliferation, tissue morphogenesis, infections by viruses, and interactions with various growth factors, cytokines, and morphogens [7–18].

Glycosyltransferases, epimerases, sulfotransferases, and related enzymes in the biosynthesis of GAGs have been cloned and characterized (Tables 1–4 and Figures 2 and 3) [6, 7, 14, 19]. Furthermore, genetic analyses using model animals including mice, zebrafish, fruit flies, and nematodes have led to new findings on different phenotypes [4, 8, 9, 12, 13]. Human genetic disorders including bone and skin diseases caused by mutations in the genes encoding the biosynthetic enzymes for GAGs have recently been reported [7, 14, 20]. This review focused on recent advances in knockout mice for GAG biosynthesis, as well as cartilage and connective tissue disorders caused by disturbances in the biosynthesis of functional GAG chains.



Symbol	Sequence
O unit	GlcUA-GalNAc
A unit	GlcUA-GalNAc(4S)
C unit	GlcUA-GalNAc(6S)
B unit	GlcUA(2S)-GalNAc(4S)
D unit	GlcUA(2S)-GalNAc(6S)
E unit	GlcUA-GalNAc(4S,6S)

(a)

Symbol	Sequence
iO unit	IdoUA-GalNAc
iA unit	IdoUA-GalNAc(4S)
iC unit	IdoUA-GalNAc(6S)
iB unit	IdoUA(2S)-GalNAc(4S)
iD unit	IdoUA(2S)-GalNAc(6S)
iE unit	IdoUA-GalNAc(4S,6S)

(b)

Symbol	Sequence
HS-0S	HexUA-GlcNAc
HS-2S	HexUA(2S)-GlcNAc
HS-6S	HexUA-GlcNAc(6S)
HS-NS	HexUA-GlcN(NS)
HS-diS$_1$	HexUA-GlcN(NS,6S)
HS-diS$_2$	HexUA(2S)-GlcN(NS)
HS-diS$_3$	HexUA(2S)-GlcNAc(6S)
HS-triS	HexUA(2S)-GlcN(NS,6S)

(c)

Figure 1: Typical repeating disaccharide units in CS, DS, HS, and heparin, and their potential sulfation sites. CS consists of GlcUA and GalNAc, whereas DS is a stereoisomer of CS including IdoUA instead of GlcUA. Both linear polysaccharides are often found as CS-DS hybrid chains in mammals. HS and heparin consist of uronic acid and GlcNAc residues with varying proportions of IdoUA. Heparin is highly sulfated and has a large proportion of IdoUA residues, whereas HS is low sulfated and has a high proportion of GlcUA. These sugar moieties are esterified by sulfate at various positions as indicated by the circled "S." The abbreviation of "i" in iO, iA, iB, iC, iD, and iE stands for IdoUA. HexUA represents hexuronic acid (GlcUA or IdoUA).

2. Biosynthesis of 3′-Phosphoadenosine 5′-Phosphosulfate

The sulfation of GAGs is required for the exertion of their physiological functions. Sulfotransferases catalyze the transfer of sulfate from the donor substrate, 3′-phosphoadenosine 5′-phosphosulfate (PAPS), to the corresponding acceptor substrates [21]. PAPS is synthesized from ATP and inorganic sulfate in the cytosol, and the reaction takes place in two sequential steps [21–23]. ATP sulfurylase firstly catalyzes the reaction between ATP and inorganic sulfate to form the biosynthetic intermediate, adenosine 5′-phosphosulfate (APS) [22, 23]. The formation of the active sulfate, PAPS, is then catalyzed by APS kinase, which involves a reaction between APS and ATP [22, 23]. ATP sulfurylase and APS kinase are encoded by the respective genes in bacteria, fungi, yeast, and plants [21]. On the other hand, both enzymes are fused in animals, resulting in a polypeptide designated PAPS synthase (PAPSS), which is a bifunctional enzyme composed of the N-terminal APS kinase domain and C-terminal ATP sulfurylase domain [21]. Following the formation of PAPS in the cytosol, PAPS is translocated into the Golgi by PAPS transporters [24].

3. Biosynthesis of GAG Chains

3.1. GAG-Protein Linkage Region. CS, DS, HS, and heparin chains are attached to serine residues in core proteins through the common GAG-protein linkage region tetrasaccharide, GlcUAβ1-3galactoseβ1-3galactoseβ1-4xyloseβ1-*O*- (GlcUA-Gal-Gal-Xyl-*O*-) (Figure 2) [1, 5]. The transfer of a Xyl residue from uridine diphosphate (UDP)-Xyl to specific serine residues in the newly synthesized core proteins of PGs in the endoplasmic reticulum and *cis*-Golgi compartments is initiated by β-xylosyltransferase (XylT) (Figure 2 and Table 2) [25, 26]. β1,4-Galactosyltransferase-I (GalT-I), which is encoded by *B4GALT7*, then transfers a Gal residue from UDP-Gal to the Xyl-*O*-serine in the core proteins [27, 28]. β1,3-Galactosyltransferase-II (GalT-II), which is encoded by *B3GALT6*, transfers another Gal residue from UDP-Gal to the Gal-Xyl-*O*-serine [29]. Finally, β1,3-glucuronosyltransferase-I (GlcAT-I), which is encoded by *B3GAT3*, transfers a GlcUA residue from UDP-GlcUA to the Gal-Gal-Xyl-*O*-serine (Figure 2 and Table 2) [30]. These enzymes may form a multienzyme complex such as the so-called GAGosome for GAG synthesizing enzymes for the construction of the linkage region [31, 32].

Several modifications including the 2-*O*-phosphorylation of the Xyl residue as well as sulfation at the C-6 position of the first Gal and at C-4 or C-6 of the second Gal residue have been reported [5]. GAG-Xyl kinase, encoded by *FAM20B*, Xyl phosphatase, encoded by *ACPL2*, and Gal-6-*O*-sulfotransferase, encoded by *CHST3 (C6ST1),* have so far been identified (Table 2) [33–35]. These modifications affect the glycosyltransferase reactions of GalT-I and GlcAT-I *in vitro* and may regulate the formation of GAG chains [36, 37].

3.2. Repeating Disaccharide Region of CS and DS. Chain polymerization of the repeating disaccharide region in CS

Table 1: Transporters for UDP-sugars and sulfate as well as biosynthetic enzymes for PAPS.

Transporters and enzymes	Coding genes (synonym)	Chromosomal location	mRNA accession number	MIM number	Human genetic disorders	Clinical features	References for the human diseases	References for the knockout mice
Solute carrier family 26 member A2 (diastrophic dysplasia sulfate transporter)	*SLC26A2 (DTDST)*	5q31–q34	NM_000112	600972 256050 222600 226900	Achondrogenesis type IB Atelosteogenesis type II Diastrophic dysplasia Multiple epiphyseal dysplasia autosomal recessive type	Lethal chondrodysplasia with severe under-development of skeleton, extreme micromelia, death before or immediately after birth. Epiphyseal dysplasia and early onset osteoarthritis.	[38–40]	[41]
Solute carrier family 35 member D1 (UDP-GlcUA/UDP-GalNAc dual transporter)	*SLC35D1 (UGTrel7)*	1p32-p31	NM_015139	269250	Schneckenbecken dysplasia	Neonatal lethal chondrodysplasia, platyspondyly with oval-shaped vertebral bodies, extremely short long bones with dumbbell-like appearance, and small ilia with snail-like appearance.	[42]	[42]
PAPS synthase 2	*PAPSS2*	10q24	NM_004670 NM_001015880	612847	Spondyloepimetaphyseal dysplasia Pakistani type (PAPSS2 type) Hyperandrogenism Brachyolmia autosomal recessive type	Short, bowed lower limbs, enlarged knee joint, kyphoscoliosis, and mild generalized brachydactyly. Androgen excess, premature pubarche, hyperandrogenic anovulation, low level of serum, dehydroepiandrosterone, short trunk, kyphosis, and scoliosis.	[43–48]	[22, 23, 49–53]
3′-Phosphoadenosine 5′-phosphate 3′-phosphatase	*IMPAD1 (PAPP)*	8q12.1	NM_017813	614078 614010	Chondrodysplasia with joint dislocations GPAPP type	Short stature, chondrodysplasia, with brachydactyly, congenital joint dislocations, cleft palate, and facial dysmorphism.	[54]	[55]

MIM: mendelian inheritance in man.
Among the several transporters and biosynthetic enzymes involved in PAPS and UDP-sugars, some of the mutations that occur have been shown to cause human genetic disorders and are listed here.

Table 2: Biosynthetic enzymes of the GAG-linkage region tetrasaccharide.

Enzymes (activity)	Coding genes (synonym)	Chromosomal location	mRNA accession number	MIM number	Human genetic disorders	Clinical features	References for the human diseases	References for the knockout mice
Xylosyltransferase (XylT)	*XYLT1*	16p12.3	NM_022166	608124	Desbuquois dysplasia type 2, Short stature syndrome	Short stature, joint laxity, advanced carpal ossification, and hand anomalies.	[56–58]	[59]
	XYLT2	17q21.33	NM_022167	608125	—	—	—	[60]
β4-Galactosyltransferase-I (GalT-I)	*B4GALT7*	5q35.2-q35.3	NM_007255	130070 604327	Ehlers-Danlos syndrome progeroid type 1 Larsen of Reunion Island syndrome	Developmental delay, aged appearance, short stature, craniofacial dysmorphism, and generalized osteopenia. Multiple dislocations, hyperlaxity, dwarfism, and distinctive facial features.	[61–69]	—
β3-Galactosyltransferase-II (GalT-II)	*B3GALT6*	1p36.33	NM_080605	271640 615349 615291	Ehlers-Danlos syndrome progeroid type 2 Spondyloepimetaphyseal dysplasia with joint laxity type 1	Sparse hair, wrinkled skin, defective wound healing with atrophic scars, osteopenia, and radial head dislocation. Spatulate finger with short nail, hip dislocation, elbow contracture, clubfeet, and mild craniofacial dysmorphism including prominent eye, blue sclera, long upper lip, and small mandible with cleft palate.	[70–72]	—
β3-Glucuronyltransferase-I (GlcAT-I)	*B3GAT3*	11q12.3	NM_012200	245600 606374	Larsen-like syndrome B3GAT3 type Multiple joint dislocations, short stature, craniofacial dysmorphism, and congenital heart defects	Joint dislocations mainly affecting the elbow, congenital heart defects such as bicuspid aortic valve, aortic root dilatation.	[73, 74]	[75, 76]
Xylose 2-*O*-kinase	*FAM20B* (*GXK1*)	1q25	NM_014864	611063	—	—	—	—
Xylose 2-*O*-phosphatase	*ACPL2* (*XYLP*)	3q23	NM_152282	—	—	—	—	—

—, not reported.
B4GALT7: xylosylprotein beta 1,4-galactosyltransferase 7; B3GALT6, beta 1,3-galactosyltransferase 6; B3GAT3, beta 1,3-glucuronyltransferase 3; FAM20B, Family with sequence similarity 20 member B; ACPL2, acid phosphatase-like 2.

Table 3: Biosynthetic enzymes of CS and DS chains.

Enzymes (activity)	Coding genes (synonym)	Chromosomal location	mRNA accession number	MIM number	Human genetic disorders	Clinical features	References for the human diseases	References for the knockout mice
Chondroitin synthase (GalNAcT-II, CS-GlcAT-II)	*CHSY1*	15q26.3	NM_014918	605282 608183	Temtamy preaxial brachydactyly syndrome Syndromic recessive preaxial brachydactyly Neuropathy	Short stature, limb malformation, hearing loss.	[77–80]	[81]
	CHSY2 (*CHSY3, CSS3*)	5q23.3	NM_175856	609963	—	—	—	—
	CHSY3 (*CHPF2, CSGLCA-T*)	7q36.1	NM_019015	608037	—	—	—	—
Chondroitin-polymerizing factor (GalNAcT-II, CS-GlcAT-II)	*CHPF* (*CSS2*)	2q35	NM_024536	610405	—	—	—	[81, 82]
Chondroitin *N*-acetylgalactosaminyltransferase (GalNAcT-I, GalNAcT-II)	*CSGALNACT1*	8p21.3	NM_018371	—	Hereditary motor and sensory neuropathy Unknown type Bell's palsy	Intermittent postural tremor, reduction in compound muscle action potentials, acquired idiopathic generalized anhidrosis, hemifacial palsy.	[83]	[81, 84–86]
	CSGALNACT2	10q11.21	NM_018590	—	—	—	—	[86]
Dermatan sulfate epimerase	*DSE*	6q22	NM_013352	615539 605942	Ehlers-Danlos syndrome musculocontractural type 2	Characteristic facial features, congenital contractures of the thumbs and the feet, hypermobility of finger, elbow, and knee joints, atrophic scarring of the skin, and myopathy.	[87]	[88, 89]
	DSEL	18q22.1	NM_032160	611125	Bipolar disorder Depressive disorder Diaphragmatic hernia Microphthalmia	Alternating episodes of depression and mania or hypomania, and congenital malformation of the diaphragm.	[90–92]	[93]
Uranyl 2-*O*-sulfotransferase	*UST*	6q25.1	NM_005715	610752	—	—	—	—
Chondroitin 4-*O*-sulfotransferase	*CHST11* (*C4ST-1*)	12q	NM_018413	610128	—	—	—	[94–96]
	CHST12 (*C4ST-2*)	7p22	NM_018641	610129	—	—	—	—
	CHST13 (*C4ST-3*)	3q21.3	NM_152889	610124	—	—	—	—

Table 3: Continued.

Enzymes (activity)	Coding genes (synonym)	Chromosomal location	mRNA accession number	MIM number	Human genetic disorders	Clinical features	References for the human diseases	References for the knockout mice
Dermatan 4-*O*-sulfotransferase	*CHST14* (*D4ST-1*)	15q15.1	NM_130468	601776 608429	Ehlers-Danlos syndrome musculocontractural type 1 Adducted thumb-clubfoot syndrome	Craniofacial dysmorphism, multiple contractures, progressive joint and skin laxities, multisystem fragility-related manifestations, contractures of thumbs and feet, defects of heart, kidney and intestine.	[97–106]	[96, 107]
Chondroitin 6-*O*-sulfotransferase	*CHST3* (*C6ST-1*)	10q22.1	NM_004273	143095 603799	Spondyloepiphyseal dysplasia with congenital joint dislocations Spondyloepiphyseal dysplasia Omani type Chondrodysplasia with multiple dislocations Humerospinal dysostosis Larsen syndrome autosomal recessive type Desbuquois syndrome	Short stature, severe kyphoscoliosis, osteoarthritis (elbow, wrist and knee), secondary dislocation of large joints, rhizomelia, fusion of carpal bones, mild brachydactyly, metacarpal shortening, ventricular septal defect, mitral and tricuspid defects, aortic regurgitations, deafness.	[108–113]	[114–116]
N-Acetylgalactosamine-4-sulfate-6-*O*-sulfotransferase	*CHST15* (*GalNAc4S-6ST*)	10q26	NM_015892	608277	—	—	—	[117]

—: not reported.
CSS: chondroitin sulfate synthase; DSEL: dermatan sulfate epimerase-like; CHST: carbohydrate sulfotransferase.

Table 4: Biosynthetic enzymes of HS and heparin chains.

Enzymes (activity)	Coding genes (synonym)	Chromosomal location	mRNA accession number	MIM number	Human genetic disorders	Clinical features	References for the human diseases	References for the knockout mice
Exostosin (GlcA and GlcNAc transferases)	*EXT1*	8q24.11	NM_000127	133700 215300 608177	Exostoses multiple type 1 Chondrosarcoma	The formation of cartilage-capped tumors (exostoses) that develop from the growth plate of endochondral bones, especially of long bones.	[118]	[119–135]
	EXT2	11p12-p11	NM_000401	133701 608210	Exostoses multiple type 2	Same as above.	[118]	[136]
Exostosin-like 2 (GlcNAc transferase-I)	*EXTL2*	1p21	NM_001439	602411	—	—	—	[137, 138]
Exostosin-like 1 (GlcNAc transferase-II)	*EXTL1*	1p36.1	NM_004455	601738	—	—	—	—
Exostosin-like 3 (GlcNAc transferase-I and -II)	*EXTL3*	8p21	NM_001440	605744	—	—	—	[139]
GlcNAc *N*-deacetylase and *N*-sulfotransferase	*NDST1*	5q33.1	NM_001543	600858	—	—	—	[140–164]
	NDST2	10q22	NM_003635	603268	—	—	—	[165–167]
	NDST3	4q26	NM_004784	603950	—	—	—	[168]
	NDST4	4q26	NM_022569	615039	—	—	—	—
HS GlcUA C5-epimerase	*GLCE*	15q23	NM_015554	612134	—	—	—	[169–172]
HS 2-*O*-sulfotransferase	*HS2ST1*	1p22.3	NM_012262	604844	—	—	—	[153, 162, 173–179]
HS 6-*O*-sulfotransferase	*HS6ST1*	2q21	NM_004807	614880 604846	Hypogonadotropic hypogonadism 15 with or without anosmia	Lack of sexual maturation and low levels of circulating gonadotropins and testosterone.	[180]	[177, 178, 181, 182]
	HS6ST2	Xq26.2	NM_147174 NM_147175	300545	—	—	—	[182, 183]
	HS6ST-3	13q32.1	NM_153456	609401	—	—	—	—
HS 3-*O*-sulfotransferase	*HS3ST1*	4p16	NM_005114	603244	—	—	—	[184]
	HS3ST2	16p12	NM_006043	604056	—	—	—	—
	HS3ST3A1 *HS3ST3B1*	17p12	NM_006042 NM_006041	604057 604058	—	—	—	—
	HS3ST4	16p11.2	NM_006040	604059	—	—	—	—
	HS3ST5	6q22.31	NM_153612	609047	—	—	—	—
	HS3ST6	16p13.3	NM_001009606	—	—	—	—	—
HS 6-*O*-endosulfatase	*SULF1*	8q13.2-q13.3	NM_015170	610012	—	—	—	[185–191]
	SULF2	20q12–q13.2	NM_018837 NM_198596	610013	—	—	—	[185–192]

—: not reported.

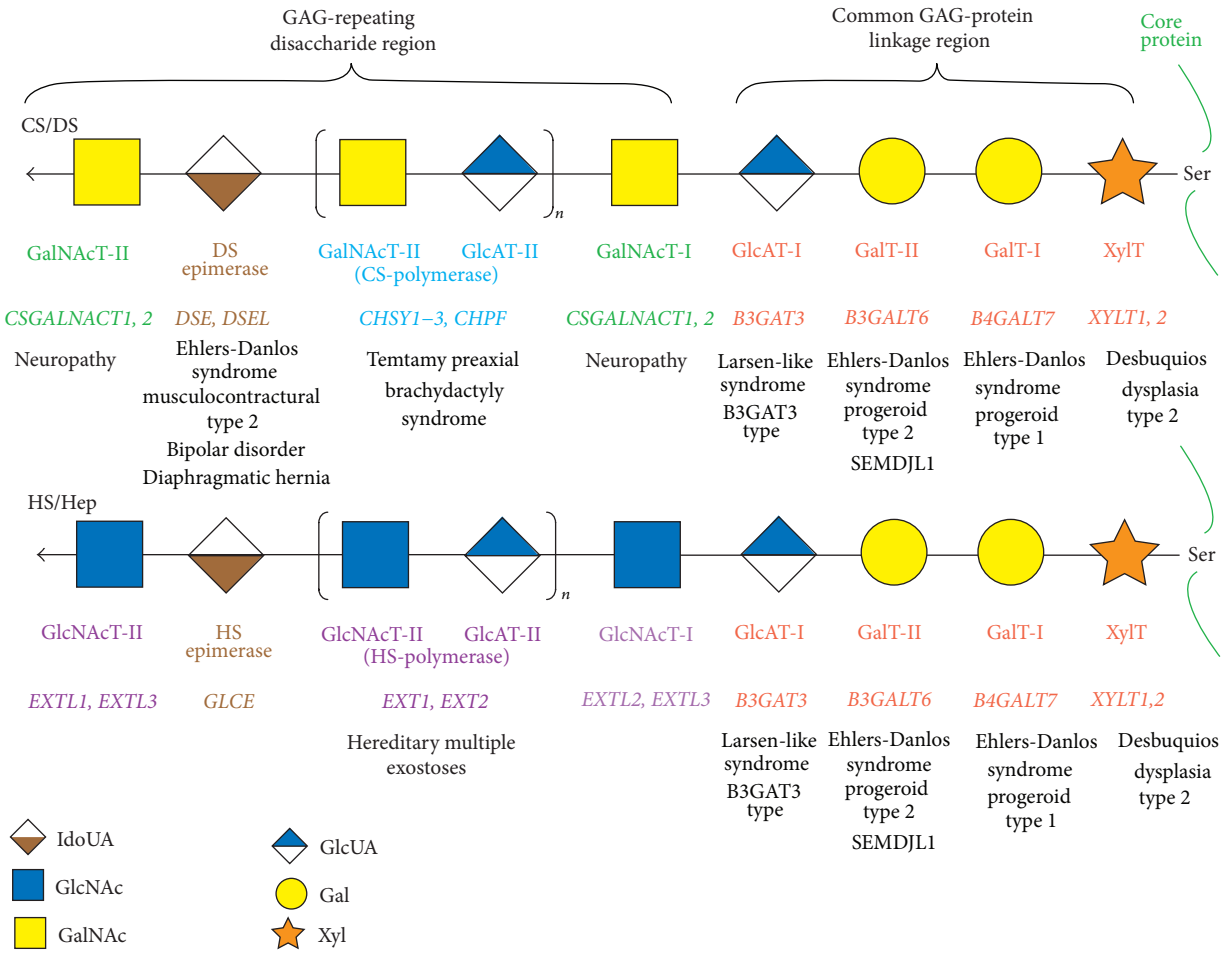


Figure 2: Biosynthetic assembly of GAG backbones by various glycosyltransferases. All glycosyltransferases require a corresponding UDP-sugar, such as UDP-Xyl, -Gal, -GlcUA, -GalNAc, and -GlcNAc, as a donor substrate. After specific core proteins have been synthesized, the synthesis of the common GAG-protein linkage region, GlcUAβ1-3Galβ1-3Galβ1-4Xylβ1-, is evoked by XylT, which transfers a Xyl residue from UDP-Xyl to the specific serine (Ser) residue(s) at the GAG attachment sites. The linkage tetrasaccharide is subsequently constructed by GalT-I, GalT-II, and GlcAT-I. These four enzymes are common to the biosynthesis of CS, DS, HS, and heparin. The first β1-4-linked GalNAc residue is then transferred to the GlcUA residue in the linkage region by GalNAcT-I, which initiates the assembly of the chondroitin backbone, thereby resulting in the formation of the repeating disaccharide region, [-3GalNAcβ1-4GlcUAβ1-]$_n$, by CS-polymerase. Alternatively, the addition of α1-4-linked GlcNAc to the linkage region by GlcNAcT-I initiates the assembly of the repeating disaccharide region [-4GlcNAcα1-4GlcUAβ1-]$_n$ of HS and heparin by HS-polymerase. Following the formation of the chondroitin and heparan backbones, both precursor chains are modified by sulfation and epimerization (see Figure 3). Each enzyme, its coding gene, and the corresponding inheritable disorder are described under the respective sugar symbols from the top of each line. SEMDJL1, spondyloepimetaphyseal dysplasia with joint laxity type 1.

and DS chains is initiated by the transfer of the first GalNAc from UDP-GalNAc to the GlcUA residue in the linkage region tetrasaccharide, GlcUA-Gal-Gal-Xyl-*O*-, by β1,4-*N*-acetylgalactosaminyltransferase-I (GalNAcT-I) (Figure 2) [193–196]. Alternatively, the transfer of a GlcNAc residue from UDP-GlcNAc to the linkage region tetrasaccharide by α1,4-*N*-acetylglucosaminyltransferase-I (GlcNAcT-I) is known to result in the initiation of the repeating disaccharide region of HS and heparin chains (Figure 2) [197–201]. Six chondroitin synthase family members have been identified including chondroitin synthases (ChSys), chondroitin-polymerizing factor (ChPF), and CSGalNAcTs (Figure 2 and Table 3) [193–196, 202–208]. ChSy1 is composed of 802 amino acids and is a bifunctional glycosyltransferase that exhibits CS-GlcAT-II and GalNAcT-II activities, which are required for the biosynthesis of the repeating disaccharide region, -4GlcUAβ1-3GalNAcβ1 (Table 3) [202]. ChSy1 itself is unable to construct the backbone of CS by the activity of polymerase, whereas the enzyme complex of ChSy with ChPF can form the repeating disaccharide region [203–205]. A precursor of CS, the chondroitin backbone, is then maturated by sulfation modified by various sulfotransferases such as uronosyl 2-*O*-sulfotransferase (UST) [209], chondroitin 4-*O*-sulfotransferases (C4ST) [210–212], chondroitin 6-*O*-sulfotransferase (C6ST) [213, 214], and GalNAc 4-sulfate 6-*O*-sulfotransferase (GalNAc4S-6ST) [215] (Figure 3 and Table 3). These transfer the sulfate group from the sulfate donor PAPS to the corresponding position of the GlcUA and

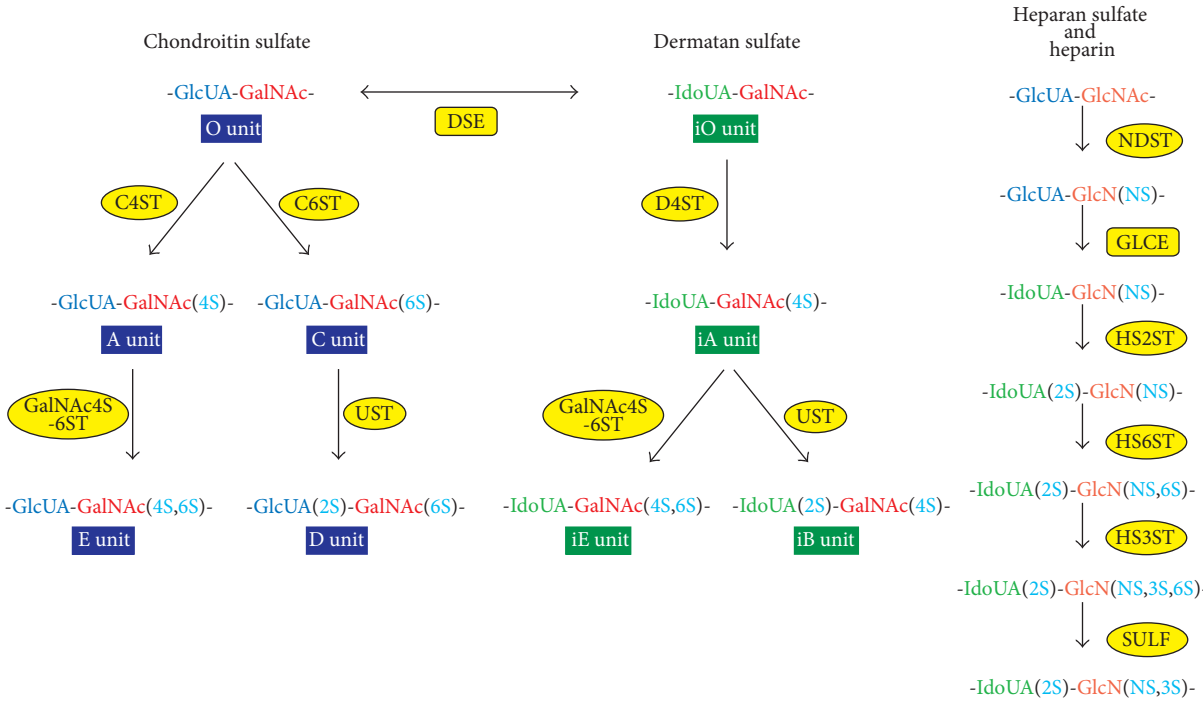


Figure 3: Modification pathways of CS, DS, HS, and heparin. After formation of the GAG backbones, including chondroitin and heparan, each sugar residue is modified by sulfation, which is catalyzed at various positions by sulfotransferases, as indicated in the figure. C4ST and C6ST transfer a sulfate group from PAPS to the C-4 or C-6 position of the GalNAc residues in the CS chain, which results in the formation of A-units and C-units, respectively. Further sulfations are catalyzed by GalNAc4S-6ST or UST, which is required for the formation of disulfated disaccharide units, E-units and D-units, respectively. DS-epimerase converts GlcUA into IdoUA by epimerizing the C-5 carboxy group in the chondroitin precursor, thereby resulting in the formation of the dermatan backbone. D4ST, which is distinct from C4ST, transfers a sulfate group from PAPS to the C-4 position of the GalNAc residues in dermatan to form the iA-units. The disulfated disaccharide units, iB and iE, are infrequently synthesized by UST and GalNAc4S-6ST, which are the same enzymes as those responsible for the biosynthesis of B and E units in CS chains. Following the synthesis of the backbone of HS or heparin by HS polymerases, the first modifications, *N*-deacetylation and *N*-sulfation, are catalyzed by NDST. Some GlcUA residues are then converted to IdoUA residues by GLCE. Thereafter, the hydroxy groups at the C-2 of IdoUA and at C-3 and C-6 of *N*-sulfated glucosamine and/or GlcNAc are sulfated by specific sulfotransferases. The 6-*O*-desulfation of the *N*-sulfated GlcN residue in the HS and heparin chains occurs by the action of SULF in order to modify the fine structure of HS for the regulation of interactions with various signaling molecules. C4ST, chondroitin 4-*O*-sulfotransferase; C6ST, chondroitin 6-*O*-sulfotransferase; D4ST, dermatan 4-*O*-sulfotransferase; DSE, dermatan sulfate C5-epimerase; GalNAc4S-6ST, GalNAc 4-sulfate 6-*O*-sulfotransferase; GLCE, heparan sulfate C5-epimerase; HS2ST, heparan sulfate 2-*O*-sulfotransferase; HS3ST, heparan sulfate 3-*O*-sulfotransferase; HS6ST, heparan sulfate 6-*O*-sulfotransferase; NDST, *N*-deacetylase/*N*-sulfotransferase; UST, uronyl 2-*O*-sulfotransferase; *N*S, 2S, 4S, and 6S, 2-*N*-, 2-*O*-, 4-*O*-, and 6-*O*-sulfate, respectively.

GalNAc residues in chondroitin. C4STs have been shown to regulate the chain length and amount of CS coordinating with CSGalNAcTs [216, 217].

Epimerization of the C-5 position of GlcUA residues in a chondroitin polymer as a precursor backbone occurs during or after the chain elongation, which results in the formation of the repeating disaccharide region, -4IdoUAα1-3GalNAcβ1-, of DS chains (Figure 3) [218–221]. The dermatan chains fully develop through sulfation catalyzed by dermatan 4-*O*-sulfotransferases (D4ST) [222, 223] and uronosyl 2-*O*-sulfotransferase (UST) [209] (Figure 3 and Table 3).

3.3. Repeating Disaccharide Region of HS and Heparin. Following the construction of the linkage region tetrasaccharide, GlcUAβ1-3Galβ1-3Galβ1-4Xylβ1-*O*-serine, on the core protein, transfer of the GlcNAc residue from UDP-GlcNAc to the tetrasaccharide induces chain polymerization of the repeating disaccharide region of HS and heparin catalyzed by GlcNAcT-I [197–201] (Figure 2). After the addition of the first GlcNAc to the linkage region, the growing pentasaccharide is further elongated by alternate additions of GlcUA and GlcNAc from UDP-GlcUA and UDP-GlcNAc by HS-β1,4glucuronyltransferase-II (HS-GlcAT-II) and α1,4-*N*-acetylglucosaminyltransferase-II (GlcNAcT-II), respectively (Figure 2). Exostosin 1 (EXT1) as well as 2 (EXT2) both exhibit HS-GlcAT-II and GlcNAcT-II activities [199, 224–226] (Table 4). Furthermore, the heterodimeric complex of EXT1 and EXT2 exhibits HS polymerase activity on a linkage region tetrasaccharide acceptor *in vitro*, which results in the biosynthesis of HS and heparin polysaccharides [227, 228]. Three homologous genes to the *EXT* have been identified [6, 14, 229]. EXTL1 and EXTL2 exhibit GlcNAcT-II and GlcNAcT-I activities, respectively, whereas EXTL3 has not only GlcNAcT-I, but also GlcNAcT-II activities (Figure 2 and Table 4) [200, 201].

After the formation of the repeating disaccharide backbone of HS chains by EXTs and EXTLs, GlcNAc residues are converted into GlcN residues by GlcNAc *N*-deacetylase (Figure 3) [6, 14, 198]. A sulfate group is subsequently transferred from PAPS to the GlcN by GlcN *N*-sulfotransferase [6, 14, 198]. Both enzymes are encoded by a single gene, *GlcNAc N-deacetylase/N-sulfotransferase* (Figure 3 and Table 4) [230–233]. The interconversion of GlcUA to IdoUA in HS and heparin is achieved by HS-glucuronyl C5-epimerase (Figure 3) [234–236]. Moreover, sulfation at the C-2 position of uronic acid as well as C-3 and C-6 positions of the GlcN residues in the HS and heparin are catalyzed by HS 2-*O*-sulfotransferase, HS 3-*O*-sulfotransferase, and HS 6-*O*-sulfotransferase, respectively (Figure 3 and Table 4) [237–244]. The desulfation of 6-*O*-sulfated GlcNS residues in HS chains by HS 6-*O*-endosulfatase modifies the fine structure of HS in order to regulate various biological events including cell signaling, tumor growth, and angiogenesis (Figure 3 and Table 4) [245–247].

4. Knockout and Transgenic Mice of GAG Biosynthetic Enzymes

4.1. Xylt1. A recessive dwarf mouse mutant (*pug*) obtained from an *N*-ethyl-*N*-nitrosourea mutagenesis screen was attributed to a missense mutation in *Xylt1*, which resulted in the substitution of an amino acid (p.Trp932Arg) [59]. XylT activity in the *pug* mutant was markedly reduced *in vitro*, which resulted in a decrease in the amount of GAGs in cartilage. Furthermore, early ossification was reported in this mutant, which resulted in a shorter body length than that of a wild-type embryo. These phenotypes may be caused by an upregulation of Indian hedgehog signaling but not MAPK signaling due to lack of GAGs [59].

4.2. B3gat3 (GlcAT-I). Mice deficient in *GlcAT-I* synthesize a smaller CS and HS chain in their blastocysts than that of the heterozygous mice [75]. In addition, these mice exhibit an embryonic lethality before the 8-cell stage due to the failure of cytokinesis, which has been attributed to a deficiency in CS, but not HS based on the findings reported in embryos treated with chondroitinase and heparinase [76]. Moreover, interaction of CS with E-cadherin, which regulates the differentiation of embryonic stem cells, may control Rho signaling pathway [76]. These findings indicated that CS, but not HS, is involved in regulating cell division in mammals.

4.3. Csgalnact1 and Csgalnact2. *CSGalNAcT1*-null mice have been shown to produce a smaller amount as well as a shorter length of CS chains than the wild-type [84, 85]. These mice also have shorter limbs and axial skeleton and a thinner growth plate in cartilage than wild-type mice, which results in a slightly shorter body length and smaller body weight [84, 85]. It is likely that the reduction in CS may affect normal chondrogenesis and formation of type II collagen fibers [84]. These findings suggest that CSGalNAcT1 is essential for the differentiation and maturation of cartilage.

A deficiency in CSGalNAcT1, but not CSGalNAcT2, has been shown to promote axonal regeneration following spinal cord injury [86]. CS-PGs function as barrier-forming molecules during axonal regeneration after damage to the nervous system [10]. Thus, the down- and upregulation of CS and HS biosynthesis, respectively, in the scars of *CSGalNAcT1*$^{-/-}$ mice led to better recovery from injuries in the nervous system than the wild type.

4.4. Chsy1. *Chsy1*-deficient mice are viable but exhibit chondrodysplasia, progression of the bifurcation of digits, delayed endochondral ossification, and reduced bone density [81]. Furthermore, a decrease in 4-*O*-sulfation and increase in 6-*O*-sulfation as well as desulfation of the GalNAc residues of CS have been reported in the cartilage of *Chsy1*$^{-/-}$ mice. The signaling of hedgehog but not of FGF, bone morphogenetic protein, or transforming growth factor-β altered in primary chondrocytes from Chsy1-deficient mice [81], which suggests that CS-PGs and hedgehog protein may coordinately regulate skeletal development and digit patterning.

4.5. Chpf. Mice deficient in *Chpf*, also known as *chondroitin sulfate synthase-2* (*CSS2*), are fertile and viable and exhibit no obvious abnormalities including osteoarthritis and cartilage development [82]. These findings are consistent with the study by Wilson et al. [81].

4.6. Dse and Dsel. The body weight of *Dse*$^{-/-}$ mutant mice, which have fewer IdoUA residues in the skin, is ~30% smaller than that of the wild type [88, 89]. Although no significant differences were observed in the content of collagen between *Dse*$^{-/-}$ and the wild type, the ultrastructure of collagen fibrils in the dermis and hypodermis was thicker in the deficient mice than in the wild type, and a decline in their mechanical strength was also noted in the deficient mice. On the other hand, no morphological or histological abnormalities have been reported in mice targeted with the disruption of DS epimerase-2 encoded by *Dsel* [93]. In addition, 4-*O*-sulfation of the DS chain was decreased in the brain of *Dse2*$^{-/-}$, whereas the adult *Dse2*$^{-/-}$ brain had normal structures in the extracellular matrix. The function of Dse2 appears to be compensated by Dse1 [93].

4.7. Chst3 (C6st1). The number of 6-*O*-sulfated disaccharide units including the C-unit (GlcUA–GalNAc6-*O*-sulfate) and D-unit (GlcUA2-*O*-sulfate–GalNAc6-*O*-sulfate) was shown to be markedly reduced in the spleens and brains of *C6st1*-deficient mice, and the number of naive T lymphocytes was also decreased in the spleen [114]. However, brain development in *C6st1*$^{-/-}$ mice is normal in spite of a decrease in D-units in the CS chains of the null mice.

CS-PGs are newly synthesized in the central nervous system following injury, and this inhibits axonal regeneration [10, 248]. Furthermore, upregulation of the expression of *C6st1* and 6-*O*-sulfated CS-PGs has been demonstrated in glial scars after a cortical injury [249]. *C6st1*$^{-/-}$ mice had

fewer or a similar number of regenerative axons after axotomy to the wild type [115].

An increase in chondroitin 6-*O*-sulfation was observed in the developing brains of *C6st1*-transgenic mice and affected the formation of the perineuronal nets and cortical plasticity [116], which are specialized structures of the dense organized matrix, which are composed of CS-PGs, hyaluronan, tenascins, and link proteins and regulate neuronal plasticity and neuroprotection [250]. Chondroitin 6-*O*-sulfate may regulate the maturation of parvalbumin-expressing interneurons through the incorporation of Otx2 [116], which regulates ocular dominance plasticity.

4.8. Chst11 (C4st1). The *C4st1* gene was identified as a target gene of bone morphogenetic protein signaling using gene trap experiments [94]. *C4st1*-mutant mice exhibit severe dwarfism and die within six hours of birth due to respiratory failure [95]. Moreover, severe chondrodysplasia with abnormalities in the cartilage growth plate and chondrocyte columns, marked reductions in GAG content and 4-*O*-sulfated CS, the downregulation of bone morphogenetic protein signaling, and the upregulation of transforming growth factor-β have been observed in these mice. These findings indicated that C4ST1 and the 4-*O*-sulfation of CS chains were essential for the signaling pathways of bone morphogenetic protein and transforming growth factor-β as well as cartilage morphogenesis.

4.9. Chst14 (D4st1). $D4st1^{-/-}$ mice have a smaller body weight, a kinked tail, and more fragile skin and are less fertile than the wild type. [107]. In addition, axonal regrowth is initially facilitated in $D4st1^{-/-}$ mice following nerve transection.

Furthermore, the impaired proliferation of neural stem cells, reduced neurogenesis, and an altered subpopulations of radial glial cells have been reported in *D4st1*-deficient mice [96]. The epitope structure recognized by the monoclonal anti-CS antibody 473HD, which contains the D-unit (GlcUA-2-*O*-sulfate–GalNAc-6-*O*-sulfate) and iA-unit (IdoUA–GalNAc4-*O*-sulfate) in the CS-DS hybrid chains on PGs, such as phosphacan, is required for the formation of neurospheres and as a marker for radial glial cells [251]. Expression of the 473HD epitope was shown to be decreased in the neural stem cells of $D4st1^{-/-}$ mice, and this resulted in the altered formation of neurospheres [96]. These findings indicated that DS chains and/or D4ST1 are essential for the proliferation and differentiation of neural stem cells.

4.10. Chst15 (Galnac4s-6st). *Galnac4s-6st*-null mice are viable and fertile and completely defective in the E-unit, GlcUA-GalNAc(4-,6-*O*-disulfates), in both CS and DS chains [117]. The activities of carboxypeptidase A and tryptase from bone marrow-derived mast cells in $Galnac4s\text{-}6st^{-/-}$ were lower than those in the wild type, which suggested that the E-unit-containing CS chain or CS-PGs may be involved in the retention of these proteases in the granules of mast cells.

4.11. Ext1 and Ext2. Gene knockout mice produced by the targeted disruption of the gene encoding *Ext1* and *Ext2* died by embryonic day 8.5–14.5 due to defects in the formation of the mesoderm and a failure in egg cylinder elongation [119–121, 136]. The GlcUA and GlcNAc transferase activities are decreased and HS chains are shorter in mice carrying a hypomorphic mutation in *EXT1* generated by gene trapping, which affect the signaling pathways of Indian hedgehog and parathyroid hormone-related peptide [120, 121]. Thus, it is difficult to analyze the *in vivo* functions of HS chains using conventional knockout mice. A growing number of conditional knockout mice produced by targeted disruption of the gene encoding HS biosynthetic enzymes has provided an insight into the physiological functions of HS and HS-PGs [14]. For example, pluripotent embryonic stem cells in which *Ext1* was disrupted fail to differentiate into neural precursor cells and mesoderm cells due to the enhancement of Fgf signaling and retention of the high expression of *Nanog* [122, 123]. Conditional *Ext1*-knockout mice selectively disrupted in the nervous system die within the first day of life and have defective olfactory bulbs, midbrain-hindbrain region, and axon guidance due to a disturbance in signaling pathways including Fgf8 and Netrin-1 [124–126]. Conditional *Ext1*-knockout mice specific for postnatal neurons exhibit a large number of autism-like phenotypes in spite of a normal morphology in the brain [127]. On the other hand, mice in which *Ext1* was specifically disrupted for chondrocytes and the limb bud, *Ext2* heterozygous mice, and compound $Ext1^{+/-}/Ext2^{+/-}$ mice display severe skeletal defects with cartilage differentiation and chondrocyte maturation, and these defects resembled an autosomally dominant inherited genetic disorder, human hereditary multiple exostoses [128–132]. Disruption of the *Ext1* gene in glomerular podocytes results in an abnormal morphology in these cells [133]. Furthermore, conditional knockout mice lacking *Ext1* in the high endothelial venules and vascular endothelium cells show a decrease in lymphocyte homing to peripheral lymph nodes and a compromised contact hypersensitivity response [134, 135]. These findings suggest that HS and HS-PGs are essential for playing a role in their physiological functions in a tissue-specific manner.

4.12. Extl2 and Extl3. Mice deficient in *Extl2* are viable and develop normally; however, they produce a larger amount of GAG chains [137, 138]. Liver regeneration was shown to be impaired in these knockout mice following liver injury induced by administration of CCl_4 due to suppression of the response to hepatocyte growth factor [137].

Mice deficient in *Extl3* are embryonically lethal, which is similar to mice lacking *Ext1* or *Ext2* [139]. In addition, selective inactivation of the *Extl3* gene in pancreatic islet β-cells caused an abnormal morphology as well as a reduction in the proliferation of the islets, which resulted in defective insulin secretion [139]. However, it remains to be determined how HS, HS-PGs, or Extl3 is involved in insulin secretion.

4.13. Ndst1, 2, and 3. Functional analyses of HS and heparin using *Ndst1*-deficient mice have been performed in approximately 20 studies to date [140–164]. Representative studies have been reviewed in this chapter. *Ndst1*-deficient mice die after birth and have cerebral hypoplasia, axon guidance errors, defects in the eye and olfactory bulbs, insufficient milk production caused by a defect in lobuloalveolar expansion in the mammary gland, and morphological abnormalities in the podocytes [140–142, 145, 156, 157, 162, 164]. *Ndst1* conditional knockout mice specific for the liver accumulated triglyceride-rich lipoproteins due to a reduction in the clearance of cholesterol-rich lipoprotein particles [148, 163]. Furthermore, mice with the endothelial-targeted deletion of *Ndst1* exhibited suppressed experimental tumor growth and angiogenesis including microvascular density and branching of the surrounding tumors due to altered responses to Fgf2 and Vegf, which resulted in reduced Erk phosphorylation [147] and attenuated allergic airway inflammation [151].

Embryos from *Ndst2*-deficient mouse are viable and fertile, whereas their mast cells are unable to synthesize heparin, which leads to changes in morphology and severely reduced amounts of granule proteases [165–167]. These findings indicated that the storage of proteases in granules is controlled by heparin or heparin-PG, such as serglycin [165–167]. On the other hand, *Ndst3*-deficient mice develop normally and are fertile [168].

4.14. Glce (HS GlcUA C5-epimerase). Mice with the targeted disruption of *HS epimerase* die immediately after birth and have agenesis of the kidney, a shorter body length, and lung defects [169, 170]. Furthermore, developmental abnormalities in the lymphoid organs, including the spleen, thymus, and lymph nodes, have been reported in the knockout mice [171, 172]. IdoUA-containing HS chains are critical for early morphogenesis of the thymus through binding with Fgf2, Fgf10, and bone morphogenetic protein 4 [171]. In addition, the interaction of HS with a proliferation inducing ligand, hepatocyte growth factor, and CXCL12α is required for B-cell maturation [172].

4.15. Hs2st. Gene trap mice lacking *Hs2st* die during the neonatal period and exhibit renal aplasia and defects in the eyes, skeleton, and retinal axon guidance [173–179]. In addition, the cell-specific disruption of *Hs2st* in the endothelial and myeloid cells enhanced the infiltration of neutrophils due to an increase in their binding to IL-8 and macrophage inflammatory protein-2 [162]. Mice with the specific disruption of *Hs2st* in the liver accumulate plasma triglycerides and the uptake of very-low-density lipoproteins is reduced, whereas mice with the specific disruption of *Hs6st* in the liver do not. These findings suggest that the clearance of plasma lipoproteins is dependent on the 2-*O*-sulfation of HS [153].

4.16. Hs3st1. *HS3ST1*$^{-/-}$ mice display normal development and anticoagulant activity [184]; however, it was previously demonstrated that the GlcN 3-*O*-sulfate structure was essential for the anticoagulant activity of heparin and HS [252]. Other HS3ST family members such as HS3ST2, HS3ST3a, HS3ST3b, HS3ST4, HS3ST5, and HS3ST6 may compensate for the loss of HS3ST1 [184].

4.17. Hs6st1 and Hs6st2. *HS6ST1*-null mice die during the late embryonic stage, are smaller than the wild type at birth, and have defective retinal axon guidance due to the disturbance of Slit-Robo signaling [177, 178, 181]. In contrast, *HS6ST2*-deficient mice develop normally [183]. However, serum levels of thyroid-stimulating hormone and the thyroid hormone, thyroxin, are higher and lower, respectively, in the deficient mice, which cause a reduction in energy metabolism with an increase in body weight [183]. The storage of mast cell proteases is altered in double knockout mice with *HS6ST1*$^{-/-}$/*HS6ST2*$^{-/-}$ [182], and their embryonic fibroblasts are partially defective in FGF signaling [253].

4.18. Sulf1 and Sulf2 (HS 6-O-endosulfatase). *Sulf1*$^{-/-}$ mice exhibit no apparent abnormalities [185]. On the other hand, *Sulf2*$^{-/-}$ mice have a smaller body size and mass [185, 192]. Mice deficient in both *Sulf1* and *Sulf2* have multiple defects including skeletal and renal malformations, which result in neonatal lethality [186]. HS 6-*O*-sulfation and/or desulfation by Sulfs are known to be involved in the cartilage homeostasis mediated by bone morphogenetic protein and Fgf [187], dentinogenesis through Wnt signaling [188], neurite outgrowth mediated by glial cell line-derived neurotrophic factor [189], muscle regeneration [190], and brain development [191]. These findings indicate that the fine-tuning of 6-*O*-sulfation by Sulfs may control multiple functions of HS chains during morphogenesis.

5. Human Disorders Affecting the Skeleton and Skin due to the Disturbance of GAGs

5.1. PAPSS2. Spondyloepimetaphyseal dysplasia of Pakistani type, which is characterized by kyphoscoliosis, generalized brachydactyly, short and bowed lower limbs, and enlarged knee joints, is caused by mutations in *PAPSS2*: p.Ser438X and p.Arg329X [43, 44].

Patients with mutations in *PAPSS2*, resulting in the substitution of corresponding amino acids (p.Thr48Arg, p.Arg329X, and p.Ser475X), also have spondylodysplasia and premature pubarche, which are accompanied by a short stature, bone dysplasia, excess androgens, hyperandrogenic anovulation, and the loss of dehydroepiandrosterone sulfate [45]. Sulfotransferase 2A1 has been shown to transfer a sulfate group from PAPS to dehydroepiandrosterone (DHEA) in the adrenal glands and liver, resulting in the formation of DHEA-sulfate [254]. The inactivation of PAPSS2 inhibits of not only the formation of PAPS but also the conversion of DHEA into DHEA-sulfate, which leads to the accumulation of DHEA in patients [45]. Excess DHEA is finally converted to testosterone through androsterone.

Autosomal recessive brachyolmia, which is a heterogeneous group of skeletal dysplasias and primarily affects the spine, is also caused by *PAPSS2* mutations [46, 47]. Brachyolmia is characterized by a short stature due to a short

trunk, irregular endoplates, a narrow intervertebral disc, calcification of cartilage in the ribs, a short femoral neck and metacarpals, and normal intelligence [46–48]. However, the excess amount of androgens cannot be detected in these patients. Furthermore, PAPS synthase activity was absent in the recombinant mutant enzymes, including p.Cys43Tyr, p.Leu76Gln, and p.Val540Asp [47].

5.2. XYLT1. Mutation in *XYLT1* causes an autosomal recessive short stature syndrome characterized by alterations in the distribution of fat, intellectual disabilities, and skeletal abnormalities including a short stature and femoral neck, thickened ribs, plump long bones, and distinct facial features [56]. The homozygous mutation in *XYLT1* gives rise to the substitution of the amino acid, p.Arg481Trp in the deduced catalytic domain, which results in decorin without a DS side chain in addition to mature decorin-PG with a DS chain from the fibroblasts of the patient [56]. In addition, the mutant XYLT1 is diffusely localized in the cytoplasm and partially in the Golgi in the fibroblasts of the patient.

Desbuquois dysplasia type 2 is a multiple dislocation group of skeletal disorders that is characterized by a short stature, joint laxity, and advanced carpal ossification [57]. Five distinct *XYLT1* mutations have been identified to date, including a missense substitution (p.Arg598Cys), nonsense mutation (p.Arg147X), truncated form mutation (p.Pro93AlafsX69), and two splice site mutations [58]. Furthermore, fibroblasts from the affected individuals synthesized a smaller amount of CS and/or DS than those from healthy controls [58].

5.3. B4GALT7 (GalT-I). Ehlers-Danlos syndrome is a heterogeneous group of heritable connective tissue disorders characterized by joint and skin laxity as well as tissue fragility. Six major types (classical, hypermobility, vascular, kyphoscoliosis, arthrochalasia, and dermatosparaxis types) and several minor types, including the progeroid type, are currently known [255]. Mutations in *B4GALT7* encoding GalT-I cause Ehlers-Danlos syndrome-progeroid type 1, which is characterized by an aged appearance, hypermobile joints, loose yet elastic skin, hypotonic muscles, craniofacial dysmorphism, a short stature, developmental delays, generalized osteopenia, and defective wound healing [61–64]. Galactosyltransferase activity is reduced in the mutant enzymes, p.Arg270Cys, p.Ala186Asp, p.Leu206Pro, and p.Arg270Cys, which results in the lack of DS side chains on decorin and biglycan core proteins and also smaller CS and HS side chains on other PGs [64–68].

A homozygous mutation in *B4GALT7* (p.Arg270Cys) causes a variant of Larsen syndrome in Reunion Island in the southern Indian Ocean, which is called Larsen of Reunion Island syndrome, and is characterized by distinctive facial features, multiple dislocations, dwarfism, and hyperlaxity [69].

5.4. B3GALT6 (GalT-II). Ehlers-Danlos syndrome-progeroid type 2 is caused by mutations in *B3GALT6* encoding GalT-II [70, 71]. GalT-II activity by the mutant enzyme (p.Ser309Thr) is significantly decreased, leading to the loss of GAG chains on the core proteins of various PGs [70]. The autosomal-recessive disorder, spondyloepimetaphyseal dysplasia with joint laxity type 1, which is characterized by hip dislocation, elbow contracture, clubfeet, platyspondyly, hypoplastic ilia, kyphoscoliosis, metaphyseal flaring, and craniofacial dysmorphisms such as prominent eyes, blue sclera, a long upper lip, and small mandible with cleft palate, is also caused by mutations in *B3GALT6* [70–72, 256]. Skeletal and connective abnormalities in both Ehlers-Danlos syndrome-progeroid type 2 and spondyloepimetaphyseal dysplasia with joint laxity type 1 overlap; however, these individuals have no common mutations among fifteen different mutations [70]. The GalT-II activities of the recombinant enzymes, p.Ser65Gly-, p.Pro67Leu-, p.Asp156Asn-, p.Arg232Cys-, and p.Cys300Ser-B3GALT6, were shown to be significantly lower than those of wild-type-B3GALT6 [70]. The mutation that affected the initiation codon, c.1A>G (p.Met1?), for *B3GALT6* resulted in a lower molecular weight of the recombinant protein than that of the wild-type protein with the deletion of 41 amino acids at the N-terminus, which indicated a shift in translation at the initiation codon at the second ATG [70]. Although wild-type B3GALT6 is expressed in the Golgi, the mutant enzyme (p.Met1?) is localized in the nucleus and cytoplasm [70], indicating that the mutant protein may not be functional due to its cellular mislocalization.

5.5. B3GAT3 (GlcAT-I). A mutation (p.Arg277Gln) in the *B3GAT3* gene encoding GlcAT-I is known to cause Larsen-like syndrome [73, 74], which is characterized by dislocations in the hip, knee, and elbow joints, equinovarus foot deformities, and craniofacial dysmorphisms such as a flattened midface, depressed nasal bridge, hypertelorism, and a prominent forehead [257, 258]. These patients mainly have elbow dislocations with congenital heart defects including a bicuspid aortic valve in addition to characteristic symptoms of Larsen-like syndrome [73]. The p.Arg277Gln mutation results in a marked reduction in GlcAT-I activity in the fibroblasts of these patients and the recombinant enzyme protein [73]. Mature decorin-PG, which is secreted by fibroblasts and has a single DS side chain, was observed in the fibroblasts of healthy controls [73]. On the other hand, fibroblasts from patients generate both a PG form of decorin and DS-free decorin [73]. Moreover, the number of CS and HS in the patients' cells is also reduced.

5.6. CSGALNACT1. Neuropathies including Guillan-Barré syndrome, chronic inflammatory demyelinating polyneuropathy, hereditary motor sensory neuropathy, and unknown etiologies are partially caused by mutations in *CSGALNACT1* encoding GalNAcT-I and GalNAcT-II [83]. The GalNAcT-II activities of the recombinant enzymes, CSGalNAcT1-His234Arg and -Met509Arg, were shown to be markedly reduced [83], which indicated that affect in CS chains on PGs in the nervous system may lead to peripheral neuropathies.

5.7. CHSY1. Patients with mutations in *CHSY1* have Temtamy preaxial brachydactyly syndrome, which is an autosomal recessive congenital syndrome characterized by facial dysmorphism, dental anomalies, brachydactyly, hyperphalangism, growth retardation, deafness, and delayed motor and mental developments [77, 78]. Their mutations result in the substitution of amino acids and truncation of the CHSY1 protein including p.Gly5AlafsX30, p.Gly19-Leu28del, p.Glu33SerfsX1, p.Gln69X, and p.Pro539Arg and a splice-site mutation [77–79]. A heterozygous mutation in *CHSY1* (p.Phy362Ser) was recently identified in a patient with neuropathy [80].

5.8. CHST3 (C6ST1). Spondyloepiphyseal dysplasia Omani type, which is characterized by severe chondrodysplasia with major involvement of the spine, is caused by a loss-of-function mutation in *C6ST1* [108–113]. Patients with the substitution of amino acid in C6ST1, p.Arg304Gln, have severe kyphoscoliosis, a short stature, mild brachydactyly, rhizomelia, fusion of the carpal bones, and osteoarthritis in the elbow, wrist, and knee joints [108, 109]. Furthermore, additional clinical features, including deafness, metacarpal shortening, and aortic regurgitations due to ventricular septal, mitral, and/or tricuspid defects, have been reported in Turkish siblings who had different mutations in *C6ST1* (p.Tyr141Met and p.Leu286Pro) [110, 111]. Mutant enzymes of the recombinant C6ST1 and enzymes from the patients' fibroblasts had markedly reduced C6ST activity, which resulted in the loss of chondroitin 6-*O*-sulfate in the fibroblasts [109–111]. Moreover, chondrodysplasia with multiple dislocations, Desbuquois syndrome, autosomal recessive Larsen syndrome, and humero-spinal dysostosis have been attributed to distinct *CHST3* mutations (p.Leu259Pro, p.Arg222Trp, p.Leu307Pro, p.Tyr201X, p.F206X, p.Glu372Lys, p.Gly363AlafsX30, and a mutation at the splice site) [112, 113]. Different pathological diagnoses may be caused by the relatively narrow clinical features and age-related descriptions of the same conditions [113].

5.9. CHST14 (D4ST1). Ehlers-Danlos syndrome musculocontractural type 1, which is characterized by progressive joint and skin laxity, multiple congenital contractures, progressive multi-system complications, and characteristic craniofacial features, is caused by mutations in *CHST14* encoding D4ST1 (p.Val49X, p.Lys69X, p.Pro281Leu, p.Cys289Ser, p.Tyr293Cys, and p.Glu334GlyfsX107) [97–102]. A recent study described a case of Ehlers-Danlos syndrome musculocontractural type 1 (p.Val49X) in which muscle hypoplasia and weakness was observed, which resulted in myopathy based on laboratory findings such as muscle biopsy, ultrasound, and electromyography [103].

The recombinant mutants of D4ST1 (p.Pro281Leu, p.Cys289Ser, and p.Tyr293Cys) and fibroblasts from affected individuals have markedly reduced sulfotransferase activity [99]. Furthermore, a single DS side chain on decorin-PG from the fibroblasts of patients was found to be replaced by a CS chain, but not dermatan [99]. Immature decorin-PG results in the dispersion of collagen bundles in the dermal tissues of patients.

The autosomal recessive disorder, adducted thumb-clubfoot syndrome, which is characterized by an adducted thumb, clubfoot, craniofacial dysmorphism, arachnodactyly cryptorchidism, an atrial septal defect, kidney defects, cranial ventricular enlargement, and psychomotor retardation, is also caused by mutations in *CHST14* (p.Val49X, p.Arg135Gly, p.Leu137Gln, p.Arg213Pro, and p.Tyr293Cys) [104–106]. The fibroblasts of these patients lack DS chains and have an excess amount of CS chains.

5.10. DSE. A mutation in *DSE* (p.Ser268Leu) has been shown to cause Ehlers-Danlos syndrome musculocontractural type 2 [87]. Clinical features including hypermobility of the finger, elbow, and knee joints, characteristic facial features, contracture of the thumbs and feet, and myopathy have been observed in these patients. Epimerase activity is markedly reduced not only in the recombinant mutant DSE (p.Ser268Leu) but also in the cell lysate from these patients [87]. In addition, a decrease in the biosynthesis of DS accompanied by an increase in that of CS has been reported in the fibroblasts of these patients. The deficiencies associated with DSE in addition to D4ST1 affect the biosynthesis of DS, which implies that both enzymes are essential for the development of skin and bone as well as the maintenance of their extracellular matrices.

6. Conclusions

The biological roles of CS, DS, and HS chains *in vivo* have been revealed by examining knockout mice in addition to nematodes, fruit flies, and zebrafish [4, 8, 12–14]. However, the mice deficient in glycosyltransferases or sulfotransferases involved in the biosynthesis of GAGs showed embryonic lethality or death shortly after the birth. These observations indicate that GAGs or PGs are essential for early development. Furthermore, studies using the conditional knockout mice have revealed the specific functions of GAGs in individual organs. Recent advances in the study of human genetic diseases in the bone and connective tissue have also clarified the biological significance of the GAG side chains of PGs [7, 14, 20]. The clinical manifestations in human disorders caused by deficiency in the biosynthetic enzymes of GAGs do not always agree with the phenotypes of the deficiency in the corresponding enzymes in knockout mice. This contradiction may be due to the residual enzymatic activity or GAGs in human patients. Although null mutant mice show severe phenotypes including embryonic lethality, human patients appear to show various symptoms depending on the degree of remaining activity of the enzymes. Further comprehensive approaches to the study of molecular pathogeneses involving CS, DS, and HS chains are required to facilitate the development of therapeutics and design of new drugs for these diseases.

Abbreviations

B3GALT6:	Beta 1,3-galactosyltransferase 6
B4GALT7:	Beta 1,4-galactosyltransferase 7
B3GAT3:	Beta-1,3-glucuronyltransferase 3
C4ST:	Chondroitin 4-*O*-sulfotransferase
C6ST:	Chondroitin 6-*O*-sulfotransferase
CHST:	Carbohydrate sulfotransferase
CHSY:	Chondroitin synthase
CS:	Chondroitin sulfate
CSGALNACT:	Chondroitin sulfate *N*-acetylgalactosaminyltransferase
D4ST:	Dermatan 4-*O*-sulfotransferase
DS:	Dermatan sulfate
DSE:	Dermatan sulfate-glucuronyl C5-epimerase
DSEL:	Dermatan sulfate epimerase-like
GAG:	Glycosaminoglycan
GalNAc:	*N*-Acetyl-D-galactosamine
GalNAc4S-6ST:	*N*-Acetyl-D-galactosamine 4-sulfate 6-*O*-sulfotransferase
GlcUA:	D-Glucuronic acid
HS:	Heparan sulfate
IdoUA:	L-Iduronic acid
PAPS:	3′-Phosphoadenosine 5′-phosphosulfate
PAPSS2:	3′-Phosphoadenosine 5′-phosphosulfate synthase 2
PG:	Proteoglycan.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Review Article

Metabolism of Cartilage Proteoglycans in Health and Disease

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Cartilage proteoglycans are extracellular macromolecules with complex structure, composed of a core protein onto which a variable number of glycosaminoglycan chains are attached. Their biosynthesis at the glycosaminoglycan level involves a great number of sugar transferases well-orchestrated in Golgi apparatus. Similarly, their degradation, either extracellular or intracellular in lysosomes, involves a large number of hydrolases. A deficiency or malfunction of any of the enzymes participating in cartilage proteoglycan metabolism may lead to severe disease state. This review summarizes the findings regarding this topic.

1. Introduction

Proteoglycans (PGs) are a class of complex macromolecules found extracellularly in most tissues. They are composed of a core protein onto which a variable number of glycosaminoglycan (GAG) side chains are attached. According to their presence in cell and tissues, interactions with other macromolecular components, and detailed structure of the core protein, PGs are divided to different families, which has been extensively reviewed [see, e.g., [1–4]]. According to the status of the cell or the tissue, the GAG chains in a selected PG may vary in number and size.

PGs studies started almost with the beginning of the 20th century; however, they became familiar in late sixties, after the application of the pioneering extracting protocol of Sajdera and Hascall [5]. A tremendous amount of work is presented during the seventies, regarding isolation, purification, and characterization of proteoglycans from extracellular matrix in healthy and pathologic states, together with studies on their biosynthesis from cell or tissue cultures and cell-free systems. In these years and due to limitations in methodology, the studies focused mainly on tissues of high PG content, that is, cartilage, aorta, and skin. In later years, through the development of micro- and nanoanalytical techniques, PG studies were extended to other tissues poorer in PGs. By combining molecular biology techniques, the research on PGs became easier and led to novel insights into the structure and function on PGs. In the middle of the eighties, new members of PGs were clearly identified and, thus, it became evident that the term proteoglycan was not sufficient to describe the sum of heterogeneous and polydisperse macromolecules isolated from tissues. Therefore, PGs were divided to five different families, according to their structure, location, or properties. The hyalectans, having the ability to interact electrostatically with hyaluronan and link protein, the basement membrane PGs, the cell-surface PGs, the intracellular PGs, and the small leucine-rich PGs (SLRPs). The name of a selected PG was coined from a characteristic property with the suffix-can. According to this, the main proteoglycan in cartilage was termed aggrecan, since it can form aggregates with hyaluronan and link protein. Similarly, a small PG of the SLRPs family that contains two GAG chains was termed biglycan. Some exceptions are known, since some PGs are present in tissue in both PG and free core protein forms, and, thus, their name came almost exclusively from their properties, that is decorin, the PG that decorate collagen fibrils formation.

With the development of bioinformatics and the sequencing of the whole genome of various organisms, new PG members, with peculiar properties, were added.

Moreover, by sequencing PGs at DNA or protein level, characteristic protein domains were revealed, indicating their existence as multifunctional macromolecules with a great impact on cell life and behavior [6, 7], making, thus, PG

Table 1: Structural proteoglycans of cartilage.

Proteoglycan	Molecular mass of core protein	Number of GAG chains	Molecular mass of GAG chain
Aggrecan	208–220	~100	~14
Decorin	36	1	~28
Biglycan	38	1-2	~28

research a significant field, employing scientists of different background.

Although basic research on PGs became familiar in the late sixties, the relation of PGs with specific diseases was well established much earlier. Examples are the various mucopolysaccharidoses (MPS), which constitute a family of lysosomal storage diseases characterized by deficiencies in lysosomal hydrolases responsible for the degradation of GAGs (mucopolysaccharides) [8].

2. Proteoglycan Structure and Populations

PGs are found in abundance in cartilage, as they represent about 10% of tissue dry weight. The typical members are shown in Table 1.

Aggrecan, formerly known as "main cartilage proteoglycan" or "large aggregating proteoglycan," consists of a protein core of about 200 kDa molecular mass, onto which about 100 chondroitin sulfate (CS), 50 keratan sulfate (KS), and 50 *N*- and *O*-linked oligosaccharide chains are attached. Dermatan sulfate (DS) is absent from aggrecan. Through its N-terminal domain, aggrecan possesses the ability to interact with hyaluronan and link protein [6]. More than 1000 aggrecan molecules may participate in each one of these giant aggregates formed and, thus, provide a stable matrix capable of absorbing high compressive loads by water desorption and resorption. Moreover, aggregates are strong inhibitors of hydroxyapatite growth. The content and composition of aggrecan seems largely to be related with the state of tissue. For example, in aged cartilage, a decrease in the absolute amount of aggrecan is observed, together with a decrease in CS content and an increase in KS content and size.

Biglycan and decorin belong to the small and rich in leucine-repeats PG family. They possess a core protein of about 40 kDa molecular mass onto which two and one, respectively, CS/DS chains are attached. Nonglycanated forms (no GAG chains) of both PGs are also observed. These PGs help to stabilize and organize collagen fibers and, in addition, they can bind growth factors [9, 10]. Decorin can bind transforming growth factor β (TGF-β), serving as a sink to keep the growth factor sequestered in the matrix. Biglycan can bind bone morphogenetic protein-4 (BMP-4), a member of TGF-β family, and it plays a significant role in cartilage and bone metabolism. Both PGs amounts are substantially increased with ageing, together with increase in DS and their nonglycanated forms.

Aggrecan represents the vast majority of PGs on weight basis, although, on molecular basis, similar amounts of aggrecan, biglycan, and decorin are present in young cartilage.

3. Biosynthesis of Proteoglycans in Health and Disease

The protein component of PGs is synthesized by ribosomes and translocated into the lumen of the rough endoplasmic reticulum. Glycosylation of the PG occurs in the Golgi apparatus in multiple enzymatic steps. First, a special link tetrasaccharide is attached to a Ser side chain on the core protein to serve as a primer for polysaccharide growth. Then sugars are added one at a time by glycosyl transferase. The completed PG is then exported in secretory vesicles to the extracellular matrix of the tissue. Due to the complexity of the macromolecules, their biosynthesis can be examined separately, that is, biosynthesis of core proteins and biosynthesis of carbohydrate chains. Both biosyntheses have a high energy demand, especially in the case of aggrecan, and involve a plethora of different enzymes in the glycosylation step.

3.1. Biosynthesis of Core Proteins and N-Linked Oligosaccharide Side Chains. The most detailed studied biosynthesis of cartilage PGs is that of aggrecan and the results are coming from cell and tissue cultures, as well as from Swarm rat chondrosarcoma. The studies have shown that core protein biosynthesis follows the general rules of protein biosynthesis, with the modifications required for protein secretion. The addition of *N*-linked oligosaccharides close to the N-terminal end becomes very early, which are synthesized onto dolichol phosphate, as it has been supported by experiments using tunicamycin as inhibitor of N-glycosylation. Therefore, all the enzymatic machinery needed for *N*-linked oligosacchride precursor biosynthesis and processing after its incorporation onto the core protein participates during the translation step. It has also been shown that once core protein is synthesized, it spends enough time to move to the Golgi apparatus for GAG biosynthesis.

In a characteristic disease state of cartilage, osteoarthritis (OA), decreased expression of aggrecan core protein is observed, which follows changes in chondrocyte phenotype [11]. However, OA is a result of cartilage destruction through depletion of aggrecan and this is discussed below. In chondrosarcoma, the cells are continuing to synthesize aggrecan [12]; however, in other types of cancer in cartilaginous tissues, aggrecan core protein expression is highly decreased [13], possibly due to the depletion of chondrocytes. Mutations of the core protein resulted in skeletal disorders, such as nanomelia and cartilage deficiency [3].

3.2. Biosynthesis of Glycosaminoglycans and O-Linked Oligosaccharide Side Chains. The most complex part of PGs biosynthesis is the addition of GAG chains (Figure 1). The main GAGs found in PGs, that is, CS, DS, heparan sulfate, and heparin, have as a prerequisite the presence of an *O*-linked tetrasaccharide, made of D-xylosyl, two D-galactosyl, and β-D-glucuronosyl residues. In the case of aggrecan, where only CS is present, the majority, if not all, of tetrasaccharides are linked to a serine residue, especially in the Ser-Gly-X sequences. The same occurs with biglycan and decorin, having CS/DS chains.

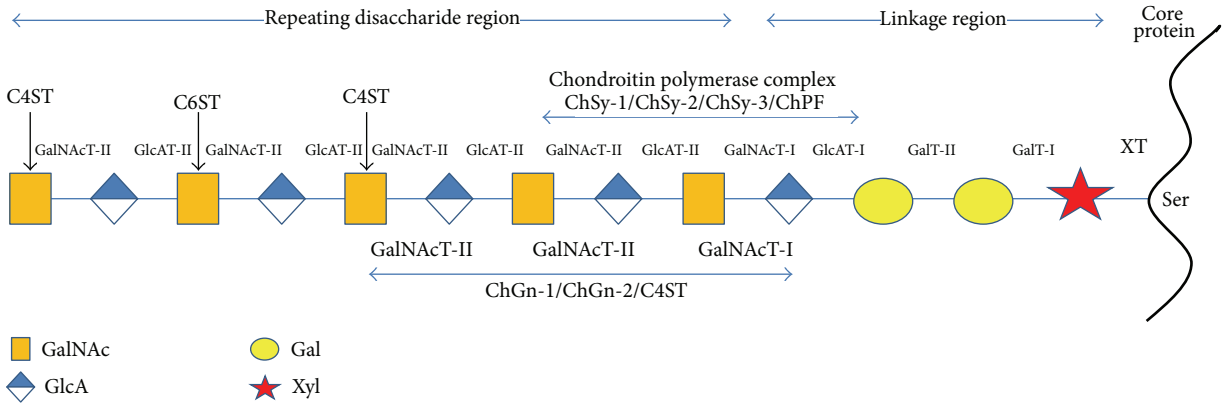

FIGURE 1: A simplified scheme for CS biosynthesis. The participation of the specific glycosyltransferases in the synthesis of the linkage region and of chondroitin chain is indicated. Chondroitin polymer is additionally modified through sulfation.

GAGs are linked to serine residues in core proteins via xylose. Xylosyl-transferase initiates the process using UDP-xylose as donor. Two isoforms of the enzyme are known in vertebrates (XT-1 and XT-2). The glycine residue in the C-terminal side of Ser contributes with its small size, and is usually followed by an acidic amino acid residue. Several PGs, especially aggrecan, contain clustered GAG attachment sites, raising the possibility that XT could act in a processive manner [14]. The extent of xylosylation of a selected PG molecule, with multiple attachment sites, varies in different cells, suggesting that xylosylation is an incomplete process. Variation in the degree of GAG substitution also might result from low levels of UDP-xylose, low activity of the XTs, or competing reactions such as Ser phosphorylation, acylation, or other forms of glycosylation, that is, addition of *O*-linked oligosaccharide side chains.

After xylose addition, the tetrasaccharide linkage region assembles by the transfer of two galactose residues catalyzed by unique members of the β1–4 galactosyl-, β1–3 galactosyl-, and β1–3 glucuronyl-transferase families of enzymes, termed GalT-I, GalT-II, and GlcAT-I, respectively [15]. This intermediate can undergo phosphorylation at the C-2 position of xylose and sulfation of the galactose residues. In general, phosphorylation and sulfation occur substoichiometrically. The lack of chain specificity for phosphorylation would seem to exclude it as a signal for controlling composition. Phosphorylation may be transient, suggesting a role in processing or sorting. Galactose sulfation is found only in CS, but its role in chain initiation, polymerization, or turnover remains unclear.

Once the linkage tetrasaccharide is formed, the biosynthesis of the entire GAG can now start through the addition of *N*-acetyl-β-D-galactosamine (D-GalNAc) linked by a β1–4 glycosidic bond. This process, that is, addition of β4-D-GalNAc or α4-D-GlcNAc, seems to be manifested at the level of enzyme recognition of the polypeptide substrate [15]. Two enzymes possessing *N*-acetyl-D-galactosaminyl-transferase (GalNAcT) activity exist, GalNAcT-I and GalNACT-II, which are involved in chain initiation, as well as in chain polymerization. Additional enzymatic activity participates in chain elongation, which is usually referred to as chondroitin synthase, because of its bifunctional role in the polymerization process; that is, it is acting as both β-D-glucuronyl-transferase (GlcAT-II) and β-*N*-acetyl-D-galactosaminyl-transferase (GalNAcT-II). Studies that took place during the last decade have identified and cloned six homologous glycosyl-transferases responsible for biosynthesis of chondroitin repeating units [15]. According to their enzymatic activity *in vitro*, the members of this family were designated as ChSy-1 (chondroitin synthase-1), ChSy-2, ChSy-3, ChPF (chondroitin-polymerizing factor), and ChGn-1 (chondroitin GalNAc transferase-1) and ChGn-2, respectively. The first three enzymes, ChSy-1, ChSy-2, and ChSy-3, are those possessing dual glycosyl-transferase activities, GlcAT-II and GalNAcT-II, but cannot polymerize chondroitin chains by themselves [16–19]. However, coexpression of any two of four proteins, ChSy-1, ChSy-2, ChSy-3, and ChPF, that has only marginal GalNAcT-II activity each [17, 20], markedly increases the ability of GlcAT-II and GalNAcT-II activities to synthesize chondroitin polymer. This indicates the importance of the chondroitin-polymerizing factor, an enzyme lacking independent activity but acting together with the transferases to enhance the formation of polymers. On the other hand, the latter two enzymes, ChGn-1 and ChGn-2, possess both GalNAcT-I and GalNAcT-II activities and, therefore, are thought to catalyze chain initiation and elongation of chondroitin [15]. Biosynthesis of chondroitin chain onto the core protein seems to occur much later than translation.

The newly synthesized chondroitin chain is subjected to modifications, such as epimerization and sulfation. Both are the very late stage in PG biosynthesis and occur at the same time and just before PG secretion. Epimerization is highly dependent to sulfation; however, in the case of aggrecan, it does not occur at all. Sulfation is a complex process, and multiple sulfotransferases are involved in 4-*O* sulfation and 6-*O* sulfation of GalNAc residues. Four 4-*O*-sulfotransferases have been characterized in mammals [21–25] and termed C4ST-1 (chondroitin 4-*O*-sulfotransferase-1), C4ST-2, C4ST-3, and D4ST-1 (dermatan 4-*O*-sulfotransferase-1). The first three preferably catalyze the 4-*O*-sulfation of GalNAc

residues in CS, whereas the latter is responsible for the 4-*O*-sulfation of GalNAc residues next to iduronate in DS. Sulfation at C-6 position of GalNAc residues in CS is mediated by C6ST-1 (chondroitin 6-*O*-sulfotransferase-1) [26]. It is questionable the presence of a sulfotransferase responsible for 6-*O*-sulfation in DS. All sulfotransferases use activated sulfate (PAPS; 3′-phosphoadenosine-5′-phosphosulfate) as a high-energy donor. In humans, sulfation occurs mainly at the C-6 of GalNAc, and only a small number of residues remain unsulfated. In other vertebrates, sulfation occurs mainly at C-4 of GalNAc. In all animal species, sulfation at C-6 of GalNAc increases with aging. Additional enzymes exist for epimerization of glucuronic acid to iduronic acid in DS, sulfation at the C-2 position of the uronic acids, but none of them act on aggrecan. In species other than human, additional sulfotransferases exist and unusual patterns of sulfation are observed.

In cell or tissue culture experiments in the presence of β-D-xyloside, a precursor of GAG biosynthesis, the PGs produced contain fewer and shorter CS/DS chains, with decreased sulfation, suggesting that the number of putative chain initiation sites controls both size and sulfation of GAGs [27]. Evidence is also presented indicating that ChGn-1 together with C4ST-II regulate the initiation of CS synthesis and, thus, control the number of CS chains attached to core protein [28]. Moreover, chain elongation is controlled by a cooperation between C4ST-1 and ChGn-2 [29]. It should also be noted that, in cell cultures in the presence of monensin, an ionophore that impairs Golgi apparatus, an accumulation of core proteins within the cells is observed, suggesting the functional role of Golgi apparatus in PGs biosynthesis [30]. However, monensin seems not to affect GalNAc-6-sulfation, but mainly epimerization of glucuronate and GalNAc-4-sulfation, providing evidence on the organization of transferases in Golgi apparatus [31].

The enzymes involved in chondroitin polymerization are indispensable for life. However, it has been revealed that knockout mice for either of *ChSy-1*, *ChPF,* or *ChGn-1* are viable and fertile, although they do synthesize reduced CS amounts with an imbalance in its sulfation [32–35]. Due to the decreased CS content and sulfation, especially in cartilaginous tissues, the animals display skeletal abnormalities including chondrodysplasia, decreased bone density, and digit-patterning defects. In addition, knockdown of *ChSy-1* in zebrafish embryos lead to skeletal anomalies and craniofacial and inner ear dysmorphogenesis. In human, mutations in *CHSY1* have been identified in syndromic recessive preaxial brachydactylies, mainly characterized by limb malformations, short stature, and hearing loss [36, 37]. These findings suggest the essential regulatory roles of ChSy-1 in skeletal development. Moreover, a mutation of C6ST-1 abolishes its activity and results in severe human chondrodysplasia with progressive spinal involvement. Mutation of PAPS synthetase results in skeletal abnormalities, known as brachymorphism [3].

Ehlers-Danlos syndrome (progeroid type) is another disorder characterized by deficiencies in biosynthetic enzymes related to glycosylation of PGs. Decorin is partially deficient since some molecules are secreted from cells as free proteins without containing the GAG chain. This is because the enzymes GalT-I and GalT-II have very low activity compared to normal [14].

In cancer of cartilaginous tissues, many changes at the expression level of almost all enzymes involved in CS/DS biosynthesis are observed [38]. Little evidence has been provided regarding the cellular pool responsible for the altered biosynthesis, which suggests that the malignant cells penetrating cartilage are responsible for at least some of these changes.

The biosynthesis of KS on aggrecan (skeletal KS or KSII) follows a different scheme. KS is linked through D-GalNAc to Ser or Thr of the core protein. Then a poly-*N*-acetyllactosamine chain follows which is sulfated somewhat randomly. At least two classes of sulfotransferases, one or more GlcNAc-6-*O*-sulfotransferases, and one galactose-6-*O*-sulfotransferase catalyze the sulfation reactions [39]. Similarly as above, these enzymes use PAPS as a high-energy donor. It seems that GlcNAc 6-*O* sulfation occurs only on the nonreducing terminal GlcNAc residue, whereas sulfation of galactose residues takes place on nonreducing terminal and internal galactose residues, with a preference for galactose units adjacent to a sulfated GlcNAc [39]. Sulfation of a nonreducing terminal galactose residue blocks further elongation of the chain, providing a potential mechanism for controlling chain length. Another mechanism for controlling KS chain elongation is the addition of a sialic acid residue. In some cases, KS seems to contain a small branch early terminated by sialic acid addition. The content and the size of KS in aggrecan molecule are highly affected by aging. Aged cartilage contains aggrecan substituted with more KS chains of higher molecular size [40, 41]. It seems that chain elongation control is affected by aging. KSII is not present in all animal species, but only in these containing an aggrecan sequence that possesses a specific KS-binding region, characterized by the presence of the amino acid sequence Glu-Glu/Leu-Pro-Phe-Pro-Ser [39]. KS biosynthesis is often markedly altered in response to metabolic, pathologic, or developmental changes in tissues.

4. Degradation of Proteoglycans in Health and Disease

Similarly as biosynthesis, degradation of PGs is a process catalyzed by different enzymes for core proteins and GAG chains. Initial degradation of PGs occurs extracellularly, due to their molecular size, and especially that of aggrecan, that does not permit endocytosis of the whole molecule. Various metalloproteinases, like MMPs (matrix metalloproteinases), ADAMTSs (A disintegrin and metalloproteinase with thrombospondin motif), and cysteine proteinases, are usually involved [42, 43]. Degradation also occurred by free radicals and mechanical stress. The fragments are endocytosed and subjected to further degradation in lysosomes (Figure 2). In various types of arthritis, increased extracellular degradation of aggrecan is observed due to increased MMPs and ADAMTS activity [42], as well as increased and secreted cathepsins activity [44].

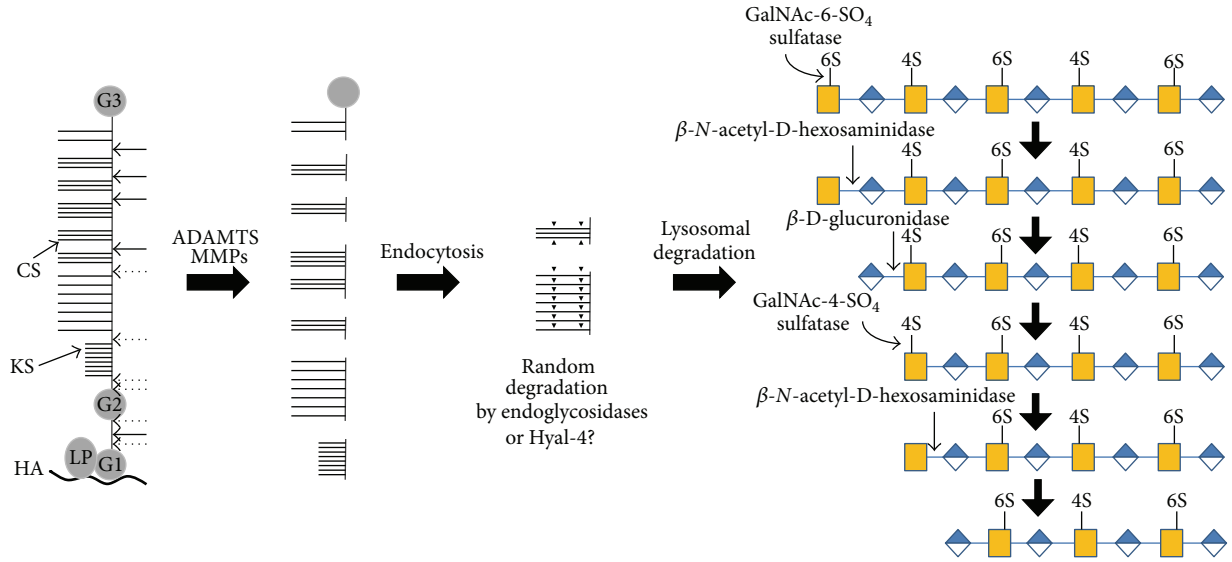

Figure 2: A simplified scheme for aggrecan degradation. Aggrecan is initially degraded by extracellular proteases, mainly ADAMTS and MMPs; the various fragments are endocytosed and the CS-containing peptides are further processed by specific glycosidases and sulfatases. Only the initial endolysosomal degradation steps are shown.

4.1. Degradation of Core Proteins. Aggrecan core protein is sensitive to proteolysis at numerous sites along its length. Cleavage at any of these sites results in the removal of a part (of different size, depending on the cleavage site) of CS chains and, thus, affecting its negative charge. Cleavage of aggrecan in the interglobular domain (IGD) between the N-terminal G1 and G2 globular domains is of greatest importance, as this releases the whole GAG bearing region of aggrecan from the cartilage matrix and so abrogates the function of the molecule [42, 45].

In healthy cartilage, the extracellular proteolytic enzymes are present in their latent forms. Moreover, the presence of specific tissue inhibitors of metalloproteinases (TIMPs) has a negative effect on their activity, although *in vitro* studies suggest that MMPs participate in normal turnover of aggrecan [42, 46]. It should be noticed that a member of TIMPs family, TIMP-3, possesses the ability to inhibit ADAMTS-4 and -5 [42]. As TIMP-3 can inhibit both MMPs and ADAMTSs, it is a central regulator of normal turnover of cartilage macromolecules. Nevertheless, in healthy cartilage, about 10–15% of the aggrecan molecules appeared to be free, in a nonaggregating state in the tissue, because they lack their N-terminal region. The presence of fragmented aggrecan is explained by the normal recycling of extracellular macromolecules. It has been proposed that m-calpain, a calcium-dependent cysteine proteinase, may be responsible for extracellular cleavage of aggrecan in CS1 region [43]. Aggrecan fragments are internalized to the cells and degraded further in lysosomes by their proteolytic machinery, the cathepsins. Cathepsins are present in lysosomes in their latent forms and are activated through their interaction with GAGs [44]. The CS chains are also degraded in lysosomes and their degradation is discussed below.

Among the pathologic states of cartilage, of particular importance are the various types of arthritis, OA being the most important. Degradation of aggrecan is an early event in the development of OA and a lot of research has been done to identify the enzymes responsible. It was initially thought that MMPs were the primary aggrecan-degrading enzymes in OA, since several MMPs, that is, MMP-1, -2, -3, -7, -8, -9, and -13, were found to be able to cleave the Asn341~Phe342 bond in the aggrecan IGD, the first one being MMP-3 [47]. However, evidence was presented revealing that the majority of aggrecan fragments present in the synovial fluid of OA patients were cleaved not at the MMP-sensitive Asn341~Phe342 bond, but at the Glu373~Ala374 bond in the IGD. Moreover, this cleavage was not blocked by the tissue inhibitors of MMPs, TIMP-1, TIMP-2, or synthetic MMP inhibitors [48], indicating that enzymes other than MMPs are responsible for the activity. These findings led to the identification of ADAMTS as the main enzymes attacking aggrecan in at least the early events of OA. ADAMTS, like all metalloproteinases, are synthesized by the cells in latent forms that require activation to express their activity. Activation proceeds mainly through the action of members of the family of proprotein convertases [49].

Activated ADAMTS-1, -4, -5, -8, -9, -15, -16, and -18 can degrade aggrecan *in vitro* [50–53], but ADAMTS-5 is the most active enzyme *in vitro*, followed by ADAMTS-4 [54], and there is some evidence suggesting that ADAMTS-4 contributes to cartilage degradation. ADAMTS-4 and ADAMTS-5 are, thus, considered to be the major enzymes responsible for pathological cleavage of aggrecan in OA, ADAMTS-5 having a pivotal role [55]. However, since aggrecan cleavage at the MMPs sensitive bond is also detectable in OA cartilage [56], MMPs may also act on aggrecan later in the progression of disease. ADAMTS can also cleave biglycan [57] and, thus,

may affect overall tissue organization, contributing even more in cartilage destruction.

It should also be noticed that secretion of lysosomal cathepsins was observed in OA. As the pH of OA cartilage is lower than normal [58], cathepsins largely participate in proteoglycan degradation extracellularly.

There is also some evidence that aggrecan depletion that occurred in cartilaginous tissues in cancer progression is mediated through the action of ADAMTS, and especially ADAMTS-5 [59]. MMPs have also shown that they participate in cartilage destruction in cancer [60], but it is not clear whether they attack aggrecan.

4.2. Degradation of Glycosaminoglycans. The CS/DS-containing peptides once they are endocytosed they are transferred to lysosome when their protein parts are further degraded by acidic proteolytic enzymes and their carbohydrate parts by specific glycosidases. The degradation process of GAGs is highly ordered and employs endo- and exoglycosidases and sulfatases, sometimes aided by noncatalytic proteins. Although the presence of lysosomal hyaluronidase, an enzyme degrading hyaluronan and, to a lesser extent, CS, is observed in cartilage extracellular matrix, no evidence has yet been presented for its involvement in extracellular degradation of CS. Hyaluronidase, an endo-β-*N*-acetyl-D-glucosaminidase, seems to be the first glycosidase that hydrolyzes CS on CS/DS-containing peptides to large oligosaccharides. There exist also enzymes, such as endo-β-D-glucuronidases or endo-β-D-hexosaminidases, cleaving CS/DS at a few specific sites. Alternatively, the specific hyaluronidase for CS, Hyal-4, may be responsible for its initial cleavage [61, 62]. However, the exact mechanism for initial CS/DS degradation is not yet clear. Then, specific sulfatases (GalNAc-6-SO_4 sulfatase and GalNAc-4-SO_4 sulfatase) remove the sulfate ester groups and the exoglycosidases β-D-glucuronidase or α-L-iduronidase and β-*N*-acetyl-D-galactosaminidase (or β-*N*-acetyl-D-hexosaminidase), acting in parallel, cleave the glycosidic linkage of terminal sugars from the nonreducing end of the oligosaccharides to liberate the monosaccharides. The tetrasaccharide linkage region is degraded by the appropriate specific glycosidases.

On the other hand, the mammalian cells do not have an endoglycosidase to cleave KS, possibly because its molecular size is small compared to that of CS/DS. Therefore, the KS-containing peptides are hydrolyzed in lysosomes by the sequential action of sulfatases and exoglycosidases. Galactose-6-SO_4 sulfatase is the same enzyme that desulfates GalNAc-6-SO_4 in CS degradation. The exoglycosidases involved are β-galactosidase and β-*N*-acetyl-D-hexosaminidase.

As it is mentioned in the Introduction, defective degradation of GAGs in lysosomes is the origin of the various mucopolysaccharidoses [63]. The absence or the malfunction of a single lysosomal hydrolase leads to the accumulation of its substrate as undegraded fragments in tissues and the appearance of related fragments in urine. The result is permanent, progressive cellular damage which affects appearance, physical abilities, organ and system functioning, and, in most cases, mental development. The pathology likely depends on the cell type and the cellular balance of synthesis and turnover rates. Hurler syndrome, Hurler-Scheie syndrome, and Scheie syndrome have a defective α-iduronidase, Hunter syndrome a defective iduronate sulfatase, Morquio syndrome A a defective GalNAc-6-SO_4 sulfatase, Morquio syndrome B a defective β-galactosidase, Maroteaux-Lamy syndrome a defective N-acetylgalactosamine-4-SO_4 sulfatase, and Sly syndrome a defective β-D-glucuronidase. The first four syndromes accumulate degradation products of DS (and of heparan sulfate), Morquio syndromes of KS and chondroitin-6-sulfate (A) and of KS (B), Maroteaux-Lamy syndrome of DS and Sly syndrome of DS/DS (and of heparan sulfate).

A very rare human disorder is the multiple sulfatase deficiency that affects all GAGs degradation.

5. Concluding Remarks

Both biosynthesis and degradation of cartilage PGs are processes involving multiple enzymes. There is increasing evidence that a deficiency or malfunction of any of the enzymes participating in any of these processes may lead to severe cellular or organ malfunction or damage. The biosynthesis of deficient PGs may affect their charge density or their interactions with other extracellular components, and it affects cartilage structure and properties. The deficient degradation of PGs may accumulate limited degradation products within the cell with harmful effects to the organism. Extended studies are performed to understand the molecular mechanisms involved and synthetic or natural drugs are applied as therapeutic tools.

For the most common cartilage disease, OA, current treatments are symptomatic and limited by side effects or lack of efficacy, and despite using them, many people with OA still have significant symptoms. New approaches to targeting pathology offer hope of new analgesic options and, for the first time, structure modification may be possible by treating a noncartilage target, the subchondral bone. In addition, gene transfer approaches allow for a long-term and site-specific presence of a therapeutic agent to reequilibrate the metabolic balance in OA cartilage and may consequently be suited to treat this slow and irreversible disorder. In an interesting clinical trial, the chondrocytes of patients have been modified to produce the transforming growth factor β1 via intra-articular injection, showing a dose-dependent trend toward efficacy. However, the distinct stages of OA need to be respected in individual gene therapy strategies, and molecular therapy appears to be most effective for early OA.

Abbreviations

ADAMTS: A disintegrin and metalloproteinase with thrombospondin motif
BMP-4: Bone morphogenetic protein-4
C4ST-1: Chondroitin 4-*O*-sulfotransferase

C6ST-1: Chondroitin 6-*O*-sulfotransferase-1
ChGn-1: Chondroitin GalNAc transferase-1
ChPF: Chondroitin-polymerizing factor
ChSy-1: Chondroitin synthase
CS: Chondroitin sulfate
D4ST-1: Dermatan 4-*O*-sulfotransferase-1
D-GalNAc: *N*-acetyl-β-D-galactosamine
DS: Dermatan sulfate
GAG: Glycosaminoglycan
GalNAcT: *N*-acetyl-D-galactosaminyl-transferase
GalT: Galactosyl-transferase
GlcAT: Glucuronyl-transferase
IGD: Interglobular domain of aggrecan
KS: Keratan sulfate
MMP: Matrix metalloproteinase
MPS: Mucopolysaccharidoses
OA: Osteoarthritis
PAPS: 3′-Phosphoadenosine-5′-phosphosulfate
PG: Proteoglycan
SLRPs: Small leucine-rich PGs
TGF-β: Transforming growth factor-β
TIMP: Tissue inhibitors of MMPs
XT: Xylosyl-transferase.

Conflict of Interests

The author has no financial or personal relationship that could inappropriately influence or bias the content of the paper.

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Research Article

ADAMTS4 and ADAMTS5 Knockout Mice Are Protected from Versican but Not Aggrecan or Brevican Proteolysis during Spinal Cord Injury

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The chondroitin sulfate proteoglycans (CSPGs) aggrecan, versican, and brevican are large aggregating extracellular matrix molecules that inhibit axonal growth of the mature central nervous system (CNS). ADAMTS proteoglycanases, including ADAMTS4 and ADAMTS5, degrade CSPGs, representing potential targets for ameliorating axonal growth-inhibition by CSPG accumulation after CNS injury. We investigated the proteolysis of CSPGs in mice homozygous for *Adamts4* or *Adamts5* null alleles after spinal cord injury (SCI). ADAMTS-derived 50–60 kDa aggrecan and 50 kDa brevican fragments were observed in *Adamts4*−/−, *Adamts5*−/−, and *wt* mice but not in the sham-operated group. By contrast *Adamts4*−/− and *Adamts5*−/− mice were both protected from versican proteolysis with an ADAMTS-generated 70 kDa versican fragment predominately observed in WT mice. ADAMTS1, ADAMTS9, and ADAMTS15 were detected by Western blot in *Adamts4*−/− mice' spinal cords after SCI. Immunohistochemistry showed astrocyte accumulation at the injury site. These data indicate that aggrecan and brevican proteolysis is compensated in *Adamts4*−/− or *Adamts5*−/− mice by ADAMTS proteoglycanase family members but a threshold of versican proteolysis is sensitive to the loss of a single ADAMTS proteoglycanase during SCI. We show robust ADAMTS activity after SCI and exemplify the requirement for collective proteolysis for effective CSPG clearance during SCI.

1. Introduction

The extracellular matrix (ECM) is important for structural and functional development and maintenance of the central nervous system (CNS). The hyalectan (hyaluronan and lectin) binding class of chondroitin sulfate proteoglycans (CSPGs) comprises aggrecan, versican, neurocan, and brevican, each sharing a common N-terminal G1 domain invariably linked to hyaluronan, and a G3 C-terminal domain [1, 2]. Large O-linked glycosaminoglycan (GAG) chains attached to

serine or threonine residues modify several splice variants of versican, aggrecan, brevican, and neurocan. Chondroitin sulphate moieties covalently attached to GAG chains confer a high negative charge attracting water into the tissue, providing rigidity and structure. Digestion of GAG chains using chondroitinase ABC creates a permissive media for axonal regrowth, in turn promoting regain of function. On the other hand, the loss of core proteins after chondroitinase ABC digestion maintains an inhibitory habitat at the injury site [1].

Adult mammalian CNS has poor plasticity to accomplish proper axon regeneration and sprouting. Glial scar formation caused by the reactive astrocyte response after traumatic injuries in the CNS is a major impediment to axonal regeneration partly due to CSPG production that is inhibitory for axonal regeneration [1]. Versican is predominately localized to nodes of Ranvier whilst aggrecan, brevican, and neurocan are present in axonal coats [3–6]. Recently, aggrecan, versican, and brevican were shown to inhibit axonal regeneration and myelination after neuronal injury [4–6]. These observations drew focus to the possibility of promoting proteolysis of hyalectans to favor gain-of-function after spinal cord injury.

ADAMTS4 and ADAMTS5 (aggrecanase-1 and aggrecanase-2, resp.) are the predominant enzymes in cartilage breakdown [7, 8], although recent studies revealed significant aggrecanolysis in articular cartilage of ADAMTS4 or ADAMTS5 knockout mice suggesting additional aggrecanase activity by one or more members of the ADAMTS aggrecanase family yet to be identified in the arthritic joint [9, 10]. Previous studies identified roles for ADAMTS4 and ADAMTS5 in spinal cord pathology; however, their role during spinal cord injury remains unclear. Tauchi et al. [6] showed that ADAMTS4 promoted functional recovery after SCI by cleaving CSPGs. We recently showed that *Adamts1*, *Adamts5,* and *Adamts9* mRNA expression were increased in mice following SCI [5]; however, the significance of this observation was not explored.

The biological importance of versican, a widely expressed transitional matrix PG [11], and its cleavage by ADAMTS aggrecanases is exemplified during developmental morphogenesis and reproduction. Despite ADAMTS5 single knockout mice being previously reported as phenotypically normal [7, 8, 12], those mice present with limb and heart abnormalities [13–15] where a reduction or absence of versican processing is also observed. An ADAMTS-generated N-terminal versican fragment rescued the ADAMTS-deficient limb-defect phenotype showing unequivocally that versican processing was required for developmental morphogenesis of the distal limb [15]. In ADAMTS1 knockout mice reduced or absent versican processing is observed in ovarian follicles and those mice have significantly lower fertility rates than wild-type mice due to anovulation [16].

The fact that ADAMTS proteoglycanases confer their substrate specificity towards the hyalectan class of CSPGs makes them ideal candidate therapeutic targets to pursue in SCI. The present study focused on ADAMTS4 and ADAMTS5 because of their previously highlighted roles in aggrecan and versican processing in contexts such as arthritis progression and developmental morphogenesis. Here, we report the proteolysis of three major substrates for these enzymes in spinal cords of *Adamts4*−/− or *Adamts5*−/− mice after spinal cord injury.

2. Material and Methods

2.1. Animals Experiments. Animal procedures were approved by the Okayama University, Okayama, Japan Animal Ethics Committee, in accordance with the International Care and Use of Animals in Research guidelines. *Adamts4* (*B6.129P2*-Adamts4^{tm1Dgen}/*J*) and *Adamts5* (*B6.129P2*-Adamts5^{tm1Dgen}/*J*) knockout mice were obtained from The Jackson Laboratory and are previously described [15, 17]. Spinal cord injury was induced in mice as previously described [5]. Briefly, mice were anesthetized with an I.P. injection of 50 mg/kg pentobarbital. Mice underwent a single level laminectomy and the dura was exposed at the middle thoracic level. A 3 g weight was dropped from 25 mm height using a modified NYU impactor to produce moderate contusion (1.3 mm) injury. This procedure was performed in three groups of 7 mice, group 1: wild-type, group 2: *Adamts4*−/−, and group 3: *Adamts5*−/−. Sham-operated (no injury) mice were also used as control ($n = 7$). For the sham group, only laminectomy was performed and surgical sites were sutured in layers.

2.2. Protein Analysis. Central lesion points in spinal cords were dissected 3 mm in length. Protein was extracted using a commercial kit (Cell lytic M Cell, sigma, St. Louis, MO). Western blot analysis was performed on days 1 (aggrecan and brevican) and 7 (versican and brevican) to examine their degradation products in all experimental groups. Protein concentration was determined using a Bradford assay kit (Biorad). Ten μg of total protein per experimental group was boiled and electrophoresed on 4–15% sodium dodecyl sulfate (SDS) reducing polyacrylamide gels. Proteins were transferred onto nitrocellulose membranes (Millipore, Billerica, MA) and blocked with 5% skim-milk in PBS for 1 h. After blocking, membranes were incubated overnight at 4°C with primary antibodies to analyze the proteoglycans that are cleaved by ADAMTS activity during the injury phase *in vivo*: anti-aggrecan NITEGE (Abcam, cat. no. ab3775), a monoclonal antibody raised to the C-terminal neoepitope NITEGE (mouse, clone BC-13), which recognizes the aggrecanase generated C-terminal neoepitope EGE373↓374A within the interglobular domain of cleaved aggrecan [8]; anti-versican DPEAAE (Abcam, cat. no. ab19345), a polyclonal antibody raised against the C-terminal neoepitope DPEAAE, which recognizes the aggrecanase generated C-terminal neoepitope DPEAAE^{441}↓A^{442} at the N-terminus of V0/V1 versican [18]; anti-brevican (Santa Cruz, cat no. sc-20555), a goat polyclonal antibody raised against a peptide mapped to the N-terminus of human brevican that recognizes full length brevican and ADAMTS aggrecanase generated C-terminal cleavage products [19]. Moreover, we next analyzed ADAMTS1 (Abcam, ab. 28284), raised to an epitope at the amino terminal end of ADAMTS1 after the

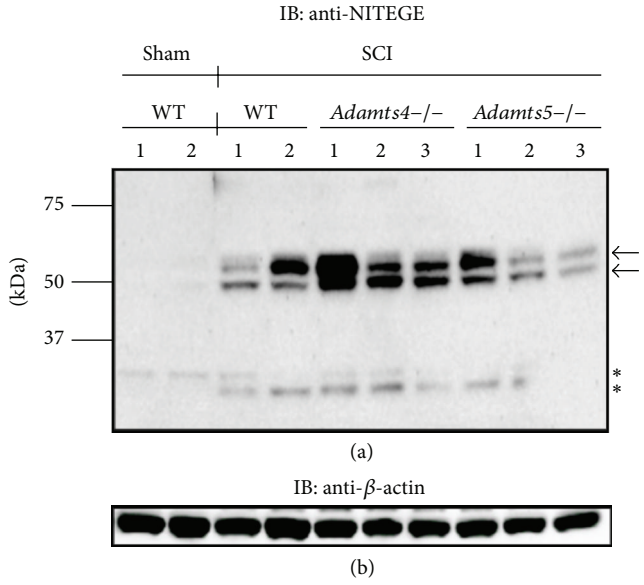

Figure 1: Aggrecan cleavage in *Adamts4*−/− and *Adamts5*−/− mice following SCI. Aggrecan fragments (~50 and 60 kDa) detected with the anti-NITEGE antibody are seen in wild-type, *Adamts4*−/−, and *Adamts5*−/− mice 1 day after SCI (arrows, top panel). Lower molecular weight species (~25 kDa) are also seen in most groups (asterisks, top panel). No aggrecanase activity was detected in sham-operated mice. β-Actin was used as a loading control (bottom panel).

second furin cleavage site in its propeptide, which detects the active form and autocatalytically processed ADAMTS1 [20]; ADAMTS9 (Abcam, ab. 28279 and it was a *kind gift from Professor Suneel Apte*) raised to the amino terminal end of ADAMTS9 previously described as detecting full-length ADAMTS9 [21]; and ADAMTS15 (Abcam, ab. 28516), raised to the cysteine-rich domain of ADAMTS15 [22]. Secondary antibodies conjugated with horseradish peroxidase followed primary antibody incubations. Enhanced chemiluminescent (ECL) substrate from Biorad (USA) was used for visualization of protein bands. Anti-β-actin antibody (Abcam) was used as a loading control.

2.3. Immunohistochemistry. Immunostaining was used to evaluate the astrocyte response to SCI. Spinal cord tissue was fixed with 4% paraformaldehyde for 20 min at room temperature, permeabilized for 10 min with 0.1% Triton X-100 in PBS, blocked with 4% skim milk (Thermo), and then incubated for 2 h at room temperature with anti-glial fibrillary acidic protein (GFAP) antibody (1 : 400; DakoCytomation, Carpinteria, CA). The tissue sections were washed in PBS Triton X-100 followed by incubation with goat anti-rabbit antibody conjugated to Alexa 488 fluorophore (1 : 400; Molecular Probes, Invitrogen). Nuclei were counterstained with Hoechst for visualization. Images were captured on Olympus BX51 microscope (Olympus Corporation).

3. Results and Discussion

3.1. Aggrecan Processing during SCI. Since we previously showed significant upregulation of aggrecan mRNA at 1 day post injury (dpi) [5], we examined aggrecanolysis at this time point. Following SCI, we observed significant ADAMTS generated aggrecan cleavage by Western blot analysis in wild-type, *Adamts4*−/−, and *Adamts5*−/− mice (Figure 1). A predominant ~60 kDa band, representing the G1-NITEGE N-terminal aggrecan fragment generated by ADAMTS proteoglycanases, with an accompanying ~50 kDa band that was readily detectable in all cases except in the sham group (Figure 1), clearly indicating that SCI was responsible for the induction of aggrecan proteolysis. In addition, lower molecular weight bands of ~25 to ~30 kDa were also differentially observed between the sham and SCI groups (Figure 1). These data suggested that ADAMTS4, ADAMTS5, or the other 5 members of the ADAMTS aggrecanase family (ADAMTS1, ADAMTS8, ADAMTS9, ADAMTS15, and ADAMTS20) might cooperate in aggrecan cleavage during spinal cord injury.

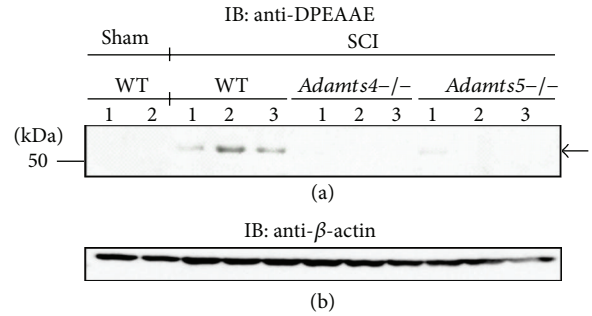

Figure 2: Versican cleavage in *Adamts4*−/− and *Adamts5*−/− mice following SCI. Versican fragments (~70 kDa) detected with the anti-DPEAAE antibody are seen in wild-type but not *Adamts4*−/− and *Adamts5*−/− mice 7 days after SCI (top panel, arrow). No versicanase activity was detected in sham-operated mice. β-Actin was used as a loading control (bottom panel).

3.2. Versican Processing during SCI. In contrast to aggrecan, versican mRNA is significantly upregulated at 7 dpi [5]. Western blot analysis on spinal cord protein lysate from 7 dpi revealed the expected ADAMTS proteoglycanase derived 70 kDa versican fragment representing the G1-DPEAAE N-terminal region of V1 versican in the wild-type group (Figure 2). However, in contrast to aggrecan at 1 dpi, little to no G1-DPEAAE fragments were observed in either case of ADAMTS4 or ADAMTS5 knockout mice that had undergone SCI. These data highlight the cooperative nature of the ADAMTS proteoglycanase family to maintain a threshold of versican cleavage during SCI, whereby neither ADAMTS4 nor ADAMTS5, or the remaining ADAMTS proteoglycanases, were able to collectively maintain threshold versican cleavage in the absence of either ADAMTS4 or ADAMTS5. In addition, we also observed an uncharacterized ~37 kDa band using the anti-DPEAAE antibody (see Figure 1 in supplementary materials available online at http://dx.doi.org/10.1155/2014/693746) in all experimental groups.

3.3. Brevican Processing during SCI. Western blot analysis to investigate brevican processing was undertaken at both

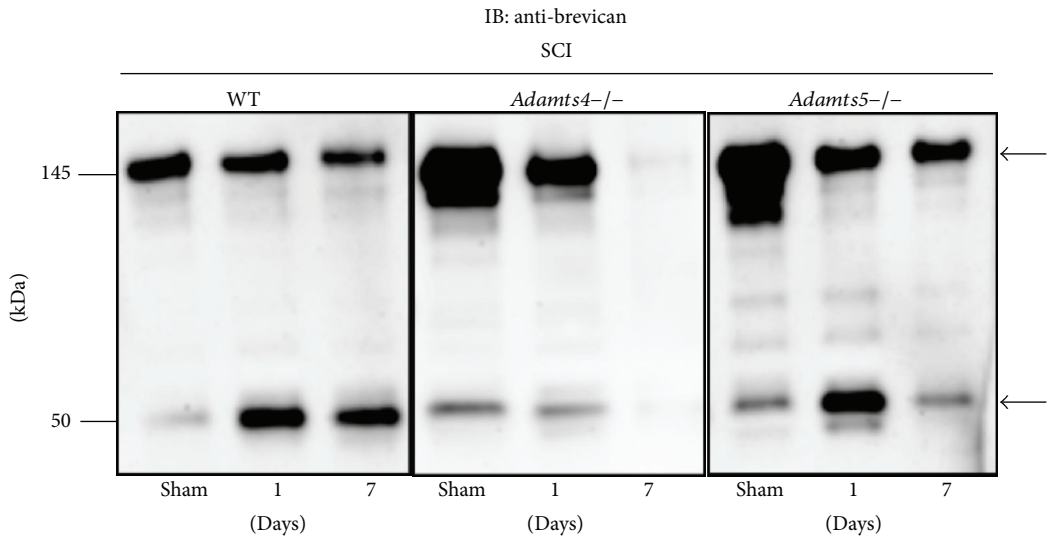

Figure 3: Brevican cleavage in *Adamts4*−/− and *Adamts5*−/− mice following SCI. Full-length brevican (~145 kDa) and brevican fragments (~50 kDa) detected with the anti-brevican antibody are seen in wild-type, *Adamts4*−/−, and *Adamts5*−/− mice 1 and 7 days after SCI (arrows). All groups showed diminished brevican cleavage at 7 days after injury. Minimal brevicanase activity was detected in sham-operated mice.

1 dpi and 7 dpi. Total brevican (~145 kDa) and the ADAMTS proteoglycanase generated 50 kDa brevican fragments were observed at days 1 and 7 in wild-type and *Adamts5*−/− mice (Figure 3), whilst *Adamts4*−/− mice showed total brevican and the ADAMTS-generated brevican fragment (G1-AVESE) [19] at 1 dpi and to a lesser extent total brevican and its fragment at 7 dpi (Figure 3). Brevican cleavage was decreased at day 7 in all groups (Figure 3).

3.4. Other ADAMTS Proteoglycanases during SCI. In the absence of ADAMTS4 + ADAMTS5 double knockout mice, we could not rule out the contribution to aggrecan and brevican proteolysis by each other or additional ADAMTS proteoglycanases. However, since aggrecanase activity was clearly present in both ADAMTS4 and ADAMTS5 knockout mice, we used *Adamts4*−/− mice to confirm the presence of a subset of other ADAMTS proteoglycanases during spinal cord injury. Western blot analysis using antibodies specific to a subset of ADAMTS proteoglycanases showed the presence of ADAMTS1, ADAMTS9, and ADAMTS15 in spinal cord tissue (Figures 4(a), 4(b), and 4(c), resp.). An 85 kDa band representing the active form of ADAMTS1 [23] as well as a smaller ~35 kDa fragment representing autocatalysis of ADAMTS1 [23] was observed at days 1 and 7 of spinal cord injury (Figure 4(a)). A ~180 kDa band representing the zymogen (unprocessed) form of ADAMTS9 [24] was observed in all experimental groups (Figure 4(b)), and a ~ 130 kDa band representing the zymogen (unprocessed) form of ADAMTS15 [22] was also observed in all experimental groups (Figure 4(c)). Lower molecular weight bands were also observed in both cases of ADAMTS9 and ADAMTS15 (Figures 4(b) and 4(c), resp.—asterisks), which may represent autocatalysis of those ADAMTS proteoglycanases [22, 24]. Although all three ADAMTS proteoglycanases were present in ADAMTS4 knockout mice, the most likely candidate for compensatory aggrecanase activity is ADAMTS1 since it was the only ADAMTS shown in its active form, and it robustly appeared in the case of spinal cord injury but not in the sham-operated group (Figure 4(a)). In addition, immunostaining demonstrated astrocyte accumulation at the injury site compared to normal tissue (Figure 4(d)), which could represent the source of ADAMTS proteoglycanase expression.

Spinal cord injury is a severe and essentially irreversible process characterized by excessive CSPGs accumulation and the enzymatic removal of chondroitin sulfate at the site of SCI can promote a regenerative process. In this current study, we induced SCI in mice and examined the activity of the ADAMTS proteoglycanases, a family of extracellular proteinases that cleave hyalectan substrates relevant to the spinal cord, namely, aggrecan, brevican, and versican, and could therefore be relevant to the regeneration process. We previously demonstrated that *Adamts1*, *Adamts5,* and *Adamts9* mRNA expressions were significantly increased in the same SCI mouse model in wild-type mice and suggested that aggrecan, versican, and brevican degradation may be mediated by these and other ADAMTS proteoglycanases produced by reactive astrocytes after SCI [5]. Here, we extended the previous study to utilize *Adamts4*−/− or *Adamts5*−/− mice and studied the consequence of SCI on proteolytic activity upon three major hyalectans that represent predominate sources of CSPGs in the spinal cord.

Aggrecan cleavage was readily detectable in injured spinal cords of both *Adamts4*−/− and *Adamts5*−/− mice. Since both ADAMTS4 and ADAMTS5 are strong aggrecanases [25, 26], this result was perhaps unsurprising. Although double *Adamts4*−/− and *Adamts5*−/− mice are required to reconcile whether one aggrecanase is compensating for the other in response to SCI, it is apparent from this current study that robust aggrecanase remodeling can occur in the

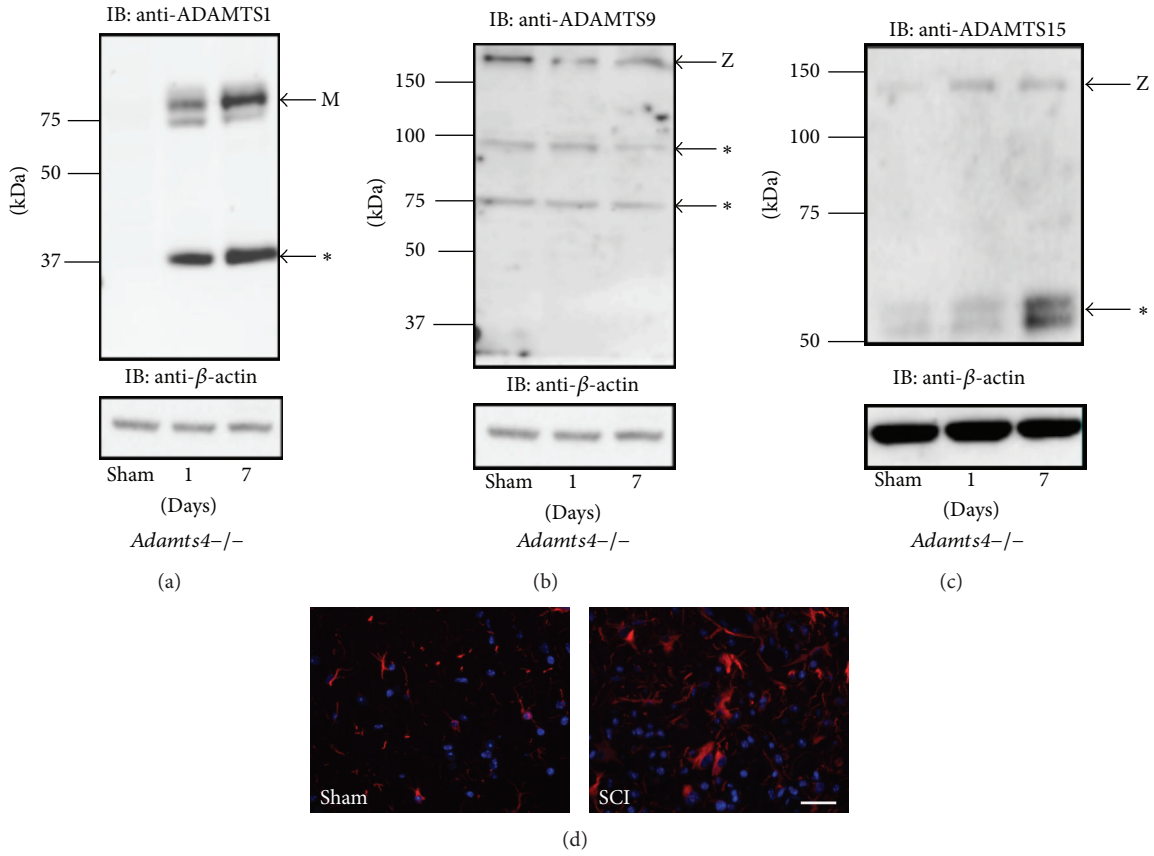

Figure 4: ADAMTS proteoglycanases are expressed during SCI. (a) ADAMTS1 is expressed in spinal cord tissue of *Adamts4*−/− mice 1 and 7 days after injury, m = mature, asterisk = autocatalytic fragment. (b) ADAMTS9 is expressed in spinal cord tissue of *Adamts4*−/− mice in the sham-operated group 1 and 7 days after injury, z = zymogen (inactive), asterisks = uncharacterized fragments. (c) ADAMTS15 is expressed in spinal cord tissue of *Adamts4*−/− mice in the sham-operated group 1 and 7 days after injury, z = zymogen (inactive), asterisk = uncharacterized fragments. In all cases β-actin was used as a loading control (bottom panels). (d) Spinal cord injury triggered astrocyte accumulation (reactive astrocytes) at the injury site compared with sham-operated mice. Red = astrocyte, blue = nucleus. Scale bar = 50 μm.

absence of either ADAMTS4 or ADAMTS5. In previous studies examining aggrecan destruction utilizing ADAMTS4 + ADAMTS5 double knockout mice, significant aggrecanolysis has been observed [9, 27] suggesting that other ADAMTS aggrecanases can contribute to aggrecan breakdown in addition to ADAMTS4 and ADAMTS5.

Versican is normally expressed in the mouse brain; its upregulation inhibits axonal regeneration after CNS injury [4]. In this current study, we observed ADAMTS proteoglycanase-specific V1 versican cleavage at DPEAAE$^{441}\downarrow$A^{442} in response to SCI in wild-type mice; however, *Adamts4*−/− or *Adamts5*−/− mice were largely protected. Several cases of severely reduced or absent versican proteolysis in single knockout mice have been reported during development including cardiac morphogenesis in ADAMTS5 knockout mice embryos [13], melanoblast colonization in ADAMTS20 knockout mice [28], and folliculogenesis in ADAMTS1 knockout mice [16]. To this end, we conclude that the absence of either ADAMTS4 or ADAMTS5, two of the strongest versicanases [29], significantly reduced the threshold of V1 versican proteolysis in SCI. ADAMTS proteoglycanases act cooperatively to process versican during several developmental processes in the mouse; ADAMTS5, ADAMTS9, and ADAMTS20 are required for interdigital web regression during limb formation, and ADAMTS9 and ADAMTS20 are required for palatal shelf closure during secondary palate formation [15, 30]. Thus it is not unreasonable to conclude this to be cooperatively required in neuronal tissue that is undergoing regeneration after injury. Although our major focus was the DPEAAE$^{441}\downarrow$A^{442} cleavage site targeted by ADAMTS proteoglycanases in V0/V1 versican, the V2 versican splice variant is the major source of versican in the CNS [31]. However the V2 splice variant does not possess the GAG-β domain containing the well-described ADAMTS cleavage site, although it is remodeled by ADAMTS proteoglycanases at an alternate site [31] not examined in detail in this current study.

Brevican, unlike versican and aggregan, is specifically expressed in the CNS. In this study, we showed that proteolytic processing of brevican was similar to that of aggrecan

with significant processing occurring in both *Adamts4*−/− and *Adamts5*−/− mice with an interesting caveat that its proteolysis was markedly reduced at 7 dpi in *Adamts5*−/− mice and essentially absent at this time-point in *Adamts4*−/− mice. Both ADAMTS4 and ADAMTS5 have previously been reported to proteolytically process brevican and *Adamts5* was found to be overexpressed in glioblastoma tissue compared to normal brain tissue [32].

We demonstrated the presence of ADAMTS1, ADAMTS9, and ADAMTS15 proteins in spinal cords of ADAMTS4 knockout mice and confirmed the active form of one of those proteoglycanases, ADAMTS1, during spinal cord injury. Proteolysis of aggrecan is exerted by ADAMTS proteases including ADAMTS1 [23], ADAMTS4 [33], ADAMTS5 [34], and ADAMTS8 [35] in cartilage tissue. The ADAMTSs that cleave aggrecan are less studied in CNS injury, and especially little is known in the context of SCI. However, accumulating evidence points out their roles in CNS pathologies. ADAMTS1 deficient mice showed efficient brevican and versican V2 cleavage in frontal cortex [36]; however, in this current study its active form was noticeably absent in the sham groups but specifically expressed during spinal cord injury in ADAMTS4 knockout mice suggesting it to be a major contributor of aggrecanase activity during this process.

We focused this study on ADAMTS4 and ADAMTS5, the major aggrecanases under investigation in cartilage destruction in arthritis. ADAMTS4 was first cloned from human brain [37] and is responsive to several CNS conditions such as beta-amyloid, which induces ADAMTS4 upregulation in rat astrocytes [38]. *Adamts4*, along with *Adamts1* mRNA, was markedly elevated in the hippocampus of rats in response to kainate-induced excitotoxic lesion [39] while *Adamts1* expression also increased in a rat model of middle cerebral artery occlusion, [40]. ADAMTS proteoglycanases may also interact with other pathways; for example, ADAMTS4 can signal through MAP ERK1/2 to promote neurite overgrowth independent of its proteolytic activity [41].

The present study was performed in single knockout mice where proteolysis of aggrecan and brevican was indicated. Given that ADAMTS activity likely benefits neurite outgrowth after CNS injury [5, 6], one might hypothesize that abolishing the activity of two or more ADAMTS aggrecanases could adversely affect the recovery process after CNS injury. Although studies in double knockout mice are feasible given that ADAMTS4 + ADAMTS5 knockout mice are reproductively viable, previous studies suggest that additional aggrecan-degrading activity is present in those mice in pathological processes [27]. However, sequentially knocking out additional ADAMTS aggrecanases in mice is not viable due to the embryonic lethality of *Adamts9*−/− mice [30], and *Adamts1* and *Adamts5* are separated by only 60 kb on mouse chromosome 16 [42], giving a low probability of segregation of these two *Adamts* genes during meiosis, making it difficult to generate ADAMTS1 + ADAMTS5 knockout mice using standard Mendelian genetic approaches. Taken together, our findings suggest that other members of the ADAMTS proteoglycanase family, such as ADAMTS1, may be responsible for turnover of aggrecan, versican, and brevican cleavage in the mouse spinal cord.

Abbreviations

ADAMTS: A disintegrin-like and metalloproteinase domain with thrombospondin-1 motifs
CNS: Central nervous system
CSPG: Chondroitin sulphate proteoglycan
ECM: Extracellular matrix
GAG: Glycosaminoglycan
PG: Proteoglycan
SCI: Spinal cord injury
SDS: Sodium dodecyl sulphate.

Conflict of Interests

There are no declarations of conflict of interests.

Acknowledgments

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Research Article

Efficacy of *Annona squamosa* L in the Synthesis of Glycosaminoglycans and Collagen during Wound Repair in Streptozotocin Induced Diabetic Rats

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The aim of this work was to find out the effects of *Annona squamosa* on the formation of glycosaminoglycans and collagen during wound healing in normal and diabetic rats. Diabetes induced rats were segregated into 4 groups, each containing six animals. Groups I and III served as the normal and diabetic control while groups II and IV served as normal and diabetic treated. The animals were treated with 200 μL of *Annona squamosa* extract topically. The granulation tissues formed were removed on the 8th day and the amount of glycosaminoglycans (GAGs) and collagen formed was evaluated by sequential extraction and SDSPAGE, respectively. Histological evaluation was also carried out using Masson's trichrome stain. *In vitro* wound healing efficacy of *A. squamosa* in human dermal fibroblast culture (HDF) was also carried out. The fibroblasts treated with varying concentrations of *A. squamosa* were examined for proliferation and closure of the wound area and photographed. *A. squamosa* increased cellular proliferation in HDF culture. The granulation tissues of treated wounds showed increased levels of glycosaminoglycans ($P < 0.05$) and collagen which were also confirmed by histopathology. The results strongly substantiate the beneficial effects of *A. squamosa* on the formation of glycosaminoglycans and collagen during wound healing.

1. Introduction

Wound healing is the process of repair that follows injury to the skin and other soft tissues. Wound healing cascade begins at the moment of injury or insult and progresses toward complete closure of the wound. Healing requires well-orchestrated integration of the complex biological and molecular events of cell migration, proliferation, and extracellular matrix (ECM) deposition [1]. The distinct interrelated phases which play a crucial role in wound healing are hemostasis, inflammation, proliferation, and remodeling [2]; the end product of wound healing is a dense connective tissue (scar) consisting predominantly of collagen [3, 4].

Diabetic wounds are defined as chronic wounds or lesions that take long time to heal or fail to heal [5]. Chronic wounds fail to progress through a normal, orderly, and timely sequence of repair. These wounds may eventually pass through the repair process without restoring sustained anatomical and functional results [6].

Proteoglycans are believed to be involved in the regulation of collagen fibrillogenesis and cell growth and act as tissue organizers [7]. Proteoglycans are the most abundant noncollagenous molecules available in the extracellular matrix, associated with collagen fibrils to form an assembly of the fibrils *in vivo*, and they may reduce diameter of fibrils by inhibiting lateral growth of fibrils [8]. Glycosaminoglycans (GAGs) are known as collagen associated proteoglycans (PGs) in the extracellular matrix of cell membranes and connective tissues. Glycosaminoglycans and proteoglycans significantly influence the cellular proliferation and rate of wound healing [9].

Proteoglycans, an essential component of mature matrix, are actively synthesized during the proliferative phase of

wound healing. Fibrin is the primary matrix at the wound site replaced by collagen and proteoglycans gradually [10].

Collagens are the major structural component of the connective tissues, composed of three identical polypeptide chains, each chain comprising Gly-X-Y, where G is glycine and X and Y are the imino acids proline and hydroxy proline, respectively. This sequence contributes to the formation of triple helical structure of collagen [11]. Both collagen and glycosaminoglycans play a key role in wound healing. Any agent which affects the normal metabolism of these important connective tissue proteins will definitely affect the normal wound healing pattern [12].

Plant extracts have been widely used as potent wound healing agents throughout the world. The role of plant extracts in glycosaminoglycans and collagen metabolism has been well documented [13, 14].

Annona squamosa L. (Annonaceae), commonly known as custard apple, is a native of West Indies and is cultivated throughout India, mainly for its edible fruit. Phytochemical screening and efficacy of this plant on wound healing in diabetic rats have already been reported by us [15].

In this paper, we report the influence of *A. squamosa* extract on the synthesis and characterization of glycosaminoglycans and collagen with detailed histological analysis during wound healing in streptozotocin-nicotinamide induced diabetic rats. This study also explores the role of *A. squamosa* in fibroblast proliferation with respect to wound closure rate.

2. Materials and Methods

2.1. Plant Collection and Extraction. *A. squamosa* leaves were procured, shade-dried, and crushed to make fine powder. 100 g of this powder was macerated with 70% ethanol in dark and filtered to harvest a viscous supernatant. The extract was dried under vacuum below 40°C. The viscous residue was collected, weighed, and kept at 4°C until use. The extract was reconstituted in phosphate buffered saline (PBS) whenever required .

2.2. Chemicals. D-Glucuronic acid, chondroitin sulphate B, chondroitinase ABC, chondroitinase AC, *Streptomyces* hyaluronidase, β-D-galactosidase, sodium dodecyl sulphate, coomassie blue R-250, N,N-methylene bisacrylamide, and ß-mercaptoethanol were purchased from Sigma Chemical Company, St. Louis, USA. Pepsin, proteinase K, and potassium acetate were procured from Sisco Research Laboratory, Mumbai, India. All other reagents were of high analytical grade.

2.3. In Vitro Studies

2.3.1. MTT Assay. For quantitative evaluation of cell viability and proliferation, MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) assay was used, in which only viable cells can reduce MTT to insoluble purple formazan [16]. Thus, the intensity of purple color represents the number of viable cells. For MTT assay, the HDF cells were cultured in 24-well microtiter plate at a density of 5×10^3 cells per 100 μL for 48 h. The culture medium was supplemented with different concentrations of *A. squamosa* like 7.8, 15.6, 31.2, 62.5, 125, 250, 500, and 1000 parts per million (ppm). The cells were then incubated in a humidified atmosphere of 5% CO_2 at 37°C and medium was changed every day (wherever applicable). MTT assay was performed at 48 h after treatment. The formazan crystals formed by living cells were solubilized with DMSO and the absorbance was measured at 570 nm with background subtraction at 690 nm using multimode plate reader (TECAN, Infinite M 200).

2.3.2. In Vitro Wound Healing Assay. The *in vitro* wound healing assay was carried out as previously described by Kheradmand et al. [17]. HDF cells were seeded on 24-well tissue culture dishes (10^6 cells/well). The cells were incubated for 48 h at 37°C with 5% CO_2. When confluence was reached, cell monolayers were incubated for 12 h in serum-free medium. The monolayers were then gently scratched with a sterile pipette tip and extensively rinsed with medium to remove all cellular debris. An indicated concentration of plant extract was added and incubated for 24, 48, and 72 h. The average extent of wound closure was evaluated by measuring the width of the wound. The rate of proliferation of cells into the wound area was examined by placing the cells under an inverted microscope and the image obtained was photographed.

2.4. In Vivo Studies

2.4.1. Experimental Design and Diabetes Induction. Healthy male Wistar albino rats (150–200 g) were used for the *in vivo* wound healing study. The rats were fed with commercial rat feed and water *ad libitum*. All clinical procedures were carried out according to the guidelines of the Institutional Animal Care and Use Committee (IACUC). A formal approval from the Animal Ethical Committee has also been obtained.

Diabetes was induced by a single intraperitoneal injection of streptozotocin (50 mg/kg b·wt) dissolved in 0.1 M of cold citrate buffer (pH 4.5), 15 min after the intraperitoneal administration of nicotinamide (110 mg/kg b·wt) in overnight fasted rats [18, 19]. Diabetic status was confirmed by tail vein blood glucose estimation using glucometer (One Touch Horizon, Johnson & Johnson, Mumbai, India) after 72 h. Two weeks after diabetic induction, rats with blood glucose level >250 mg dL^{-1} were deemed diabetic and used for the experiment.

The rats were divided into four groups comprising six rats in each group as given below:

group I: control rats left untreated,

group II: rats treated with *A. squamosa* (200 μL) at a concentration of (100 mg/kg b·wt),

group III: diabetic control rats also left untreated,

group IV: diabetic rats treated with *A. squamosa* (200 μL) at a concentration (100 mg/kg b·wt).

2.4.2. Excision Wound and Drug Administration. Wounds were created after the confirmation of diabetes. Rats were

anaesthetized by the intraperitoneal injection of thiopentone (50 mg/kg b·wt) dissolved in sterile distilled water [20]. A 2 cm^2 (4 cm) full thickness open excision wound was made on the back of the rat as reported in our earlier studies [15]. The control rats were left untreated and the treated rats were administered once daily with 200 μL (100 mg/kg b·wt reconstituted in PBS) of the extract for 8 days. The animals were sacrificed and the wound tissues were removed on day 8 after wounding and used for the glycosaminoglycan and collagen analyses.

2.4.3. Glycosaminoglycans (GAGs). Total GAGs from the wound tissues were extracted as described by Smith et al. [21]. The amount of GAGs was determined by estimating uronic acid content [22]. Individual GAGs were estimated using GAG degrading enzymes and nitrous acid treatment as described by Breen et al. [23].

2.4.4. Collagen. Fractionation of collagen was performed by the method of Piez [24]. The susceptibility of insoluble collagen to denaturing agents like urea and potassium thiocyanate was analyzed by the method of Adam et al. [25]. The aldehyde content of the acid soluble collagen was estimated according to the method of Paz et al. [26].

2.4.5. SDS-PAGE. The subunit composition of the isolated collagen was investigated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) [27]. Briefly, collagen bands were separated by SDS-PAGE using 3% stacking gel with 5% separating gel and Coomassie brilliant blue staining. Interrupted SDS-PAGE was used for the separation of type III collagen.

2.5. Histopathology. The rats were sacrificed and the tissues were removed from the wound site periodically. These samples were then separately fixed in 10% formalin-saline, dehydrated through graded alcohol series, cleared in xylene, and embedded in paraffin wax (melting point 56°C). Serial sections of 5 μm were cut and stained with Masson's trichrome. The sections were examined under light microscope and photomicrographs were taken for the analysis.

2.6. Statistical Analysis. Data were expressed as mean ± SD of six animals in each group and the results were statistically evaluated using one-way ANOVA and Student's paired t-test. All statistical analyses were performed using GraphPad prism (version 5.0; GraphPad software Inc., San Diego, CA, USA). Values corresponding to $P < 0.05$ were considered as significant.

3. Results

3.1. Cell Viability Assay. The proliferation and viability of the cells in the presence of different concentrations of the extract (7.8–1000 ppm) were observed for 48 h, by MTT assay. A significant increase in the cell viability was observed up to a concentration of 125 ppm as depicted in Figure 1. The proliferation was found to increase within the same cells day by day (data not shown). Culture supplemented with high concentrations like 250, 500, and 1000 ppm showed slightly lesser number of viable cells than the concentrations up to 250 ppm. Based on this, concentrations between 7.8 and 125 ppm of *A. squamosa* extract were used for further study.

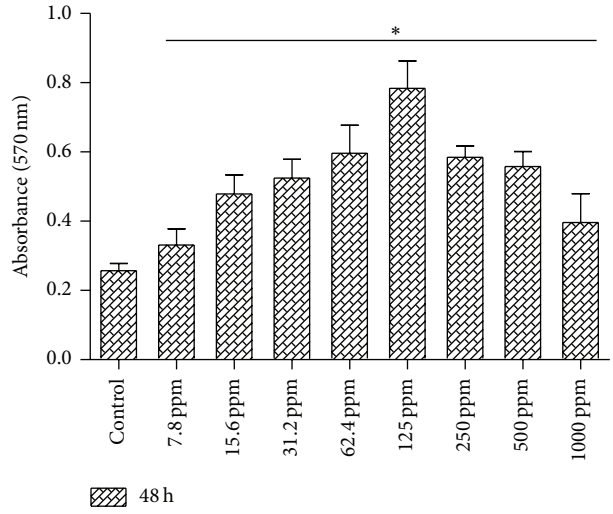

Figure 1: Efficacy of *A. squamosa* extract on the proliferation of HDF cells for 48 hrs. HDF cells were cultured with graded concentrations of *A. squamosa* extract (7.8 ppm–1000 ppm) and the number of viable cells was determined by MTT assay after 48 hrs. The results revealed that the *A. squamosa* extract increased the viable cells with increasing concentrations and there is no toxic or adverse effect at any concentration. All the data were expressed as mean ± SD ($n = 6$) and statistically significant ($^*P < 0.05$) when compared to control culture.

3.2. Efficacy of A. squamosa in In Vitro Wound Healing. *In vitro* wound healing assay was carried out using scratch wound model in confluent HDF culture. The proliferation and wound closure were evaluated until the completion of healing. After making the scratch, the cells were supplemented with 7.8–125 ppm of *A. squamosa* extract and photomicrographs were taken using inverted microscope to assess wound healing at different time point intervals. Figure 2 shows the images of the scratch wounds treated with 125 ppm of *A. squamosa*, taken on days 0, 1, and 2, as this concentration showed the best results. *A. squamosa* supplementation showed enhanced proliferation of HDF cells and wound closure was completed within two days, whereas part of incomplete wound was observed in control culture and it took almost three days to complete healing. For each concentration and each time frame, three scratched wells were used to assess wound healing. These results suggested that *A. squamosa* has no cytotoxic effect and has significant effect on proliferation of HDF cells in *in vitro*, which would be advantageous for reepithelialization during wound healing.

3.3. Effect of A. squamosa on Glycosaminoglycans. Levels of total GAGs evaluated on 8th day wound tissues of

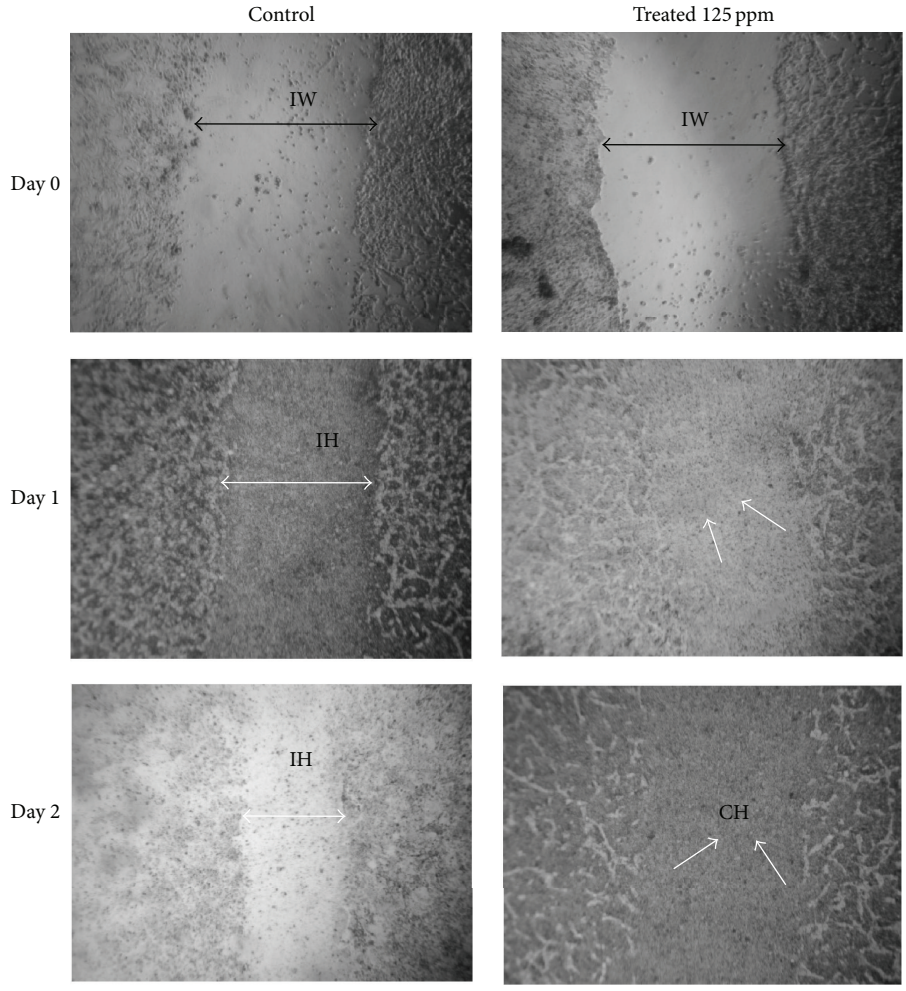

Figure 2: *In vitro* wound healing of HDF cells of control culture and *A. squamosa* treated (supplemented with 7.8–125 ppm) cultures. Proliferation of HDF cells was observed for 2 days after wound creation. The double headed black arrow shows the margin of scratched area on day 0 as initial wound (IW), and double headed white arrow shows the incomplete healing (IH), whereas white arrow shows the complete healing (CH). *A. squamosa* treatment shows complete healing on day 2, which clearly indicates that the *A. squamosa* accelerates the rate of proliferation faster in treated culture than the control.

the control and treated rats are shown in Figure 3. The values are expressed as uronic acid equivalence. Synthesis of ground substances was found to be high in treated groups. The amount of total GAGs was significantly ($P < 0.05$) higher (53%) in normal treated (group II) than control. In group IV, the increase was around 46% when compared to group III diabetic control (Figure 3(a)). Percentage of individual GAGs determined in the wound tissues of control and *A. squamosa* treated groups is shown in Figure 3(b). Among the various GAGs formed in the wound tissues, hyaluronic acid levels have a major proportion in both untreated and treated groups. In normal treated group, about 11% increase was observed when compared to control. Similar trend (8%) was observed in diabetic treated group for hyaluronic acid. A 7% and a 6% increase in chondroitin sulfates were observed in normal treated and diabetic treated groups as compared to their respective controls. Dermatan sulfate was also significantly increased in normal treated group (7%) and diabetic treated group (11%) when compared to control. But, keratan sulfate content was significantly reduced in both diabetic treated (29%) and normal treated groups (40%) when compared to their respective controls.

3.4. Effect of A. squamosa on Collagen. Table 1 illustrates the solubility pattern of collagen of day 8 tissues of control and treated rats in terms of neutral salt soluble (NSS), acid soluble (AS), and pepsin soluble (PS) collagen and insoluble collagen (IS). In all groups, pepsin digestion showed a significantly higher amount of collagen particularly in *A. squamosa* treated rats. The amount of AS fraction was greatly improved in *A. squamosa* treated groups. The difference between normal and diabetic treated group was found to be 18%. A similar kind of trend was observed for IS in treated groups. It shows that there was abundant and earlier maturation of collagen fibers in *A. squamosa* treatment.

The susceptibility AS to different denaturing agents like 2 M KCNS and 6 M urea was carried out in both control

Table 1: Effect of *A. squamosa* on solubility pattern of collagen on day 8 wound tissues.

Group	Neutral salt soluble collagen	Acid soluble collagen	Pepsin soluble collagen	Insoluble collagen
Control	84.5 ± 10.4	590.5 ± 42.4	2289 ± 187.5	976.7 ± 10.2
Treated	97.8 ± 9.6	898.8 ± 86.5*	2812 ± 195.8*	1039.8 ± 17.4*
Diabetic control	64.9 ± 8.2	335.5 ± 66.1	1467 ± 173.4	841.3 ± 7.0
Diabetic treated	55.3 ± 9.2	465.0 ± 62.6*	1766 ± 90.4*	883.7 ± 13.2

Values (μg/100 mg wet tissue) are expressed as mean ± SD ($n = 6$ animals).
* $P < 0.05$ is considered as significant compared to corresponding control.

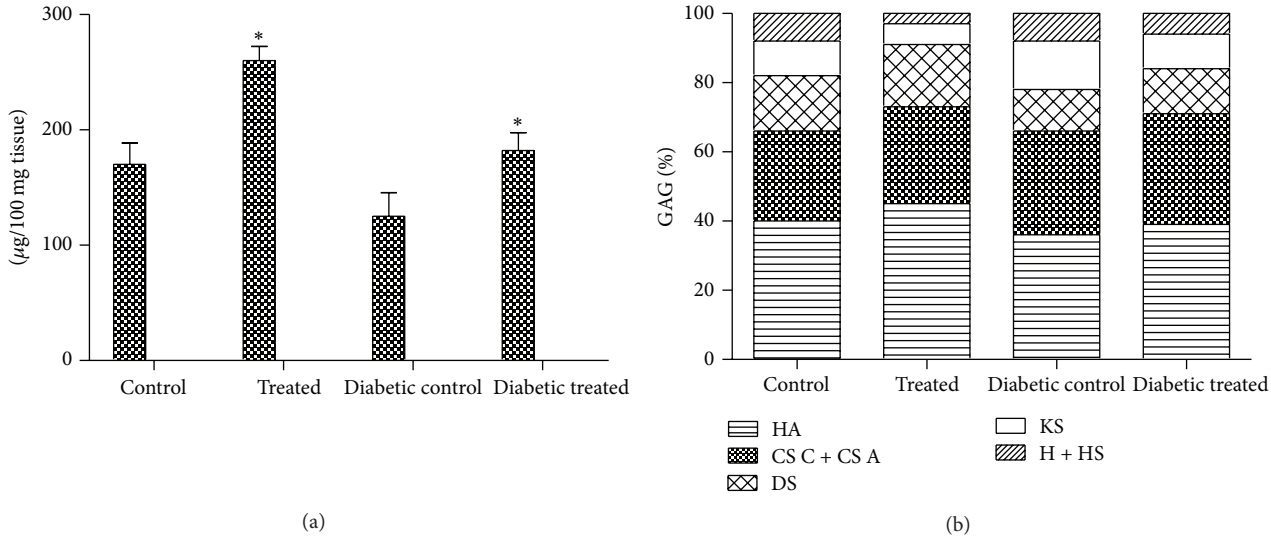

Figure 3: (a) Glycosaminoglycans of control and *A. squamosa* treated wound tissues on day 8 after wounding. Values are expressed as mean ± SD for six animals. * $P < 0.05$ is considered as significant compared with the control. (b) Percentage proportions of various glycosaminoglycans formed in control and *A. squamosa* treated wound tissues on day 8 after wounding. HA: hyaluronic acid; CS C + CS A: chondroitin sulphate A and C; DS: dermatan sulphate; KS: keratan sulphate; H + HS: heparin and heparin sulphate.

and treated wound tissues. The amount of collagen released by these agents was significantly ($P < 0.05$) reduced in *A. squamosa* treated rats. 2 M KCNS treatment showed 46% release for control and 33% for extract treated AS. Similarly, 41% and 34% of decreased collagen release were obtained for both diabetic control and diabetic treated (Table 2).

A. squamosa treated wound tissues showed high aldehyde content in the pepsin soluble collagen. There were about 46% increase in normal and 38% increase in diabetic treated groups when compared to control (Table 3). The increase in aldehyde content and reduction of susceptibility pattern in *A. squamosa* treated rats significantly explain the formation and maturation of collagen.

3.5. SDS-PAGE. Figure 4(a) shows the SDS-PAGE pattern of pepsin soluble collagen from control and treated wound tissues. From the banding pattern, we could observe a significant increase in type I collagen in *A. squamosa* treated normal and diabetic rats. Figure 4(b) depicts the interrupted gel electrophoresis of pepsin soluble collagen, from which we could observe a marked increase in type III collagen in the treated groups.

Table 2: Effect of *A. squamosa* on susceptibility of insoluble collagen on day 8 wound tissues.

Group	2 M KCNS	6 M urea
Control	5.75 ± 0.79	6.70 ± 0.56
Treated	3.08 ± 0.58*	3.98 ± 0.63*
Diabetic control	3.49 ± 0.79	3.47 ± 0.79
Diabetic treated	2.33 ± 0.76*	2.29 ± 0.69*

Values (mg/100 mg collagen) are expressed as mean ± SD ($n = 6$ animals).
* $P < 0.05$is considered as significant compared to corresponding control.

Table 3: Aldehyde content of acid soluble collagen on day 8 wound tissues of control and *A. squamosa* treated rats.

Control	4.59 ± 1.01
Treated	8.51 ± 1.09*
Diabetic control	3.22 ± 0.90
Diabetic treated	5.20 ± 1.08*

Values (μM/100 mg collagen) are expressed as mean ± SD ($n = 6$ animals).
* $P < 0.05$ is considered as significant compared to corresponding control.

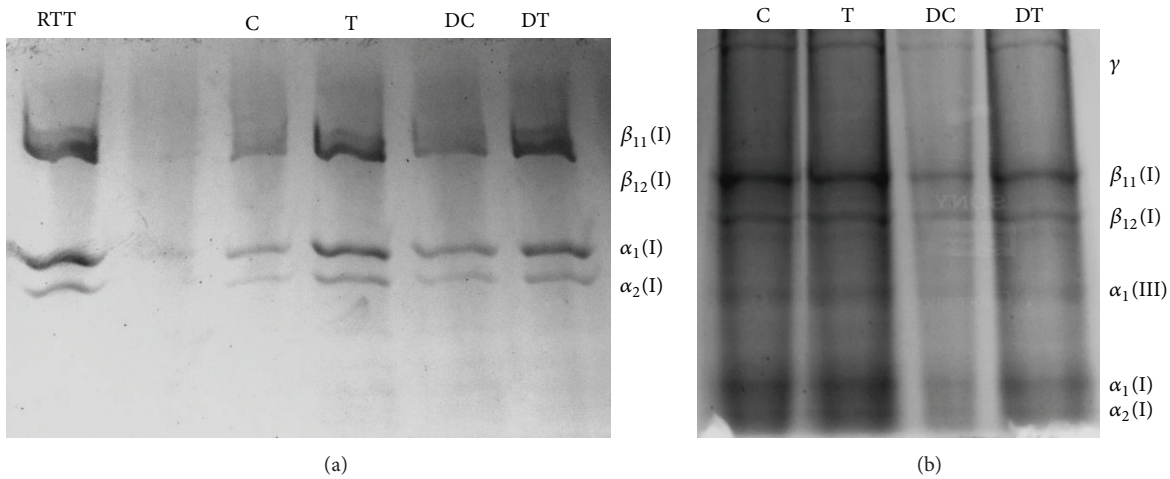

Figure 4: Normal (a) and interrupted (b) SDS-PAGE photograph showing the distribution of collagen. All lanes were loaded with equal concentration of collagen (25 μg). RTT: rat tail tendon; C: control; T: treated; DC: diabetic control; DT: diabetic treated.

3.6. Histopathology of Wound Tissues. A detailed histological evaluation was carried out to screen the collagen maturation using Masson's trichrome staining. Masson's trichrome stained wound tissue of the first week normal control showed less collagen fibers, proliferating blood capillaries, and incomplete epithelialization (Figure 5(a)). Whereas, complete fibrous tissue, more number of collagen bundles and large number of blood vessels were observed in treated tissue (Figure 5(b)). In the second week, the amount of collagen deposited on the wound surface in control tissue was found to be inadequate (Figure 5(c)). But, more accumulation of collagen fibers was seen in treated tissue (Figure 5(d)).

In diabetic group, the first week of control tissue showed large number of blood vessels with minimal cellular infiltrates on the wounded site (Figure 6(a)). In treated tissue, large amount of collagen has been observed with an early epithelialization process (Figure 6(b)). The second week of control tissue showed equal amount of blood capillaries and collagen bundles at the wound site (Figure 6(c)). Thick collagen fiber deposition under the completed epithelial layer on the wound surface was seen in treated tissue (Figure 6(d)). In the third week, fewer amounts of loosely packed collagen molecules were accumulated in untreated tissue depicting incomplete wound closure (Figure 6(e)). But, highly organized thick collagen fibers as bundles and thick uniform epithelial layer were formed in treated tissue (Figure 6(f)).

4. Discussion

In this investigation, efficacy of *A. squamosa* extract in *in vitro* cell viability, proliferation of HDF cells, and its wound closure was studied to confirm the therapeutic activity of this extract in wound healing.

Even though various models have been proposed to study reepithelialization process in *in vitro*, the simple one is fibroblast monolayer scratch wound, in which injury was mechanically created in confluent fibroblast culture using sterile microtip and recovery of wound area has been used as an indicator for wound reepithelialization [28]. After scratch wounding, the neighboring intact mesenchymal cells start to migrate rapidly over the wounded area until they cover the wound surface with cells. Then the cells begin to proliferate to increase number of viable cells that restore the normal epithelial structure. Later, the fibroblasts start to differentiate on the wounded surface area of the healed site and become soft and smooth, and a well-layered architecture is restored [29]. In our results, we found that all the concentrations of *A. squamosa* extract (7.8 ppm–1000 ppm) increased the number of viable cells and proliferation of fibroblasts and cover the wound surface rapidly. There is no significant toxicity and adequate antiproliferative effect observed in *A. squamosa* treatment.

Cell proliferation is an essential event during reepithelialization, so proliferating fibroblasts at the wound site ensure an adequate supply of cells to migrate and cover the wound surface. Synthesis of extracellular matrix (ECM) is a key feature of wound healing. Dermal reconstruction is characterized by the formation of granulation tissue, which includes cell proliferation, ECM deposition, wound contraction, and angiogenesis [30].

After tissue injury, synthesis of ground substances plays an important role during wound healing process. They are mainly PGs and GAGs. GAGs have been found to be regulators of cellular proliferation, migration, and differentiation [31]. Synthesis of these substances and their degradation has great impact on the healing process. The GAGs are the first components of the extracellular matrix to be synthesized during wound healing and form the scaffold for collagen and elastin deposition [32]. Our results revealed that *A. squamosa* extract significantly improved the total GAGs in normal and diabetic treated rats more than the control.

HA, a known glycosaminoglycan, is an important physiological substance that plays a vital role in the healthy formation of connective tissue [33]. HA has also been shown to stimulate collagen synthesis in fetal fibroblast cultures [34]. Fibroblasts are the major components which

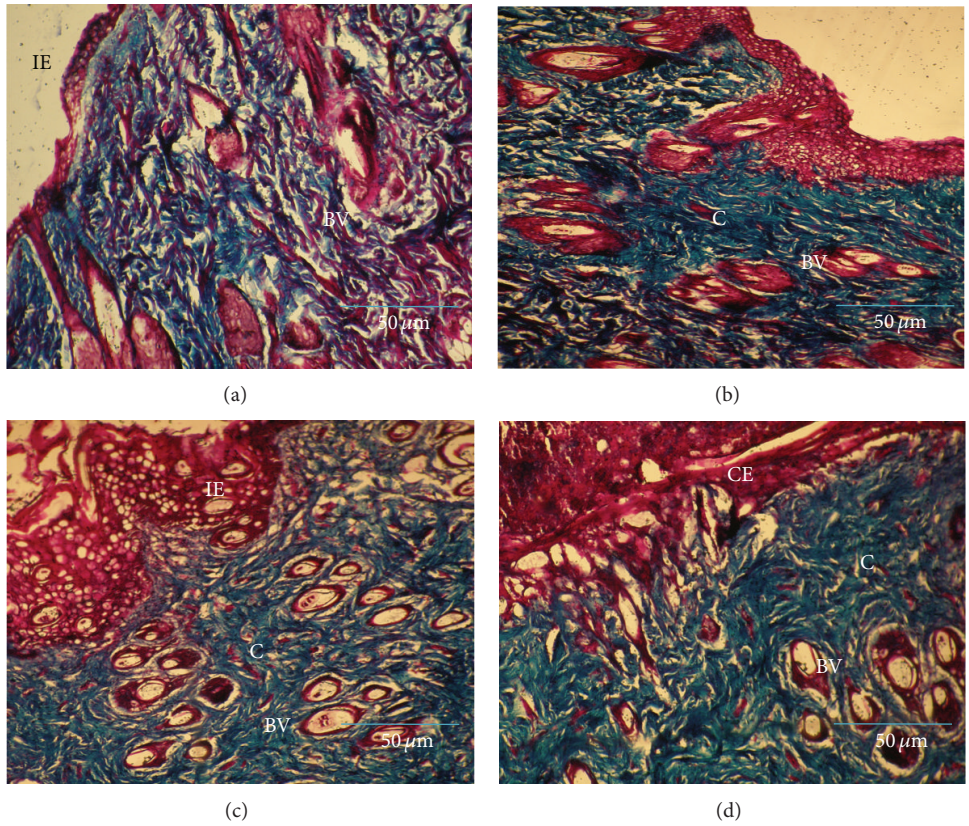

FIGURE 5: Masson's trichrome staining for collagen in control and *A. squamosa* treated wound tissues from first and second week, respectively (magnification 20x). Control (a) shows collagen fibers with incomplete epithelialization whereas (b) treated tissue shows dense collagen deposition. In the second week, control (c) depicts thin collagen layer at the wound site and (d) treated tissue shows complete epithelialization with uniform collagen deposition. IE: incomplete epithelialization, CE: complete epithelialization, E: epithelialization, BV: blood vessels, and C: collagen. Scale bar: 50 μm.

secrete hyaluronic acid into the extracellular matrix [35]. The role of hyaluronate gel in wound healing in streptozotocin induced diabetic rats has already been reported [36].

HA might be the rationale behind the availability of excess fluid and flexible matrix on the wound site that facilitates greater cell mobility and easier and faster regeneration of damaged tissue. The increased content of HA in *A. squamosa* treated wounds may result in the formation of a more stable scar [13]. Chondroitin sulfates A and C have a crucial role in *in vitro* proliferation of fibroblasts to accelerate the wound closure rapidly through their location of sulfation group [37]. Dermatan sulphate proteoglycans are closely associated with collagen fibers [38]. They influence the collagen fibril formation *in vitro* and may therefore contribute to the organization and strength of the collagen fibrillar assembly in wound tissues [39].

A significant reduction in the levels of keratan sulfate in the treated groups was observed. Funderburgh has reported that myofibroblasts exhibited reduced expression of keratocan, a keratan sulfate linked proteoglycan. The increased rate of contraction in the treated groups suggested that *A. squamosa* extract accelerates the transformation of fibroblasts to myofibroblasts. Reduced level of keratan sulfate in the wound tissue is associated with inflammation, suggesting the role of proinflammatory cytokines involved in the downregulation of keratan sulfate biosynthesis [40, 41]. Cintron et al. have shown that corneal wound healing resulted in a reduction of keratan sulfate and in accumulation of highly sulfated chondroitin/dermatan sulfate in the scar. The results we obtained also substantiate this observation [42].

Collagen, a principal component of connective tissue, plays a major role in the healing of wounds by providing a structural architecture for the remodeling tissue. Collagen molecules contain aldehydic groups that cross-link with amino acids to form collagen fibers. Normally, an increase in collagen synthesis would result in an increase in aldehyde content which leads to a greater potential for cross-linking [43, 44].

The present investigation shows that the collagen obtained from *A. squamosa* treated wounds has a higher content of aldehydic groups (Table 3) than collagen from controls. This observation indicates that the collagen in treated wounds must have undergone a greater degree of cross-linking resulting in an ultimate increase in wound

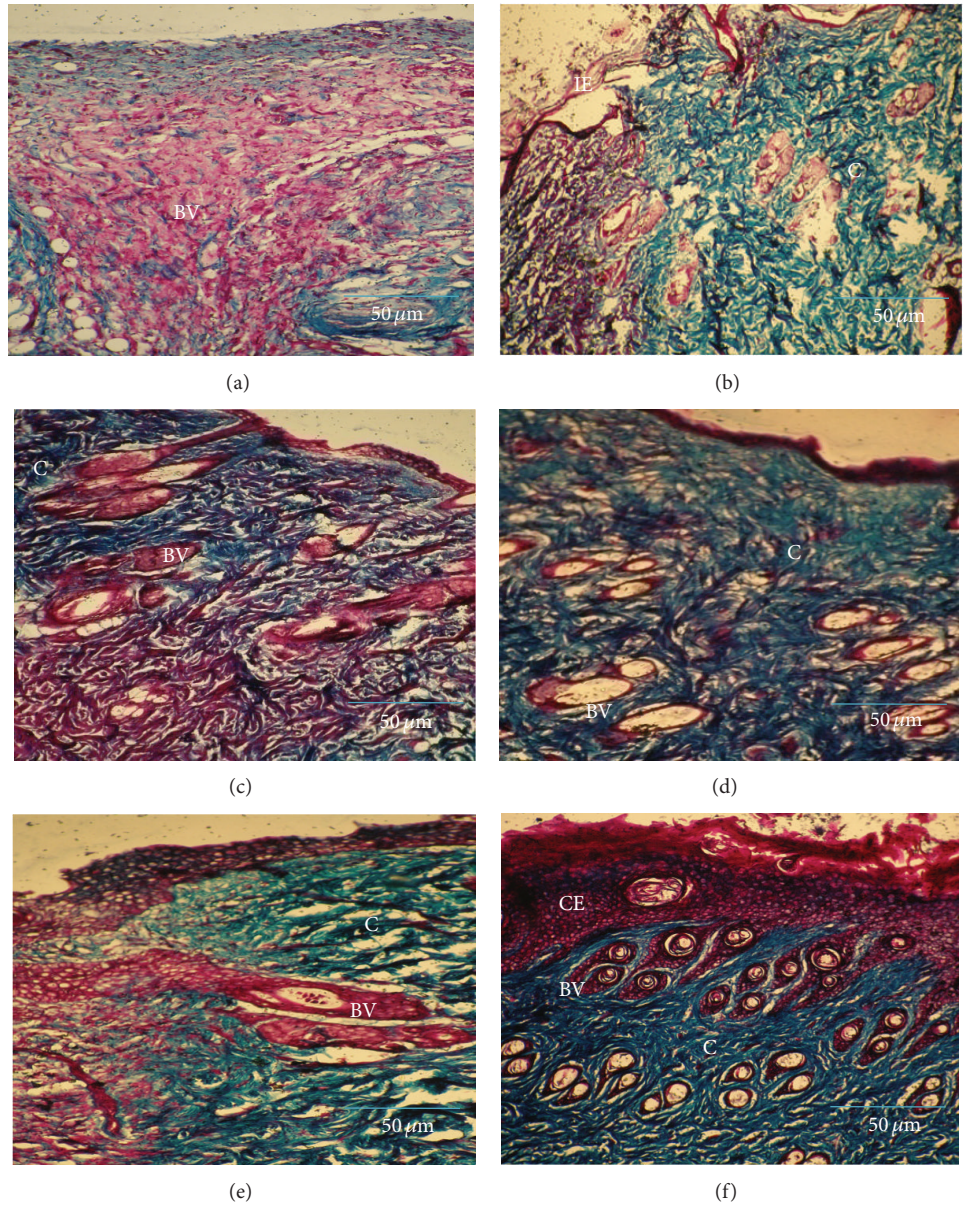

Figure 6: Masson's trichrome staining for collagen in control and *A. squamosa* treated diabetic wound tissues from first, second, and third week, respectively (magnification 20x). Control (a) shows large number of proliferating blood vessels, whereas (b) treated tissue shows less collagen formation. In the second week, control (c) depicts adequate amount of blood vessels formation and collagen fibers and (d) treated tissue shows thick collagen fiber deposition under thin epithelial layer. In the third week, control (e) shows accumulation of loosely arranged collagen bundles at the wound site and (f) explores the completed epithelial layer with highly organized collagen deposition. IE: incomplete epithelialization, E: epithelialization, C: collagen, and BV: blood vessels. Scale bar: 50 μm.

strength, which was further confirmed by the tensile strength of the wounds.

Solubility pattern of collagen in neutral salt buffer and acid solution mainly depends on the cross-linking of collagen. Highly cross-linked collagen becomes less soluble in neutral salt buffer and acid solution which can be released only in pepsin digestion. *A. squamosa* treatment showed decreased percentage of solubility in neutral buffer and in dilute acid solution. A significantly larger amount was solubilized in pepsin digestion and this is an indication of increased levels of cross-linking in treated groups. The insoluble collagen content of treated groups is also higher than that of the control group.

Type I collagen is the most abundant type of collagen observed in normal dermis (approximately 80% to 90%). But during the early phases of wound healing, fibroblasts actively produce type III collagen, which may account for 30% of the total collagen in a healing wound. By week 2, type I collagen again becomes the principal collagen produced by fibroblasts. Type I and type III collagen are formed in skin in

a higher proportion relative to other types and are maintained in a fixed proportion relative to one another in normal skin tissue [45]. *A. squamosa* treated wounds synthesize greater amounts of type III collagen when compared to controls. The presence of higher levels of type III collagen may have beneficial effects on early wound healing process and result in better organization of type I collagen in the final scar [32].

Histological evaluations using specific stains strongly support these results. A greater degree of epithelialization and collagen deposition observed in *A. squamosa* treated wounds reveals the healing efficacy of the plant extract. Proliferated blood capillaries and dense collagen fibers were uniformly distributed, significantly increased at the wound site in normal treated and diabetic treated rats, especially during second and third week, respectively. These changes indicate a shift in wound healing from the proliferative phase to the maturation phase.

Earlier, we have reported that the rate of wound contraction was significantly higher and period of epithelialization was shorter in normal and diabetic treated rats, when treated with *A. squamosa* [15]. These results further strongly substantiate the efficacy of *A. squamosa* in collagen maturation during wound repair.

5. Conclusion

We have shown that the wound healing efficacy of *A. squamosa* in human dermal fibroblast by assessing cell proliferation and wound closure through scratch wound assay and the topical administration of ethanolic extract of *A. squamosa* promotes active synthesis of GAGs and collagen maturation during wound healing in normal and diabetic rats. This in turn is a positive sign of better wound healing.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

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