Changes in Perlecan Expression During Vascular Injury
Role in the Inhibition of Smooth Muscle Cell Proliferation in the Late Lesion

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Objective—Vascular smooth muscle cells (SMCs), activated by growth factors after arterial injury, migrate and proliferate to expand the intima of the blood vessel. During intimal expansion, proliferation is suppressed and an increasingly large proportion of the neointimal mass is composed of newly synthesized extracellular matrix (ECM). We sought to determine whether the ECM heparan sulfate proteoglycan (HSPG) perlecan, which inhibits SMC proliferation in vitro, also accumulates and limits SMC proliferation during neointimal expansion.

Methods and Results—Perlecan expression and accumulation were analyzed by immunohistochemistry and in situ hybridization during neointima formation after balloon catheter injury to the rat carotid artery. Perlecan expression was low in uninjured vessels and up to 7 days after injury, during maximal SMC proliferation. By 14 days after injury, perlecan was dramatically increased, and immunostaining remained heavy throughout the advanced lesion, 35 to 42 days after injury. Finally, explants of intimal tissue from 35- to 42-day neointimal lesions were digested with glycosaminoglycanases to determine whether endogenous HSPGs inhibit intimal SMC proliferation. SMCs within HS-depleted, but not chondroitinase ABC–treated or mock-incubated, explants were found to proliferate in response to platelet-derived growth factor BB.

Conclusions—HSPGs, such as perlecan, may inhibit the proliferative response of SMCs after vascular injury. (Arterioscler Thromb Vasc Biol. 2003;23:608-614.)

Key Words: heparan sulfate ■ perlecan ■ neointimal hyperplasia ■ vascular smooth muscle ■ proliferation

Accumulated evidence indicates that extracellular matrix (ECM) proteins provide both permissive and inhibitory modulation of the cellular responses to growth factors. Thus, changes in the amounts and types of ECM proteins that are deposited during the development of the atherosclerotic lesion may modify the effects of growth factors during vascular pathogenesis. However, mechanisms by which the ECM may inhibit intimal smooth muscle cell (SMC) proliferation in the ECM-rich advanced lesion remain unresolved. Considerable previous work has suggested that the glycosaminoglycan chains of heparan sulfate proteoglycans (HSPGs), and heparin, are potent inhibitors of SMC proliferation in vitro. Conversely, HS lyases appear to accelerate the conversion of the SMC phenotype from a quiescent, contractile state to a rapidly growing, “synthetic” state. Pioneering studies demonstrated that heparin infusion dramatically suppresses the early wave of medial SMC proliferation that is required for the formation of a neointima after injury to the rat carotid artery. Moreover, heparin was found to inhibit SMC cell growth in vitro, suggesting that heparinlike molecules, such as vascular HSPGs, may be endogenous inhibitors of vascular SMC proliferation.

Proteoglycans are among the ECM proteins that are deposited within the late neointimal lesion. Perlecan is an ECM HSPG that is a potent modulator of cellular phenotype and proliferation and a major vascular wall basement membrane component. The induction of perlecan expression in developing vessels has been correlated with developmental downregulation of SMC proliferation and is associated specifically with nontdividing cells. The mechanism by which perlecan downregulates cell proliferation and supports the maintenance of a more fully differentiated cellular state is unclear. However, it has been proposed that the HS chains of perlecan may bind and modify the effects of heparin-binding growth factors and directly or indirectly influence cellular interactions with the ECM that are important in the control of cell behavior and phenotype.

SMCs that populate the neointima of the balloon-injured rat carotid artery progressively lose proliferative capacity and...
growth factor responsiveness, such that by 4 to 6 weeks after injury, SMC proliferation is almost completely refractive to further stimulation by growth factors. Because perlecan may modify SMC proliferation, we determined whether perlecan expression and accumulation in the neointima is altered. We found that perlecan expression, as assayed by in situ hybridization, was low in the media and early neointima at times of maximal SMC proliferation, was maximal at 2 weeks after injury, and was downregulated thereafter. Despite decreased perlecan mRNA expression, the late neointimal lesion (5 to 6 weeks after injury) remained perlecan rich, as determined by immunohistochemistry. Although convincing studies suggest that perlecan can inhibit SMC proliferation in vitro and application of exogenous perlecan inhibits intimal hyperplasia, it is not known whether endogenous HSPGs, such as perlecan, limit intimal SMC proliferation in the developing neointima. Therefore, perlecan-rich, 5- to 6-week intimal lesion tissue explants were treated with heparin lyases before growth factor stimulation in vitro. Removal of endogenous HSs from late lesion explants caused a dramatic recovery of the proliferative responsiveness of neointimal SMCs within the explant to platelet-derived growth factor (PDGF). These observations suggest that the regulation of perlecan expression after injury to the vessel may modify SMC response to cytokines.

**Methods**

**Experimental Injury, Harvest, and Tissue Preparation of Rat Carotid Arteries**

Sprague-Dawley rats weighing 350 to 400 g and 3 to 4 months of age were anesthetized, and the left or both carotid arteries were denuded of endothelium with a 2F Fogarty balloon catheter, which was introduced through the external branch as previously described. The balloon was inflated and passed through the common carotid artery introduced through the external branch as previously described.

**Immunohistochemistry**

Sections were treated for 10 minutes with H2O2 to block endogenous peroxidase activity and then with 1% normal goat serum (Sigma Immunochemicals) in PBS overnight at 4°C. Sections were incubated 1 hour at room temperature and then overnight at 4°C with primary antibodies, including rabbit anti-perlecan polyclonal antibody EY-9 provided by Dr John Hassell (Shriners Hospital for Children, Tampa, Fla) or with mouse anti-HS monoclonal antibody (HepSS-1; Seikagaku America, Inc) and, as a control, either normal rabbit serum (Zymed Laboratories, Inc) or rat IgG/IgM (Sigma) that was diluted 1:200 to 1:1000 in 1% normal goat serum in PBS. Epitope unmasking was done by treatment with 0.1% trypsin in 0.1% CaCl2, for 15 minutes at 37°C. Sections were incubated 1 hour at 21°C with biotin-conjugated goat anti-rabbit IgG or anti-mouse IgM (Jackson ImmunoResearch Laboratories, Inc) and then developed by standard immunoperoxidase procedures. Immunoperoxidase-stained sections were lightly counterstained with Mayer's hematoxylin.

**In Situ Hybridization**

A cDNA (clone 5/BPG5) that partially encodes mouse perlecan was provided by Dr S. Ledbetter (Upjohn, Kalamazoo, Mich). A 661-bp BamHI/BstX1 fragment representing base pairs 3713 to 4374 of mouse perlecan was excised and subcloned into the pcRII vector (Invitrogen Corp). This sequence contains no known homologies to sequences other than perlecan and, when used as a probe on Northern blots of total RNA isolated from cultured rat SMCs, recognizes a single ~13-kb message. S-labeled sense and antisense riboprobes were prepared by using the T7 and Sp6 promoters and 32S-UTP and were used for in situ hybridizations. Tissue sections were deparaffinized, rehydrated, and incubated in 0.2% Triton X-100 in PBS followed by 50 µg/mL proteinase K and acetic anhydride in 0.1 mol/L triethanolamine. Samples were then dehydrated, air dried, prehydrized for 2 hours, and hybridized overnight to the 32P-labeled riboprobe (1X106 counts per minute per section). Sequential sections were used for hybridization with either sense or antisense probes. After hybridization, the sections were washed in 2X standard saline citrate (SSC), incubated with RNAase A (Sigma Chemical Co), and washed several times with 2X SSC at both room temperature and 55°C, and washed several times with 0.1X SSC at 55°C. The sections were then dehydrated through a graded ethanol series, air dried, and dipped in NTB-2 emulsion (Eastman Kodak Co). Slides were developed after 21 days and counterstained with hematoxylin and eosin.

**Northern Blotting**

Uninjured and balloon-catheterized carotid arteries were excised, stripped of adventitia, and frozen in liquid nitrogen. Total RNA was isolated from pooled aortas that had been powdered by impact in liquid nitrogen, by the single-step method. Ten micrograms of total RNA was loaded per lane and resolved by electrophoresis overnight on 1% wt/vol agarose/formaldehyde gels. After electrophoresis, RNA was transferred to Zetaprobe GT (Bio-Rad) and UV cross-linked (Stratagene Cloning Systems). Filters were prehybridized and then hybridized with a 32P-labeled mouse perlecan domain I cDNA probe (19 J, kindly provided by Dr John Hassell, Shriners’ Hospital, Tampa, Fla) as previously described. Autoradiographs were developed after exposure on Kodak XAR2 film at −70°C.

**Organ Culture of Rat Carotid Artery and Assay for Proliferation**

In 2 separate experiments, transverse sections (3 to 5 mm) were prepared from unfixed carotid arteries that were removed from animals 5 or 6 weeks after balloon catheter injury. Before organ culture, the tissue was suspended for 30 minutes at 37°C in 10 U/mL each heparin lyase I (EC 4.2.2.7) and heparin lyase II (EC 4.2.2.7.5), or 2 U/mL chondroitin ABC lyase (all 3 enzymes from Sigma) in 100 mmol/L Tris-HCl with 5 mmol/L CaCl2 and 15 mmol/L sodium acetate, pH 7.4, or in buffer alone. Explants were washed 3 times in PBS and then suspended in Dulbecco’s modified Eagle’s medium (DMEM) with 0.1% fetal bovine serum with or without 10 ng/mL PDGF-BB. Eighteen hours after suspension in DMEM, 50 µg/mL of the thymidine analogue 5-bromo-2’-deoxyuridine (BrdU, Boehringer Mannheim Corp) was added to the culture medium. After an additional 30 hours (48 hours total), explants were rinsed twice with PBS and then fixed with 3% paraformaldehyde in PBS at 4°C for 30 minutes. After being rinsed with PBS, fixed tissue was dehydrated, embedded in paraffin, and sectioned. The nuclei of proliferating intimal SMCs that had incorporated BrdU were stained with immunoperoxidase by using an anti-BrdU monoclonal antibody (G342, acquired from Developmental Studies Hybridoma Bank, University of Iowa, Iowa City). The sections were lightly counterstained with hematoxylin or methyl green. Labeled nuclei of intimal SMCs were counted in explant pieces, and the area of intima was calculated with the TAXAN image analysis program. Statistical comparison of
Expression of Perlecan RNA in Rat Carotid Arteries After Experimental Injury

A perlecan-specific antisense riboprobe was used for in situ hybridization of a temporal series of sections of injured rat carotid arteries and uninjured contralateral controls to determine the pattern of expression of perlecan mRNA in the developing lesion (Figure 1). Little mRNA for perlecan was detectable in the media and adventitia of uninjured control vessels (Figure 1a). However, by 4 to 7 days after injury, significantly elevated hybridization with the perlecan probe was found within SMCs in the media, particularly in those cells nearest the vessel lumen (Figure 1b and 1c). Between 9 and 14 days after injury, abundant perlecan mRNA was evident within the neointima as well as within the media of the artery (Figure 1d and 1e). By 21 days after injury, however, perlecan mRNA expression by cells within both the extensive intima and in the media was greatly diminished and remained low at 42 days after injury (Figure 1f and 1g). These observations suggest that perlecan mRNA is actively regulated after arterial injury, with an increase in levels during intimal formation subsequent to the peak of medial SMC proliferation that occurs 2 days after injury,26 and a decrease in perlecan mRNA expression by 21 days after injury. Perlecan mRNA levels, as assessed by Northern blotting of total RNA from pooled carotid arteries, were low in uninjured vessels and at times up to 7 days after injury, consistent with the in situ hybridization data of perlecan mRNA expression. The highest perlecan mRNA levels were apparent in intima samples 14 days after injury, but perlecan mRNA was also detectable in the late (42-day) lesion samples (Figure 2). The presence of perlecan mRNA in both midstage and late intimal lesions was additionally confirmed by polymerase chain reaction of the perlecan sequence from reverse-transcribed RNA isolated from pooled 14-day and 42-day carotid intimas by using primers to a 365-bp sequence of perlecan domain I (not shown).

Perlecan Deposition in the ECM of Injured Carotid Arteries

A series of injured rat carotid artery sections was immunostained with a polyclonal antibody33 against perlecan to examine the localization of perlecan during the time course of development of the intimal lesion (Figure 3). The small amount of staining for perlecan that was evident in sections of uninjured control vessels was associated with the endothelial cell monolayer and the adventitia (Figure 3A). However, by 24 hours after injury, there were foci of immunostaining for perlecan within the vessel media, particularly in the first layer of cells below the internal elastic lamina (Figure 3B). Perlecan immunoreactivity was more generally present within the small neointima and at the luminal region of the media at 4 days after injury (Figure 3C). The more extensive neointima present at 14 days after injury had distinct regions of intense immunostaining for perlecan (Figure 3D), consistent with the high levels of perlecan expression that were detected at this stage by in situ hybridization (see Figure 1). Perlecan immunostaining in 42-day lesions was diffuse throughout the intima and media (Figure 3E). The staining of 42-day lesion sections was amplified by epitope unmasking27 (Figure 3F), indicating that abundant perlecan remained deposited within the advanced lesion despite the downregulation of perlecan mRNA expression.

Heparin Lyase Treatment Restored Intimal SMC Responsiveness to PDGF-BB in Explants From 35- to 42-Day Neointimas

SMCs in intimal lesions of balloon-injured rat carotid arteries progressively lose the ability to proliferate in response to growth factors.34 Because previous studies had indicated that perlecan can regulate the proliferative responsiveness of SMCs in vitro,16,35 we tested whether the responsiveness to PDGF could be restored by removal of endogenous HS from the neointimal extracellular matrix. Thus, 35-day or 42-day intimal lesions explants were preincubated for 30 minutes with heparin lyases, chondroitin ABC lyase, or buffer (see Methods) and then cultured in the presence or absence of 10 ng/mL PDGF-BB. Treatment with heparin lyases effectively removed HS from the explants, as indicated by...
immunoperoxidase staining of sections of the 35-day neointimal explants with an antibody that recognizes HS chain epitopes (Figure 4). Explants treated with either buffer (Figure 4A) or chondroitin ABC lyase (Figure 4B) retained staining for HSs, whereas staining was almost absent in sections of heparan lyase–treated explants (Figure 4C). The continued presence of the large proteoglycan versican in heparin lyase–treated explants (Figure 4D) indicates that the enzyme treatment did not indiscriminately remove other ECM proteins. Explants cultured after enzyme treatment in the presence or absence of PDGF-BB were exposed to BrdU and stained for BrdU-positive nuclei to identify proliferating cells within the explants (Figure 5). PDGF-BB did not significantly stimulate proliferation of SMCs within the buffer- (Figure 5A) or chondroitin ABC lyase–treated (Figure 5E) explanted neointimases when compared with explants cultured in 0.1% serum without PDGF-BB (Figure 5B and 5D). In contrast, large numbers of nuclei within sections of heparin lyase–treated explants incorporated BrdU (Figure 5C), indicating that the removal of HS restores responsiveness of the intimal SMCs to PDGF-BB. When BrdU-positive nuclei of intimal SMCs within sections were counted and normalized to intimal area, the increase in PDGF-BB–responsive intimal SMCs within heparin lyase–treated explants was >4-fold when compared with either unstimulated explants or explants exposed to other treatments (Figure 6).

Discussion

At early times during the process of neointimal hyperplasia after experimental injury, medial SMCs proliferate and migrate in response to growth factors, such as fibroblast growth factor (FGF)-2 and PDGF, that are released or activated at the site of injury.36–38 The induction of medial SMC proliferation and migration is a prerequisite for the formation of the neointima. During this early response of the vascular wall, activated SMCs upregulate the synthesis of cellular and extracellular (ECM) proteins that encourage cellular migration, such as hyaluronan and versican.39,40 In the rat carotid artery model of neointimal formation after balloon catheter injury, cell proliferation is maximal 48 hours after injury. As cells migrate to form and populate the neointima, however, the ECM comprises a progressively larger proportion of the neointimal volume. The attendant decrease in both endogenous and induced cell proliferation among intimal cells in the neointimal lesion after 4 weeks (in the rat carotid model)9 is not correlated with a decrease in receptors for cytokines such as PDGF and FGF-2.34 This observation has led to the hypothesis that macromolecules deposited within the ECM modify the responsiveness of neointimal SMCs to cytokines.

Perlecan is a large ECM HSPG that is synthesized by vascular cells.13,19,20,35,41–43 Increased perlecan expression is associated with noncycling SMCs in the developing vasculature,19,20 and direct inhibition of SMC proliferation in vitro by perlecan has been demonstrated.16,35 Several
inhibitors of SMC proliferation, such as heparin,\textsuperscript{14} apolipoprotein E,\textsuperscript{35} and transforming growth factor-β1,\textsuperscript{44} increase perlecan expression. Perlecan inhibits SMC proliferation in vitro, and exogenous perlecan may inhibit intimal hyperplasia.\textsuperscript{23,25} However, the pattern of perlecan expression and distribution in the later stages of lesion formation after vascular injury has not been previously described, nor has endogenous HSPG been shown to act as an inhibitor of cell proliferation in the neointimal lesion. Our results indicate that perlecan accumulation is correlated with the attenuation of neointimal SMC proliferation after vascular injury. Specifically, expression and deposition of perlecan are low as SMCs proliferate and migrate in response to growth factors at early times after vascular injury. In contrast, large amounts of perlecan remain deposited during later stages of lesion development, when SMC proliferation decreases and cellular responsiveness to infused growth factors is attenuated.\textsuperscript{45} Our observation of perlecan upregulation within 2 weeks after injury agrees with a previous report of perlecan mRNA expression in the rat carotid injury model\textsuperscript{13} and is consistent with the relative HS distribution during neointima formation in a rabbit model of acute arterial injury.\textsuperscript{46} In contrast, other cell-associated HSPGs, such as syndecans, are upregulated shortly after arterial injury, during the SMC proliferative and migratory phase in this model.\textsuperscript{13,47} Somewhat surprisingly, our results also indicate that perlecan mRNA levels are relatively decreased in the neointima 3 to 6 weeks after injury. Despite the decreased perlecan mRNA expression at these later times, perlecan core protein remains abundant, suggesting that turnover of perlecan is slow in the advanced lesion. Our results also indicate that removal of HS, but not chondroitin sulfate, from the ECM of dissected, advanced, carotid intimal lesions restores the proliferative response of intimal SMCs to PDGF in organ cultures. This observation supports the hypothesis that perlecan negatively regulates SMC proliferation in situ, as suggested by other reports.\textsuperscript{16,19,20,35}

The effect of perlecan on proliferation is not confined to SMCs, because the expression of perlecan antisense in fibrosarcoma cells increased both the growth rate and metastatic potential of these cells.\textsuperscript{48} However, other work suggests that some cell types may require perlecan for proliferative and migratory responses. For example, the level of expression of perlecan by metastatic melanoma cells is correlated with the invasiveness of the tumor.\textsuperscript{49} Antisense-mediated downregulation of perlecan in melanoma cells causes decreased proliferation and invasion of these cells.\textsuperscript{50} Interestingly, antisense targeting of perlecan in tumor cells also inhibited tumor growth and neovascularization of tumors in vivo. This duality of response is not unexpected, because the HS chains of perlecan bind heparin-binding growth factors and may serve either as a ECM repository or as a required cofactor in high-affinity receptor binding and signaling by these factors.\textsuperscript{51-53} Indeed, high levels of expression of HSPGs, which are required for cellular replicative response to FGF-2, can also suppress cell proliferation. For example, overexpression of cell-surface HSPG inhibits the response of cells to

Figure 5. Immunoperoxidase staining for BrdU to localize proliferating cells in carotid sections explanted 35 days after injury and cultured in vitro. Rat carotid arteries were harvested 35 days after balloon injury. Explants containing neointima were treated with buffer alone (A), heparin lyases I and II (B, C), or chondroitin ABC lyase (D, E) for 30 minutes before organ culture in medium with 0.1% serum alone (B, D) or including 10 ng/mL PDGF-BB (A, C, E). After 48 hours, sections of the explants were prepared, and nuclei of proliferating cells were localized by immunohistochemical staining for incorporated BrdU. Note that dramatically increased BrdU incorporation into intimal SMC nuclei occurred only after PDGF-BB stimulation of heparin lyase-treated explants.
this growth factor, as does the addition of endothelial cell HSPGs. The involvement of perlecan in FGF-2 signaling is supported by the observations that perlecan promotes the binding of FGF-2 to a high-affinity receptor and that antisense-mediated perlecan downregulation inhibits FGF-2 signaling and mitogenesis. The correlation of enhanced perlecan deposition both in heparin-treated injured carotid arteries and in the neointima 3 to 4 weeks after injury (Nikkari et al.) and vide infra), with attenuation of FGF-2–mediated SMC replication, may suggest a role for perlecan in the downregulation of the growth factor proliferative response that occurs in the untreated neointima after injury in this experimental model. In our experiments, the proliferative response of intimal SMCs in explants to PDGF was restored after heparin lyase treatment. FGF-2 is also induced by PDGF treatment of SMCs, raising the possibility that FGF-2 induction or release from ECM sites is responsible for the proliferative competence of the SMCs in the lesion. Perlecan may also modulate the signaling of other heparin-binding growth factors, such as heparin-binding epidermal growth factor, which is clearly involved in the heparin blockade of thrombin-induced SMC migration. In our studies, we do not favor the hypothesis that HS chains of perlecan inhibit interaction of the growth factor with cell surface receptors, because SMCs in heparin lyase–treated explants do not proliferate after FGF-2 treatment, probably because the HS chains of obligate cell-surface low-affinity receptors for FGF-2 have also been degraded. As an alternative mechanism for perlecan activity, interactions of perlecan with HS-binding domains of ECM proteins such as fibronectin and laminin may modulate cell-matrix interactions that are important to the control of cell proliferation and migration. Perlecan inhibits mesangial and vascular SM cell adhesion to fibronectin. Therefore, it is probable that under these conditions, fibronectin–dependent integrin signaling, which facilitates growth factor–stimulated cell proliferation, is also inhibited. The core protein of perlecan also appears to interact with the cell surface by both integrin-dependent and -independent mechanisms. This direct interaction raises the possibility that perlecan receptors at the cell surface may signal inhibitory pathways that block downstream from growth factor receptor signals that upregulate proliferative pathways. Indeed, interaction of SMCs with perlecan has also been found to suppress expression of Oct-1, which is a transcription factor induced during the induction of cell proliferation. Suppression of cell proliferation by perlecan during the formation of the intimal lesion can clearly serve to limit the extent of intimal expansion and stenosis of the vessel.

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