Heparanase Alters Arterial Structure, Mechanics, and Repair Following Endovascular Stenting in Mice


Abstract—Heparan sulfate proteoglycans (HSPGs) are potent regulators of vascular remodeling and repair. Heparanase is the major enzyme capable of degrading heparan sulfate in mammalian cells. Here we examined the role of heparanase in controlling arterial structure, mechanics, and remodeling. In vitro studies supported that heparanase expression in endothelial cells serves as a negative regulator of endothelial inhibition of vascular smooth muscle cell (vSMC) proliferation. Arterial structure and remodeling to injury were also modified by heparanase expression. Transgenic mice overexpressing heparanase had increased arterial thickness, cellular density, and mechanical compliance. Endovascular stenting studies in Zucker rats demonstrated increased heparanase expression in the neointima of obese, hyperlipidemic rats in comparison to lean rats. The extent of heparanase expression within the neointimal thickness following injury. To test the effects of heparanase overexpression on arterial repair, we developed a novel murine model of stent injury using small diameter self-expanding stents. Using this model, we found that increased neointimal formation and macrophage recruitment occurs in transgenic mice overexpressing heparanase. Taken together, these results support a role for heparanase in the regulation of arterial structure, mechanics, and repair. (Circ Res. 2009;104:380-387.)

Key Words: heparanase ■ vascular remodeling ■ restenosis ■ stenting ■ arterial compliance

Vascular smooth muscle cell (vSMC) proliferation and hypertrophy are common pathophysiological mechanisms underlying clinical cardiovascular disorders including hypertension, atherosclerosis, and restenosis. Arterial structure is maintained by a dynamic interplay between growth inhibitory factors produced primarily by endothelial cells and growth stimulatory factors produced principally by vSMCs, inflammatory cells, and dysfunctional endothelial cells. Disease states can compromise endothelial function and disturb this balance, leading to local inflammation, thrombosis, and vasoconstriction. Heparan sulfate proteoglycans (HSPGs) derived from endothelial cells are potent regulators of vSMC growth and vascular remodeling. Both heparin and endothelial cell-derived HSPGs are potent inhibitors of vSMC proliferation and mitogenesis. This regulation is dependent on the overall health and growth state of the endothelial cells. Subconfluent cultures of endothelial cells stimulate vSMC growth whereas postconfluent cultures inhibit vSMC growth. Furthermore, these endothelial-derived HSPGs are essential to inhibiting and resolving the neointimal response to vascular injury.

Although there is evidence that HSPGs participate in the control of vascular remodeling, it remains unclear how these molecules are regulated in context of disease and injury. Heparanase is the major mammalian enzyme capable of digesting heparan sulfate chains. This enzyme is an endo-β-D-glucuronidase that cleaves at a specific motif in the heparan sulfate chains to create fragments 10 to 20 sugar units long and biologically active. Heparanase has been intensely studied for its role in angiogenesis and cancer metastasis, yet the role of this enzyme in regulating arterial structure and remodeling remains poorly defined. We hypothesized that heparanase plays a key role in regulating arterial structure, mechanics, and vascular remodeling. In the present work, we show that heparanase expression in endothelial cells serves as a negative feedback regulator of paracrine inhibition of vSMC proliferation. Mice overexpressing the human heparanase transgene had increased arterial thickening, cellularity, and arterial compliance. We found that stent-induced vascular injury in obese, hyperlipidemic rats had increased neointimal heparanase expression and that the magnitude of this increase directly related to the extent of neointimal expansion. Finally, we developed a novel minimally invasive murine arterial stenting model that demonstrated increased neointimal formation in response to endovascular stenting in transgenic heparanase mice.
Materials and Methods

Cell Culture
Human aortic vSMCs and human umbilical cord vascular endothelial cell (HUVEC) lines were purchased from Cambrex (Walkersville, Md). Cells were maintained in MCDB-131 media (Invitrogen, Carlsbad, Calif) supplemented with EGM-2 growth supplements (Cambrex). Cells were cultured at 37°C under 5% CO₂ and were used between passages 3 and 5.

Transfection and Small Interfering RNA Vectors
Four short hairpin RNA expression vectors were screened for gene silencing activity toward the heparanase gene. The following sequences were used in the pRS expression vector (Origene, Rockville, Md): (1) TTATGTGGCGTGATGAATTTGGGCCTGTC; (2) GTGTTGATGCGGCAATGCTATCTTTGGACG; (3) TCGTTCCT-GCCTGTCACCATGTAGCCGCAA; (4) GTCACAGAACAGCAGCACTACTGCAAGAAGC. Overexpression of heparanase was performed using a vector with constitutive gene expression under control of the CMV promoter (Origene).

Cell Lysis and Western Blotting
Cells were lysed in 1 mL of lysis buffer containing 20 mmol/L Tris, 150 mmol/mL NaCl, 1% Triton X-100, 0.1% deoxycholate, 0.1% sodium dodecyl sulfate, 1 mmol/L sodium orthovanadate, 50 mmol/L NaF, 2 mmol/L phenylmethylsulfonyl fluoride, and complete protease inhibitor cocktail (Roche, Nutley, NJ). After 10 minutes of incubation, the plates were scraped, and the lysates were pipetted into a centrifuge tube. The samples were then centrifuged at 14 000g for 15 minutes before western blotting. Samples were run on 4% to 15% polyacrylamide gradient gels and transferred to polyvinylidene difluoride membranes. The membranes were blocked for 1 hour in 5% nonfat milk in PBS with 0.01% Tween-20 (PBST) and probed with the primary antibody (1:100, Cell Sciences, Canton, Mass) at 4°C overnight in 1% nonfat milk. The membranes were washed with PBST, incubated at room temperature for 2 hours with a 1:3500 dilution of a horseradish peroxidase–linked secondary antibody and detected using chemiluminescence (Perkin Elmer, Boston, Mass).

Immunocytochemical Staining
Cells were washed 3 times in PBS and fixed for 5 minutes with methanol at −20°C. The cultures were then blocked with 20% goat serum in PBS for 45 minutes at room temperature. A primary antibody heparanase (Cell Sciences) was added at 1:100 dilution in PBS containing 1% BSA. A secondary antibody conjugated with Alexa Fluor 594 or Oregon Green dyes (Invitrogen) was added at 1:200 dilution in PBS containing 1% BSA. The cell surfaces were blocked with 20% normal goat serum for 45 minutes. The sections were then washed twice in PBS-BSA solution. A secondary antibody conjugated with Alexa Fluor 594 or Oregon Green dyes (Invitrogen) was added at 1:200 dilution in PBS-BSA solution. The samples were allowed to cool for 20 minutes and were then incubated in 3% hydrogen peroxide for 10 minutes. The samples were rinsed 3 times with PBST between each of the following steps. The sections were blocked with 20% normal goat serum for 3 hours at room temperature. The primary antibodies were diluted in PBS containing 1% BSA, applied to slides, and incubated in a humid chamber overnight at 4°C. Secondary antibody staining at detection was performed using the LSAB 2 kit (DakoCytomation, Carpinteria, Calif) according to the instructions of the manufacturer. An AEC substrate (DakoCytomation) was used for detection of the horseradish peroxidase conjugate. The samples were counterstained in Mayer’s hematoxylin for 3 minutes, washed with tap water, and mounted in aqueous mounting medium (DakoCytomation).

Histochemical Analysis
Aortae were harvested from wild-type and heparanase transgenic mice. The aortae were fixed in neutral buffered formalin at 4°C overnight, paraffin-embedded, and sectioned using standard methods. The aortae were stained with hematoxylin/eosin or with an elastin Movat stain as previously described.15 Arterial thickness, circumference, and nuclei were counted on 3 aortic sections from 4 mice for each group of animals using Adobe Photoshop (Adobe, San Jose, Calif).

Smooth Muscle Cell Proliferation Assay
Human smooth muscle cells were passaged into 48-well plates at low density. Smooth muscle cells were serum-starved in 0.5% calf serum for 24 hours. The cells were then washed twice in culture medium with no growth supplements, and endothelial cell conditioned medium was applied. After 24 hours, 1 μCi of [3H]-thymidine was added. Twenty-four hours later, the cells were washed 3 times with PBS at 4°C and then incubated with 10% trichloracetic acid for 30 minutes. The cultures were then washed twice in 95% ethanol and solubilized in 1 mL of 0.25 mol/L NaOH with 0.1% sodium dodecyl sulfate for 1 hour. The samples were added to scintillation cocktail, and radioactivity was measured using a liquid scintillation counter.

Animal Models of Endovascular Stent Injury
All experimental procedures and protocols used in this investigation were reviewed and approved by the Animal Care and Use Committee of the Massachusetts Institute of Technology and conformed to the Guiding Principles in the Care and Use of Animals of the American Physiological Society and the NIH Guide for the Care and Use of Laboratory Animals. Zucker obese (Crl:ZUC-fbBR) and Zucker lean rats were used in an animal model of vascular injury and stenting in the presence of metabolic syndrome and insulin resistance.16 At the time of stenting, the rats were 12 and 14 weeks old for the obese and lean rats, respectively. The rats were anesthetized using isoflurane and given 100 U/kg dose of heparin, and a small incision was made to expose the right femoral artery. This artery was ligated, and an arteriotomy was performed proximal to the ligature. A 0.014-inch angioplasty guide wire was passed into the aorta, and a 9-mm-long endovascular stent (Nirflex; Medinol Inc, Tel Aviv, Israel) that was mounted on a 15×2.5-mm angioplasty balloon (Crossail; Guidant Inc, Santa Clara, Calif) was passed into the abdominal aorta. The stent was deployed with a 15-second inflation at 8-atm inflation pressure. Poststenting the animals were given aspirin via drinking water at an approximate dose of 5 mg/kg per day.16 After 14 days, the stents were harvested for analysis.
Wild-type and heparanase transgenic mice were derived directly from those previously described. The animals were given aspirin via drinking water for 24 hours before stent implantation and thereafter. Immediately before stenting, the mice were given a subcutaneous dose of heparin. Vascular access was obtained through the right femoral artery in a manner similar to that used for the rat studies. A coronary stent system with a 1.5-mm constrained diameter and 12-mm length was used (CardioMind, Sunnyvale, Calif). The catheter was passed into the abdominal aorta through the femoral artery. The stent was deployed by removing an external sheath, allowing the self expanding stent to deploy. The average diameter of the aortic lumen in the mice was 1.3 mm, and the deployed stent diameter was 1.5 mm, leading to 15.4% dilation on stent deployment. After seven days the stents were explanted and analyzed by histo-
graphy.

The average blood pressure for each group was 113.6±4.2 and 106.8±3.6 mm Hg for wild-type and heparanase transgenic mice, respectively (n=4, P=NS).

Mechanical Testing of Aortae

Before mechanical testing aortae from heparanase, transgenic and wild-type mice were harvested and adventitial tissue removed. The longitudinal compliance and ultimate tensile strength of whole arteries was measured using an ElectroForce Biodynamic Test Instrument (Bose Corporation, Framingham, Mass). The samples were measured in uniaxial tensile configuration using a 22 N load cell (Interface Inc, Scottsdale, Ariz) with a displacement rate of 0.05 mm/sec and maximal displacement of 4.0 mm. WinTest 3.0 software (Bose) was used to record data during the testing. Circumferential testing was performed on aortic ring samples cannulated with 2 sutures and tested using the Biodynamic Test Instrument. To examine mechanical properties of the elastin networks, we digested aortae in 0.1 N NaOH at 75°C for 1 hour. The solution was neutralized and removed with multiple washes with PBS. This procedure has been shown to remove cellular components and extracellular matrix but leave the elastin network intact.

Statistics

All results are shown as means±SEM. An ANOVA followed by Student–Newman–Keuls post hoc test. A 2-tailed probability value of <0.05 was considered statistically significant was used to make comparisons between groups of continuous variables. The Pearson product moment correlation statistic was used as a measure of correlation between variables.

Results

Heparanase Provides Negative Feedback Control to Paracrine Growth Inhibition of vSMCs by Endothelial Cells

Endothelial cells were transfected with an expression vector for heparanase (pHPA), 1 of 4 small interfering (si)RNA vectors targeted to the heparanase (siHPA-1 to -4), a siRNA control vector (pRS), or an empty overexpression vector (pcDNA). After 2 days in culture, the cells were lysed and assayed for heparanase by immunoblotting to confirm alterations in heparanase expression (Figure 1a). The most effective siRNA sequence (siHPA-3) was selected and used in further studies. This siRNA reduced heparanase expression to 13.2±7.2% from 100.0±18.7% for control cells (n=3, P<0.05). Transfected cells under similar conditions were fixed, immunostained for heparanase, and imaged using fluorescent microscopy (Figure 1b and 1c). The effect of heparanase on the glycosaminoglycans of endothelial cells was measured through metabolic labeling and analysis by ion exchange chromatography. In these graphs, the early peaks represent predominantly protein, whereas later peaks are from highly charged glycosaminoglycans (ie, fractions 9 through 20). These studies demonstrated that enhanced endothelial expression of heparanase led to reduction in both cell surface and soluble heparan sulfate glycosaminoglycans (Figure 1d). For the surface HSPGs, the relative area of the glycosaminoglycan peak was 100.0±6.4%, 36.2±3.3%, and 154.9±4.9% for pRS, pHPA, and siHPA-transfected cells, respectively (P<0.05 for comparisons between all groups). Conversely, reduction of heparanase expression increased heparan sulfate in cell surface and conditioned media fractions. Notably, there was also a large reduction in cell surface protein with heparanase overexpression, likely attributable to heparanase-induced shedding of syndecans.

Endothelial cells produce soluble factors that inhibit vSMC proliferation. This process is essential for arterial health and requires endothelial cell derived HSPGs. We took conditioned media from endothelial cells and applied it to vSMCs in culture to examine the role of heparanase in controlling endothelial inhibition of vSMC proliferation. Endothelial cells transfected with a control vector inhibited vSMC proliferation to about half of the baseline vSMC proliferation in growth media (Figure 1e). This effect was absent in cells overexpressing heparanase and markedly enhanced in cells with reduced heparanase levels (Figure 1e). Previous studies have implicated the release of soluble factors, in particular fibroblast growth factor (FGF)-2, as an effector mechanism of heparanase tissue action. However, in our experiments, FGF-2 levels remained relatively constant irrespective of heparanase expression (data not shown).

Heparanase Overexpression Leads to Arterial Thickening, Increased Arterial Compliance, and Incidence of Spontaneous Aneurysm

Transgenic mice overexpressing heparanase were used to examine the effects of heparanase excess on arterial structure and mechanics. The aortae of mice with the heparanase transgene had increased medial thickness in comparison to wild-type controls (Figure 2a and 2b). This thickening appeared to be attributable to enhanced cellular density rather than hypertrophy in the heparanase transgenic animals when compared to their wild-type counterparts (Figure 2c). Aortae were harvested from both groups and mechanical properties of the aorta were measured using a mechanical testing device with environmental control. Heparanase overexpression led to a profound alteration in the force–displacement relationship of the aorta. This led to a nearly 3-fold reduction in longitudinal arterial stiffness (Figure 3a) and ultimate tensile strength (Figure 3b). We also measured circumferential stiffness and ultimate strength and found a similar reduction in arterial mechanical properties for the heparanase transgenic mice (Figure 3c and 3d). To test whether heparanase expression affected the integrity of the elastin network, arteries were digested with NaOH at elevated temperature. This treatment has been shown to destroy most cellular and extracellular matrix components while leaving the elastin network intact. We mechanical tested the remaining elastin...
networks and found that the heparanase transgenic mice had elastin with dramatically reduced mechanical integrity (Figure 3e and 3f). Consistent with the reduced stiffness and reduction in ultimate strength, localized spontaneous aneurysms were found in the transgenic heparanase mice but not in the wild-type (Figure 3g).

Heparanase Expression Is Increased in Stent Injury on Obese, Hyperlipidemic Zucker Rats and Is Associated With Increased Intimal Thickness

In the clinical setting, diabetic patients are at increased risk of atherosclerosis and restenosis with both bare metal and drug-eluting stents.23–25 We examined the expression of heparanase in stent induced restenosis by implanting stents in the aortae of normal and obese Zucker rats using minimally invasive femoral access surgery.16 The obese Zucker rat is a model of type 2 diabetes with obesity, insulin resistance, hyperinsulinemia, hypertriglyceridemia, and hypercholesterolemia. Following stent injury and restenosis, we found that obese Zucker rats had increased neointimal thickness and expression of heparanase (Figure 4a through 4c). The expression of heparanase within the neointima correlated strongly with the final neointimal area, giving a correlation of \( R^2 = 0.50 \) (\( P<0.001 \)) for lean rats and \( R^2 = 0.77 \) (\( P<0.001 \)) for fatty rats (Figure 4d).

Heparanase Overexpression Enhances Neointimal Proliferation and Macrophage Recruitment in Response to Endovascular Stent Implantation

To examine arterial repair in the presence of heparanase overexpression, we developed a minimally invasive method of stent placement in mice. Using a novel small diameter...
stenting system (CardioMind, Sunnyvale, Calif), we performed minimally invasive endovascular stenting of transgenic mice overexpressing heparanase. This method takes advantage of self-expanding stents that have a diameter identical to that of the catheter wire before deployment. Nitinol self-expanding stents were deployed in the abdominal aorta of wild-type and heparanase transgenic mice via femoral access (Figure 5a). In the heparanase transgenic mouse, the neointimal lesion was rich in macrophages, staining strongly for the Mac-1 surface marker (Figure 5b). Heparanase transgenic mice had markedly increased neointimal formation with increased neointimal area (Figure 5c) and intima to media ratio (Figure 5d), with no significant change in medial area (Figure 5e). We examined release of monocyte chemoattractant protein (MCP)-1 1 hour after vascular injury and found increased amounts of MCP-1 in the heparanase transgenic mice (Figure 5f).

Discussion

Despite the discovery of heparanase-like activity in endothelial cells nearly 2 decades ago, the functional role of this expression in arterial biology has been explored by only a limited number of studies. Previous studies have shown that heparanase is expressed in endothelial cells and can be regulated by high glucose and oxidized lipoproteins. However, the functional significance of these findings to arterial biology was unknown. The present study adds to these findings by defining a functional role for heparanase in controlling paracrine inhibition of vSMCs by endothelial cells. Endothelial inhibition of vSMCs is an essential process for maintaining arterial health and its loss is a precursor to atherosclerosis, restenosis and arterial thickening. We and others have shown that HSPGs, specifically perlecan, are essential for endothelial inhibition of vSMC proliferation. In this study, we demonstrated that overexpression of heparanase can abolish this effect and, further, that knock down of heparanase expression enhances endothelial cell inhibition of vSMC growth. We found that heparanase expression within endothelial cells led to degradation of surface and soluble

Figure 2. Transgenic mice overexpressing heparanase demonstrate altered arterial structure and cellular density. Thoracic aortae were harvested from transgenic and wild-type animals (n = 8 for each group) and formalin-fixed, paraffin-embedded, and sectioned. Aortic sections were taken from 4 animals of each group at 3 different locations on the aorta. a, Histological analysis of heparanase transgenic (HPA Tg) and wild-type (WT) mice stained with hematoxylin/eosin and elastic Movat pentachrome stain (EMP). b, Aortic thickness is increased in heparanase transgenic mice. *Statistically significant difference between samples (n = 4, P < 0.05). Scale bar = 50 μm.

Figure 3. Overexpression of heparanase alters the mechanical compliance and ultimate strength of the mouse aorta. The thoracic aortae were harvested from transgenic and wild-type animals and mechanical properties measured using a Bose BioDynamic test instrument. a, Aortic stiffness expressed as the slope of the force-displacement curve was reduced in HPA Tg animals. b, Average aortic ultimate strength was reduced in mice expressing the heparanase transgene. c, Circumferential aortic stiffness of wild-type and HPA Tg animals. d, Circumferential ultimate force for aortae isolated from wild-type and HPA Tg animals. e, Circumferential stiffness of the elastin network remaining after digestion with NaOH solution. f, Circumferential ultimate strength of the elastin network remaining after digestion with NaOH solution. g, Example of a local, spontaneous aneurysm found in the aorta of a transgenic heparanase mouse compared to a wild-type control. The sample was stained with elastic Movat's pentachrome stain. Scale bar = 50 μm.
heparan sulfate chains. The heparan sulfate motif recognized by heparanase may be critical to inhibition of vSMCs. Thus, even with small changes in total HSPGs, the inhibitory properties may be dramatically reduced. This finding implies that inhibitors of heparanase may be potential therapeutics for diseases that compromise endothelial dysfunction and induce aberrant vascular remodeling.

One known mechanism of heparanase activity is the cleavage of extracellular matrix HSPGs, leading to a release of matrix bound growth factors. Heparanase isolated from platelets has been shown to release FGF-2 and increase cell proliferation. Here we examined the effects of endothelial cell expression of heparanase in contrast to direct application of the active enzyme. This allows for the endogenous control mechanism for regulating heparanase activity in endothelial cells to remain intact while increasing gene expression. The levels of FGF-2 in the conditioned media of samples under the various treatments were constant, implying a differential mechanism in relation to direct application of active heparanase protein in comparison to increased gene expression.

Diabetes, infection, and inflammation can lead to arterial states with heightened heparanase expression. Heparanase isolated from platelets has been shown to release FGF-2 and increase cell proliferation. Here we examined the effects of endothelial cell expression of heparanase in contrast to direct application of the active enzyme. This allows for the endogenous control mechanism for regulating heparanase activity in endothelial cells to remain intact while increasing gene expression. The levels of FGF-2 in the conditioned media of samples under the various treatments were constant, implying a differential mechanism in relation to direct application of active heparanase protein in comparison to increased gene expression.

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neointimal formation. Heparin and PI-88 (a synthetic heparin analog) have been used in animal models to reduce neointimal formation. Both of these compounds can inhibit heparanase activity but also have other activities including growth factor binding and direct activity on vSMCs. A neutralizing antibody to heparanase has also been used to reduce neointima formation following carotid balloon injury. In our studies, we used a novel murine stent injury model that provided deep vascular injury, permanent stretch, and indwelling device, providing injury similar to clinical interventions. Our results showed increased macrophage infiltration in heparanase transgenic mice in concert with enhanced intimal formation. Consistent with these findings, we also found that the concentration of MCP-1 was increased in the transgenic heparanase mice following acute vascular injury.

The cellular production of MCP-1 is decreased by heparin/ heparan sulfate, and this may be a potential mechanism for heparanase causing an increase in MCP-1 following vascular injury. The murine model of endovascular stenting represents a significant advancement in the animal models of vascular injury, allowing for clinically relevant stent-induced injury in the powerful genetic models available in mice. Stent injury provides deeper vascular injury, thus generating a distinct inflammatory response and endothelial dysfunction not present in balloon or wire injury models. Taken together, our results demonstrate that heparanase is a potent regulator of vascular remodeling, both on the level of paracrine regulation of vascular homeostasis and as an effector molecule in vascular response to injury. Our study suggests that heparanase can act through multiple mecha-
nisms to alter vascular remodeling and response to injury. Heparanase can serve as a control point allowing endothelial cells to modulate between inhibition and stimulation of vascular smooth muscle cells. Furthermore, overexpression of heparanase can alter elastin fiber integrity, as well as increase response to vascular injury through enhancing macrophage recruitment. A common unifying feature of these processes is the involvement of heparan sulfate and, thus, vulnerability to disruption by heparanase. Consequently, aberrant heparanase may serve as a common pathophysiological mechanism governing vascular remodeling under different pathological disease states. Although effective and specific small molecule inhibitors of heparanase have long been sought after for the treatment of cancer, our study indicates that these molecules would also be useful in disease states leading to pathophysiologic arterial remodeling.

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Disclosures
None.

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