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Heparan Sulfate in Perlecan Promotes Mouse Atherosclerosis: Roles in Lipid Permeability, Lipid Retention, and Smooth Muscle Cell Proliferation

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Abstract

Heparan sulfate (HS) has been proposed to be anti-atherogenic through inhibition of lipoprotein retention, inflammation, and smooth muscle cell proliferation. Perlecan is the predominant HS proteoglycan in the artery wall. Here, we investigated the role of perlecan HS chains using apoE null (ApoE0) mice that were cross-bred with mice expressing HS-deficient perlecan (*Hspg2*^{Δ3/Δ3}). Morphometry of cross-sections from aortic roots and *en face* preparations of whole aortas revealed a significant decrease in lesion formation in ApoE0/*Hspg2*^{Δ3/Δ3} mice at both 15 and 33 weeks. *In vitro*, binding of labeled mouse triglyceride-rich lipoproteins and human LDL to total extracellular matrix, as well as to purified proteoglycans, prepared from ApoE0/*Hspg2*^{Δ3/Δ3} smooth muscle cells was reduced. *In vivo*, at 20 min influx of human ¹²⁵I-LDL or mouse triglyceride-rich lipoproteins into the aortic wall was increased in ApoE0/*Hspg2*^{Δ3/Δ3} mice compared to ApoE0 mice. However, at 72 hours accumulation of ¹²⁵I-LDL was similar in ApoE0/*Hspg2*^{Δ3/Δ3} and ApoE0 mice. Immunohistochemistry of lesions from ApoE0/*Hspg2*^{Δ3/Δ3} mice showed decreased staining for apoB and increased smooth muscle α-actin content, whereas accumulation of CD68-positive inflammatory cells was unchanged. We conclude that the perlecan HS chains are pro-atherogenic in mice, possibly through increased lipoprotein retention, altered vascular permeability, or other mechanisms. The ability of HS to inhibit smooth muscle cell growth may also influence development as well as instability of lesions.

Keywords

Atherosclerosis; Lipoproteins; Perlecan; Heparan sulfate; Smooth muscle cells

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Disclosures

None.

Introduction

Proteoglycans in the extracellular matrix (ECM) influence both cellular and molecular processes in atherogenesis. Chondroitin and dermatan sulfate (CS/DS) proteoglycans, like versican and biglycan, are considered pro-atherogenic because of their ability to retain low-density lipoproteins (LDL).¹ By contrast, heparan sulfate (HS) proteoglycans have been proposed to be anti-atherogenic,^{2, 3} because decreased HS is associated with increased atherosclerosis in different species.^{4–8} Perlecan is the predominant HS proteoglycan in the arterial ECM, and it is found in basement membranes underneath the endothelial layer and around medial SMCs.^{9, 10} Perlecan consists of a core protein with three attachment sites for HS glycosaminoglycan (GAG) side chains in domain I^{11–13} and an additional site in domain V.^{14, 15} We have recently demonstrated that perlecan is downregulated in human carotid atherosclerosis.⁸ On the other hand perlecan has, together with biglycan, been shown to be the predominant proteoglycan in atherosclerotic lesions in mice.¹⁶

Anti-atherogenic properties of HS may involve several mechanisms. Enzymatic removal of HS has been shown to increase binding of LDL to endothelial matrix *in vitro*,¹⁷ indicating that HS may interfere with lipoprotein retention. HS also reduces endothelial permeability for LDL^{18, 19} and removal of HS has been shown to increase monocyte binding.^{18, 20} In addition, heparin and HS are potent inhibitors of smooth muscle cell (SMC) proliferation, which may influence plaque stability and size.^{21–26} However, HS proteoglycans have also been shown to bind LDL *in vitro*, indicating a possible pro-atherogenic role.²⁷

A recent study shows that partially reduced perlecan expression, achieved by cross-breeding perlecan null heterozygotes with either apoE null (ApoE0) or LDL receptor null mice, is associated with reduced atherosclerosis in young males.²⁸ However, that study does not separate the roles of the perlecan core protein and its HS chains. In a previous study, we investigated the role of the perlecan HS chains in vascular disease using mice expressing HS-deficient perlecan, generated by targeted deletion of exon 3 in domain I of the perlecan gene (*Hspg2*^{Δ3/Δ3}).²⁹ The mutation leads to a significant depletion of the arterial HS content and promotes increased SMC proliferation and formation of intimal hyperplasia.²⁴

Here, to test directly whether the perlecan HS chains play a role in atherosclerosis, *Hspg2*^{Δ3/Δ3} mice were cross-bred with ApoE0 mice. We show that depletion of endogenous perlecan HS is associated with significantly reduced atherosclerosis at early and late time points in both females and males. We investigate the underlying mechanism, and the results indicate how perlecan HS may be both pro- and anti-atherogenic. The role of perlecan in atherosclerosis may however be species specific.

Materials and methods

Animals

ApoE0 mice (M&B Breeding and Research Center A/S, Ry, Denmark) were crossed with mice lacking exon 3 of the perlecan gene (*Hspg2*^{Δ3/Δ3}),²⁹ which had been backcrossed for ten generations to C57BL/6. ApoE0/*Hspg2*^{Δ3/Δ3} mice were fed a regular chow diet, developed normally, and were fertile. Animal care and experimental procedures were performed according to the regulations of the local animal care committee.

Lipid measurements

Blood for lipid analyses was collected, and sera were stored at –20°C. Total cholesterol and triglycerides were determined by standard enzymatic methods (Roche Molecular Biosystems, Indianapolis, IN). Size fractionation of lipoproteins was performed by fast performance

liquidchromatography gel filtration (ÄKTA Explorer; GE Healthcare BioSciences AB, Uppsala, Sweden) with a Superose 6 HR 10/30 column.³⁰

Quantitative and qualitative analysis of atherosclerosis

The mice were euthanized at 15 and 33 weeks of age. The heart and ascending aorta were frozen following cryo-protective treatment with sucrose. The aortic roots were sectioned according to a standardized protocol.³¹ Sections were collected starting 100 µm from the appearance of the aortic valve. Eight 10 µm sections at 100 µm intervals were fixed with formaldehyde and stained with Oil Red-O and hematoxylin. Lesion size was measured by a blinded observer using Easy Image Measurement 2000 (Rainfall Image Analysis, Vange, Sweden). Mean lesion area per section was calculated for each mouse. Sections from the aortic root were also stained with hematoxylin-eosin, Movat's pentachrome, and picro-sirius red for further qualitative assessment of the lesions.

Aortas were also prepared en face as previously described.³² In brief, the aortas were pinned on plates and stained with Sudan IV (Merck AG, Darmstadt, Germany). Lesions were measured and expressed as percentage of total aortic area.

Real time PCR

Total RNA was isolated from aortas of 35-week-old ApoE0 mice and matched C57BL/6 wild-type controls using the RNeasy kit (Qiagen Inc, Valencia, CA). RNA quality was verified with a BioAnalyzer (Agilent Technologies Deutschland GmbH, Waldbronn, Germany). cDNA was made using Superscript-II (Life Technologies Inc, Rockville, MD) and random primers. Amplification was performed in an ABI 7700 Sequence Detector (Perkin-Elmer Applied Biosystems, Foster City, CA).

Immunohistochemistry

Acetone-fixed frozen sections were used for immunohistochemistry. Antibodies used were: rat anti-mouse CD68 (1:20000; Serotec Ltd, Oxford, UK), rat anti-mouse CD3 (1:200; Southern Biotechnology Associates, Birmingham, AL), alkaline phosphatase conjugated anti- α -smooth muscle actin (1:100; Sigma-Aldrich, St Louis, MO), rabbit anti-mouse versican (1:500; Chemicon International, Temecula, CA), polyclonal rabbit anti-mouse apoB48 (1:10000; Abcam, Cambridge, UK). Rabbit anti-mouse biglycan (1:1000; LF-106) was a gift from Dr Larry Fisher (Bethesda, MD). For perlecan core protein, a rabbit polyclonal antibody was used (1:500; R14).³³

For perlecan staining, sections were pretreated with hyaluronidase and for versican and biglycan with chondroitinase. CD3 epitopes were unmasked using 0.1% saponin. The sections were incubated with primary antibodies followed by incubation with biotinylated secondary antibodies (Vector Laboratories, Burlingame, CA). Staining was visualized using biotin-avidin-peroxidase and diaminobenzidine (Vector). Staining with anti- α smooth muscle actin was visualized with Vector Red substrate kit (Vector) and biglycan with Alexa-555 (Invitrogen, Carlsbad, CA). CD68, apoB and α -smooth muscle actin staining was quantified by a blinded observer using Easy Image 2000 Analysis.

Cell culture

The thoracic aorta was separated in situ from its adventitia and digested for 4–5 h in 0.1% collagenase in medium F-12 with 50 µg/mL L-ascorbic acid, 50 µg/mL streptomycin, 50 IU/mL penicillin, and 0.1% bovine serum albumin (F-12/0.1% BSA). The cell suspension was filtered, centrifuged, washed in F-12/0.1% BSA, and seeded in F-12/20% FCS. Passages 2–5

were used for experiments. Cell culture reagents were from Invitrogen and collagenase type II from Worthington (Freehold, NJ)

Metabolic labeling and proteoglycan extraction

SMCs were grown to subconfluence (10 000 cells/cm²), synchronized for 24 h in F-12/0.5% FCS, labeled for 48 h with 75 μ Ci/mL ³⁵S-sulfate (GE Healthcare) in F-12/0.5% FCS, and medium collected. The cell-layer was rinsed and solubilized in 8 mol/L urea, 50 mmol/L Tris-HCl (pH 7.4), 0.5% Triton X-100, 2 mmol/L EDTA, and 0.25 mol/L NaCl (8 mol/L urea/0.25 mol/L NaCl) for 20 minutes, scraped off the dishes, and protease and phosphatase inhibitors added (5 mmol/L benzamidine-HCl, 1 mmol/L phenylmethylsulfonylfluoride, 100 mmol/L 6-aminocaproic acid). Proteoglycans were concentrated by anion chromatography on DEAE-sephacel columns (Bio-Rad Laboratories, Hercules, CA), washed with 8 mol/L urea/0.25 mol/L NaCl, and eluted with 8 mol/L urea/3 mol/L NaCl.

Enzymatic deglycosylation and SDS-PAGE

Proteoglycans were precipitated twice overnight at -20 °C in 70% ethanol and 1.0% potassium acetate, and 100 μ g chondroitin sulfate was used as carrier (Sigma-Aldrich). Pellets were digested with 0.05 U chondroitinase ABC lyase (MP Biomedicals, Irvine, CA) in 30 μ l of 16 mmol/L Tris-HCl (pH 8.0), 17 mmol/L sodium acetate, 0.1% BSA or 0.8 U each of heparinase I and heparinase II (Sigma-Aldrich), with or without 0.05 U chondroitinase, in 30 μ l of 33 mmol/L Tris-HCl (pH 7.0), 3 mmol/L calcium acetate, and 6 mmol/L sodium acetate for 2 h at 37°C and 1 h at 42°C. Equal amounts digested materials were loaded, 67 000 and 37 000 cpm/lane, for medium and cell-layer, respectively. SDS-PAGE was performed on a 4–12% gradient gel with a 3.5% stacking gel. The gel was fixed, treated for 45 min with enhancer, rinsed, dried onto Whatman filter paper, and analysed after exposure to a PhosphorImager screen.

ECM preparation and binding of lipoproteins

12-well-plates were coated overnight with 0.02% type I collagen. SMCs were seeded in F-12/20% FCS (23 000 cells/well for ApoE0/*Hspg2* ^{Δ 3/ Δ 3} and 28 000 for controls to compensate for the difference in growth rate) and grown for six days. Wells without cells were used as background control. Cell-free ECM was prepared by solubilization with 0.5% Triton X-100 in PBS and 25 mmol/L ammonium hydroxide in PBS followed by 4 washes with PBS, as previously described.²⁴ Human LDL (1.02–1.05 g/mL) isolated from healthy subjects and triglyceride-rich lipoproteins (1.006–1.05 g/mL) isolated from plasma of ApoE0 mice on regular chow diet by sequential ultracentrifugation,³⁴ were radiolabeled with ¹²⁵I using Iodogen (Pierce).³⁵ Dilutions of radiolabeled lipoproteins in the physiological Earle's balanced salt solution (EBSS; Gibco) with 1% BSA (EBSS/BSA) were incubated with extracellular matrix preparations for 2 h at 37°C, after which the lipoproteins were removed and the matrix was washed three times with EBSS/BSA.³⁶ The radiolabeled lipoproteins were measured in a 1470 Wallac Wizard (Perkin Elmer, Massachusetts, USA). Curve-fit analysis was performed in GraphPad Prism version 3.00 for Windows using a nonlinear curve fit assuming 1-site binding (GraphPad Software, San Diego, CA).

Proteoglycan purification and LDL binding

SMCs were grown to subconfluence (10 000 cells/cm²). For each cell type, the cell-layer proteoglycans (materials from intact cells and ECM) from two 150 cm² tissue culture flasks were extracted in 8 mol/L Urea, 2 mmol/L EDTA, 20 mmol/L Tris-HCl (pH 7.5), 0.5% CHAPS (Calbiochem, San Diego, CA), supplemented with Complete Mini Protease Inhibitor (Roche, Mannheim, Germany). The extracts were filtered through 0.22 μ m filters (Millipore, Billerica, MA), and concentrated on DEAE-sephacel columns (Hitrap Q, 5mL, Amersham Pharmacia).

The columns were washed with 0.25 mol/L NaCl, and proteoglycans eluted with 1.5 mol/L NaCl. The eluates were dialyzed (Slide-A-Lyzer 3500 MWCO, Pierce) against distilled H₂O at 4°C, and lyophilized.

96-well plates (Maxisorp-immunoplate, Nunc) were coated with cell-layer proteoglycans (10 µg/mL) resuspended in HBS-buffer (20 mM HEPES, 0.15 M NaCl, pH 7.4), 50 µL/well, overnight at room temperature (RT). The plates were washed 3 times with HBS buffer, blocked with 1% BSA in HBS-buffer for 1 h, then washed 3 times with 0.02% Tween-20 in HBS buffer, and incubated with different concentrations (0, 0.625, 1.25, 2.5, 5, 10, 25 µg/mL) of human LDL (1.02–1.05 g/mL) diluted in binding buffer (10 mM HEPES, 20 mM NaCl, 2 mM CaCl₂, 2 mM MgCl₂, pH 7.4) for 1 h at RT. The plates were then washed 2 times with 0.02% Tween-20 in HBS buffer, and incubated with lipoprotein-deficient serum diluted 1:50 in binding buffer 30 min at RT, followed by two washes in 0.02% Tween-20 in HBS and incubation with horseradish peroxidase-conjugated polyclonal antibody against human apoB (Immunkemi, Sweden) 1:750 in 0.02% Tween-20 in HBS-buffer with 0.1% BSA for 1.5 h at RT. Detection was performed after 3 washes with HBS buffer by addition of 75 µL of 1-Step Turbo TMB-ELISA (Pierce) substrate, the reaction stopped with 75 µL 4 mol/L H₂SO₄, and absorbance measured at 450 nm.

Uptake of mouse triglyceride-rich lipoproteins and human LDL in vivo

Radiolabeled mouse triglyceride-rich lipoproteins and human LDL were prepared as described for *in vitro* binding and injected retro-orbitally in 8 to 10-week-old mice. After 20 min (for both types of lipoproteins) or 72 h (for human LDL), the mice were perfusion fixed, the aortas dissected and measured for radioactivity. An identical 20 min experiment was also performed twice with ¹²⁵I-albumin (PerkinElmer, Billerica, MA) instead of lipoproteins.

Statistical Analysis

Comparisons between the experimental groups were performed using a two-tailed Student's *t*-test. *P* < 0.05 was considered significant.

Results

Reduced atherosclerosis in ApoE0/*Hspg2*^{Δ3/Δ3} mice

Aortic sinus atherosclerosis was analyzed at 15 and 33 weeks. Compared with ApoE0 control mice, lesion size was reduced in female ApoE0/*Hspg2*^{Δ3/Δ3} by 53% and 20% at 15 and 33 weeks, respectively (Figures 1A–1C). En face preparations of whole aortas revealed a 59% reduction in lesion area in female ApoE0/*Hspg2*^{Δ3/Δ3} mice compared with controls at 33 weeks (Figures 1D and 1E). Similar reductions in lesion sizes were observed in ApoE0/*Hspg2*^{Δ3/Δ3} males, 53% at 15 weeks in the aortic root, and 59% at 33 weeks by en face (not shown). Total cholesterol and triglyceride levels did not differ significantly between the two genotypes (Online Table I). Size fractionation of lipoproteins was also performed and there was no difference in cholesterol (Figure 2A–D) or triglyceride profiles (not shown).

Proteoglycan distribution in ApoE0/*Hspg2*^{Δ3/Δ3} mice

No differences in staining patterns for perlecan, versican, or biglycan were observed between ApoE0/*Hspg2*^{Δ3/Δ3} mice and ApoE0 controls at 33 weeks (Online Figure I). Prominent staining for perlecan core was detected throughout the intima and underneath the endothelium. Staining was also found in the media but was sparse underneath large lesions with medial thinning (Online Figure I-A and I-B). Versican was virtually absent from the lesions whereas some staining was seen in the media and strong staining was seen in valves (Online Figure I-D and

I-E). Biglycan was detected in intimal lesions as well as in the media and most prominently in aortic valves (Online Figure I-G and I-H).

Perlecan gene expression is unchanged

Because apoE can be involved in the regulation of perlecan expression,³⁷ perlecan mRNA levels in extracts from ApoE0 mouse aortas were compared with C57BL/6 controls using real time PCR. No difference was detected between the two groups at 35 weeks of age, demonstrating that the atherosclerosis model used retained intact perlecan expression in the aorta (not shown).

Depletion of HS in ApoE0/Hspg2 $\Delta 3/\Delta 3$ mice

ApoE0 and ApoE0/Hspg2 $\Delta 3/\Delta 3$ SMCs were labeled *in vitro* with ³⁵S-sulfate, and proteoglycan production was analyzed by concentration over DEAE columns, digestion with chondroitinase or heparinases, and SDS-PAGE. This protocol yields proteoglycans with attached GAG side-chains only, and no non-glycosylated material is recovered. After chondroitinase digestion of samples from ApoE0 controls, large HS proteoglycans were seen as a smear in the stacking gel and an intense band at the top of the resolving gel (Figure 3, lane 3 for both medium and cell-layer). In samples from ApoE0/Hspg2 $\Delta 3/\Delta 3$ mice, almost no large HS proteoglycans were seen (Figure 3, lane 4 for both medium and cell-layer). No obvious differences in other proteoglycans were detected between ApoE0/Hspg2 $\Delta 3/\Delta 3$ and controls (Figure 3).

Reduced lipoprotein binding to ECM and purified cell-layer proteoglycans from ApoE0/Hspg2 $\Delta 3/\Delta 3$ SMCs

Binding of mouse triglyceride-rich lipoproteins to ECM from cultures of aortic SMCs isolated from ApoE0/Hspg2 $\Delta 3/\Delta 3$ mice showed reduced binding capacity compared with controls (Figure 4A). Similar experiments were performed with human LDL, which also showed reduced binding to ECM from ApoE0/Hspg2 $\Delta 3/\Delta 3$ SMCs (data not shown). Consistent with this observation, purified cell-layer proteoglycans from ApoE0/Hspg2 $\Delta 3/\Delta 3$ SMCs exhibited reduced affinity and maximal binding capacity for human LDL, compared with ApoE0 controls (Figure 4B).

Lipoprotein uptake and apoB accumulation in the vessel wall

Subendothelial lipoprotein content *in vivo* in 8 to 10-week-old ApoE0/Hspg2 $\Delta 3/\Delta 3$ and ApoE0 mice was compared after infusion of ¹²⁵I-labeled mouse triglyceride-rich lipoproteins or human LDL (Table 1). Analysis 20 min after injection showed significantly more of both types of lipoproteins in the aortas of ApoE0/Hspg2 $\Delta 3/\Delta 3$ mice compared with ApoE0 mice. However, when human LDL was analysed after 72 h, no significant difference remained between the two groups. ¹²⁵I-Albumin injections showed no difference in subendothelial albumin content between the groups after 20 min (Table 1). Immunohistochemistry demonstrated significantly less apoB in aortic root lesions from ApoE0/Hspg2 $\Delta 3/\Delta 3$ mice compared with ApoE0 controls (Figure 5).

Accumulation of SMCs in lesions from ApoE0/Hspg2 $\Delta 3/\Delta 3$ mice

Except for lesion size, no gross morphological differences were observed between the two genotypes at 33 weeks. Staining for smooth muscle α -actin within the lesions was significantly increased in ApoE0/Hspg2 $\Delta 3/\Delta 3$ mice compared with controls (Figures 6A–C). Most α -actin staining was located in the luminal part of the lesions corresponding to fibrous cap regions. Medial atrophy was readily observed underneath large lesions (Figures 6A–B). In contrast, collagen content quantified after detection with picro-sirius red showed no significant difference between the genotypes (Figures 6D–F) and CD68 staining showed no difference in macrophage content between the two groups (Figures 6G–I). CD3 staining demonstrated the

presence of T-cells in lesions of both genotypes but the staining was too sparse to allow quantification (data not shown).

Discussion

Here, we studied the role of the perlecan HS chains in atherosclerosis by cross-breeding *Hspg2*^{Δ3/Δ3} mice with ApoE0 mice. A significant reduction in atherosclerosis was demonstrated in ApoE0/*Hspg2*^{Δ3/Δ3} mice compared with ApoE0 controls. *In vitro*, we observed reduced binding of labeled mouse triglyceride-rich lipoproteins to total ECM prepared from ApoE0/*Hspg2*^{Δ3/Δ3} SMCs. Similarly, binding of human LDL to total ECM and purified proteoglycans from ApoE0/*Hspg2*^{Δ3/Δ3} mice was reduced. *In vivo*, influx of lipoproteins into the vessel wall of ApoE0/*Hspg2*^{Δ3/Δ3} mice was increased, but retention was decreased and lesions contained less apoB. In addition, an increased accumulation of SMCs was observed.

A reduction in aortic root lesion area in ApoE0/*Hspg2*^{Δ3/Δ3} mice was observed at both 15 and 33 weeks in both females and males. The difference between ApoE0/*Hspg2*^{Δ3/Δ3} mice and ApoE0 controls was less at 33 weeks, probably because of suppressed expansion of plaque volume in the aortic roots of the ApoE0 controls at this late time-point. A larger reduction in lesion area was measured at 33 weeks when whole aortas were analysed en face, which has previously been reported as a better method for late time points.^{32, 38}

These results support a pro-atherogenic role for the HS chains of perlecan in mouse atherosclerosis. Our results confirm findings from an earlier study that showed a reduction of atherosclerosis in ApoE0 mice with partially reduced perlecan expression but did not discriminate between the roles of the perlecan core protein and its HS chains.²⁸ However, other studies suggest that HS and perlecan prevent atherosclerosis,^{2, 3} and several groups have shown an inverse relationship between HS proteoglycan levels and atherosclerosis in different species.^{4–7} Reduced atherosclerosis has also been observed in both hypercholesterolemic rabbits and ApoE0 mice treated with glucosamine to increase the HS proteoglycan content of the vessel wall.^{19, 39} However, since glucosamine treatment would affect not only perlecan but also other HS-containing molecules, those studies are more difficult to interpret.

The lesion reduction in ApoE0/*Hspg2*^{Δ3/Δ3} mice observed in our study could not be explained by altered serum lipid levels, in agreement with previous findings.²⁸ It is likely that other cell-surface-bound HS molecules, such as syndecans, are more important than perlecan for lipid uptake in the liver and in peripheral tissues.⁴⁰ However, it has been shown that perlecan plays a role in lipid uptake in cells lacking other proteoglycans.⁴¹

We explored other possible mechanisms behind the observed reduction of atherosclerosis in ApoE0/*Hspg2*^{Δ3/Δ3} mice. Lipoprotein binding to total ECM and to purified proteoglycans from ApoE0/*Hspg2*^{Δ3/Δ3} mice was significantly reduced compared with control, indicating that perlecan HS is important for lipoprotein binding and retention in the vessel wall. A reduced amount of staining for apoB in the ApoE0/*Hspg2*^{Δ3/Δ3} lesions supported this conclusion.

Our findings are in agreement with an earlier study showing that HS binds LDL *in vitro*,²⁷ and is consistent with the response-to-retention hypothesis of early atherogenesis.⁴² Furthermore, incubation of SMCs with non-esterified fatty acids increases both perlecan production and LDL binding,⁴³ and atherosclerosis is reduced in mice expressing apoB with a defective binding capacity for proteoglycans.⁴⁴ The reduced lipoprotein-binding capacity of HS-deficient ECM may thus be responsible for the observed reduction in lesion development. In this context it should be noted perlecan and biglycan are the most abundant proteoglycans in mouse atherosclerosis.¹⁶

The uptake of lipoproteins in aortas of ApoE0/*Hspg2*^{Δ3/Δ3} mice compared with controls was faster shortly after injection. It is possible that the permeability for lipoproteins in the subendothelial basement membrane is higher in the absence of perlecan HS, thereby allowing a more rapid diffusion into the vessel wall. The uptake of labeled albumin was similar in ApoE0/*Hspg2*^{Δ3/Δ3} mice and controls, suggesting a selective, rather than a general, increase in basement membrane permeability. However, the amount of human LDL retained at 72 h was the same in ApoE0/*Hspg2*^{Δ3/Δ3} mice and controls, which suggests that the efflux of lipoproteins from the vessel wall of ApoE0/*Hspg2*^{Δ3/Δ3} mice is greater because of a reduced retention capacity of the HS-deficient ECM. In support of these findings, the rate of LDL entry into the normal artery wall has previously been shown to vastly exceed LDL accumulation⁴⁵, indicating that changes in endothelial permeability are relevant for atherogenesis only if the lipoproteins are subsequently retained. In support of our findings, HS in the endothelial basement membrane has previously been reported to reduce endothelial permeability for LDL *in vitro*.^{18, 19} Our observations suggest that perlecan can both promote and prevent atherogenesis by influencing transport of lipoproteins across the endothelial barrier and retention in the interstitial ECM, even though retention is most essential at least for early atherogenesis.

In order to evaluate whether our results may be relevant also in human atherosclerosis, both mouse apoB48-containing triglyceride-rich lipoproteins (elevated in ApoE0 mice) and human apoB100-containing LDL was used. Analysis of lipoprotein binding *in vitro* and *in vivo* using both mouse and human lipoproteins gave identical results confirming that both apoB48 and apoB100 bind proteoglycans in a similar way, although binding of apoB48 is mediated via a proteoglycan binding sequence that is exposed only in carboxyl-truncated forms of apoB⁴⁶.

Increased α-actin staining was seen in lesions of ApoE0/*Hspg2*^{Δ3/Δ3} mice, mostly in the fibrous cap. This is in agreement with our earlier report of increased SMC proliferation and intimal hyperplasia in *Hspg2*^{Δ3/Δ3} mice, and is an expected finding as HS and heparin are potent inhibitors of SMC proliferation.^{21–24} Perlecan HS may thus control SMC proliferation in lesion development and thereby influence plaque stability.^{47–49} We cannot exclude the possibility that the ability of perlecan HS to influence SMC proliferation may also contribute to accumulation of lipoproteins and lesion development through mechanisms not dependent on the composition of the ECM. Nevertheless, an increased proliferation of SMCs ApoE0/*Hspg2*^{Δ3/Δ3} mice is not sufficient to normalize vessel wall HS content since no HS is detectable even after substantial SMC proliferation in intimal hyperplasia of *Hspg2*^{Δ3/Δ3} mice.²⁴ Although HS and heparin have been reported to influence inflammation,² we did not observe any differences in the accumulation of CD68-positive inflammatory cells. It is, however, possible that the reduced atherosclerosis observed in ApoE0/*Hspg2*^{Δ3/Δ3} mice may be influenced by inflammatory processes such as cytokine bioavailability rather than accumulation of leukocytes.⁵⁰

In summary, we conclude that the HS chains of perlecan promote atherosclerosis in mice, most likely through increased retention of lipoproteins. In addition, the ability of perlecan HS to regulate SMC proliferation was found to influence SMC content in lesions, thus implicating a role for perlecan in plaque stability. Due to differences in proteoglycans expressed in mice and humans, it is difficult to determine a role for perlecan in human disease. However, the observed ability of perlecan to influence central processes in atherogenesis such as lipoprotein transport across the endothelial barrier, lipoprotein retention, and SMC proliferation should stimulate further studies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Wight TN, Merrilees MJ. Proteoglycans in atherosclerosis and restenosis: key roles for versican. *Circ Res* 2004;94:1158–1167. [PubMed: 15142969]
2. Engelberg H. Endogenous heparin activity deficiency: the 'missing link' in atherogenesis? *Atherosclerosis* 2001;159:253–260. [PubMed: 11730804]
3. Pillarisetti S. Lipoprotein modulation of subendothelial heparan sulfate proteoglycans (perlecan) and atherogenicity. *Trends Cardiovasc Med* 2000;10:60–65. [PubMed: 11150731]
4. Hollmann J, Schmidt A, von Basewitz DB, Buddecke E. Relationship of sulfated glycosaminoglycans and cholesterol content in normal and arteriosclerotic human aorta. *Arteriosclerosis* 1989;9:154–158. [PubMed: 2493783]
5. Murata K, Yokoyama Y. Acidic glycosaminoglycan, lipid and water contents in human coronary arterial branches. *Atherosclerosis* 1982;45:53–65. [PubMed: 7159490]
6. Murata K, Murata A, Yoshida K. Heparan sulfate isomers in cerebral arteries of Japanese women with aging and with atherosclerosis--heparitinase and high-performance liquid chromatography determinations. *Atherosclerosis* 1997;132:9–17. [PubMed: 9247354]
7. Edwards IJ, Wagner JD, Vogl-Willis CA, Litwak KN, Cefalu WT. Arterial heparan sulfate is negatively associated with hyperglycemia and atherosclerosis in diabetic monkeys. *Cardiovasc Diabetol* 2004;3:6. [PubMed: 15117408]
8. Tran PK, Agardh HE, Tran-Lundmark K, Ekstrand J, Roy J, Henderson B, Gabrielsen A, Hansson GK, Swedenborg J, Paulsson-Berne G, Hedin U. Reduced perlecan expression and accumulation in human carotid atherosclerotic lesions. *Atherosclerosis* 2007;190:264–270. [PubMed: 16620836]
9. Iozzo RV, Cohen IR, Grassel S, Murdoch AD. The biology of perlecan: the multifaceted heparan sulphate proteoglycan of basement membranes and pericellular matrices. *Biochem J* 1994;302 (Pt 3): 625–639. [PubMed: 7945186]
10. Timpl R, Brown JC. Supramolecular assembly of basement membranes. *Bioessays* 1996;18:123–132. [PubMed: 8851045]
11. Noonan DM, Fulle A, Valente P, Cai S, Horigan E, Sasaki M, Yamada Y, Hassell JR. The complete sequence of perlecan, a basement membrane heparan sulfate proteoglycan, reveals extensive similarity with laminin A chain, low density lipoprotein-receptor, and the neural cell adhesion molecule. *J Biol Chem* 1991;266:22939–22947. [PubMed: 1744087]
12. Kallunki P, Tryggvason K. Human basement membrane heparan sulfate proteoglycan core protein: a 467-kD protein containing multiple domains resembling elements of the low density lipoprotein receptor, laminin, neural cell adhesion molecules, and epidermal growth factor. *J Cell Biol* 1992;116:559–571. [PubMed: 1730768]
13. Dolan M, Horchar T, Rigatti B, Hassell JR. Identification of sites in domain I of perlecan that regulate heparan sulfate synthesis. *J Biol Chem* 1997;272:4316–4322. [PubMed: 9020150]
14. Friedrich MV, Gohring W, Morgelin M, Brancaccio A, David G, Timpl R. Structural basis of glycosaminoglycan modification and of heterotypic interactions of perlecan domain V. *J Mol Biol* 1999;294:259–270. [PubMed: 10556044]
15. Tapanadechopone P, Hassell JR, Rigatti B, Couchman JR. Localization of glycosaminoglycan substitution sites on domain V of mouse perlecan. *Biochem Biophys Res Commun* 1999;265:680–690. [PubMed: 10600481]

16. Kunjathoor VV, Chiu DS, O'Brien KD, LeBoeuf RC. Accumulation of biglycan and perlecan, but not versican, in lesions of murine models of atherosclerosis. *Arterioscler Thromb Vasc Biol* 2002;22:462–468. [PubMed: 11884291]
17. Pillarisetti S, Paka L, Obunike JC, Berglund L, Goldberg IJ. Subendothelial retention of lipoprotein (a). Evidence that reduced heparan sulfate promotes lipoprotein binding to subendothelial matrix. *J Clin Invest* 1997;100:867–874. [PubMed: 9259586]
18. Duan W, Paka L, Pillarisetti S. Distinct effects of glucose and glucosamine on vascular endothelial and smooth muscle cells: evidence for a protective role for glucosamine in atherosclerosis. *Cardiovasc Diabetol* 2005;4:16. [PubMed: 16207378]
19. Guretzki HJ, Gerbitz KD, Olgemoller B, Schleicher E. Atherogenic levels of low density lipoprotein alter the permeability and composition of the endothelial barrier. *Atherosclerosis* 1994;107:15–24. [PubMed: 7945555]
20. Sivaram P, Obunike JC, Goldberg IJ. Lysolecithin-induced alteration of subendothelial heparan sulfate proteoglycans increases monocyte binding to matrix. *J Biol Chem* 1995;270:29760–29765. [PubMed: 8530367]
21. Clowes AW, Karnowsky MJ. Suppression by heparin of smooth muscle cell proliferation in injured arteries. *Nature* 1977;265:625–626. [PubMed: 859561]
22. Bingley JA, Hayward IP, Campbell JH, Campbell GR. Arterial heparan sulfate proteoglycans inhibit vascular smooth muscle cell proliferation and phenotype change in vitro and neointimal formation in vivo. *J Vasc Surg* 1998;28:308–318. [PubMed: 9719326]
23. Lindner V, Olson NE, Clowes AW, Reidy MA. Inhibition of smooth muscle cell proliferation in injured rat arteries. Interaction of heparin with basic fibroblast growth factor. *J Clin Invest* 1992;90:2044–2049. [PubMed: 1430226]
24. Tran PK, Tran-Lundmark K, Soininen R, Tryggvason K, Thyberg J, Hedin U. Increased intimal hyperplasia and smooth muscle cell proliferation in transgenic mice with heparan sulfate-deficient perlecan. *Circ Res* 2004;94:550–558. [PubMed: 14739157]
25. Hedin U, Daum G, Clowes AW. Heparin inhibits thrombin-induced mitogen-activated protein kinase signaling in arterial smooth muscle cells. *J Vasc Surg* 1998;27:512–520. [PubMed: 9546238]
26. Kinsella MG, Irvin C, Reidy MA, Wight TN. Removal of heparan sulfate by heparinase treatment inhibits FGF-2-dependent smooth muscle cell proliferation in injured rat carotid arteries. *Atherosclerosis* 2004;175:51–57. [PubMed: 15186946]
27. Iverius PH. The interaction between human plasma lipoproteins and connective tissue glycosaminoglycans. *J Biol Chem* 1972;247:2607–2613. [PubMed: 4336380]
28. Vikramadithyan RK, Kako Y, Chen G, Hu Y, Arikawa-Hirasawa E, Yamada Y, Goldberg IJ. Atherosclerosis in perlecan heterozygous mice. *J Lipid Res* 2004;45:1806–1812. [PubMed: 15258195]
29. Rossi M, Morita H, Sormunen R, Airenne S, Kreivi M, Wang L, Fukai N, Olsen BR, Tryggvason K, Soininen R. Heparan sulfate chains of perlecan are indispensable in the lens capsule but not in the kidney. *Embo J* 2003;22:236–245. [PubMed: 12514129]
30. Flood C, Gustafsson M, Pitas RE, Arnaboldi L, Walzem RL, Boren J. Molecular mechanism for changes in proteoglycan binding on compositional changes of the core and the surface of low-density lipoprotein-containing human apolipoprotein B100. *Arterioscler Thromb Vasc Biol* 2004;24:564–570. [PubMed: 14726411]
31. Nicoletti A, Kaveri S, Caligiuri G, Bariety J, Hansson GK. Immunoglobulin treatment reduces atherosclerosis in apo E knockout mice. *J Clin Invest* 1998;102:910–918. [PubMed: 9727059]
32. Tangirala RK, Rubin EM, Palinski W. Quantitation of atherosclerosis in murine models: correlation between lesions in the aortic origin and in the entire aorta, and differences in the extent of lesions between sexes in LDL receptor-deficient and apolipoprotein E-deficient mice. *J Lipid Res* 1995;36:2320–2328. [PubMed: 8656070]
33. Miller JD, Cummings J, Maresh GA, Walker DG, Castillo GM, Ngo C, Kimata K, Kinsella MG, Wight TN, Snow AD. Localization of perlecan (or a perlecan-related macromolecule) to isolated microglia in vitro and to microglia/macrophages following infusion of beta-amyloid protein into rodent hippocampus. *Glia* 1997;21:228–243. [PubMed: 9336237]

34. Boren J, Lee I, Zhu W, Arnold K, Taylor S, Innerarity TL. Identification of the low density lipoprotein receptor-binding site in apolipoprotein B100 and the modulation of its binding activity by the carboxyl terminus in familial defective apo-B100. *J Clin Invest* 1998;101:1084–1093. [PubMed: 9486979]
35. Schwenke DC. Gender differences in intima-media permeability to low-density lipoprotein at atherosclerosis-prone aortic sites in rabbits. Lack of effect of 17 beta-estradiol. *Arterioscler Thromb Vasc Biol* 1997;17:2150–2157. [PubMed: 9351384]
36. Chang MY, Potter-Perigo S, Wight TN, Chait A. Oxidized LDL bind to nonproteoglycan components of smooth muscle extracellular matrices. *J Lipid Res* 2001;42:824–833. [PubMed: 11352990]
37. Paka L, Kako Y, Obunike JC, Pillarisetti S. Apolipoprotein E containing high density lipoprotein stimulates endothelial production of heparan sulfate rich in biologically active heparin-like domains. A potential mechanism for the anti-atherogenic actions of vascular apolipoprotein e. *J Biol Chem* 1999;274:4816–4823. [PubMed: 9988721]
38. Daugherty A, Rateri DL. Development of experimental designs for atherosclerosis studies in mice. *Methods* 2005;36:129–138. [PubMed: 15893934]
39. Stender S, Astrup P. Glucosamine and experimental atherosclerosis. Increased wet weight and changed composition of cholesterol fatty acids in aorta of rabbits fed a cholesterol-enriched diet with added glucosamine. *Atherosclerosis* 1977;26:205–213. [PubMed: 836356]
40. Fuki IV, Kuhn KM, Lomazov IR, Rothman VL, Tuszyński GP, Iozzo RV, Swenson TL, Fisher EA, Williams KJ. The syndecan family of proteoglycans. Novel receptors mediating internalization of atherogenic lipoproteins in vitro. *J Clin Invest* 1997;100:1611–1622. [PubMed: 9294130]
41. Fuki II, Iozzo RV, Williams KJ. Perlecan heparan sulfate proteoglycan. A novel receptor that mediates a distinct pathway for ligand catabolism. *J Biol Chem* 2000;275:31554. [PubMed: 11013268]
42. Williams KJ, Tabas I. The response-to-retention hypothesis of early atherogenesis. *Arterioscler Thromb Vasc Biol* 1995;15:551–561. [PubMed: 7749869]
43. Camejo G, Hurt-Camejo E, Olsson U, Bondjers G. Lipid mediators that modulate the extracellular matrix structure and function in vascular cells. *Curr Atheroscler Rep* 1999;1:142–149. [PubMed: 11122703]
44. Skalen K, Gustafsson M, Rydberg EK, Hultén LM, Wiklund O, Innerarity TL, Boren J. Subendothelial retention of atherogenic lipoproteins in early atherosclerosis. *Nature* 2002;417:750–754. [PubMed: 12066187]
45. Carew TE, Pittman RC, Marchand ER, Steinberg D. Measurement in vivo of irreversible degradation of low density lipoprotein in the rabbit aorta. Predominance of intimal degradation. *Arteriosclerosis* 1984;4:214–224. [PubMed: 6324731]
46. Flood C, Gustafsson M, Richardson PE, Harvey SC, Segrest JP, Boren J. Identification of the proteoglycan binding site in apolipoprotein B48. *J Biol Chem* 2002;277:32228–32233. [PubMed: 12070165]
47. Littlewood TD, Bennett MR. Apoptotic cell death in atherosclerosis. *Curr Opin Lipidol* 2003;14:469–475. [PubMed: 14501585]
48. Johnson JL, George SJ, Newby AC, Jackson CL. Divergent effects of matrix metalloproteinases 3, 7, 9, and 12 on atherosclerotic plaque stability in mouse brachiocephalic arteries. *Proc Natl Acad Sci U S A* 2005;102:15575–15580. [PubMed: 16221765]
49. Schonbeck U, Libby P. CD40 signaling and plaque instability. *Circ Res* 2001;89:1092–1103. [PubMed: 11739273]
50. Whitelock JM, Iozzo RV. Heparan sulfate: a complex polymer charged with biological activity. *Chem Rev* 2005;105:2745–2764. [PubMed: 16011323]

Figure 1.

Quantification of atherosclerosis in ApoE0 and ApoE0/*Hspg2*^{Δ3/Δ3} mice. Oil-RedO/Htx staining of aortic root sections from female mice at 15 (A and B; n=10 and 9) and 33 weeks (C; n=11 and 10). D and E shows en face analysis of whole aortas at 33 weeks (n=12 and 10).

Figure 2.
Size fractionation of lipoproteins. Males 15 weeks (A), females 15 weeks (B), males 33 weeks (C), and females 33 weeks (D). ApoE0 (●), ApoE0/*Hspg2* Δ 3/ Δ 3 mice (○).

Figure 3.

Gradient gel electrophoresis of proteoglycans produced by aortic SMCs from ApoE0 (A) and ApoE0/*Hspg2*^{Δ3/Δ3} (Δ3) mice. Proteoglycans were labeled with ³⁵S-sulfate, DEAE-purified and digested with chondroitinase and/or heparinase I and II as described in Methods. Note lack of smear in the stacking gel of chondroitinase-digested samples from ApoE0/*Hspg2*^{Δ3/Δ3} SMC cultures indicating lack of large HS proteoglycans.

Figure 4. Binding of mouse triglyceride-rich lipoproteins to total ECM prepared from aortic SMCs from ApoE0 (■) and ApoE0/*Hspg2* $^{\Delta 3/\Delta 3}$ (□) mice (A; n=4) and binding of human LDL to proteoglycans purified from ApoE0 (■) and ApoE0/*Hspg2* $^{\Delta 3/\Delta 3}$ (□) SMCs (B; one of two experiments with identical results).

Figure 5. ApoB staining of aortic roots (female 15 weeks; n=10 and 9) with adjacent sections stained with Oil Red-O (A–F). Quantification of apoB staining (G).

Figure 6.

Qualitative differences between aortic root lesions from ApoE0 (A, D, and G) and ApoE0/*Hspg2*^{Δ3/Δ3} (B, E and H) mice. Immunohistochemical demonstration and quantification of α-actin for SMCs (A, B and C; n=9 and 7) and CD68 for macrophages (G, H and I; n=7). Quantification of staining (C,F and I) expressed as % of total lesion area. The arrowhead in B indicates α-actin staining in a fibrous cap region. Also note prominent medial thinning underneath large lesions. D, E and F show picrosirius red staining for collagen (n=10 and 13) analyzed and quantified using polarized light.

TABLE 1

Lipoprotein and albumin uptake in vivo

Injected	Time	Genotype	n	Uptake [CPM]	P-value
Mouse TG-rich Lipoproteins	20 minutes	apoE0	7	734 ± 225	0.039
		apoE0/Hspg2 ^{A3/A3}	8	1159 ± 451	
Human LDL	20 minutes	apoE0	5	205 ± 55	0.028
		apoE0/Hspg2 ^{A3/A3}	5	327 ± 85	
	72 hours	apoE0	5	669 ± 189	NS
		apoE0/Hspg2 ^{A3/A3}	5	655 ± 192	
Albumin	20 minutes	apoE0	6	6054 ± 732	NS
		apoE0/Hspg2 ^{A3/A3}	5	6400 ± 1640	

Values normalized to blood concentration and animal weight. Presented as mean ± SD.