Atherosclerosis in perlecan heterozygous mice

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Abstract The hypothesis that lipoprotein association with perlecan is atherogenic was tested by studying atherosclerosis in mice that had a heterozygous deletion of perlecan, the primary extracellular heparan sulfate proteoglycan in arteries. We first studied the expression of perlecan in mouse lesions and noted that this proteoglycan in aorta was found in the subendothelial matrix. Perlecan was also a major component of the lesional extracellular matrix. Mice with a heterozygous deletion had a reduction in arterial wall perlecan expression. Atherosclerosis in these mice was studied after crossing the defect into the apolipoprotein E (apoE) and LDL receptor knockout backgrounds. At 12 weeks, chow-fed apoE null mice with a heterozygous deletion had less atherosclerosis. However, at 24 weeks and in the LDL receptor heterozygous background, the presence of a perlecan knockout allele did not significantly alter lesion size. Thus, it appears that loss of perlecan leads to less atherosclerosis in early lesions. Although this might be attributable to a decrease in lipoprotein retention, it should be noted that perlecan might mediate multiple other processes that could, in sum, accelerate atherosclerosis.—Vikramadithyan, R. K., Y. Kako, G. Chen, Y. Hu, E. Arikawa-Hirasawa, Y. Yamada, and I. J. Goldberg. **Atherosclerosis in perlecan heterozygous mice. *J. Lipid Res.* 2004.** 45: **1806–1812.**

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Much of atherosclerosis research in the 20th century focused on the cholesterol hypothesis. The evidence that plasma cholesterol-containing lipoproteins cause atherosclerosis is indisputable. However, as was nicely summarized nearly 50 years ago (1), how these lipid-containing particles accumulate within the artery and then produce inflammatory reactions is still unclear. A refinement to the "infiltrative theory" has been an attempt to define the biochemical interactions that lead to this process. Because the lipoproteins are initially found in the extracellular space, they are thought to bind to protein or carbohydrate components of the extracellular matrix. Among the molecules that have been most studied in this respect are proteoglycans (2).

Within the artery are a number of classes of proteoglycans, complex proteins that contain highly negatively charged carbohydrates. There are several observations that support the proteoglycan-lipoprotein association hypothesis. *1*) Dermatan/chondroitin sulfate proteoglycans are found in greater amounts in lesions (3, 4), and some classes of these proteins are found in regions that also contain apolipoprotein B (apoB) (5), the major protein component of LDL and remnant lipoproteins. *2*) Complexes of proteoglycans and LDL have been isolated from arteries (6). *3*) Prolonged incubations of proteoglycans and LDL will, in vitro, produce aggregates (7).

Of all the proteoglycans, the most heavily charged and most avid LDL binding are the heparan sulfate proteoglycans (8). Heparin, a highly charged carbohydrate of this class, has been studied because it interacts with apoB-containing lipoproteins (9, 10). This reaction, however, is most evident in low ionic strength solutions; in several reports, the proteoglycan-LDL complexes are dissociated by physiologic saline (9). Moreover, increasing LDL charge (e.g., via oxidation) decreases the proteoglycan-lipoprotein interaction (11).

One way to study the role of proteoglycans in atherosclerosis is to use genetically manipulated mice that have an alteration in proteoglycan production. Perlecan is a 450 kDa core protein containing three heparan sulfate chains of 70 kDa attached in domain I and an additional chain associated with domain V. Perlecan is the major heparan sulfate proteoglycan in the subendothelial matrix (12). This protein has a variety of actions and is essential for normal bone formation and neurological development; a homozygous deletion of perlecan in mice is lethal as a result of bone and neurological malformations (13). Within the vasculature, perlecan helps to stabilize the endothelial barrier and decrease the proliferation of smooth muscle cells (14); these are potentially antiatherogenic actions of perlecan. We first studied perlecan expression

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in normal and atherosclerotic mouse arteries and then tested whether a partial loss of perlecan alters atherosclerosis in the mouse.

METHODS

Mouse housing and diets

Mice were maintained in a temperature-controlled (25°C) facility with a 12 h light/dark cycle and given free access to food and water, except when fasting blood specimens were obtained. Mice were fed either laboratory rodent chow (PMI Nutrition International, Inc.) or a Paigen diet (C13002; Research Diets, Inc.). Rodent chow contained 4.5% (w/w) fat; the Paigen diet contained 15% (w/w) fat, 1.25% (w/w) cholesterol, and 0.5% cholic acid.

Genetically altered mice

Heterozygous perlecan-deficient mice (13) were backcrossed to C57BL/6 mice for seven generations. These animals were crossed onto apolipoprotein E null (apoE0) mice or LDL receptor knockout mice in the same background that were purchased from Jackson Laboratory (Bar Harbor, ME).

Immunohistochemical staining for aortic perlecan

Aortas from male apoE0 mice fed a chow diet at different time points were embedded in OCT and snap frozen on dry ice. Ten micrometer frozen sections were fixed in cold acetone, and endogenous peroxidase was quenched with 3% H_2O_2/methanol. The sections were then stained with 1:100 rat monoclonal antibody against perlecan (Neo Marks, Inc.) at 37°C for 1 h, then at 4°C overnight. The antibody was detected with the standard Avidin Biotin Complex method (Vector Laboratories, Inc.) and 3-amino-9-ethyl carbazole (Zymed Laboratories) as the substrate. Positive staining is shown as red deposits. For a negative control, slides were incubated with nonimmune rat IgG (Sigma) in place of the first antibody.

Quantitative real-time PCR for the aortic perlecan gene

Total RNA was isolated from aortas of heterozygous LDL receptor-deficient mice and heterozygous perlecan mice in the heterozygous LDL receptor-deficient background using the RNeasy mini kit (Qiagen). The mRNA levels for perlecan were determined by SYBR green (Applied Biosystems) real-time PCR using 10–100 ng of total RNA. Primer sequences were selected from domain I, the potential heparan sulfate attachment domain (PubMed sequence NM008305; 201–408). The primer sequences are as follows: Prl-RT (forward), 5′-TACGCGGTCCATTGAG-3′; Prl-RT (reverse), 5′-AGATCCGTCCGCATTC-3′. The real-time PCR standard curve was constructed by using serial dilutions of mouse total RNA isolated from aortas. Data were normalized to mouse β-actin.

Blood sampling

Fasting blood samples were obtained from mice after removal of food for 6 h in the morning. The animals were anesthetized with methoxyflurane and bled by retro-orbital phlebotomy into tubes containing anticoagulant (5 mM EDTA) using heparinized capillary tubes. Cholesterol and triglyceride were measured using kits from Sigma. Lipoproteins—VLDL ($d < 1.006$ g/ml), intermediate density lipoprotein/LDL ($d = 1.006$–1.063 g/ml), and HDL ($d = 1.063$–1.21 g/ml)—were separated by sequential density ultracentrifugation of plasma in a TLA 100 rotor (Beckman Instruments) (15).

Quantitative atherosclerosis analysis

Mice were killed and atherosclerosis assays were performed on the aortic roots as described previously (15, 16). Hearts were perfused with PBS, fixed in 10% phosphate-buffered formalin, embedded in OCT compound, and sectioned with a cryostat at 10 μm thickness. Slides containing the aortic tissue were stained with Oil Red O and hematoxylin and counterstained with light green. Lesions of the proximal aorta were measured in 80 μm intervals. The mean lesion area of six sections was calculated and shown as lesion area (μm^2). The atherosclerotic lesions were assessed by en face assays (17).

Statistical analysis

Comparisons between two genotypes were performed using a two-tailed Student's *t*-test.

RESULTS

Perlecan expression in blood vessels

The expression of perlecan within the aorta of apoE mice fed a chow diet differed markedly as a function of

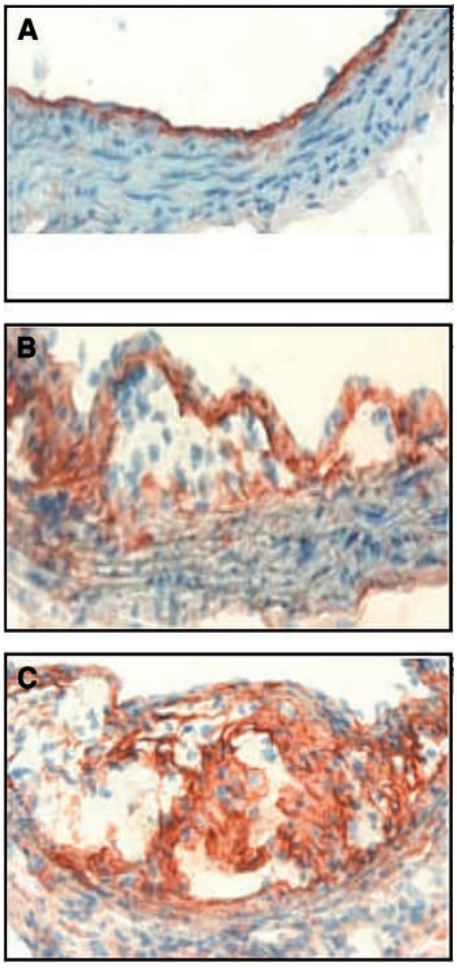

Fig. 1. Perlecan expression in lesions of atherosclerosis varies with lesion severity. A: In normal aorta, perlecan was distributed in the subendothelial regions. B,C: Perlecan expression increased markedly in advanced lesions, in the fibrous cap as well as in the plaque core.

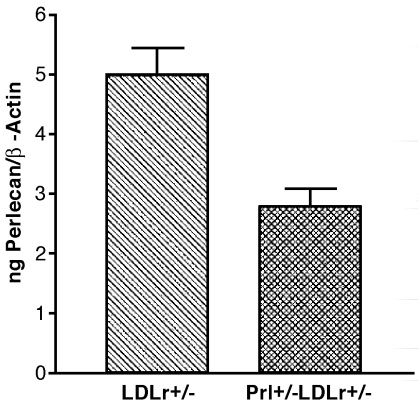

Fig. 2. Perlecan expression in aortas by quantitative real-time PCR. Aortas from heterozygous LDL receptor (LDLr)-deficient mice and heterozygous perlecan (Prl) mice in the heterozygous LDL receptor-deficient background were used for real-time PCR. Aortas from three mice from each group were pooled for RNA per set. The data shown are averages of two sets. Values are expressed as mean ± SEM.

disease within the vessel. In normal, nondiseased aortas, perlecan staining was primarily localized to the subendothelial regions (**Fig. 1A**). In more advanced lesions, additional staining was found within the fibrous cap that would be expected to have proliferated smooth muscle cells (Fig. 1B). Most remarkably, in advanced lesions with lipid-filled cores, intense staining was within the lipid core (Fig. 1C). Thus, perlecan is a major matrix protein within the lesions.

Quantitative real-time PCR for the aortic perlecan gene

To determine whether heterozygous perlecan knockout mice have an alteration of perlecan expression, the aortic content of perlecan mRNA was compared with that of control mice. A shown in **Fig. 2**, deletion of one allele reduced perlecan expression.

Lipids in perlecan heterozygous knockout mice

Because lipoprotein uptake by the liver is thought to be mediated, in part, by "capture" by proteoglycans, we assessed whether the loss of perlecan would alter plasma lipoproteins. **Table 1** shows plasma and lipoprotein lipids of wild-type and perlecan heterozygous mice eating chow and a Paigen diet. Both strains of mice had identical levels of plasma cholesterol and triglyceride, VLDL, LDL, and HDL. This was confirmed by FPLC analysis (data not shown). When these mice were placed on a Paigen diet, both the controls and perlecan heterozygous mice had a similar increase in plasma apoB-containing lipoproteins. However, loss of perlecan did not affect lipids.

Lipoprotein uptake via receptors might have obscured any uptake as a result of liver or peripheral tissue heparan sulfate proteoglycan-mediated processes. Therefore, we next examined lipids and lipoproteins in apoE0 and apoE0/perlecan heterozygous mice. As expected, compared with wild-type mice, apoE deficiency led to marked increases of cholesterol and triglyceride at 12 weeks of age (**Table 2**). However, perlecan deficiency did not significantly alter plasma cholesterol, which was 420 ± 105 mg/dl for apoE0 mice and 359 ± 114 mg/dl for apoE0/perlecan heterozygous mice. A second group of mice was maintained on this diet for 24 weeks (Table 2). Again, the lipids and lipoproteins were not different. Therefore, perlecan deficiency did not alter either plasma or lipoprotein lipids in the apoE0 background.

Perlecan-deficient mice were then crossed with LDL receptor knockouts. Because our primary objective was to study atherosclerosis, we created a colony of heterozygous LDL receptor knockouts and fed them the Paigen diet as reported by van Haperen et al. (18). Male LDL receptor heterozygous ($LDL^{+/-}$) and perlecan-deficient $LDL^{+/-}$ mice were begun on a Paigen diet at 4 weeks of age and maintained on this diet for 16 weeks. Plasma cholesterol in these mice averaged 579 ± 33 and 595 ± 32 mg/dl in $LDL^{+/-}$ and perlecan-deficient/LDL receptor-deficient mice, respectively (**Table 3**).

Atherosclerosis in control and perlecan heterozygous apoE0 mice

In the chow-fed apoE knockout mice, both early lesions (12 weeks) and later lesions (24 weeks) were studied. In 12 week old male mice, perlecan deficiency led to a dramatic and significant reduction in lesion size, which averaged 23,961 μm^2 in controls and 7,796 μm^2 in perlecan heterozygous animals ($P = 0.02$) (**Table 4**). Lesion sizes for individual animals are shown in **Fig. 3A**, graphed on a semi-log scale. Lack of perlecan led to a >70% reduction

TABLE 1. Lipid profiles of wild-type and heterozygous perlecan-deficient mice fed chow and Paigen diets

				Cholesterol				Triglyceride	
Genotype	Diet	Age	n	Plasma	VLDL	LDL	HDL	Plasma	VLDL
		weeks		*mg/dl*				*mg/dl*	
Wild-type	Chow	15	5	83 ± 12	21 ± 13	23 ± 4	55 ± 10	50 ± 14	17 ± 8
$Prl^{+/-}$	Chow	15	5	95 ± 20	17 ± 4	27 ± 10	63 ± 24	52 ± 19	13 ± 5
Wild-type	Paigen	19	4	236 ± 36	123 ± 23	68 ± 6	48 ± 8	48 ± 2	25 ± 2
$Prl^{+/-}$	Paigen	19	5	248 ± 36	151 ± 34	59 ± 4	48 ± 7	50 ± 10	25 ± 5
Wild-type	Paigen	40	4	198 ± 27	114 ± 27	33 ± 4	41 ± 13	44 ± 9	27 ± 9
$Prl^{+/-}$	Paigen	40	5	199 ± 2	112 ± 15	34 ± 3	45 ± 9	49 ± 6	31 ± 6

Blood samples were obtained at 15 weeks of age for the chow diet-fed state and at 19 weeks and 10 months of age for the Paigen diet-fed state. Prl+/− denotes mice that have a heterozygous deletion of the perlecan gene.

TABLE 2. Lipid profiles of heterozygous perlecan-deficient mice in the apoE0 background

Genotype	Gender	n	Cholesterol				Triglyceride	
			Plasma	VLDL	LDL	HDL	Plasma	VLDL
			mg/dl				*mg/dl*	
12 weeks of age								
apoE0	Male	13	420 ± 105	311 ± 115	82 ± 23	23 ± 7	120 ± 35	88 ± 27
apoE0/Prl$^{+/-}$	Male	13	359 ± 114	257 ± 113	71 ± 19	23 ± 7	115 ± 20	89 ± 16
apoE0	Female	18	373 ± 101	264 ± 62	76 ± 29	14 ± 5	58 ± 12	38 ± 9
apoE0/Prl$^{+/-}$	Female	13	392 ± 87	288 ± 55	84 ± 22	14 ± 5	67 ± 14	44 ± 16
24 weeks of age								
apoE0	Male	10	487 ± 160	404 ± 156	79 ± 25	19 ± 7	127 ± 30	106 ± 27
apoE0/Prl$^{+/-}$	Male	19	418 ± 162	320 ± 158	65 ± 21	15 ± 7	116 ± 52	92 ± 49
apoE0	Female	10	401 ± 109	302 ± 96	73 ± 19	8 ± 6	49 ± 12	29 ± 11
apoE0/Prl$^{+/-}$	Female	17	405 ± 95	308 ± 82	65 ± 2	6 ± 4	50 ± 12	32 ± 10

Lipoprotein profiles were assessed by ultracentrifugation. apoE0, apolipoprotein E knockout mice; apoE0/Prl$^{+/-}$, heterozygous perlecan-deficient mice in the apoE knockout background.

in lesion size. This amount of atherosclerosis was too small to allow for en face quantification. Lesion size in female mice is also shown in Table 4 and Fig. 3A. Although a trend toward reduced lesions was noted in the perlecan heterozygous females, this was not significant.

After 24 weeks, male perlecan heterozygous mice still had, on average, less atherosclerosis, 121,961 μm^2 in controls and 89,167 μm^2 in perlecan heterozygous animals, but this difference was not significant (Fig. 3B). Female mice had larger lesions, but there was no difference with the presence of a perlecan knockout allele. En face assays also showed no difference in lesion area with perlecan deficiency, 2.8 ± 2% in apoE0 mice and 1.9 ± 1.2% in apoE0/perlecan heterozygous mice (average ± SD, $P = 0.2$).

Atherosclerosis in heterozygous LDL receptor-deficient mice

It has been hypothesized that the interaction of lipoproteins with proteoglycans might differ in mice that express apoE (19), a stronger proteoglycan binding protein than apoB. For this reason, we assessed whether a similar degree of atherosclerosis in heterozygous LDL receptor knockout mice would still show the effects of perlecan deficiency on atherosclerosis. For this reason, male mice were studied at 20 weeks. As shown in **Fig. 4**, perlecan deficiency did not alter atherosclerosis in this model. The average lesion areas in heterozygous LDL receptor-deficient and heterozygous perlecan-deficient mice in the LDL receptor heterozygous background are shown in **Table 5**.

DISCUSSION

Crossing perlecan heterozygous mice into an atherosclerotic background tested the hypothetical role for heparan sulfate proteoglycans in atherogenesis. Our data show the following. *1*) Although perlecan is primarily found in the subendothelial matrix in normal vessels, it is found in core regions of advanced atherosclerotic lesions. *2*) Despite a possible role of perlecan in the removal of lipoproteins from the circulation, a heterozygous deletion of perlecan did not alter plasma lipoprotein profiles. *3*) Young perlecan heterozygous knockout/apoE0 male mice had reduced atherosclerosis. A similar trend occurred in females. However, older mice and heterozygous LDL receptor-deficient mice had no difference in lesion size.

Endothelial cells produce a number of heparan sulfate proteoglycans when assessed in cultured cells. These include syndecans, which are a component of the cell membrane (20). Perlecan is the major heparan sulfate proteoglycan within the subendothelial matrix; some perlecan is also found associated with the apical side of the cells. Cultured smooth muscle cells express and secrete perlecan (14). Our immunohistological data suggest that both cells express perlecan in vivo, but at different times and under different stimuli. In normal vessels, perlecan was prominently found within the subendothelial matrix. However, with the development of lesions, perlecan staining was decreased in the subendothelial region. In humans, this may result in the decrease in arterial heparan sulfate proteoglycans reported with atherosclerosis (21).

TABLE 3. Lipid profiles of heterozygous perlecan-deficient mice in the LDL receptor heterozygous background on the Paigen diet for 16 weeks

Genotype	n	Cholesterol				Triglyceride	
		Plasma	VLDL	LDL	HDL	Plasma	VLDL
		mg/dl				*mg/dl*	
LDLr$^{+/-}$	13	579 ± 33	353 ± 44	219 ± 31	59 ± 5	55 ± 3	31 ± 2
LDLr$^{+/-}$/Prl$^{+/-}$	13	551 ± 30	307 ± 24	167 ± 11	62 ± 4	48 ± 3	23 ± 2

Lipoprotein profiles were assessed by ultracentrifugation. LDLr$^{+/-}$, heterozygous LDL receptor knockout mice; LDLr$^{+/-}$/Prl$^{+/-}$, heterozygous perlecan-deficient mice in the heterozygous LDL receptor background.

TABLE 4. Atherosclerotic lesions of heterozygous perlecan-deficient mice in the apoE0 background

Genotype	Gender	12 Weeks of Age n	12 Weeks of Age Lesion Size (μm^2)	24 Weeks of Age n	24 Weeks of Age Lesion Size (μm^2)
apoE0	Male	13	23,961 ± 26,075	11	121,961 ± 71,643
apoE0/Prl$^{+/-}$	Male	11	7,796 ± 7,949[a]	19	89,167 ± 49,973
apoE0	Female	13	28,940 ± 45,369	10	205,004 ± 107,623
apoE0/Prl$^{+/-}$	Female	14	19,789 ± 22,302	17	180,142 ± 63,035

[a] $P < 0.05$ compared with apoE0 mice.

The reasons for this decrease might be altered perlecan expression or an increase in its degradation. The latter might result from the actions of heparanase, which is secreted by endothelial cells in response to atherogenic stimuli (22).

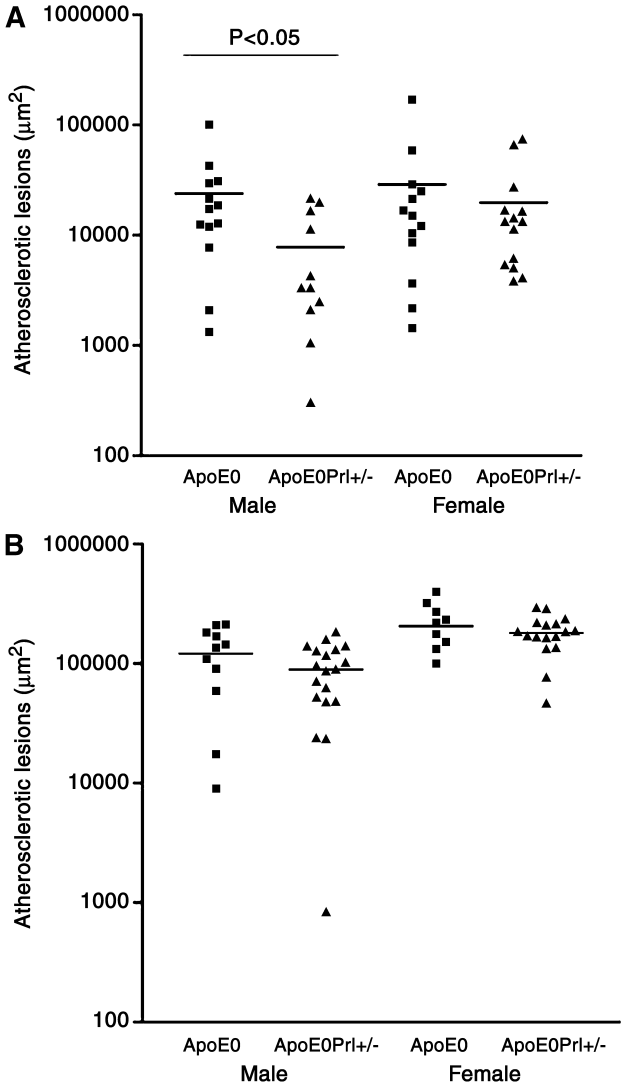

Fig. 3. Effects of heterozygous perlecan deficiency on atherosclerotic lesions. Heterozygous perlecan deficiency was crossed into the apoE knockout background. Male and female chow-fed mice were assessed for lipids and atherosclerosis after 12 weeks (A) and 24 weeks (B). The data obtained from sections of the aortic root are shown on a log scale. $P < 0.05$.

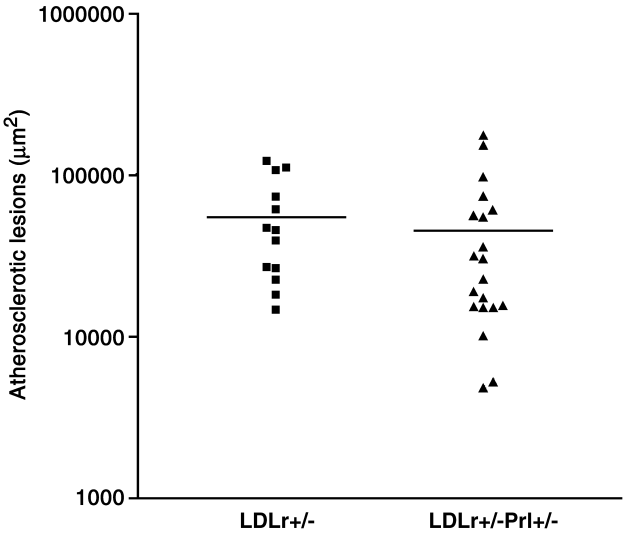

Fig. 4. Atherosclerosis in LDL receptor (LDLr) heterozygous knockout mice. Perlecan (Prl) heterozygous knockout mice were bred with LDL receptor knockout mice. The offspring, all with a heterozygous deficiency of LDL receptor, were fed a high-fat and cholesterol/cholic acid-containing diet (Paigen) for 16 weeks, and atherosclerotic lesion size at the aortic root was quantified.

Kunjathoor et al. (23) have demonstrated that the major proteoglycans detected in murine atherosclerosis are perlecan and biglycan. Perlecan was present extensively in the lesions of apoE-deficient and LDL receptor-deficient mice. Perlecan was also detected in intermediate and advanced lesions of hypercholesterolemic nonhuman primates and in cultures of medial smooth muscle cells from human atherosclerotic tissue. Our data comparing early and advanced lesions confirm their observations. Like others, we also found perlecan staining surrounding the lipid core (24). Moreover, we found remarkable amounts of perlecan in very advanced lesions containing necrotic lipid cores. Because these regions are distant from endothelial cells, we speculate that either smooth muscle cells or macrophages are the source of this perlecan. Because macrophages do not appear to express perlecan in great quantities (25), either smooth muscle cells or some other cells are the likely origin of most perlecan in mouse atherosclerotic lesions.

We had anticipated that perlecan deficiency would result in a defect in lipoprotein removal from the bloodstream, similar to that found in some diabetic models (26). However neither fasting nor postprandial lipemia differed between control and perlecan heterozygous mice.

TABLE 5. Atherosclerotic lesions of heterozygous perlecan-deficient mice in the LDL receptor heterozygous background

Genotype	Gender	20 Weeks of Age n	20 Weeks of Age Lesion Size (μm^2)
LDLr$^{+/-}$	Male	13	55,653 ± 10,505
LDLr$^{+/-}$/Prl$^{+/-}$	Male	20	45,361 ± 10,622

Mice were maintained on the Paigen diet for 16 weeks.

In fact, the rationale for the use of apoE0 mice was, in part, to allow us to assess the effect of perlecan deficiency on the removal of remnant lipoproteins. We hypothesized that the apoB-48-containing lipoproteins in these mice were unable to interact with either the LDL receptor or the LDL receptor-related protein and relied on heparan sulfate proteoglycan binding for removal. However, partial loss of perlecan was not physiologically significant. Perhaps the apoE-deficient lipoproteins were unable to bind to heparan sulfate proteoglycans, and therefore, perlecan deficiency had no effect.

To test some of these hypotheses, we performed a study in heterozygous LDL receptor knockout mice fed an atherogenic diet. These mice have sufficient hypercholesterolemia to develop atherosclerotic lesions, as noted by others (18); however, perlecan deficiency did not alter the lipoprotein profiles. Using heterozygous LDL receptor knockout mice, we attempted to create lesions that were similar in size to those of the 12 week old male apoE0 mice. Although the lesions were somewhat larger, perlecan deficiency did not alter the lesion size. Perhaps these lesions in the cholic acid-fed mice were more inflammatory and, hence, less affected by a gene deletion that would alter lipoprotein retention. Alternatively, the lesions studied might have been too advanced to find differences. Finally, the pathogenesis of lesions might differ in these two models as a result of the presence of apoE.

Although perlecan was a likely candidate to have affected atherosclerosis progression, the heterozygous mutation could have been expected to increase or decrease lesions. A central role for lipoprotein accumulation attributable to its binding to matrix molecules within the arterial walls was proposed almost 50 years ago by Page (1). The "infiltrative" theory of atherosclerosis suggested that plasma cholesterol-containing lipoproteins accumulate in vessels either because they are present in greater concentrations or that matrix is altered to increase the propensity for the lipoproteins to be retained within the artery. A refinement of this theory termed the "response to retention" suggested that proteoglycans are the matrix component that "traps" atherogenic lipoproteins (27). However, the biochemical evidence supporting an important role for lipoprotein-proteoglycan complexes has been questioned (28). Non-apoE-containing lipoproteins have relatively weak affinity for proteoglycans in physiological ionic conditions. This has led us (28, 29) and others (30) to suggest that lipoprotein-proteoglycan interaction requires an intermediary molecule such as lipoprotein lipase. The other lipoprotein-associating molecule that would be expected to increase lipoprotein-proteoglycan interaction, apoE, is antiatherosclerotic.

Our studies show that perlecan deficiency leads to less atherosclerosis. This was found in early, but not late, lesions of apoE0 mice. Most notable is the comparison of our data with those of Skalen et al. (19), who studied atherosclerosis in mice with an apoB mutation that decreases proteoglycan affinity. Like these authors, we found that later lesions were no longer significantly different from those of controls. In addition, like Skalen et al. (19), we failed to find a major difference in atherosclerosis when perlecan deficiency was assessed in the heterozygous LDL-deficient background. These authors postulated that apoE-containing LDL, found in mice but not in humans, has greater affinity for proteoglycans. Hence, the effects of alterations in apoB structure, and perhaps the amount of vessel wall perlecan, might be less evident in LDL receptor knockout mice.

We conclude that perlecan is a major component of advanced atherosclerotic lesions in the mouse. A heterozygous deficiency of perlecan led to reduced atherosclerosis in apoE0 mice. This effect was found in young males without advanced lesions. Although it is possible that perlecan deficiency alone reduced atherosclerosis as a result of reduced lipoprotein retention, the complexity of the process and the multiple biological actions of perlecan do not allow one to make that conclusion with certainty.

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