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Decorin and biglycan retain LDL in disease-prone valvular and aortic subendothelial intimal matrix

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Abstract

Objective—Subendothelial LDL retention by intimal matrix proteoglycans is an initial step in atherosclerosis and calcific aortic valve disease. Herein, we identify decorin and biglycan as the proteoglycans that preferentially retain LDL in intimal matrix at disease-prone sites in normal valve and vessel wall.

Methods—The porcine aortic valve and renal artery ostial diverter, initiation sites of calcific valve disease and renal atherosclerosis, respectively, from normal non-diseased animals were used as models in these studies.

Results—Fluorescent human LDL was selectively retained on the lesion-prone collagen/proteoglycan-enriched aortic surface of the valve, where the elastic lamina is depleted, as previously observed in lesion-prone sites in the renal ostium. iTRAQ mass spectrometry of valve and diverter protein extracts identified decorin and biglycan as the major subendothelial intimal matrix proteoglycans electrostatically retained on human LDL affinity columns. Decorin levels correlated with LDL binding in lesion-prone sites in both tissues. Collagen binding to LDL was shown to be proteoglycan-mediated. All known basement membrane proteoglycans bound LDL suggesting they may modulate LDL uptake into the subendothelial matrix. The association of purified decorin with human LDL in an *in vitro* microassay was blocked by serum albumin and heparin suggesting anti-atherogenic roles for these proteins *in vivo*.

Conclusions—LDL electrostatic interactions with decorin and biglycan in the valve leaflets and vascular wall is a major source of LDL retention. The complementary electrostatic sites on LDL or these proteoglycans may provide a novel therapeutic target for preventing one of the earliest events in these cardiovascular diseases.

Keywords

LDL retention; decorin; biglycan; aortic valve; renal ostia; collagen; elastin; two photon microscopy

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Disclosures

None

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Introduction

Cardiovascular lesions, including arterial atheroma and valvular calcification, though divergent in their pathogenic pathways, may share a common mechanism of initiation, namely LDL retention¹⁻⁵. LDL retention by proteoglycans in the arterial wall is currently thought to lead to maladaptive responses by cells residing in the subendothelial matrix and consequent matrix remodeling and calcification⁶⁻⁸. Although a considerable number of studies support the role of LDL retention in atheroma initiation, to date there is scant evidence for a role in valvular disease. Heart valve leaflets are markedly enriched with proteoglycans and subendothelial LDL accumulation is an early event in valvular degenerative disease, preceding calcification⁹. Valvular calcific disease is associated with elevated plasma LDL levels^{10, 11} and lowering plasma cholesterol has been shown to halt the progression of aortic valve disease in a mouse model¹². Thus, LDL retention by valvular proteoglycans may also serve as an initial step in valvular disease^{2, 3}.

The role of LDL-proteoglycan interactions in atherogenesis has been extensively discussed and documented¹³⁻¹⁵. We hypothesize that specific electrostatic interactions with a subset of proteoglycans within the normal vascular wall and valve structures is responsible for the majority of subendothelial LDL retention. This hypothesis is based on numerous LDL binding assays and presence of specific proteoglycans within these vascular structures¹⁶. LDL affinity chromatography has shown that the chondroitin/dermatan sulfate chains of arterial proteoglycans bind to LDL¹⁷ *in vitro*. Conversely, aortic proteoglycan affinity chromatographic studies have identified LDL particle characteristics that promote LDL-proteoglycan binding¹⁸. Differential proteomic screening of atherosclerosis-prone vs. non-prone arteries found lumican to be the sole proteoglycan enriched in atherosclerosis-prone arterial intima¹⁹. Immunohistochemical and biochemical analyses identified a number of proteoglycans that are enriched in both atherosclerotic lesions and calcific aortic valves, including decorin, biglycan, and versican^{15, 20}. Angiotensin II, which is known to potentiate the development of atherosclerosis, has recently been shown to increase vascular biglycan and perlecan content in the LDL receptor-deficient mouse, preceding atherosclerotic lesion development²¹.

In the present study, we conducted proteomic analyses by affinity chromatography of the entire soluble protein fraction of normal aortic valves and renal artery ostia to identify the proteoglycans that specifically bind to native LDL prior to onset of disease. Once identified, the topological distribution of these LDL-interacting proteins was evaluated using histological techniques. In addition, a variety of affinity columns were created that are capable of screening drug or other molecular interactions with the LDL or proteoglycan electrostatic interaction sites.

Material and Methods

Histological Analyses

Aortic valve leaflets (N=6) and renal ostia (N=6) harvested from anesthetized and heparinized (10,000 U IV) 16- to 32-week-old pigs were immediately placed in 10% buffered formalin, embedded in paraffin and 4 µm thick serial sections were stained with hematoxylin and eosin, Masson's trichrome, Elastic Van Gieson's and Movat stains. Images were taken by using Leica macro-(M420) and microscopes (DMRXA) with DC500 digital camera. All animal experiments were performed in accordance with a research protocol (ASP# 0123) approved by the Animal Care and Use Committee of the National Heart, Lung, and Blood Institute of the National Institutes of Health.

LDL Preparation

Briefly, LDL was isolated from 250 mL of freshly collected donor human plasma by sequential ultracentrifugation (100,000 rpm @ 4°C for 5 hr) after density adjustment with KBr (d=1.019 to 1.063 g/mL) with a TLA-100.2 rotor on a Beckman tabletop ultracentrifuge (Beckman Instruments, Palo Alto, Calif). After extensive dialysis against PBS containing EDTA and sodium azide, particle integrity was confirmed by native agarose gel electrophoresis. LDL concentration was estimated by measuring protein concentration. For microscopy studies, LDL was labeled with Alexa Fluor 647 protein labeling kit (Invitrogen), which has a succinimidyl ester moiety that reacts with primary amines of proteins to form stable dye-protein conjugates, according to manufacturer's instructions, as previously described^{22, 23}. At the last step of the labeling process, LDL was eluted from the separation column using binding buffer (BB: 10mM Hepes, 20 mM NaCl, pH 7.0). For affinity chromatography, LDL extensively dialyzed against coupling buffer (0.1M NaHCO₃, 0.5M NaCl, pH 8.3) was coupled to Sepharose 4B beads (GE Healthcare Biosciences AB, Uppsala, Sweden), according to manufacturer's instructions. At the last step of the coupling process, LDL was washed, and resuspended in BB. LDL preparations were used immediately to minimize oxidation.

Two-photon Excitation Microscopy

Two-photon images were taken at room temperature with a Zeiss LSM 510 META^{22, 23}. LDL labeled with Alexa Fluor 647 was bound to freshly isolated porcine aortic valve leaflets (N=3), and imaged as previously described^{22, 23}. Briefly, two-photon images were taken at room temperature with an LSM 510 META microscope (Zeiss, Thornwood, NY) with a X10, 0.3-numerical aperture, or, a X40 1.3- numerical aperture oil-immersion objective (Zeiss). A pulsed Ti:sapphire Laser (Mai-Tai) set at 860 nm was used for excitation, and emission spectra were collected to optimize the separation of collagen and elastin signals, as previously described^{22, 23}. Collagen second harmonic generation (SHG) was detected at 415–430 nm, elastin fluorescence at 500 to 550 nm, and Alexa Fluor 647 (LDL) at 650–710 nm. Three-dimensional images were collected as a stack of images at 2 µm spacing starting just above the luminal surface. Arrays of image stacks were collected using the MultiTime Series Macro (Zeiss). The individual z-series were rendered as maximum projection images using Zeiss software and then manually tiled. LDL binding was quantified by measuring the integrated pixel intensity in each section of the z-stack using MetaMorph software.

Protein Extraction

Fresh or frozen porcine aortic valve leaflets in 1 ml cold proteoglycan extraction buffer (50 mM Tris, pH 7.4, containing 7 M urea, 0.1 M NaCl, complete protease inhibitor mixture (Roche Applied Science) and 5 mM dithiothreitol) were mechanically ground (MPbio), extracted for 24 hrs at 4 °C, then centrifuged and the supernatant collected. The pellet was resuspended, mechanically ground, extracted for 24 hrs at 4°C, and centrifuged. Combined supernatants from all three leaflets were used for analyses. Porcine caudal renal ostia (renal diverter) were dissected from the aorta on ice, and then frozen at –80°C. Prior to extraction, the adventitia and the bulk of the media were dissected from frozen ostia on an aluminum block on dry ice, under a dissecting scope. Protein extracts from nine dissected renal diverter samples were combined prior to application to LDL affinity columns.

LDL Affinity Column Chromatography

Analysis of aortic valve and renal ostial diverter proteins—LDL (60 mg) was coupled to sepharose 4B (3 gm) according to the manufacturer's instructions generating a column bed of ~10 ml. Protein extracts were dialyzed against BB for 48 hrs prior to

application to LDL-sepharose 4B affinity columns. Protein extract in 10 ml BB was allowed to bind to the coupled LDL-sepharose 4B columns. Following flow-through (FT) collection, columns were washed with 100 ml BB, and then eluted with 100 ml of 250 mM NaCl. We used 250 mM NaCl since no additional protein eluted with 500mM or 1M NaCl, consistent with previous reports¹⁸. FT and eluate (E) samples were concentrated using centrifugal filters (Amicon). No proteins were detected in eluates of protein extracts run on end-capped Sepharose 4B bead columns (blocked using Tris buffer), confirming the specificity of the LDL columns.

Microassays

LDL mini-affinity columns—Human LDL, or, human albumin were coupled to sepharose 4B (0.28 gm), generating a column bed of ~0.25 ml containing 4 mg LDL, or, 4 mg albumin. 50 µg of purified decorin (Sigma), lumican (R&D), heparin (Sigma) or IgG dissolved in 500 µl BB was applied to the column and incubated for 15 min at RT prior to centrifugation at 2000 x g for 1 min to generate the initial FT. Columns were then washed 4X with 500 µl BB and centrifuged. The first two washes were added to the initial FT. Columns were eluted with 250 mM NaCl (500 µl, 2X) and the filtrates were combined. FT (non-LDL-binding protein) and E (LDL-binding protein) were concentrated and digested with chondroitinase ABC prior to SDS-PAGE analysis.

Collagen I mini-affinity columns—Human recombinant collagen I (1 mg) was coupled to sepharose 4B (0.28 gm) as above, generating a column bed of ~0.25 ml containing 1 mg collagen. 10 or 50 µg of LDL, and, 50 µg of purified decorin (Sigma) in 500 ml of BB were applied to the columns, alone or in combination, and then processed as above. Following the 250 mM NaCl elution, a portion of the columns were washed, and then additionally eluted with 7M urea (50 mM Tris, pH 7.4, 0.1 M NaCl; 2x, 500 µl), or, 0.1% SDS (25 mM Tris, pH 8.3, 192 mM glycine; 2x, 500 µl), then washed with binding buffer (500 µl, 4X).

Decorin Immunoblotting

Flow-through and eluates of purified decorin (100 µg) applied to LDL mini-columns, and treated as above, were loaded onto 10% SDS-PAGE, transferred to nitrocellulose blotting paper, incubated overnight at 4°C in polyclonal mouse anti-human decorin antibody (LF-122; diluted 1:2000), and then with fluorescent rabbit anti-mouse secondary antibody (diluted 1:10000) for 1 hr. Immunoblots were imaged with a Typhoon 9400 Variable Mode Imager (GE).

Proteomic Analyses

iTRAQ Labeling—Paired flow-through and eluate samples were collected from three different LDL affinity column runs using porcine aortic valve protein extracts from three different animals, or, three different pools of dissected renal diverter protein extracts, and prepared for iTRAQ 8-plex labeling (ABSCIEX, Foster City, CA), per manufacturers instructions. Approximately 100 µg of each sample was acetone precipitated by using 6 volumes of chilled acetone placed in -20 C for overnight precipitation. Vials were centrifuged at 10,000 g for 10 minutes at 4 C. The acetone was removed and the pellets were air dried briefly before starting the iTRAQ 8-plex labeling (ABSCIEX), per the manufacturer's instructions. LDL affinity column flow-through (FT) samples 1–3 were labeled with iTRAQ tags 118,119,121 (valve) or 114,115,116 (diverter), and LDL affinity column eluate (E) samples 1–3 were labeled with tags 114,115,116 (valve) or 118,119,121 (diverter), respectively. After stopping the labeling, the resulting peptide mixtures were combined and dried until the final volume of 100 µL was achieved. The combined peptide digest was resuspended in 900 µL of 0.1%FA and desalted using Waters Oasis HLB 1 cm³

cartridges (Milford, MA) per the manufacturer's instructions using acetonitrile instead of methanol. Eluent was dried and resuspended with 100 μ L SCX buffer A (10mM KH_2PO_4 /25% acetonitrile, pH 3.0). The combined peptide digest was fractionated offline by strong cation exchange chromatography (SCX) followed by online reverse phase liquid chromatography - tandem mass spectrometry.

Strong Cation Exchange Chromatography (SCX)—Agilent 1200 series HPLC was used to separate the peptides by their charge using a polysulfoethyl A column; 200×2.1 mm, particle size 5 μ m, 200 $\text{\AA}$ (Poly LC, Columbia MD). A 60 min linear ramp from 0%–40% Buffer B (10mM KH_2PO_4 /500mM KCL/25% ACN, pH3.0) was used to separate the peptides. Column temperature was maintained at room temperature and a flow rate of 200 μ L/min throughout the run. Fractions were collected at 1 min intervals on a 96 well microtiter plate for a total of 60 fractions. The chromatographic peaks were monitored using the built in UV detector (214nm) and fractions were combined for a final count of 28 fractions. Each fraction was desalted using Waters Oasis HLB 1 cm^3 cartridges (Milford, MA) per the manufacturer's instructions using acetonitrile instead of methanol. Liquid chromatography - tandem mass spectrometry was performed using an Eksigent nanoLC-Ultra 1D plus system (Dublin, CA) coupled to an LTQ Orbitrap Velos mass spectrometer (Thermo Fisher Scientific, San Jose, CA). 10 μ L of the peptide digest was first loaded onto an Zorbax 300SB-C18 trap column (Agilent, Palo Alto, CA) at a flow rate of 6 μ L/min for 6 min, and then separated on a reversed-phase PicoFrit analytical column (New Objective, Woburn, MA) using a 40-min linear gradient of 5–40% acetonitrile in 0.1% formic acid at a flow rate of 250 nL/min. LTQ-Orbitrap Velos settings were as follows: spray voltage 1.5 kV; full MS mass range: m/z 300 to 2000, operated in a data-dependent mode. A single full-scan MS in the Orbitrap (30,000 resolution, 300–2000 m/z) was followed by six data-dependent MS2 scans for precursor ions above a threshold ion count of 50000, using the HCD cell with the resolution set to 7500 and 45% normal collision energy.

All mass spectrometry data were generated using an LTQ-Orbitrap Velos (Thermo Fisher Scientific, San Jose, CA) mass spectrometer and analyzed with Proteome Discoverer v.1.2 software (Thermo Fisher Scientific, San Jose, CA) using Mascot search engine (Matrix Science, Boston, MA). For protein identifications to be considered, a minimal of 2 unique peptides or more with a false discovery rate (FDR) of less than 1% was required. The raw files generated from the LTQ Orbitrap Velos were analyzed using Proteome Discoverer v1.2 software (Thermo Fisher Scientific, LLC) using our six-processor Mascot cluster at NIH (<http://biospec.nih.gov>, version 2.3) search engine. The search criteria was set to: database, Swiss-Prot (Swiss Institute of Bioinformatics); taxonomy, human; enzyme, trypsin; miscleavages, 2; variable modifications, oxidation (M), deamidation (NQ), iTRAQ 8plex tyrosine; fixed modifications, (MMTS) methyl methanethiosulfonate (C), N-terminal iTRAQ8plex, iTRAQ8plex lysine, MS peptide tolerance 10 ppm; MS/MS tolerance as 0.05 Da. The automatic decoy database search option was selected and the high confidence (FDR, 0.01) peptides were only accepted for protein identification. Briefly, every time a peptide sequence search is performed on a target database a random sequence of equal length is automatically generated and tested. The statistics for matches are calculated and a peptide significance is generated, an in depth explanation can be found at the Matrix Science website (www.matrixscience.com).

The calculated ratio (E/FT) of the total protein signal in E relative to that in FT was used as a measure of the relative binding affinity of the extracted tissue proteins to LDL on the columns. The E/FT values for the renal diverter were corrected for protein losses during processing by normalization to total E and FT peptide signal intensities. We determined that the porcine and human protein sequence databases provided similar values for the E/FT ratios for the aortic valve and renal diverter proteins that were identified by both databases.

Searching the human sequence database identified a markedly greater number of proteins (valve: 239 vs 771; diverter: 282 vs 1012, porcine vs human, respectively), insofar as the human sequence database is complete, while that of the pig is not. For these reasons we present the findings using the human sequence database analyses.

Decorin Immunohistochemistry—Deparaffinized sections treated with chondroitin ABC lyase (to digest GAG chains) and then with 0.3% H₂O₂/methanol (to block endogenous peroxidase) were incubated with mouse anti-bovine decorin 6D6 antibodies (Developmental Studies Hybridoma Bank), followed by biotinylated IgG, ABC, and DAB, as previously described²⁴, then counterstained with hematoxylin.

Results

Our previous studies showed the presence of a dense collagen/proteoglycan matrix in the absence of a luminal elastin barrier contributes to increased retention of LDL in the arterial intima at numerous sites^{22, 23}. To characterize LDL binding sites on the lesion-prone aortic surface of the aortic valve, we performed conventional histological analyses and two-photon excitation *en face* microscopy. As seen in Fig. 1, the ventricular surface of the valve is relatively collagen-poor with a thick elastic lamina. In contrast, the aortic surface of the valve (Fig. 1) is characterized by dense collagen bands embedded in proteoglycan-enriched regions that are covered by a very thin elastic lamina. These histological analyses were consistent with exposed LDL binding sites on the aortic surface of the aortic valve, as previously observed on lesion-prone arterial wall sites^{22, 23}. To directly assess LDL binding, endothelium-denuded valves were incubated with fluorescent-tagged human LDL at 37°C for 3 hrs in a low ionic strength buffer to facilitate binding. As seen in Fig. 1N, LDL binding is markedly enhanced on the lesion-prone aortic surface of the valve. LDL binding at this site is likely underestimated since reaction of Alexa-fluor with lysine residues on apo B-100 may reduce the positive charge of its proteoglycan binding groups.

We performed quantitative iTRAQ proteomic analysis of aortic valve and renal diverter tissue protein extracts to identify the proteins that are electrostatically retained on LDL-affinity columns. We calculated the ratio of the amount of protein present in the eluate (E) compared to that in the flow-through (FT) as a measure of relative LDL binding affinity. This analysis identified a total of 771 proteins in the aortic valve and 1012 proteins in the renal diverter having 2 or more unique peptide fragments, and revealed that a subset of aortic valve and renal diverter proteins are enriched in the LDL-affinity column eluate (Fig. 2; Supplemental Tables 1&2). The vast majority of the aortic valve and renal diverter proteins (>95%) had a mean E/FT ratio value of ~3.0 or less. We arbitrarily selected the E/FT value of 3.0 or greater (top 5%) to represent relatively high LDL binding affinity. A number of nuclear and cytosolic cellular proteins were found to be relatively enriched in the LDL affinity column eluates (Supplemental Tables 1&2). Since these proteins were extracted from lysed cells during protein solubilization prior to application of tissue extracts to the LDL-affinity columns, we assumed these to be an artifact of the *in vitro* protein association assay, representing proteins that LDL would not likely access *in vivo*, and were thus disregarded.

Several extracellular proteins, including proteoglycans and collagens, were enriched in the LDL affinity column eluates (Table 1; Supplemental Tables 1&2). All of the proteoglycans known to be present in the aortic valve and renal diverter were identified (Fig. 2). Many proteoglycans displayed little or no enrichment in the eluate including fibromodulin, lumican, keratocan, prolargin, chondroadherin, mimican, and aggrecan (Table 1). Simple abundance of a protein did not correlate with LDL binding affinity ($R^2 = 0.011$) suggesting significant binding specificity. The proteoglycans that exhibited enhanced binding to LDL

(E/FT = 3.0) represent two distinct tissue compartments, namely, the subendothelial basement membrane (BM), and subendothelial intimal matrix. The intimal matrix proteoglycans, decorin and biglycan showed a notable degree of enrichment in the LDL affinity column eluates in both the aortic valve (mean E/FT ratio of 3.3 and 4.0, respectively; Table 1) and renal diverter (mean E/FT ratio of 4.6 and 3.6, respectively; Table 1). The BM proteoglycans agrin and perlecan, as well as the BM collagens XV and XVIII, which are chondroitin sulfate²⁵ and heparin sulfate²⁶ proteoglycans, respectively, were also notably enriched in LDL affinity column eluates. Chondroitin sulfate proteoglycan 4 (CSPG4), a membrane spanning cell surface protein that binds to collagens V and VI competitively with decorin²⁷, was also found to bind to LDL with relatively high affinity.

A subset of non-proteoglycan collagen proteins was also retained on LDL columns, including the fibrillar collagens I and II, which bind to both decorin and biglycan core proteins²⁸, as well as collagens V and XI, which are essential for the assembly of collagen I and collagen II²⁹ fibrils, respectively. In addition, fibronectin type III domain-containing protein and tenascin-X (which contains fibronectin type III repeats) were retained. Both of these proteins bind to collagens^{29, 30} and proteoglycans^{31, 32}.

As shown in Fig. 3, decorin content is enhanced in lesion-prone subendothelial intimal matrices. Immunohistochemical analyses revealed that decorin is widely abundant in the aortic valve with increased decorin content on the aortic surface of the valve, and, is abundant in the caudal renal ostium (diverter), but not in the non-lesion prone cranial renal ostium²³.

We developed a rapid and accurate microassay to monitor proteoglycan-LDL interactions *in vitro*. When purified decorin was applied to the large bed LDL affinity columns used to identify LDL-binding proteins in the aortic valve and renal diverter, LDL binding sites were present in excess, and nearly all the decorin was electrostatically associated with LDL (not shown). LDL in the microassay column bed was decreased such that a detectable amount of decorin would occupy all of the LDL sites, allowing us to identify competitive inhibitors of this potentially important binding interaction (Fig. 4A). We balanced the ratio of column LDL with applied decorin to attain a ~35% retention of decorin to perform this task. Little, if any, purified lumican bound to LDL columns (Supplemental Fig. 1A), consistent with the observed specificity of the interaction of aortic valve and diverter decorin with LDL (Table 1). Heparin, which has been shown to interfere with LDL-proteoglycan interactions³³, inhibited the binding of purified decorin to LDL by ~60% at a ratio of heparin:decorin of 1:1 (Fig. 4A). Higher concentrations of heparin nearly eliminated decorin retention, thus validating the microassay as a means of screening for inhibitors (Fig. 4B).

To assess the specificity of the decorin binding, we compared the binding of decorin to LDL vs albumin sepharose 4B minicolumns. Although decorin binding to LDL surpassed decorin binding to albumin, decorin binding to albumin was substantial (Fig. 4C). Albumin blocked the ability of decorin to bind LDL minicolumns (Fig. 4D). Albumin itself did not bind to the LDL minicolumns (Fig. 4D, Supplemental Fig. 1C), and no albumin was detected on LDL prepared for coupling to the beads (Supplemental Fig. 1B). These findings suggest that albumin binding to decorin blocks decorin binding to LDL.

Proteoglycan binding sites identified on LDL apoB include lysine residues³⁴. To determine whether lysine-mediated binding of decorin is specific to apoB, we blocked lysine residues on sepharose 4B-bound LDL and albumin via acetylation, using sulfo-NHS-acetate. Sulfo-NHS-acetate pre-treatment completely blocked decorin binding to both LDL and albumin minicolumns (Fig. 4C), suggesting that lysine residues on both LDL and albumin are required for decorin binding. To further assess the specificity of plasma protein binding to

LDL, we determined the ability of plasma IgG proteins to compete with decorin binding to both LDL and albumin minicolumns. As seen in Supplemental Fig. IB, IgG proteins did not bind to either LDL or albumin (not shown) minicolumns, and did not alter the ability of albumin to bind to either type of column. Thus, albumin, but not all plasma proteins, can bind to decorin.

As noted above, fibrillar collagens I and II as well as fibril assembly collagens V and XI in tissue protein extracts were retained on large-scale LDL affinity columns. It is likely that these collagens assembled into fibrils that were retained on the LDL columns by virtue of their association with the proteoglycans decorin and biglycan, and possibly others. Collagen VI, which assembles into microfibrils forming networks that link cells to the matrix³⁵, was also retained on the LDL columns. Insofar as both biglycan and decorin bind to the collagen VI triple helix³⁶, its retention on the column might also be proteoglycan-mediated.

To gain insight as to whether proteoglycans might mediate retention of collagens on LDL columns, we assessed the effect of decorin on LDL binding to collagen I affinity minicolumns, a model for arterial subendothelial intima. As seen in Fig. 5A, a large fraction of the applied decorin was electrostatically retained on collagen I columns. Very little LDL bound to collagen I columns when applied alone. However, LDL retention on collagen columns was markedly increased when decorin was pre-bound, consistent with LDL binding to decorin retained on the column. This point is dramatically illustrated in Fig. 5B, where a large bolus of LDL was applied. LDL binding to decorin on the collagen columns was confirmed to be electrostatic in nature since little, if any decorin or LDL could be eluted from collagen I columns by the protein denaturing agents SDS, or, urea following salt elution (Supplemental Fig. 2). These findings suggest that the retention of collagen contained in aortic valve and diverter tissue extracts on LDL affinity columns was likely mediated by tissue proteoglycans bound to LDL.

Discussion

One of the earliest stages of atherosclerosis⁸ and likely some forms of valve disease^{2, 3} is the subendothelial retention of LDL in the macromolecular matrix. Herein we have identified decorin and biglycan as the major sources of LDL retention in normal, non-diseased aortic wall and valve leaflets via electrostatic interactions mediated by lysine residues on apoB. Potentially, this electrostatic interaction modified at either the LDL or proteoglycan site may provide a new therapeutic target for the prevention of atherosclerosis and associated valve disease at its earliest stages.

We have previously shown that in normal, non-diseased tissue, LDL binds to exposed proteoglycan-rich regions of dense collagen at lesion-prone arterial branch points where the internal elastin membrane is thin and porous^{22, 23}. In the present study, we have observed, in a similar manner, that LDL binding to the aortic valve in normal, non-diseased tissue, is markedly enhanced on the lesion-prone, collagen and proteoglycan-dense, elastin-poor aortic surface. Thus, we have extended our previous observation that the macromolecular structure of arterial subendothelial matrix is itself a determinant of disease susceptibility to the subendothelial matrix of the aortic valve, and, have shown for the first time that LDL can be retained in lesion-prone sites in normal valve tissue, prior to the onset of disease. This finding is consistent with the proposed role of lipid retention in the initiation of valvular disease.

Decorin has been shown to bind to collagen³⁷, to mediate LDL binding to collagen³³, and, to play a role in regulating collagen fibrillogenesis³⁸. Biglycan binds to collagen *in vitro* and both of these proteoglycans organize collagen networks by interconnecting microfibrillar

collagens (i.e., collagen VI) with fibrillar collagens^{36, 39}. The significant retention of collagens I and II as well as fibril associated collagens V and XI from the aortic valve and renal ostia on LDL columns appeared to be proteoglycan-mediated insofar as LDL retention on collagen columns was nearly completely dependent on pretreatment with decorin (Fig. 5). This finding provides a molecular basis for our histological, immunohistochemical, and 2-photon microscopic observations that LDL-retention in the aortic valve and renal diverter occurs at discrete disease-prone sites that are collagen-dense and enriched with proteoglycans

In addition, we have discovered that all of the BM proteoglycans bind LDL with relatively high affinity, raising the possibility that the subendothelial BM may play a role in modulating the entry of LDL into the subendothelial intimal matrix. BM proteoglycans may restrict the access of LDL to decorin and biglycan in the intimal matrix and in this regard may be atheroprotective. Consistent with this notion, loss of collagen XVIII enhances vascular permeability to lipids and increases the content of atheroma lipids in an atherosclerotic mouse model⁴⁰. Further studies are needed to provide evidence to support this hypothesis.

We have identified the proteoglycans decorin and biglycan as the major subendothelial intimal LDL binding proteins in both the vessel wall and valve leaflets based on their high retention on LDL affinity columns coupled to their high copy number (Table 1) and immunohistochemical localization (Fig. 3). Decorin and biglycan retained their binding affinity to LDL even in the presence of competing extracellular and intracellular proteins (the latter of which they are not normally exposed to), suggesting an effectively high affinity of these glycans to the LDL molecule. We confirmed that the electrostatic interaction of decorin with both LDL and albumin is dependent on the positive charge of their lysines since selective elimination of these sites with sulfo-NHS-acetate eliminated the interactions. The lysines responsible for this electrostatic interaction have been shown to be in the site B region (3359–3369) of apo B on the native LDL particle³⁴. Exposed Lys 195 and Lys 199^{41, 42} on the albumin molecule may be responsible for the interaction with decorin, consistent with the well characterized electrostatic interactions of albumin with numerous plasma proteins and drugs⁴³. The other major plasma protein, gamma globulin, did not bind to LDL or decorin.

What might be the best target for interfering with the electrostatic interaction between LDL lysines and the decorin and biglycan glycans in disease-prone cardiovascular sites? *In vivo* validation for this therapeutic approach has in part been provided by studies which showed that mutations in the proteoglycan binding site on LDL⁴⁴, or conversely, the administration of antibodies that bind to extracellular matrix glycosaminoglycans⁴⁵, markedly reduce the development of atherosclerotic lesions in mouse disease models. However, due to their abundance and wide range of overlapping functions, glycans are unlikely to be feasible drug targets. Moreover, based on our findings, the lysines of proteoglycan-bound serum albumin would likely shield these sites from LDL. A specific agent that would shield the lysines on LDL might be a viable therapeutic target. In this study we have provided a simple and robust assay for LDL association with proteoglycans using a human LDL micro-affinity column. One model drug, heparin, was shown to shield the LDL sites from the interaction with decorin. A screen for drugs with high affinity and specificity for the lysine binding sites on LDL might prove to be fruitful, if interaction with these sites does not negatively impact cellular uptake or metabolism of the LDL particle.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

LDL	low density lipoprotein
E/FT	effluent/flow-through ratio
BM	basement membrane

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Highlights

- Lesion-prone vascular LDL-binding sites are collagen and proteoglycan enriched
- Decorin and biglycan retain LDL in lesion-prone arteries and heart valve leaflets
- Collagen binding to LDL is proteoglycan-mediated
- Basement membrane proteoglycans may modulate LDL entry into vascular intima
- Blocking the LDL-proteoglycan interaction may prevent arterial and valve disease

Figure 1. LDL binds to the elastin-poor, collagen/proteoglycan rich aortic surface of the aortic valve

Histological analysis. The aortic valve was stained with Elastic Van Gieson's stain for elastin (*black*; **A, D, E, F**), Masson's trichrome stain for collagen (*blue*; **B, G, H, I**) and Movat stain for proteoglycans (*green*; **C, J, K, L**). Regions boxed in **A, B**, and **C** are shown enlarged in **D–F, G–I**, and **J–L**, respectively. Note the aortic surface (**Ao**) of the valve has regions containing dense collagen bands that are embedded in proteoglycan, while the ventricular surface (**V**) of the valve is relatively collagen-poor, and has a much thicker elastic lamina. Scale bars = 400 μm (**A–C**); 100 μm (**D–L**). Representative sections of six porcine valves. (**M–O**) Distribution of LDL binding to the aortic valve. Porcine aortic valve leaflets were incubated with fluorescent LDL and imaged ($N=6$). Tiled (8X8) and (8X10) two-photon maximum projection of z-series images through 170 μm and 162 μm of the luminal surface of the aortic valve, for (**m**) and (**n**), respectively. (LDL shown in *white*). (**M**) Ventricular surface of valve. Scale bar = 500 μm . (**N**) Aortic surface of valve. Scale bar = 500 μm . (**O**) Distribution of LDL pixel intensity in images of aortic (**N**) and ventricular (**M**) surfaces of aortic valve. Note the signal is much greater on the aortic surface of the valve leaflet.

Figure 2. Proteomic analysis of LDL binding proteins in the aortic valve and renal ostial diverter Histograms of the distribution of LDL affinity column Eluate/Flow through (E/FT) values of all the tissue proteins detected by iTRAQ analysis. Aortic valve (**A**) and (**B**) renal ostial diverter protein enrichment in LDL affinity column eluates. The 5% of total proteins with E/FT values ≥ 3.0 (55 proteins in the valve, 64 proteins in the diverter) were arbitrarily designated as relatively high LDL binding affinity proteins.

Figure 3. Decorin is enriched in lesion-prone subendothelial matrices

Immunohistochemical analyses. (A–E). Decorin distribution in the renal ostium. (A) Ao denotes aortic lumen. Decorin (*brown*) is abundant in the caudal renal ostium (Ca; box, enlarged in (C) and (E)), but is nearly absent in the cranial renal ostium (Cr, box enlarged in (B) and (D)). Arrows in (D) denote the inner elastic membrane which delineates the subendothelial matrix. Decorin immunostaining in the caudal renal ostium is extracellular and fibrillar in appearance (E). Decorin is also abundant in the adventia (small arrows in (A)) outside of and surrounding the aorta. (F–I) Decorin distribution in the aortic valve. Decorin is widely abundant in the aortic valve. (F) Low magnification of aortic valve. Region in box (F) shown at higher magnification in (G). Decorin in the subendothelial matrix of aortic surface directly underlies the endothelium (I) whereas decorin on the ventricular surface is deeper below the endothelium (H). Scale bar = 500 μ m (A); 250 μ m (F); 100 μ m (B,C); 25 μ m (B,C,E); 5 μ m (D,E,H,I).

Figure 4. Decorin binding to LDL in vitro

SDS-PAGE analysis of purified decorin binding to LDL affinity microcolumns.

Chondroitinase ABC-digested flow-through (FT; non-LDL-binding protein) and the high salt (250mM) eluate (E; LDL-binding protein) were stained with Sypro-Ruby. **(A)** Heparin blocks decorin binding to LDL. Decorin (50 μg) was applied to LDL minicolumns in the absence (-Hep) or presence (+Hep) of 50 μg heparin. Note heparin markedly reduced decorin binding to LDL. **(B)** Dose response curve of heparin-mediated inhibition of decorin-LDL interaction. **(C)** Lysine residues on both LDL apoB and albumin mediate their binding to decorin. Decorin (50 μg) was applied to non-treated (Control) or sulfo-NHS-acetate pre-treated ((+)S-NHS-acetate) LDL or albumin minicolumns. Note that sulfo-NHS-acetate pre-treatment prevented decorin binding to both LDL and albumin. **(D)** Decorin (50 μg) and albumin (50 μg) were applied alone (Dec, and Alb, respectively), or, in combination (Dec + Alb) to LDL minicolumns. Note albumin markedly reduced decorin binding to LDL and that albumin itself does not bind to LDL columns. The albumin bands appearing in the lanes in which albumin had not been applied to the columns (i.e., Dec E), represent albumin present in the chondroitinase ABC reaction mixture. **(E)** Albumin (50 μg) applied to LDL affinity columns in the absence of decorin is not retained. Note the flow through (FT) and eluate (E) were not digested with chondroitinase ABC prior to SDS-PAGE.

Figure 5. Decorin mediates LDL binding to collagen I

SDS-PAGE analysis of LDL and purified decorin binding to collagen I affinity microcolumns. The flow-throughs (FT, FT-L, FT-D) and high salt (250mM) eluates (E) were concentrated, digested with chondroitinase ABC, run on SDS-PAGE, and stained with Sypro-Ruby. Decorin (50 µg) alone, LDL alone ((**A**) 10 µg, or (**B**) 50 µg), or, Dec then LDL, [decorin (FT-D) followed by LDL (FT-L)], was applied to collagen I minicolumns. Note: Decorin binding to collagen I columns is considerable (**A**; Dec); LDL binding to the columns is markedly increased when decorin is pre-bound (**A**); and, the LDL binding capacity is massively increased when 50 µg LDL is applied to collagen columns pre-bound with decorin (**B**). The decorin signal appears weak in (b) because the imaging conditions for the gel were optimized for the LDL protein signal.

Table 1
Relative LDL binding affinities of aortic valve and renal ostial diverter extracellular proteins

<u>PROTEIN</u>	<u>VALVE</u>	<u>DIVERTER</u>
Decorin	3.3±0.1	4.6±0.8
Biglycan	4.0±0.2	3.6±0.3
Fibromodulin	2.2±0.5	2.0±0.1
Lumican	1.1±0.1	1.1±0.2
Keratocan	1.2±0.1	N.D.
Prolargin	2.2±0.4	1.8±0.3
Chondroadherin	N.D.	1.9±0.3
Mimecan	1.7±0.1	2.4±0.8
Versican	2.7±0.4	2.4±0.1
Aggrecan	1.2±0.4	1.2±0.1
Agrin	3.1±0.5	3.0±0.7
Perlecan	3.4±0.2	4.3±0.6
CSPG4*	2.7±0.4	3.4±0.6
Fibronectin	2.3±0.3	2.2±0.1
Fibronectin Type III domain-containing protein	2.8±0.1	3.9±0.7
Tenascin	2.9±0.5	1.6±0.2
Tenascin-X	3.2±0.1	N.D.
Putative Tenascin-XA	3.7±0.1	N.D.

<u>COLLAGEN α CHAIN</u>	<u>VALVE</u>	<u>DIVERTER</u>
1(I)	3.2±0.7	3.2±1.1
2(I)	3.9±0.7	3.9±1.6
1(II)	4.3±0.9	N.D.
1(III)	2.2±0.3	2.3±0.4
1(IV)	N.D.	1.4±0.2
2(IV)	N.D.	2.4±0.6
3(IV)	N.D.	2.3±0.5
5(IV)	N.D.	1.4±0.2
6(IV)	N.D.	1.0±0.1

<u>COLLAGEN α CHAIN</u>	<u>VALVE</u>	<u>DIVERTER</u>
1(V)	3.4±0.2	3.5±0.9
2(V)	3.6±0.8	N.D.
1(VI)	0.8±0.2	0.9±0.1
2(VI)	1.7±0.2	2.6±0.7
3(VI)	2.8±0.4	3.3±0.6
6(VI)	2.5±0.6	0.4±0.1
1(VII)	N.D.	1.5±0.1
1(XI)	3.8±0.4	2.3±0.3
1(XII)	2.8±0.4	2.3±0.5
1(XIV)	2.9±0.7	2.5±0.3
1(XV)	3.9±0.6	4.3±1.4
1(XVIII)	N.D.	3.4±1.0

E/FT values represent mean ± S.D. of three different paired aortic valve and renal ostial diverter LDL affinity column effluent/flow-through samples determined by iTRAQ analysis.
N.D.:none detected.

* Note: CSPG4 is a plasma membrane proteoglycan.