Contribution of Macromolecular Structure to the Retention of Low-Density Lipoprotein at Arterial Branch Points

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Background—Extracellular deposition of low-density lipoprotein (LDL) in the arterial wall is an essential early step in atherosclerosis. This process preferentially occurs at arterial branch points, reflecting a regional variation in lipoprotein–arterial wall interactions. In this study, we characterized the submicron microstructure of arterial wall collagen and elastin to evaluate its potential role in regional LDL deposition.

Methods and Results—With 2-photon microscopy, we used the intrinsic optical properties of collagen and elastin to determine the arterial wall microstructure in fresh porcine and murine arteries. This optical approach generated unique nondestructive en face 3-dimensional views of the wall. The collagen/elastin microstructure was found to vary with the topology of the arterial bed. A nearly confluent elastin surface layer was present throughout but was missing at atherosclerosis-susceptible branch points, exposing dense collagen-proteoglycan complexes. In LDL binding studies, this luminal elastin layer limited LDL penetration, whereas its absence at the branches resulted in extensive LDL binding. Furthermore, LDL colocalized with proteoglycans with a sigmoidal dose dependence (inflection point, \( \approx 130 \text{ mg LDL/dL} \)). Ionic strength and competing anions studies were consistent with the initial interaction of LDL with proteoglycans to be electrostatic in nature.

Conclusions—This optical sectioning approach provided a robust 3-dimensional collagen/elastin microstructure of the arterial wall in fresh samples. At atherosclerosis-susceptible vascular branch points, the absence of a luminal elastin barrier and the presence of a dense collagen/proteoglycan matrix contribute to increased retention of LDL. (Circulation. 2008;117:2919-2927.)

Key Words: aorta • atherosclerosis • collagen • imaging • lipoproteins • pathology

A rterial wall mechanical properties and susceptibility to pathological vascular remodeling such as atherosclerosis are determined by the macromolecular structure of collagen, elastin, and proteoglycans.1–3 At low pressures, the high compliance of elastin dominates the wall displacement. At higher pressures, displacement is limited by the stiffer collagen. Changes in these mechanical properties with aging,4 smoking,5 diabetes,6 hypertension,7 and atherosclerosis are associated with modifications of the collagen and elastin scaffold. Although these macromolecules generate the biphasic mechanical response of the wall to pressure, the specific nature of their microstructure remains an important missing element in the modeling of wall mechanics.9,10

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In addition to its influence on mechanical properties, the extracellular matrix plays an essential role in low-density lipoprotein (LDL) accumulation in atherogenesis. Extracellular deposition of LDL in the arterial intima and media is an essential early step in this process.11 In both humans and animals,13,14 atherosclerosis develops preferentially at arterial branch points and certain regions of the aortic arch, including aortic branch points. The most popular hypothesis addressing this heterogeneity is that the distribution of low wall shear stress and turbulence determines susceptibility.15,16 However, the absolute magnitude of wall stress and turbulence alone is inadequate to fully explain this heterogeneity because both mice and humans have similar atherogenic regions with much different flow characteristics.17,18 Another nonexclusive hypothesis for LDL accumulation involves the direct interaction of macromolecules with LDL. The internal elastic lamina (IEL) serves as the major permeability barrier to water19 and LDL20 and limits LDL access to the wall, whereas direct binding of LDL by collagen and proteoglycans is important in LDL retention.21–23 Thus, the presence of the IEL and the collagen/proteoglycan content may be important factors in
determining susceptibility. In this study, we used 2-photon microscopy and lumen-to-intima–registered, en face optical sectioning through the intima and media to evaluate the distribution and structure of arterial wall macromolecules and their potential contribution to regional LDL retention.

Methods

Selection of Tissue for Imaging

We attempted to characterize 3 arterial beds in this study: the coronary, carotid, and aorta. We selected the porcine coronary and carotid because they were of the appropriate size for this optical approach. The porcine aorta was too thick to permit transmural examinations and too large to screen different regions; thus, the murine aorta was used as a substitute.

Mouse Aorta Preparation

Aorta samples were harvested from 20- to 28-week-old female C57BL/6J mice. Blood was removed by perfusion of the left ventricle of the heart with 1× PBS without calcium and magnesium (2.6 mmol/L KCl, 1.4 mmol/L KH2PO4, 136.8 mmol/L NaCl, 8 mmol/L Na2HPO4, 7H2O) (Quality Biological, Inc, Gaithersburg, Md). The aorta was dissected from the origin of the heart to the ileal bifurcation, cut longitudinally, and carefully placed endothelial cell surface down on the coverslip. A glass cover was secured to the coverslip with surgical tape on all 4 sides, and the sample was immediately moved to the microscope for study. Samples were placed longitudinally across the stage and oriented in the same direction throughout the study. All experiments performed on animals were performed according to a research protocol (H-0050) approved by the Animal Care and Use Committee of the National Heart, Lung, and Blood Institute of the National Institutes of Health.

Porcine Coronary Artery and Carotid Artery Preparation

Pig hearts were harvested from anesthetized and heparinized (10 000 U IV) animals. Pig left carotid arteries were dissected and collected after the hearts were harvested and placed in normal saline (154 mmol/L NaCl) (Quality Biological, Inc). The isolated heart was regradually perfused via the aorta with 500 mL cold buffer C (0.28 mol/L sucrose, 10 mmol/L HEPES, and 0.2 mmol/L EDTA, pH 7.21) to remove blood. The coronary arteries were isolated from the heart and immediately placed in normal saline. A 1-cm portion of the coronary artery was dissected from its aortic origin and cut longitudinally. After extra fat and myocardial tissue were trimmed off the artery, the vessel was placed luminal side down on a microscope cover and covered by a glass slide. A 1-cm piece of the left carotid artery also was cut open longitudinally, placed luminal side down on a microscope cover, and covered by a glass slide. The cover glass was loosely secured to the slide with surgical tape on all 4 sides and immediately moved to the microscope for study. Samples were placed longitudinally across the stage and oriented in the same direction throughout the study. All procedures performed were in accordance with the guidelines listed in the Animal Care and Welfare Act (7 US C. 2142 §13).

Two-Photon Excitation Microscopy

All images were taken from the beginning of the intimal surface to the deeper medial layers. Two-photon images were taken at room temperature with an LSM 510 META microscope (Zeiss, Thornwood, NY) with a ×40, 1.3-numerical aperture oil-immersion objective (Zeiss). Pig coronary arterial images were taken with a ×40, 1.2-NA, water objective (Zeiss) to measure relative LDL binding concentrations. A pulsed Ti:sapphire laser (coherent) set at 860 nm was used for excitation unless a different frequency is noted. Emitted light from the sample was passed through an infrared-blocking BG39 filter, a spectral dispersion grating residing in the Zeiss META system, and was detected by an array of photomultiplier tubes generating a 10.7-nm spectral resolution. Emission spectra were collected to optimize the separation of collagen and elastin signals. Emission spectra (Figure I of the online Data Supplement) reveals that at <840-nm excitation the near-ultraviolet second-harmonic-generation (SHG) signal was suppressed, likely because of the transmission characteristics of the optics and the inner filter properties of the tissues. Thus, an excitation >840 nm (ie, 420-nm single photon) was required to detect the collagen SHG. The elastin fluorescence emission was found to shift with excitation frequency, suggesting multiple chromophores within elastin. The inset in Figure I shows a plot of the difference in peak frequency of elastin fluorescence versus the collagen SHG as a function of excitation frequency. The spectral difference between elastin and collagen decreased with increasing excitation frequency. Thus, the lower the excitation frequency was, the better the separation of the collagen and elastin signals was. With the lower end of the detection of the second harmonic at 840 nm, we selected an excitation frequency of 860 nm for these studies. Elastin fluorescence was measured from 500 to 533 nm; collagen SHG was measured from 393 to 436 nm. The collagen signal was completely excluded from the elastin channel, and the contamination of elastin in the collagen channel was <2%.

LDL was labeled with Alexa Fluor 647 (Invitrogen, Carlsbad, Calif), and images of the pig coronary were acquired with dichroic mirrors and bandpass filters. Collagen SHG was detected at 415 to 430 nm, elastin fluorescence at 500 to 550 nm, and Alexa Fluor 647 (LDL) at 650 to 710 nm. Three-dimensional images were generally collected as a stack of images at 1-μm spacing starting at the luminal surface of the vessel. For qualitative studies, the gain and laser power were generally increased with increasing depth into the tissue to optimize the signal-to-noise ratio. More specific imaging parameters for each image may be found in the supplementary Methods section.

Relative Quantification of Collagen SHG Signal in the Mouse Aortic Intima-Media

Using two-photon microscopy, we quantified the relative superficial collagen content within atherosclerosis-prone aortic arch branch points and resistant areas in the murine aortic wall by measuring the collagen SHG signal/volume from the luminal surface to the beginning of the collagen-rich adventitia. For 4 to 5 images were acquired in each region in 5 mouse aortas and averaged for analysis. The backscattered SHG pixel intensity was integrated from the surface to 15 to 20 μm deep. Normalization was performed by dividing the SHG signal integrals by the measurement volume to correct for overall wall thickness. The calculated SHG pixel densities were then squared to get the relative collagen content.

To determine the amplitude of the orientation effect of collagen fibrils within different parts of the aortic wall, the SHG signal of 2 random locations in the murine lower abdominal aortic wall was quantified by integrating the entire SHG signal in paired studies from the surface to 20 to 30 μm deep, with the fibrils arranged grossly parallel to the laser and the sample then rotated 90°. The 90° rotation resulted in only an ~6% (P=0.01) increase in calculated collagen content. The extended Methods section in the online Data Supplement provides further discussion.

LDL Preparation

LDL was isolated from 250 mL blood freshly collected from donor human plasma by sequential ultracentrifugation (4°C, 100,000 rpm for 5 hours) 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 phosphate-buffered saline containing EDTA and sodium azide, particle integrity was confirmed by native agarose gel. LDL concentration was estimated by measuring total protein concentration. LDL was labeled with the Alexa Fluor 647 protein labeling kit (Molecular Probes, Eugene, Ore), which has a succinimidyl ester moiety that reacts with primary amines of proteins to form stable dye-protein conjugates. At the last step of the labeling process, labeled LDL was eluted from the separation column using PBS with 2 mmol/L azide provided in the kit.
or was eluted with normal saline, dH2O, or buffer B (120 mmol/L NaCl, 3 mmol/L KCl, 2 mmol/L CaCl2, 1 mmol/L MgCl2, 1 mmol/L KPO4, and 2 mmol/L HEPES). LDL was concentrated with a centrifugal filter device that had a nominal molecular weight limit of 10 kDa (Millipore, Billerica, Mass).

**LDL Binding Studies**

The endothelial cell layer was denuded from 1-cm segments of pig coronary arteries by gentle scraping with a cell scraper. Coronary segments were incubated in 1.5-mL Eppendorf tubes at room temperature with various concentrations of labeled LDL prepared in the appropriate buffer. They were then washed in a matching buffer solution for 1 hour to remove any free dye. Steady-state binding studies at branch points were done in PBS with a 2:1 ratio of unlabeled LDL to Alexa Fluor–labeled LDL. Experiments done in bicarbonate buffer B had 25 mmol/L NaHCO3 added immediately before incubation, and the incubation was done in a 5% CO2 incubator at 37°C with the sample tube open.

**Figure 1.** Collagen and elastin microstructure of the mouse aorta. A, Two-photon z-series images through the thoracic aortic wall, starting from the luminal surface (4 μm) to the adventitia (70 μm). Elastin autofluorescence is red; the collagen SHG is green. A nearly confluent, wavy sheet of elastin envelopes the luminal surface of the aortic wall. Collagen fibrils are radially arranged within the inner folds of the elastin lamellae and between lamellar units. Further from the lumen, tortuous collagen fibrils make up the adventitia. Scale bar=50 μm. This series demonstrates that both the intima-media interface, classically marked by the IEL, and the media-adventitia interface, marked by the collagen-rich connective tissue layer, can be effectively demarcated with this approach. B, Detailed image of the aortic wall showing bunched-up individual collagen fibrils radially arranged within elastin folds. Scale bar=10 μm. C, Three-dimensional surface reconstructions of atherosclerosis-susceptible intervertebral branch points with a ring of exposed collagen immediately surrounding the ostia. Scale bar=50 μm. D, Aortic arch branch point composed of a dense, knotted sheet of collagen. Scale bar=50 μm.

**Figure 2.** Collagen and elastin microstructure of the porcine carotid artery. Selected 2-photon z-series images through the porcine carotid intima, starting from the luminal surface (11.80 mm) to the first lamellar unit. Elastin autofluorescence is in red; the collagen SHG is in green. A wavy sheet of elastin with circular holes throughout envelops the luminal surface of the artery. Individual, bunched-up collagen fibrils are radially arranged within the inner folds of the elastin lamellae. Scale bar=50 μm.
Relative LDL signal per volume was measured with 2-photon microscopy by integrating the LDL pixel intensity from the luminal surface to the point in the wall where its signal had nearly disappeared (15 to 30 μm). Normalization was performed by dividing the LDL signal integral by the measurement volume.

Proteoglycan Labeling
For labeling proteoglycan distribution, anti-heparan antibody (Seikagaku Corp, East Falmouth, Mass) was labeled with an Alexa Fluor 564 antibody labeling kit (Molecular Probes). The endothelial cell layer was denuded from 1-cm segments of pig coronary artery by gentle scraping with a cell scraper. Coronary segments were blocked with 5% BSA in buffer B at room temperature for 1 hour. Alexa Fluor 564–labeled heparan antibody was diluted (1:50) in a 5% BSA solution, and arterial samples were incubated with the antibody at room temperature for 1 hour and then at 4°C overnight. Samples were washed with buffer B for 30 minutes and subsequently incubated with LDL in physiological ionic conditions as stated above.

Colocalization Analysis of LDL With Collagen or Proteoglycans
The first 20 slices of the z-series images were analyzed for LDL colocalization with collagen. The first 2 to 3 slices of the z-series images were analyzed for LDL colocalization with surface proteoglycans. Colocalization was quantified with the Imaris 5.7 software package (Bitplane AG, Zurich, Switzerland). Pixel codistribution was calculated for the falsely colored blue collagen or falsely colored green proteoglycan with falsely colored red LDL throughout the 3-dimensional data sets. Approximately 20% of the field of view was reduced into a region of interest for analysis selected by the collagen or proteoglycan distribution. The colocalized pixels (voxels in 3 dimensions) were displayed in a white mask overlapping the fluorescence channels. Two-dimensional histograms (fluorograms) showed the distribution of pixel intensities. The percentage of colocalized material (intensity and volume) was analyzed, and Pearson correlation coefficients in the region of interest (R; 1 = perfect correlation, 0 = no correlation, and −1 = perfect inverse correlation) were compared.

Imaging LDL Binding to Mouse Aortas
Wide field-of-view images of the mouse aorta were obtained with an OV110 Small Animal Imaging System (Olympus, Center Valley, Pa). Four parfocal and parcentered objectives provide a zoom range of several orders of magnitude (×0.1 to ×16) with high numerical aperture (0.43 to 0.07). A xenon bulb with a bandpass excitation filter of 610 to 645 nm (Olympus) was used as a light source, and fluorescence was measured with a high-sensitivity charge-coupled device camera (Hamamatsu, Shizuoka, Japan) filtered by a 675- to 715-nm bandpass emission filter (Olympus). Images (512×512×8) were obtained with a 0.89×/0.04-N.A. objective. For higher-magnification objectives (>×1), a dichroic mirror reflecting below 665 nm was used, whereas with lower-magnification objectives, no dichroic was used, and excitation and emission followed separate beam paths. Images were automatically optimized for brightness and contrast with a linear look-up table.

Statistical Analysis
All data are expressed as mean±SD. The Wilcoxon rank-sum test was performed to analyze the differences in collagen content between the aortic arch and the abdominal aortic wall. Statistically significant differences in collagen content between the parallel and perpendicular sample orientations were assessed by 2-tailed Student’s t test because the orientation of the collagen fibrils was not uniformly aligned to the laser polarization. The sigmoid binding

Figure 3. Collagen and elastin microstructure of the porcine coronary artery. A, Selected 2-photon z-series images from the luminal surface (12 μm) to the deeper layers of the arterial media (115 μm). Elastin autofluorescence is in red; collagen SHG is in green. A net-like IEL covers the luminal surface of the coronary free wall. The areas at which images were taken along a coronary arterial branch point are illustrated. B, Images were taken from the edge of the free wall. C, Densely knotted collagen structure not covered by the IEL surrounds the ostia. D, Portion of the cut branching artery. Scale bar=50 μm.
curve obtained from steady-state binding studies of LDL was fitted and a 3-parameter equation was derived with SigmaPlot 8.0.

The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

## Results

### Mouse Aorta

The collagen and elastin microstructure was determined for various regions in the C57BL/6J mouse aorta (n=6). Along the free wall of the thoracic aorta, elastin formed lamellar layers (Figure 1A). The most superficial layer was a continuous sheet covering the luminal surface. With no intraluminal pressure, the arterial wall surface compressed in the radial direction into longitudinal wavy folds, reflecting the residual circumferential stress, or residual strain, within the intima-medial layers. The wavy nature of these folds indicated that both radial and longitudinal residual strain was occurring. The spacing of the lamellar folds was ≈50 to 100 μm in the upper thoracic aorta and 30 to 50 μm in the lower abdominal aorta. Coiled collagen fibers were arranged radially within the compressed inner folds of the elastin lamellae and between each lamellar unit (Figure 1A and 1B). Individual collagen fibers were coiled with a mean periodicity of 2 to 5 μm (Figure 1B). Moving out to the adventitia, the abundant SHG signal from tortuous collagen fibers overwhelmed the image. At atherosclerosis-prone intervertebral branch points, collagen fibers were circumferentially organized in a knotted ring surrounding the ostia (Figure 1C). Unlike in the free wall, the ostia were mostly not covered by an elastin layer. Further from the ostia, the insertion of the branch deflected parallel collagen fibers, generating denser collagen structures. These collagen fibers did not show sinusoidal compression, suggesting minimal residual stress at branch regions consistent with the high collagen content. Similarly, collagen at aortic arch branch points formed dense knotted structures (Figure 1D). Relative quantification of superficial collagen content with 2-photon microscopy showed the mean±SD collagen density in the aortic arch to be 53.8±5.6 arbitrary units/μm³ and the mean collagen density in the abdominal aortic wall to be 41.0±3.6 arbitrary units/μm³ in 5 paired experiments. There was a 24% higher density of collagen in the aortic arch branch regions than in the free abdominal wall (P=0.05, Wilcoxon rank-sum test).

### Porcine Carotid Artery

Similar elastin and collagen microstructure was seen in the porcine carotid artery and the mouse distal aorta. The porcine carotid artery had a superficial layer of elastin covering the luminal surface (n=6) (Figure 2). This layer was compressed in the radial direction into longitudinal wavy folds spaced ≈40 to 80 μm apart. In contrast to the murine aorta, the surface elastin in the porcine carotid artery had larger circular openings throughout. There was ≈0.002 to 0.004 opening per 1 μm², and each opening ranged in size from small circular holes that were 3 μm in diameter to larger oval holes that were 25×9 μm. Similar to the murine aorta, collagen fibers in the porcine carotid artery were radially arranged within the inner folds of the elastin layer and were coiled with a mean periodicity of 5 to 10 μm.
Porcine Coronary Artery

Macromolecular images of the porcine coronary artery were collected from the free wall and branch points (n=6). In the free coronary arterial wall, the elastin was limited to the single IEL layer, in contrast to the porcine carotid artery and the murine aorta (Figure 3A). The IEL was fenestrated, with individual elastin fibers arranged into a net-like structure over the luminal surface. The residual strain resulted in longitudinal folds with a spacing of 30 to 50 \( \mu \text{m} \), implying a dominant radial stress. Collagen associated with the surface of the IEL was amorphous and ran roughly parallel to the longitudinal elastin fibers. Beneath the IEL, collagen fibrils were radially arranged throughout the tunica media, with few elastic fibrils intermixed between collagen fibers. The fact that the elastin fibers of the IEL and its folds were parallel indicated that the deeper layers running perpendicular to the IEL fibers dominated the residual strain. A close examination of a coronary artery branch point is presented in Figure 3B through 3D. Serial images taken through coronary branch points were taken from the free wall (Figure 3B) through the annulus (Figure 3C) to the edge of the cut branch artery (Figure 3D). The IEL thinned and ended at the annulus, exposing a dense mesh of collagen and its associated proteoglycans. This branch point structure was qualitatively similar to the branch point structures observed in the mouse aorta (Figure 1C), where the surface elastin layer was absent at the branch point ostia.

LDL Binding

LDL binding to specific extracellular matrix components was monitored in arterial samples with 4 mg/mL fluorescently labeled LDL in physiological buffer B in the absence of convective flow. By performing these studies in vitro with no flow, we eliminated the physical contribution of flow and pressure gradients in LDL binding distribution. The endothelial cell layer was removed for these studies to eliminate the potential contribution of localized endothelial cell damage during preparation to influence the binding assay. LDL bound most abundantly within the exposed collagen at porcine coronary arterial branches (Figure 4A) but not in the free wall where the elastin layer apparently restricted the penetration of LDL (Figure 4B) (n=6). Similarly, wide-field single-photon excitation fluorescence imaging of the murine aorta showed extensive LDL binding within the structures surrounding the intervertebral branch points, whereas LDL was excluded from the free wall of the aorta (Figure 5) (n=3).

On the basis of these observations, we focused on the characterization of LDL binding to the porcine coronary branch points. Washout studies showed a complete washout of free Alexa Fluor from branch points after 1 hour, whereas the Alexa Fluor–labeled LDL was unaffected by extensive washing, consistent with a very tight association of LDL with the macromolecules (supplementary Figure II). It has previously been reported that the association of LDL to vascular wall macromolecules is an electrostatic interaction.\(^{27}\) We found that low-ionic-strength media such as water significantly increased LDL binding (Figure 6A) over normal saline (Figure 6B). In contrast, the addition of competing anions such as 2 mmol/L azide to PBS almost completely blocked LDL binding (Figure 6C). These results are consistent with the notion that LDL binding is electrostatic in nature, increasing with decreasing ionic strength, and is in competition with multivalent anions.

The steady-state kinetics of LDL binding at coronary branch points was determined by varying the LDL concentration in the bath. Time courses revealed a plateau at nearly 100 minutes (Figure 4C). Thus, 120-minute incubation times were used for the LDL binding experiments. The steady-state binding kinetics of LDL revealed a sigmoid binding curve.
with an inflection point near 130 mg LDL/dL, suggesting a cooperative binding mechanism (Figure 4D).

Colocalization analysis in the coronary branch point revealed very little correlation between LDL and collagen \( (n=3; R_{\text{coloc}}=0.056 \pm 0.075) \) (Figure 7A). In contrast, the degree of colocalization between LDL and immune-labeled proteoglycans was relatively high \( (n=3; R_{\text{coloc}}=0.469 \pm 0.030) \) (Figure 7B and 7C). The correlation of LDL and proteoglycans was not perfect, and inspection of the images suggested that only a subset of the labeled proteoglycans were interacting with LDL. This is consistent with the nature of the antibody used, which is rather nonspecific for the different proteoglycan subtypes.

**Discussion**

These data provide the 3-dimensional microstructure of arterial wall collagen and elastin in different arterial beds.

![Figure 6. LDL binding to pig coronary arterial branch points in various buffered solutions. A, LDL binding in dH2O was very high, and LDL formed insoluble complexes along the surface of the exposed collagen. B, LDL binding in normal saline was lower than that seen in dH2O above or in bicarbonate buffer B. C, LDL in a highly anionic buffer composed of 1× PBS with 2 mmol/L azide showed very low binding to the surface of the branch point. Scale bar=50 \( \mu \)m.](image1)

![Figure 7. Colocalization analysis of LDL binding at porcine coronary arterial branch points. A, Quantification of colocalization between collagen and LDL. Pixel codistribution was calculated for blue (collagen) and red (LDL) channels for z-series 2-photon data sets. Two-dimensional histogram (fluorogram) shows the distribution of pixel intensities for collagen vs LDL with poor colocalization \( (n=3) \). B, Quantification of colocalization between proteoglycans and LDL. Pixel codistribution was calculated for green (proteoglycan) and red (LDL) channels. Two-dimensional histogram showed that the distribution of pixel intensities for proteoglycans vs LDL reveals a positive spatial correlation. The Pearson correlation coefficient demonstrates significantly greater colocalization between LDL and proteoglycans \( (0.469 \pm 0.030) \) vs LDL and collagen \( (0.056 \pm 0.075) \) \( (1=\text{perfect correlation}, 0=\text{no correlation}, \text{and } -1=\text{perfect inverse correlation}) \). C, Two-photon image of LDL (red) colocalization with immunolabeled proteoglycans (green).](image2)
This information is useful for the quantitative modeling of arterial wall mechanics and the analysis of water and macromolecular permeability. The nondestructive optical sectioning methods presented here should aid in the future characterization of pathological vascular remodeling by providing fully registered, high-resolution 3-dimensional images of the vascular microstructure.

A major difference among various arterial beds was the structure of the surface elastin layer, a barrier believed to play a key role in the water and LDL permeability. Structural differences in elastin are highlighted by observing 3 different arterial structures from the pig. A surface rendering of the surface elastin layer in the porcine aorta, carotid, and coronary vessels is presented in Figure 8. Despite the large thickness of the porcine aorta, the surface elastin can be examined closely with this optical approach. In contrast to the nearly confluent murine aorta elastin layer, the porcine aorta elastin layer was clearly not confluent and revealed a significant amount of the underlying collagen. The large differences in macromolecular structure in the porcine and murine aorta suggest that species differences need to be considered when these data are extrapolated. On a macroscopic view, the predicted relative permeability would be highest in the porcine aorta and then in the coronary and carotid arteries. The predicted permeability of the aorta and coronary correlates well with the general susceptibility of these structures to lipid deposition in young men. Another striking feature was the absence of this elastin barrier and the exposure of a dense collagen/proteoglycan network at arterial branch points in both the porcine and murine models. These alterations in microstructure likely reflect the different mechanical stress encountered by these arterial branch points. LDL preferentially bound within the proteoglycan structures of these branch points, consistent with the absence of the elastin barrier. The interaction in this dense collagen/proteoglycan network was consistent with an initial electrostatic interaction with proteoglycans followed by a hydrophobic self-association of LDL, resulting in the cooperative kinetics of binding (Figure 6D). The 2 models of early LDL deposition in atherosclerosis involve low shear stress and turbulent flow characteristics, influencing both transport and gene expression alterations or macromolecule retention of LDL. These 2 models are brought together by our observations because both low shear flow and modifications of macromolecular structure that promote LDL retention are occurring in the same atheroma-prone regions. It is possible that the macromolecular structure observed in this study is a direct result of molecular signaling associated with low shear stress. For example, Platt et al. demonstrated that laminar flow may protect the elastic lamina by inhibiting cathepsins, a potent stimulants of elastin breakdown, whereas others have demonstrated that low shear stress activates several other potential elastin proteases. These enzymatic alterations associated with low shear stress in vitro could explain the compromise of the elastin layer at arterial branch points. However, other mechanical aspects of the vascular branch point may be important in generating this macromolecular structure. Independently of the mechanism of generation, it is unclear what physiological role the removal of the surface elastin layer may play at these vascular branch points. In any event, the macromolecular structure of arterial branch points potentiates the retention of LDL, along with many other documented physiological effects associated with low shear flow occurring in these regions.

It is of interest to attempt to correlate the steady-state binding kinetics of LDL at coronary branch points with clinical risk factors and outcomes. First, the highly nonlinear, cooperative nature of the binding is consistent with the notion that small changes in plasma concentration of LDL will result in large differences in binding and potential atherogenesis. Notably, the inflection point of the LDL binding curve at 130 mg/dL (Figure 4D) is near the LDL concentration at which the American Heart Association recommends starting medical therapy in patients with elevated lipids. Although this value was determined from clinical outcomes, the association of LDL with vascular wall proteoglycans in this highly nonlinear fashion may provide a molecular mechanism explaining the significance of this empirical value as an important risk factor in atherogenesis and the high sensitivity of clinical outcome to small alterations in plasma LDL levels.

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Disclosures
None.

References


**CLINICAL PERSPECTIVE**

We characterized the microstructure of the major macromolecules, elastin and collagen, in the arterial wall using state-of-the-art optical microscopy techniques. This structural examination of macromolecular structures revealed a surprising result, namely the near absence of the internal elastic lamina layer at vascular branch points, which are among the sites most prone to atherosclerosis. The reason for the lack of a surface elastic layer at the branch points is unknown but could be due to the unique flow or mechanical characteristics at the branch. We hypothesized that the lack of an elastic layer may contribute to the initial infiltration and/or retention of low-density lipoprotein (LDL) at these sites and the subsequent development of atherosclerosis. Consistent with this hypothesis, we found that LDL binding was most extensive in the arterial branch points where the elastic layer was absent. Steady-state LDL binding studies in branch points revealed a highly nonlinear cooperative binding characteristic that resulted in a disproportionate increase in LDL retention with only relatively small increases in LDL beyond a certain LDL threshold. This highly nonlinear dependence of LDL retention in the arterial branch is similar to the clinical observation of a certain threshold of LDL needed to initially develop atherosclerosis and the further marked increased risk for coronary artery disease with increasing levels of LDL.
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