Early Human Atherosclerosis: Accumulation of Lipid and Proteoglycans in Intimal Thickening Followed by Macrophage Infiltration

Yutaka Nakashima, Hiroshi Fujii, Shinji Sumiyoshi, Thomas N. Wight, Katsuo Sueishi

Objective—The present study was designed to clarify the morphological features of early human atherosclerosis and to determine whether specific extracellular matrix proteoglycans play a role in early atherogenesis.

Methods and Results—Step and serial sections were obtained from right coronary arteries with no or early atherosclerosis. Atherosclerosis was classified into 4 grades according to the amount of lipid deposition. Coronary arteries with Grade 0 showed diffuse intimal thickening (DIT) with no lipid deposits. The extracellular matrix proteoglycans, biglycan and decorin, were localized in the outer layer of DIT. Most cases of Grade 1 and Grade 2 exhibited fatty streaks with extracellular lipids colocalizing with biglycan and decorin in the outer layer of the intima. As lipid grades increased, macrophages increased in number and were present in the deeper layers. Most cases of Grade 3 exhibited pathologic intimal thickening (PIT) with extracellular lipids underneath a layer of foam cell macrophages.

Conclusions—In early human coronary atherosclerosis, fatty streaks develop via extracellular deposition of lipids associated with specific types of proteoglycans in the outer layer of preexisting DIT. As the amount of the lipid increases in fatty streaks, macrophages infiltrate toward the deposited lipid to form PIT with foam cells. (Arterioscler Thromb Vasc Biol. 2007;27:000–000-)

Key Words: early human atherosclerosis — diffuse intimal thickening — fatty streak — lipid retention — biglycan

Little is known as to how early human atherosclerosis develops. We previously reported that diffuse intimal thickening (DIT) develops from an early age in human arteries before atherosclerosis evolves. DIT, also known as “nonatherosclerotic” intimal thickening, is a thickened intima mainly composed of smooth muscle cells (SMCs), elastin, and proteoglycans, and devoid of lipid deposition. As DIT is strongly expressed in atherosclerosis-prone arteries, such as coronary arteries and abdominal aorta, we suggested that DIT plays an important role in human atherogenesis. In the classic pathological study, Holman et al showed that the fatty streak, a nonraised sudanophilic lesion, is the earliest lesion that appears in the aorta of children and adolescents and some fatty streaks convert into the advanced raised lesion in later life. This fact was also recently confirmed in the coronary artery by McGill et al in The Pathobiological Determinants of Atherosclerosis in Youth (PDAY) study. However, as microscopic examinations were not performed in these studies, it is not clear how fatty streaks develop in normal arteries and covert into advanced lesions. Based on microscopic findings, Virmani et al defined the pathologic intimal thickening (PIT) as a preatheromatous lesion that is composed of extracellular lipid pools with an overlying layer of SMCs and lipid-laden macrophages. These studies have contributed to our understanding of the microscopic features of human atherosclerosis before the stage of advanced lesions. Thus, PIT is thought to be an intermediate stage that represents the link from early to advanced lesions, but the nature of the early lesion and how the early lesion is converted into PIT are yet to be clarified. Furthermore, Williams and Tabas proposed the response-to-retention hypothesis in early atherogenesis in 1995, which states that atherogenic lipoproteins are retained in the intima by binding to extracellular proteoglycans. This hypothesis further states that lipoprotein–proteoglycan complexes exhibit increased susceptibility to oxidation and lead to uptake by macrophages to form foam cells. Recent biochemical and molecular biological studies support this hypothesis. However, it is still not clear whether this hypothesis is applicable to human atherogenesis, partly because of the lack of morphological evidence of early lesions documenting the presence and location of these macromolecules.

In the present study, we examined human coronary arteries, aiming to clarify the morphological features of the early phases of atherosclerosis and the relationships between DIT, fatty streaks, and PIT. Furthermore, we wished to determine...
whether specific extracellular matrix (ECM) proteoglycans such as biglycan and decorin are associated with lipid deposits in the early lesion in support of the response-to-retention hypothesis.

**Methods**

An expanded Methods section is available online at http://atvb.ahajournals.org.

**Autopsy Subjects and Coronary Arteries**

A middle segment of the right coronary artery (RCA) together with acute marginal branch was obtained from 38 Japanese autopsied subjects who died between 7 and 49 years of age. The average of serum total cholesterol and triglyceride levels were within normal limits.

**Definitions of DIT and Atherosclerotic Lesions**

In the present study, DIT was defined as a concentric intimal thickening composed of SMCs, elastin, and proteoglycans and devoid of lipid deposits. The fatty streak was defined as a nonraised sudanophilic lesion. PIT was defined as a lesion with an accumulation of extracellular lipids underneath a layer of lipid-laden macrophages.

**Two-Dimensional Image of the Arterial Cross Section**

Step and serial cryostat sections were stained with elastica van Gieson (EVG) and Sudan IV stains and immunostained with anti-CD68 antibody. Intima, media, and lipids and macrophages existing in the intima and media, were extracted from the digital images of the stained sections (Figure 1a, 1b, 1c), and composed into a single two-dimensional (2D) image (Figure 1d).

**Reconstruction of a Three-Dimensional Image**

A three-dimensional (3D) image of the artery was reconstructed from a series of the 2D images (Figure 1e, 1f, 1g; supplemental Movie I, available online at http://atvb.ahajournals.org). The ratio of the lipid volume to the arterial wall (intima + media) volume was calculated.

**Vertical Distribution of Lipids and Macrophages**

The total lipid density (the ratio of the total lipid area to the total arterial wall area) was calculated in 2D images. The arterial wall was divided into 4 layers, ie, inner intima, outer intima, inner media, and outer media (Figure 2a), to calculate the percentage of the lipid area in the respective layer. The same measurements and calculations were performed for macrophages.

**Topographical Relationship Between Lipids and Intimal Components**

Cryostat sections were immunostained with antibodies to apolipoprotein B (apoB), α-smooth muscle actin (α-SMA), α-elastin, biglycan (LF-121), decorin, monocyte chemoattractant protein-1 (MCP-1, F9), and oxidized phosphatidylcholine (ox-PC, DLH3).

**Results**

**Grading of Lipid Deposition and Spatial Distributions of Lipids and Macrophages**

According to the ratio of the lipid volume to the arterial wall volume (×100) analyzed in 3D images, the grade of lipid deposition in the whole arterial segment was classified into 4 grades: Grade 0, 0 (n = 6); Grade 1, 0.01 to 0.50 (n = 12); Grade 2, 0.51 to 5.00 (n = 13); Grade 3, 5.01 or more (n = 7). Lipid grades correlated positively with mean ages (ANOVA, P < 0.0001), but not with serum total cholesterol, triglyceride levels, or smoking status (supplemental Table I). One of the two cases with diabetes mellitus (27-year-old female) was in Grade 1 and the other (49-year-old male) was in Grade 2. In most cases of Grade 1 through Grade 3, lipids were deposited eccentrically and more strongly in the proximal and branching portions than the distal portion (Figure 1f; supplemental Movie I). These results demonstrate that this lipid deposition is an early stage in human atherosclerosis. Macrophages were sparsely distributed throughout the arterial wall in Grade 0 and Grade 1, and increased in Grade 2 and Grade 3 particularly around the deposited lipids (Figure 1g).

**Vertical Distributions of Lipids and Macrophages**

Figure 2b and 2c shows the total lipid density and the vertical distribution of lipids in the arterial wall, respectively. No lipid deposits were seen in Grade 0. Lipids occupied small areas of the arterial wall in Grade 1. Most of the lipid existed in the outer intima (hatched bar) and only a small proportion was found in the inner intima (dotted bar) and inner media (open bar). In Grade 2, the total lipid density was increased, and at the same time, the proportion of the lipid area in the inner intima was increased. This trend was more remarkable in Grade 3 than Grade 2, although a large proportion of the lipids was still present in the outer intima.

Figure 2d and 2e shows the total macrophage density and the vertical distribution of macrophages in the arterial wall, respectively. Although occupying only small areas, macro-
Phagocytes were seen even in Grade 0 and their distribution was largely confined to the inner intima (dotted bar). The total macrophage density and their distribution in Grade 1 were almost the same as those of Grade 0. In Grade 2, the total macrophage density was increased and so was the proportion of the macrophage area in the outer intima (hatched bar). This trend was greater in Grade 3 than Grade 2, although a large proportion of the macrophages was still present in the inner intima. A small proportion of macrophages was seen in the outer media (cross-hatched bar) in Grade 0 through Grade 3. The ratio of total macrophage area to total lipid area tended to be larger in smokers than nonsmokers, although there was no statistical significance (Mann–Whitney U test, \( P < 0.001 \)).

**Microscopic Findings**

Figure 3 shows representative micrographs of lipids and macrophages. The arteries in Grade 0 exhibited DIT. There were no lipid deposits, but a few macrophages in the superficial layer of the thickened intima (Figure 3a, 3b, 3c). All 12 cases in Grade 1 exhibited the mildest form of the fatty streak. Mild lipid deposition was seen in the deep layer of the intima, whereas macrophages showed no differences from those of Grade 0 (Figure 3d, 3e, 3f). Ten of 13 cases in Grade 2 and 1 of 7 cases in Grade 3 also exhibited fatty streaks, but lipids were more intensely and widely stained and macrophages were slightly increased in number and infiltrated deeper than Grade 1 (Figure 3g through 3l). Most of the lipids were localized in the outer layer of the intima but small amounts were seen in the inner intima and inner media. Higher magnification revealed that the lipids were localized extracellularly and suggested that some lipids were associated with elastin (supplemental Figure I). Even in cases with greater amount of lipid deposits, the region affected by the fatty streak was not raised as shown in Figure 1a and 1b. Three of 13 cases in Grade 2 and 6 of 7 cases in Grade 3 exhibited PIT. Extracellular lipids accumulated in the outer intima and were overlaid by a layer of lipid-laden macrophages (Figure 3m through 3r). Compared with the fatty streak, extracellular lipids were more widely stained and macrophages were more increased in number and more deeply infiltrated in PIT. Foam cell macrophages were found in 8 of 9 cases of PIT. In 7 cases (2 cases in Grade 2 and 5 cases in Grade 3), foam cells were present in and around the interface between infiltrating macrophages and extracellularly deposited lipids (Figure 3p, 3q, 3r). One case in Grade 2 showed a small aggregate of foam cells in the superficial layer of the intima. The region affected by the PIT lesion was generally more thickened than the region without the lesion.

The correlation between lipid grades and atherosclerotic lesions is summarized in supplemental Table I. All cases in Grade 0 showed DIT. In Grade 1 through Grade 3, the fatty streak and PIT were seen significantly in lower and higher grades, respectively (Mann–Whitney U test, \( P < 0.001 \)).

**Topographical Relationship Between Lipids and Intimal Proteoglycans**

Topographical relationships between extracellular lipids and ECM proteoglycans were examined in 21 cases. First, the distribution of biglycan and decorin was examined in the area with no lipid deposits in 12 cases (Figure 4a). In 9 cases, biglycan was concentrically and extracellularly localized predominantly in the outer layer of DIT (Figure 4b, 4c). Similar distribution was seen for decorin in 3 cases, but the positive area in the intima was generally smaller than those of biglycan (Figure 4d). No staining was observed in 3 cases for biglycan and 9 cases for decorin. The average age of the positive cases was older than the negative cases, although no statistical significance was found (29.3 ± 10.4 versus 15.7 ± 8.3 for biglycan, 36.3 ± 7.5 versus 22.4 ± 10.5 for decorin).

Second, correlations between the distribution of lipids and that of biglycan and decorin were examined in 9 cases with severe and/or diffuse extracellular lipid deposits in fatty streaks. In all 9 cases, the distribution of apoB coincided with that of the Sudan IV positive area (supplemental Figure IIa, IIb). Biglycan was colocalized with apoB in all 9 cases.
Localizations of Oxidized Lipoproteins and MCP-1

Ox-PC was present extracellularly in the intima and tended to be colocalized with apoB when relatively large amount of lipids were deposited (supplemental Figure IIIa, IIIb). Ox-PC was also seen intracellularly (supplemental Figure IIIa, IIIc). MCP-1 was found in most of the macrophages and some SMCs in the intima (supplemental Figure IIId).

Discussion

Initial Event of Human Atherosclerosis: Extracellular Lipid Deposition in DIT and Fatty Streaks

Only a few microscopic studies are available that examine the earliest stage of human atherosclerosis, and the results are inconsistent. For example, isolated macrophage foam cells were believed to be the earliest sign of atherosclerosis, whereas other studies suggested that intimal fibroplasia was a first stage. In the present study, we used step and serial sections of coronary arteries to detect subtle pathological changes, and found that the earliest stage of human coronary atherosclerosis is the fatty streak that develops via extracellular deposition of apoB-containing lipids in the outer layer of preexisting DIT. As the lesion progresses, lipids continue to accumulate in the outer layer of the thickened intima of the fatty streak without causing a remarkable change in intimal thickness. Recently, extracellular lipid deposits in the intima of human early lesions were also found by other investigators.11,12

It is generally believed that extracellular lipids originate from dead foam cell macrophages and accumulate to form a lipid core in the advanced lesion. However, a few studies have reported that lipids accumulate in the intima before macrophages infiltrate in the early lesion. Napoli et al found that lipids accumulated in the intima of human fetal aorta in the absence of macrophages.13 Investigating the human aorta of young and middle-aged adults, Guyton and Klemp sug-
The present study also suggests that the transformation from macrophages to foam cells is achieved by phagocytizing deposited lipids, because foam cells were primarily formed in and around the interface between infiltrating macrophages and extracellular lipids. Phagocytizing lipid by macrophages is believed to be an essential mechanism to form foam cells in animal models as well, but microscopic features of the lesions containing foam cells are considerably different from those of humans. In animal models, foam cell macrophages appear in the initial stage of atherosclerosis and occupy the whole thickness of the intima as a predominant component of the lesion. As shown in the present study, foam cells are formed in human PIT lesions subsequently to the deposition of a certain amount of extracellular lipid, together with SMCs and proteoglycans.

Role of Proteoglycans in Extracellular Lipid Deposition

A number of biochemical and molecular biological studies suggest that the lipid binding capacity of proteoglycans contributes to retaining atherogenic lipoproteins in the intima. However, morphological evidence and specific location of the components involved in proteoglycan-lipoprotein accumulation is lacking. The present study illustrates that biglycan occurs extracellularly in the outer layer of DIT in the exact location as the early distribution of lipids (note the similarity of the prelesional distribution of biglycan in DIT in Figure 4b and the early distribution of lipids in the fatty streak in Figure 3h). However, it is of interest that lipids deposit eccentrically, whereas biglycan is localized concentrically. It is believed that structural changes in the glycosaminoglycan (GAG) chains on proteoglycans are the initial proatherogenic step that leads to increased binding properties of proteoglycans for atherogenic lipoproteins. For example, proteoglycans produced by transforming growth factor-β1–treated cultured SMCs show longer GAG chains and greater binding affinity to LDL than control SMCs. Interestingly, patchy distribution of TGF-β1 is found in DIT. It is also noteworthy that mechanical strain, which is thought to be unevenly distributed in the arterial wall, upregulates biglycan mRNA expression in cultured SMCs. These results suggest that regional differences in the quality and quantity of biglycan exist in the intima and possibly lead to regional differences in the amount of lipid deposition. Two other mechanisms that may cause regional differences in the lipid distribution are uneven plasma lipoprotein concentration and the permeability of the arterial wall. Deng et al reported that luminal surface concentration of LDL was increased in areas where wall shear stress was low and suggested that increased surface LDL concentration results in an increased lipid infiltration rate into the intima. However, the relationship between permeability of the arterial wall and susceptibility of atherosclerosis is debatable. The permeability to LDL was greater in the atherosclerosis-susceptible areas than atherosclerosis-resistant areas of the rabbit aorta, but opposite results were obtained in white Carneau pigeon aorta.

Correlations between the distribution of lipids and proteoglycans have been investigated in early and advanced atherosclerotic lesions. A consistent finding, including that of...
the present study, is the colocalization of biglycan and apolipoproteins.\textsuperscript{25-26} It is also noteworthy that oxidized LDL is capable of stimulating biglycan expression by SMCs and enhancing the interaction of this proteoglycan with lipoproteins.\textsuperscript{28} It is tempting to speculate that the accumulation of biglycan in specific regions of the early lesions may result from the presence of the lipoproteins associated with the SMCs. These molecular interactions may result in a vicious cycle of atherosclerosis. We found that decorin appears to colocalize with lipid in some instances but much less consistently than biglycan. Versican is another important extracellular proteoglycan in human atherogenesis, as it accumulates in human atherosclerotic lesions.\textsuperscript{25,27} but not in mouse models.\textsuperscript{26} The role of versican may be different from that of biglycan and decorin, because its distribution is different from biglycan and decorin.\textsuperscript{25,29} In our preliminary study, versican was predominantly localized in the inner layer of DIT and fatty streaks. Diffuse distribution of versican across the intima was also seen in some cases. In advanced human lesions, versican is present at the plaque thrombus interface, suggesting a possible role in thrombosis.\textsuperscript{27}

Summary
The present study supports the response-to-retention hypothesis as being involved in the early phases of human coronary atherosclerosis. Furthermore, this study highlights the importance of the DIT and the fatty streak as a reservoir for lipid retention and identifies a family of ECM molecules that may be involved in lipid retention, contributing to the early phases of lesion formation before the stage of the PIT. Although only coronary arteries were targeted in the present study, the same mechanisms are expected to occur in other atherosclerosis-prone arteries, such as abdominal aorta, because well-developed DIT is present in their intima as well.\textsuperscript{1}

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Disclosures
None.

References


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Methods

Autopsy subjects and coronary arteries

A middle segment of the right coronary artery (RCA) together with acute marginal branch was obtained from 38 Japanese autopsied subjects who died between 7 and 49 years of age (32.3 ± 11.2 years, 25 males and 13 females). The arteries with no or early stage of atherosclerosis were examined in this study. The autopsy subjects died of diseases, such as malignancies and liver cirrhosis, but not coronary heart diseases. The average of serum total cholesterol and triglyceride levels were within normal limits (150.0 ± 41.1 mg/dl, n=38 and 120.6 ± 58.0 mg/dl, n=34, respectively). Serum triglyceride levels were unknown in 4 cases. Diabetes mellitus was found in two cases. Twenty subjects were smokers and 16 were nonsmokers. Smoking status was unknown in 2 cases. The study was approved by the ethics committee of the Department of Pathology, Kyushu University and performed in accordance with the guidelines.

Definitions of diffuse intimal thickening (DIT) and atherosclerotic lesions

In the present study, DIT was defined as a concentric intimal thickening composed of smooth muscle cells (SMCs), elastin and proteoglycans and devoid of lipid deposits. According to the definition of Holman et al., the fatty streak was defined as a non-raised sudanophilic lesion. Pathologic intimal thickening (PIT) was defined according to the definition of Virmani et al. as a lesion with an accumulation of extracellular lipids underneath a layer of lipid-laden macrophages.

Antibodies

Rabbit polyclonal antisera for human biglycan (LF-121) were generously provided by Larry W. Fisher, Craniofacial and Skeletal Disease Branch, National Institute of Dental and Craniofacial Research, National Institutes of Health, Bethesda, MD and used at a 1/500 dilution. Mouse monoclonal antibodies for monocyte chemoattractant protein-1 (MCP-1, F9) and
oxidized phosphatidylcholine (ox-PC, DLH3) \(^7\) that specifically recognize oxidized lipoproteins including oxidized low density lipoprotein (ox-LDL) were generously provided by Motohiro Takeya, Department of Cell Pathology, Graduate School of Medical Sciences, Kumamoto University and Hiroyuki Itabe, Laboratory of Biological Chemistry, School of Pharmaceutical Sciences, Showa University and used at 1/7 and 1/3200 dilutions, respectively. Mouse monoclonal antibodies for anti-human CD68, α-smooth muscle actin (α-SMA) and MCP-1 were purchased from Dako (Glostrup, Denmark), Sigma (St. Louis, MO) and R & D systems (Minneapolis, MN) and used at 1/100, 1/1000 and 1/50 dilutions, respectively. Rabbit antisera for human α-elastin and goat antisera for human decorin and apolipoprotein B (apoB) were purchased from Biogenesis (Poole, England), R & D systems (Minneapolis, MN) and Rockland (Gilbertsville, PA) and used at 1/100, 1/20 and 1/400 dilutions, respectively.

### Two-dimensional image of the arterial cross section

Three serial cryostat sections of 10 micrometers thick were cut from the artery and grouped in one unit. Twenty-nine to 50 units were obtained every 200 or 250 micrometers, which made a total length ranging from 6.0 to 12.3 mm. Two sections in each unit were stained with elastica van Gieson (EVG) and Sudan IV stains to identify the arterial intima, media, and lipids, respectively. The other section was immunostained with anti-CD68 antibody to identify macrophages. Digital images of the stained sections were captured with a charge-coupled device (CCD) camera. Intima, media, and lipids and macrophages existing in the intima and media, were extracted from these digital images using digital imaging software (Adobe Photoshop, Adobe Systems Inc.) (Figure 1a, 1b, 1c), and composed into a single two-dimensional (2D) image (Figure 1d).

### Reconstruction of a three-dimensional image

A three-dimensional (3D) image of the artery was reconstructed from a series of the 2D images using 3D reconstruction software (TRI, RATOC System Engineering, Tokyo, Japan) to observe the spatial distribution of lipids and macrophages (Figure 1e, 1f, 1g, supplemental Movie I), and to calculate the ratio of the lipid volume to the arterial wall (intima + media) volume.

### Vertical distribution of lipids and macrophages

A 2D image with the most severe lipid deposits was selected from each case. The image with a branching site was excluded. The total lipid density, which was defined as the ratio of the total lipid area to the total arterial wall (intima + media) area, was calculated using digital
imaging software. Then, the arterial wall was divided into four layers, i.e., inner intima, outer intima, inner media and outer media, by two lines drawn circumferentially at the middle of the intima and media, respectively (Figure 2a), to calculate the percentage of the lipid area in the respective layer. The same measurements and calculations were performed for macrophages. The ratio of total macrophage area to total lipid area was calculated to determine the effect of smoking and diabetes mellitus on macrophage infiltration.

**Topographic relationship between lipids and intimal components**

In 21 cases (31.9 ± 11.6 years of age, 14 males and 7 females), seven cryosections of 4 micrometers thick were obtained from the adjacent portion of RCA. Two sections were stained with EVG and Sudan IV stains. The other five sections were immunostained with antibodies to apoB, α-SMA, α-elastin, biglycan and decorin. The distributions of ox-PC and MCP-1 were also investigated by immunohistochemistry with two other cryosections.

**Statistical analysis**

Results were given in mean ± SD, and analyzed by ANOVA, Student’s t-test, and Mann-Whitney’s U test. P<0.05 was considered significant.

**References**


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**Table**

**Table I.**
Grading of lipid deposition and correlations of the grade with the atherosclerotic lesions and clinical data.

<table>
<thead>
<tr>
<th>Grade (x100)</th>
<th>ratio of lipid volume to arterial wall volume*</th>
<th>n†</th>
<th>atherosclerotic lesion‡</th>
<th>age (years)</th>
<th>total cholesterol (mg/dl)</th>
<th>triglyceride (mg/dl)</th>
<th>smoking status¶</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>6</td>
<td>DIT (6)§</td>
<td>18.2±11.0†</td>
<td>175.2±57.9 (6)</td>
<td>148.8±76.9 (6)</td>
<td>S (2) NS (4)</td>
</tr>
<tr>
<td>1</td>
<td>0.01 - 0.50</td>
<td>12</td>
<td>FS (12)</td>
<td>29.7± 9.3</td>
<td>142.3±30.0 (12)</td>
<td>114.2±60.8 (11)</td>
<td>S (7) NS (4) UK (1)</td>
</tr>
<tr>
<td>2</td>
<td>0.51 - 5.00</td>
<td>13</td>
<td>FS (10) PIT (3)</td>
<td>35.2± 7.0</td>
<td>142.4±44.2 (13)</td>
<td>93.5±37.3 (11)</td>
<td>S (5) NS (8)</td>
</tr>
<tr>
<td>3</td>
<td>5.01 - 7</td>
<td>7</td>
<td>FS (1) PIT (6)</td>
<td>43.4± 6.8</td>
<td>155.7±33.9 (7)</td>
<td>153.7±45.9 (6)</td>
<td>S (6) NS (0) UK (1)</td>
</tr>
</tbody>
</table>

* arterial wall volume = intimal volume + medial volume
† n: number of cases in each grade
‡ DIT: diffuse intimal thickening, FS: fatty streak, PIT: pathologic intimal thickening
§ ( ): number of cases
¶ mean ± SD
‖ S: smoker, NS: nonsmoker, UK: unknown
**Movie**

**Movie I.** (Please see the supplemental movie.) Spatial distribution of lipids in the arterial wall. The movie is an animation of the model shown in Figure 1f. Lipids deposited on the epicardial surface and not on the myocardial surface of the coronary artery in this case. 39 years of age, female.

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**Figures**

**Figure 1.** Serial sections showing the extracellular localization of lipids. Lipids deposited in the extracellular space of the deep intima (a) shown as an unstained area in a section immunostained for smooth muscle cells (b). Some lipids were aggregated to demonstrate wavy appearance (a) similar to that of elastic fibers (c), suggesting the association of lipids with elastin. (a): Sudan IV stain. (b): Immunostaining with anti-α-SMA antibody. (c): Immunostaining with anti-α-elastin antibody. 44 years of age, male. Arrowheads indicate internal elastic lamina. Bars represent 20 µm.
Figure II. Serial sections showing the correlations between the localization of apoB and that of biglycan and decorin in the fatty streak. The distribution of apoB (b) coincided with that of the Sudan IV positive area (a). Biglycan (c) and decorin (d) were colocalized with apoB (b). (a): Sudan IV stain. I: intima, M: media. (b): Immunostaining with anti-apoB antibody. (c): Immunostaining with anti-biglycan antibody. (d): Immunostaining with anti-decorin antibody. 43 years of age, male. Arrowheads indicate internal elastic lamina. Bars represent 100 µm.
Figure III. Serial sections showing the localization of ox-PC and MCP-1. Ox-PC was present extracellularly in the outer layer of the intima (a) and colocalized with apoB (b). Ox-PC also existed in the cells (a). Round cells in the upper layer were macrophages, as shown in figure c, and polygonal and spindle cells in the deeper layer were SMCs. MCP-1 was found in most of the macrophages infiltrating in the upper layer of the intima and some SMCs (arrows) in the deeper layer (d). (a, b, c, d): Immunostaining with anti-ox-PC (a), anti-apoB (b), anti-CD68 (c) and anti-MCP-1 (d) antibodies, respectively. The magnification in figure d is twice as high as that in other figures so that the positive cells are clearly shown. 33 years of age, male. Arrowheads indicate internal elastic lamina. Bars represent 50 µm (a, b, c) and 20 µm (d).