Early Human Atherosclerosis

Accumulation of Lipid and Proteoglycans in Intimal Thickenings Followed by Macrophage Infiltration

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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;27:1159-1165.)**

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.[1] DIT, also known as "nonatherosclerotic" intimal thickening,[2] 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.[3] This fact was also recently confirmed in the coronary artery by McGill et al in The Pathobiological Determinants of Atherosclerosis in Youth (PDAY) study.[4] 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.[2,5] 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,[5] 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.[6] 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.[7] 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.

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In the present study, we examined human coronary arteries, aiming to clarify the morphological features of the early

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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.[1,2] The fatty streak was defined as a nonraised sudanophilic lesion.[3] PIT was defined as a lesion with an accumulation of extracellular lipids underneath a layer of lipid-laden macrophages.[2,5]

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),[8] decorin, monocyte chemoattractant protein-1 (MCP-1, F9),[9] and oxidized phosphatidylcholine (ox-PC, DLH3).[10]

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).

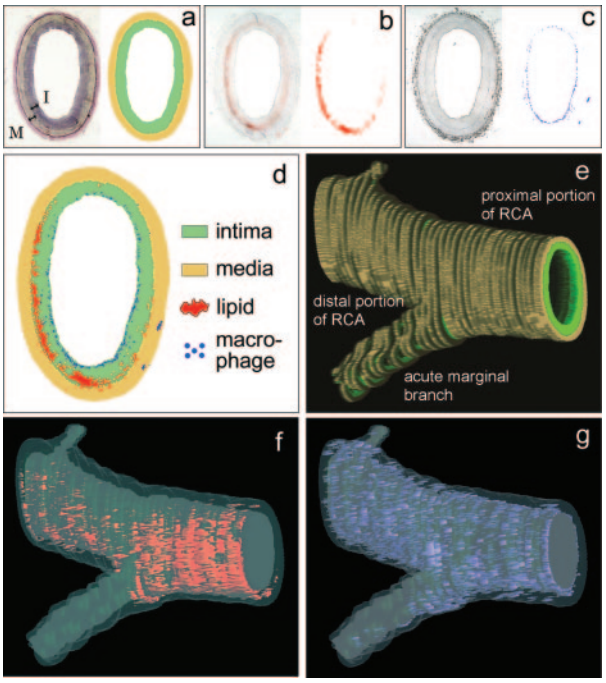

Figure 1. Construction of 2D and 3D images. a, A digital image of intima and media (right) extracted from a EVG stained section (left). I indicates intima; M, media. b, A digital image of lipids in the intima and media (right) extracted from a Sudan IV stained section (left). c, A digital image of macrophages in the intima and media (right) extracted from an immunostained section with anti-CD68 antibody (left). d, A 2D image composed of the digital images of intima, media, lipids and macrophages. e, A 3D image of the artery reconstructed from a series of 2D images. f and g, The arterial wall was made translucent to observe the distribution of lipids and macrophages, respectively. Lipid grade in Grade 2, fatty streak. 39 years of age, female.

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.

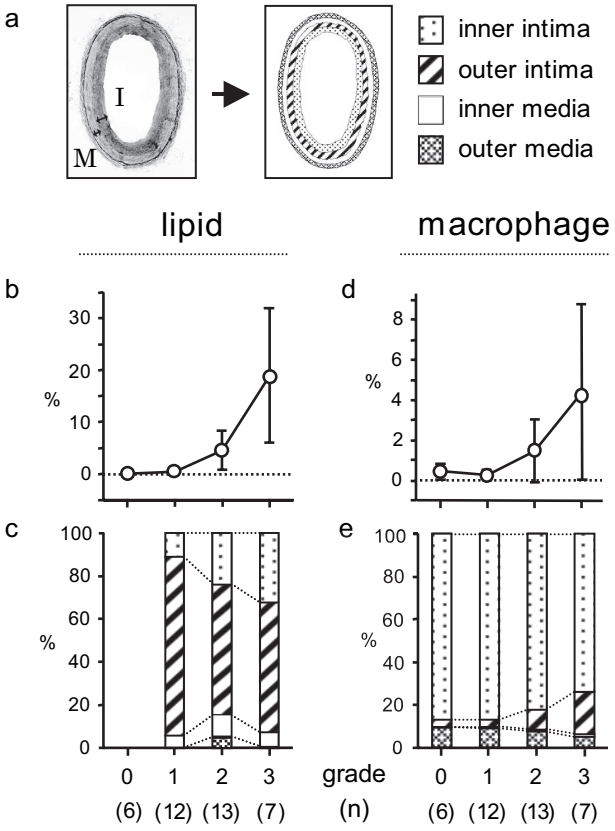

Figure 2. Density and vertical distribution of lipids and macrophages in the arterial wall. a, Four layers of the arterial wall divided as described in supplemental Methods section. I indicates intima; M, media. b, Total lipid density defined as the ratio of total lipid area to total arterial wall (intima + media) area. ANOVA, $P<0.0001$. c, The percentage of lipid area in the inner intima, outer intima, inner media, and outer media (from top to bottom) to total lipid area, which was averaged over the number of cases in each grade. d, Total macrophage density defined as the ratio of total macrophage area to total arterial wall area. ANOVA, $P<0.005$. e, The percentage of macrophage area in the inner intima, outer intima, inner media, and outer media (from top to bottom) to total macrophage area, which was averaged over the number of cases in each grade.

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, macrophages 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, $P=0.075$). The ratio in 2 diabetic patients was not different from that in nondiabetic patients of similar age.

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

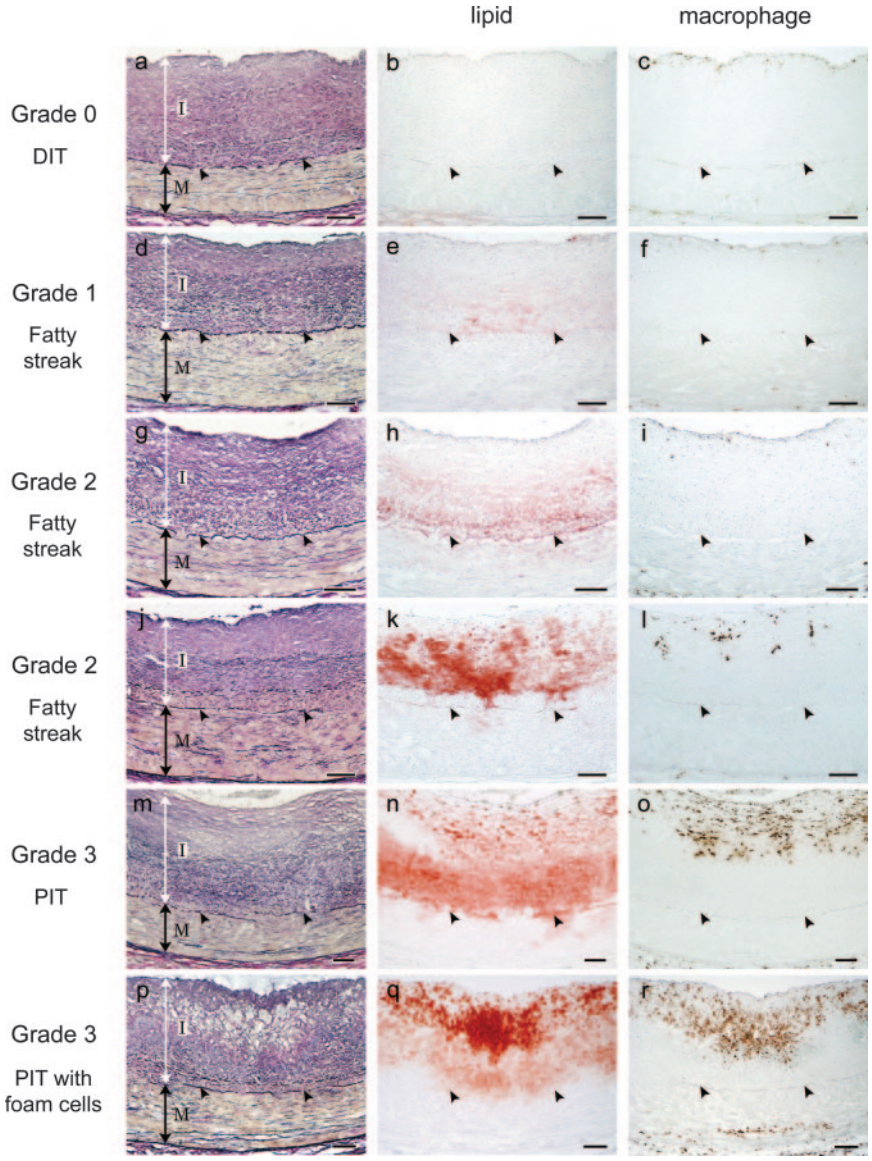

Figure 3. Representative histology of deposited lipids and infiltrating macrophages in the arterial wall. a, b, and c, Grade 0, DIT, 36 years of age, male. d, e, and f, Grade 1, fatty streak, 32 years of age, male. g, h, and i, Grade 2, fatty streak, 39 years of age, female. j, k, and l, Grade 2, fatty streak, 44 years of age, male. The total lipid density was greater in this case than the case of Figure g, h and i. m, n, and o, Grade 3, PIT, 49 years of age, male. An accumulation of lipid-laden (but not foamy) macrophages was seen in the upper to middle layer of the intima. p, q, and r, Grade 3, PIT with foam cells, 29 years of age, male. Foam cells with clear cytoplasm in EVG stained section and abundant intracellular lipids in Sudan IV stained section were accumulated in the upper to middle layers of the intima. a, d, g, j, m, and p, EVG stain. I indicates intima; M, media. b, e, h, k, n, and q, Sudan IV stain. c, f, i, l, o, and r, Immunostaining with anti-CD68 antibody. Arrowheads indicate internal elastic lamina. Bars represent 100 μm. DIT indicates diffuse intimal thickening; PIT, pathologic intimal thickening.

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 (supplemental Figure IIc). The same finding was observed for decorin in 6 cases (supplemental Figure IId), and no staining in 3 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 pathologic 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

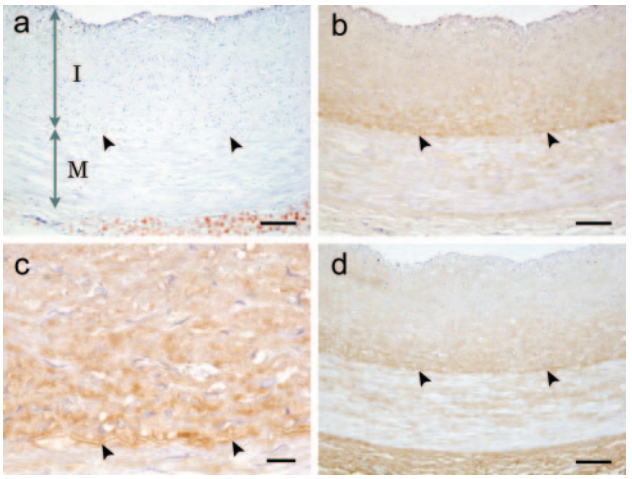

Figure 4. Serial sections showing the localization of biglycan and decorin in DIT. a, DIT with no lipid deposits. Sudan IV stain. I indicates intima; M, media. b, Immunostaining with anti-biglycan antibody. c, Higher magnification of the outer layer of DIT in b, showing extracellular localization of biglycan. d, Immunostaining with anti-decorin antibody. 36 years of age, male. Arrowheads indicate internal elastic lamina. Bars represent 100 μm (a, b, d) and 20 μm (c).

the absence of macrophages.[13] Investigating the human aorta of young and middle-aged adults, Guyton and Klemp suggested that an early lipid core arises from lipids accumulating gradually in the ECM of the deep intima.[14] In the present study, we confirmed that the initial lipid deposition occurred independently of foam cell death, as no such cells existed around the deposited lipids. Accumulation of lipids prior to the macrophage infiltration is also reported in animal models,[15] suggesting that the same molecular mechanism occurs both in experimental animals and humans. However, it is noteworthy that the environment where lipids deposit is considerably different. In experimental animals, lipids deposit in the narrow subendothelial space that consists almost exclusively of ECM and contains few SMCs.[15] However, in humans, the lipid deposits initially in the deep layer of the thickened intima of DIT which contains abundant ECM and SMCs.[1,2]

Macrophage Infiltration and Formation of PIT With Foam Cells

The present study suggests that macrophages are stimulated to accumulate in the fatty streak, infiltrate toward the deposited lipids, and subsequently form the PIT, as extracellular lipids increase in amount. According to in vitro studies, chemotactic movements of macrophages are induced via modification of low-density lipoprotein (LDL) and stimulation of MCP-1 production by cells in the lesion.[16] These mechanisms are expected to occur in vivo as well, because the presence of oxidized lipoproteins and MCP-1 was revealed in the intima in the present study. The existence of oxidized and enzymatically-modified LDL and MCP-1 is also demonstrated in the human arterial wall in other studies.[9,11,12] Cigarette smoking may enhance macrophage infiltration in the intima as shown in the present study. This enhancement may be related to the modification of LDL and/or inflammatory factors such as adhesion molecules by toxic substances in the smoke.

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.[17] 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.[2,5]

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 increase binding properties of proteoglycans for atherogenic lipoproteins.[18] For example, proteoglycans produced by transforming growth factor (TGF)-β1–treated cultured SMCs show longer GAG chains and greater binding affinity to LDL than control SMCs.[19] Interestingly, patchy distribution of TGF-β1 is found in DIT.[20] 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.[21] 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.[22] 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,[23] but opposite results were obtained in white Carneau pigeon aorta.[24]

Correlations between the distribution of lipids and proteoglycans have been investigated in early and advanced atherosclerotic lesions.[25–27] A consistent finding, including that of the present study, is the colocalization of biglycan and apolipoproteins.[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.[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,[25,27] but not in mouse models.[26] The role of versican may be different from that of biglycan and decorin, because its distribution is different from biglycan and decorin.[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.[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.[1]

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Disclosures

None.

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