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Natural Progression of Atherosclerosis from Pathologic Intimal Thickening to Late Fibroatheroma in Human Coronary Arteries: A Pathology Study

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Abstract

Objective—Smooth muscle cells, macrophage infiltration and accumulation of lipids, proteoglycans, collagen matrix and calcification play a central role in atherosclerosis. The early histologic changes of plaque progression from pathologic intimal thickenings (PIT) to late fibroatheroma lesions have not been fully characterized.

Methods—A total of 151 atherosclerotic coronary lesions were collected from 67 sudden death victims. Atherosclerotic plaques were classified as PIT without macrophage infiltration, PIT with macrophages, and early and late fibroatheromas. Presence of macrophages and proteoglycans (versican, decorin and biglycan) were recognized by specific antibodies while hyaluronan was detected by affinity histochemistry. Lipid deposition was identified by oil-red-O, and calcification was assessed following von Kossa and alizarin red staining.

Results—Lesion progression from PIT to late fibroatheroma was associated with increase in macrophage accumulation ($p<0.001$) and decreasing apoptotic body clearance by macrophages (ratio of engulfed-to-total apoptotic bodies) ($p<0.001$). Lipid deposition in lipid pool of PIT had a microvesicular appearance whereas those in the necrotic core were globular in nature. Overall, the accumulation of hyaluronan ($p<0.001$), and proteoglycan versican ($p<0.001$) and biglycan ($p=0.013$) declined along with lesion progression from PIT to fibroatheromas. Microcalcification was first observed only within areas of lipid pools and its presence and size increased in lesions with necrotic core.

Conclusions—PIT to fibroatheroma lesions are accompanied by early lipid accumulation, followed by macrophage infiltration with defective clearance of apoptotic bodies along with decrease in proteoglycan and hyaluronan in lipid pools that convert to necrotic cores. Calcification starts in PIT and increases with plaque progression.

Keywords

Atherosclerosis; pathology; macrophage; apoptosis; extracellular matrix; proteoglycans; calcification

1. Introduction

The natural history of atherosclerosis in humans is a dynamic process involving the progression of early lesions to advanced plaques that are responsible for the majority of acute ischemic cardiovascular events. The early development of atherosclerotic lesion in coronary arteries starts in industrialized nations in the second decade of their life [1]. However, the early atherosclerotic lesions do not always progress to late stage to cause catastrophic acute vascular events such as myocardial infarction or sudden cardiac death

[2-4]. Since the mouse models do not mimic the progression of atherosclerotic plaque seen in man, it is essential that lesion progression be studied in greater detail in human arteries to advance our knowledge such that new diagnostic modalities can be developed to identify lesions that are precursors of the lesions that lead to catastrophic events of thrombosis.

The earliest feature of progressive atherosclerosis as described by the American Heart Association (AHA) classification is pathologic intimal thickening (PIT), which is characterized by extracellular lipid accumulation (lipid pools) that are rich in proteoglycans and hyaluronan. Inflammation plays a pivotal role in the progression and destabilization of atherosclerotic lesions [5]. The infiltration of macrophages is a distinctive characteristic of progression of PIT to fibroatheromas; however, the processes involved are poorly understood. It has been reported both in animal models and in man that macrophages apoptosis is an integral process of plaque progression [6, 7]. Furthermore, increase in macrophage apoptosis, but also a decreased uptake of apoptotic bodies is thought to be of utmost importance in necrotic core formation in fibroatheroma [8].

Extracellular matrix (ECM) molecules such as proteoglycans and collagen play an important role in plaque mass but also govern lipid accumulation and the trafficking of inflammatory cells within the plaque. Many different proteoglycans and hyaluronan, which are non-fibrillar components of the ECM, have been identified in atherosclerotic plaques and are synthesized by vascular smooth muscle cells (SMCs) and influenced by growth factors [9-11]. The response-to-retention hypothesis in early atherogenesis states that atherogenic lipoproteins are retained in the intima by binding to ECM proteoglycans [12]. This hypothesis further states that lipoprotein-proteoglycan complexes exhibit increased susceptibility to oxidation and lead to uptake by macrophages to form foam cells. Proteoglycans have been reported to play a fundamental role in cellular and extracellular events associated with the pathogenesis of vascular lesions, such as thrombosis [13], lipid metabolism [14], and vascular SMC proliferation and migration [11]. Nakashima, et al. [15] showed that in early human coronary atherosclerotic lesions, biglycan and decorin were distributed in the outer layer of diffuse intimal thickening when no lipids were detected. In PIT, decorin co-localized with the lipid in some instances but much less than biglycan; however, the presence and localization of hyaluronan and versican were not evaluated in their study.

In the current study, we not only studied the accumulation of hyaluronan and other proteoglycans in progression of plaque from PIT to late fibroatheroma but also showed how these relate to the presence of lipid, macrophage and apoptosis along with calcification to further our understanding of their relationship to one another. Elucidating the involvement of these pivotal processes of atherosclerotic progression, through which lipid pool convert to the necrotic core, is of utmost importance to prevent or slow the progress of advanced lesion development.

2. Methods

2.1. Study Population

The coronary arteries for this study were obtained from the hearts of 67 patients (60 men, mean age = 47.6 ± 11.1 years) who had died suddenly of coronary causes (n=41 cases) or non-coronary causes (n=26 cases) (Table 1). A total of 151 coronary plaques of various phenotypes from early atherosclerosis lesions, PIT to fibroatheroma, were selected for further analysis. We selected 1 section from 1 lesion to represent the lesion morphology. The 151 lesions included 128 paraffin-embedded and 23 frozen sections. Morphometric analysis was performed in all paraffin-embedded lesions. Select 60 paraffin-embedded lesions were used for immunostaining for macrophages (CD68), T-lymphocytes (CD45RO), vasa vasorum (CD31/34), and nick-end in situ labeling for apoptosis, while 40 paraffin-embedded lesions were submitted for additional proteoglycan staining (10 of 40 lesions had been used in previous report [16]). In addition, 28 non-decalcified paraffin-embedded sections with lesion morphology PIT or early fibroatheroma were used for von Kossa and alizarin red staining. The additional 23 frozen sections were used for oil red O staining (Table 1, Supplemental Figure 1).

2.2. Classification of Lesions

Early coronary plaques were classified using our modified AHA classification, to include adaptive intimal thickening (AIT), fatty streak, PIT, and fibroatheromas [17]. AIT consists mainly of SMCs and proteoglycan matrix with little or no infiltration of inflammatory cells. Fatty streak represent lesions primarily composed of foamy macrophages and to a lesser extent lipid laden SMCs within the intima. PIT is characterized by the presence of SMCs interspersed within ECM towards the lumen and areas of extracellular lipid accumulation with absence of SMCs (lipid pool) close to the media, and were further divided into 2 groups based on the presence or absence of macrophage infiltration (PIT without macrophages and PIT with macrophages). Fibroatheromas were characterized by a dense fibrous cap overlying a necrotic core, and this lesion was further divided into 2 different stages; early and late fibroatheromas [18]. Early fibroatheroma was defined as a lesion consisting of macrophage infiltration into the lipid pool forming focal areas of necrosis, with loss of proteoglycans as assessed by the Movat pentachrome staining, and few free cholesterol crystals. Late fibroatheroma consists of necrotic core with discrete collections of cellular debris, increase in free cholesterol crystals, and complete depletion of ECM (proteoglycans and collagen).

2.3. Histological Preparation

Coronary arteries (except those for frozen sectioning) were fixed in 10% neutral buffered formalin, sectioned serially at 3- to 4-mm thickness, and submitted for paraffin embedding. Histologic sections were cut at 6 μ m, mounted on charged slides, and stained with hematoxylin-eosin (H&E), Movat pentachrome, von Kossa, and alizarin red. Frozen sections were cut at 6 μ m and stained with oil red O stain as previously described [18].

2.4. Immunohistochemistry

Immunohistochemistry was carried out using an anti-CD68 antibody (KP-1 clone, Dako, Carpinteria, CA) for the identification of macrophages, an anti-CD45RO antibody (Dako) for T-lymphocytes, and an anti-CD31/34 antibody (Dako) for endothelial cells. All primary antibodies were labeled with a biotinylated linked antibody directed against mouse antigen with the use of a peroxidase-based kit (LSAB, Dako) and visualized by a 3-amino-9-ethylcarbazole substrate. The sections were counterstained with Gill's hematoxylin (Sigma-Aldrich).

2.5. Apoptosis Staining

Apoptotic nuclei were identified by in situ end labeling (ISEL) DNA fragmentation staining using terminal deoxyribonucleotide transferase (TdT)-mediated nick end-labeling (TACS; Trevigen, Gaithersburg, MD) as described previously [19]. Co-localization of apoptotic nuclei and macrophages were evaluated by combined ISEL and immunostaining using an anti-CD68 antibody. Tissue sections were initially stained for DNA fragmentation substituting, followed by immunostaining of macrophages.

2.6. Proteoglycan Staining

Accumulation of hyaluronan was identified by affinity histochemistry and that of specific proteoglycans (versican, decorin and biglycan) were evaluated using specific antibodies. For proteoglycan immunohistochemistry, mouse IgG (versican) or rabbit IgG (biglycan and decorin) was used as negative controls, and little to no non-specific staining was observed with isotype controls (data not shown). A rabbit antibody specific for the poly E region of human versican (VC-E) was kindly provided by Richard LeBaron (University of Texas at San Antonio). The biotinylated hyaluronan-binding protein region of aggrecan was used as a specific probe for the detection of hyaluronan (4 g/mL) and was kindly provided by Charles Underhill (Department of Anatomy and Cell Biology, Georgetown University, Washington, DC). Specificity of hyaluronan staining was verified by abolition of staining by pretreatment of the sections with Streptomyces hyaluronidase (data not shown). The reactions were visualized by using streptavidin conjugated to horseradish peroxidase according to a method described previously. Color development with diaminobenzidine was contrast-enhanced with NiCl₂. Rabbit polyclonal antisera for the core proteins (amino-terminal peptides) of human biglycan (LF-51) and decorin (LF-136) were generously provided by Larry Fisher, National Institute of Dental Research, Bethesda, MD [20].

2.7. Morphometric Analyses

Morphometric measurement of coronary sections was performed using image-processing software (IPLabs, Scanalytics, Rockville, MD) on slides stained with Movat pentachrome. Quantitative planimetry included area analysis of the internal elastic lamina (IEL), lumen, and necrotic core size. Plaque area was defined as IEL minus lumen area, and the percent stenosis was obtained as plaque area divided by IEL area. Computer-assisted color image analysis segmentation with background correction was used to quantify immunohistochemical stains of macrophages and proteoglycans within regions of interest, and the percentage of positive staining in regions of interest was determined. The extent of

T-cell infiltration was evaluated as cell count in a select high power field ($\times 400$ magnification) where the greatest infiltration of T-cells in the section was observed. The extent of vasa vasorum was assessed as the number of CD31/34 positive vessels in each section. Apoptotic body density was evaluated as the mean ratio of ISLE-positive nuclei to total number of nuclei in 2 different high power fields ($\times 400$ magnification). In addition, the number of free and engulfed apoptotic bodies was counted in 2 different high power fields ($\times 400$ magnification) on slides with dual immunostaining for macrophages and apoptotic bodies, and the extent of apoptotic body clearance was assessed as the ratio of engulfed to total (free and engulfed) apoptotic bodies.

2.8. Statistical Analysis

Continuous variables with normal distribution were expressed as mean $\pm$ SD. Variables with non-normal distribution were expressed as median and 25th to 75th percentiles. Comparisons of continuous variables with normal distribution were tested by the oneway analysis of variance (ANOVA) followed by all pairs Tukey HSD (honestly significant difference) test for all differences among means. A Wilcoxon Kruskal-Wallis test was used for comparisons of non-normally distributed continuous variables. Normality of distribution was tested with the Shapiro-Wilk test. Categorical variables were analyzed by the chi-square test. All analyses were performed with the use of JMP5 (SAS Institute, Cary, NC). A value of $p < 0.05$ was considered statistically significant.

3. Results

3.1. Lesion Characteristics

The paraffin-embedded lesions ($n=128$) included 27 PIT without macrophages, 37 PIT with macrophages, 41 early fibroatheroma, and 23 late fibroatheroma (Table 1, Supplemental Figure 1). Selected coronary lesions for the assessment of inflammation, vasa vasorum, and apoptosis ($n=60$ lesions) consisted of 10 PIT without macrophages, 18 PIT with macrophages, 19 early fibroatheroma, and 13 late fibroatheroma, while additional sections for proteoglycan staining included 40 lesions (10 lesions for each morphology). Twenty-eight non-decalcified sections consisted of 7 PIT without macrophages, 9 PIT with macrophages, and 12 early fibroatheromas, for von Kossa and alizarin red staining (calcium stain). There were no difference in the location of the lesions, i.e., the differential coronary arteries and the geometry (proximal or mid or distal), among the 4 groups (Table 2). The 23 frozen sections for oil red O staining included 3 adaptive intimal thickening, 3 fatty streak, 3 PIT without macrophages, 4 PIT with macrophages, 4 early fibroatheromas and 6 late fibroatheromas (Table 1, Supplemental Figure 1).

3.2. Plaque Progression, Inflammation, Vasa Vasorum, and Apoptosis

Representative histologic images of coronary plaques showing macrophages and T-lymphocytes infiltration and vasa vasorum in association with lesion progression are illustrated in Figure 1. Morphometric analyses showed no significant difference in IEL area among the 4 groups, whereas PIT with macrophages had smaller plaque area as compared to early and late fibroatheromas (Figure 2, Supplemental Table 1). Stenosis severity increased in concordance with lesion progression from PIT (mean, PIT without macrophages=41.4%,

PIT with macrophages=47.2%) to fibroatheromas (early fibroatheroma=59.4%, late fibroatheroma=70.9%), while necrotic core size did not differ significantly between early and late fibroatheromas (Figure 2, Supplemental Table 1).

Macrophage infiltration increased in association with lesion progression from PIT to late fibroatheroma, where a significant difference was observed between late fibroatheroma (median, 2.99%) versus PIT with macrophages (1.29%) and early fibroatheroma (1.51%) (Figure 1, 2, 3A, Supplemental Table 1). T-cell infiltration was the greatest in PIT with macrophages, followed by early fibroatheroma, late fibroatheroma, and was the least in PIT without macrophages (Figure 1, Supplemental Table 1). The extent of vasa vasorum increased from PIT without macrophages to early and late fibroatheromas (Figure 1, Supplemental Table 1). Apoptotic body density was low in PIT with and without macrophages; however, it increased from PIT to early fibroatheromas, and was the greatest in late fibroatheromas, where a significant difference was observed between late fibroatheroma (median, 18.7%) versus PIT without macrophages (6.98%), PIT with macrophages (6.22%), and early fibroatheroma (10.65%) (Figure 2, 3A, Supplemental Table 1). Dual immunostaining for macrophages (CD68) and apoptotic bodies demonstrated that most apoptotic bodies co-localized with macrophages and were engulfed in PIT lesions with macrophages, whereas the proportion of free apoptotic bodies increased and engulfed macrophages decreased through the process of lesion progression from PIT with macrophages to early and late fibroatheromas (mean percentage of engulfed apoptotic bodies, PIT with macrophages=67.5%, early fibroatheroma=41.5%, late fibroatheroma=32.1%) (Figure 2, 3B, Supplemental Table 1).

3.3. Proteoglycan Accumulation

Representative histologic images of ECM molecules hyaluronan and proteoglycans (versican, biglycan, and decorin) in PIT and early and late fibroatheromas are shown in Figure 4A. The accumulation of hyaluronan and proteoglycan in the lipid pool/necrotic core declined in concordance with progression of lesions from PIT to early and late fibroatheroma (Figure 4A and 4B, Supplemental Table 2). Hyaluronan accumulated in PIT without macrophages (median, 33.4%) and PIT with macrophages (54.4%), thereafter it sharply declined in early fibroatheroma (28.1%) and was significantly less in late fibroatheroma (3.5%) ($p<0.001$). Versican showed a similar pattern as hyaluronan, with high accumulation in PIT without macrophages (median, 59.4%) and PIT with macrophages (41.1%), and a sharp decline to early fibroatheroma (21.3%) and near absence in late fibroatheroma (1.0%) ($p<0.001$). The accumulation of biglycan was less compared to hyaluronan and versican, but similar patterns were observed; however, decorin did not show similar decline (Figure 4A and 4B, Supplemental Table 2).

The localization of the proteoglycans in PIT was dispersed throughout the lesions, while in early and late fibroatheromas, the decline in accumulation was mainly observed in the region of the necrotic core and was patchy around areas of macrophage infiltration in lipid pools. In late fibroatheroma, the accumulation of proteoglycans was very low and was effectively non-existent in the necrotic core (Figure 4A and 4B).

3.4. Lipid Accumulation

Frozen sections of adaptive intimal thickening did not show any presence of lipid, while fatty streak showed globular (relatively large) lipid droplets within macrophages, with absence of microvesicular (tiny) lipid droplets in the surrounding neointimal tissue (Figure 5). In the case of PIT without macrophages, microvesicular lipid droplets were seen in the region of the lipid pools but the overlying neointima rich in SMCs failed to show any presence of lipid. On the other hand, in PIT with macrophages, the areas of the lipid pool showed microvesicular lipid droplets whereas areas of the macrophages showed globular lipid droplets within macrophages. Early fibroatheromas in the area of the early necrotic core showed a mixture of microvesicular and globular lipid droplets, while the late necrotic core showed a heavy infiltration of globular lipid droplets, along with cholesterol cleft that did not stain with oil red O (Figure 5).

3.5. Microcalcification

Calcification was assessed in sections that were not decalcified and therefore both alizarin red and von Kossa stains showed the presence of microcalcification (Figure 6), varying in size 0.5 μ m and <15 μ m (early microcalcification) in the areas of lipid pool in 4 of the 7 sections (57%) of PIT without macrophages, where none of the calcifications measured $\geq$ 15 μ m (punctate microcalcification) (Table 3). On the contrary, in the lesions of PIT with macrophages, 7 of the 9 sections (78%) showed presence of microcalcification; 3 sections (33%) with early microcalcification (< 0.5 μ m, <15 μ m) and 4 with punctate microcalcification ($\geq$ 15 μ m in diameter). In early fibroatheromas, all sections showed presence of microcalcification and majority (10 of 12 sections, 83%) had punctate calcification ($\geq$ 15 μ m) (Table 3). The extent of microcalcification (both circumferential and in depth within the intima) increased from PIT without macrophages to early fibroatheroma, although the difference was not statistically significant in this limited number of sections evaluated.

Because late fibroatheromas almost uniformly show some degree of confluent areas of calcification, it is not possible to cut paraffin section without decalcification and have quality section, therefore special stains for the identification of calcium were only carried out in early lesions. Nevertheless, the decalcified arterial sections from late fibroatheromas showed fragment or sheets of calcification as identified by H&E and Movat pentachrome stains (Supplemental Figure 2).

4. Discussion

The current study demonstrates the progression of human coronary plaques from PIT to late fibroatheroma that is associated with increasing macrophage infiltration and buildup of apoptotic bodies, as well as a decrease in the phagocytic clearance of these bodies. The accumulation of ECM molecules such as versican and hyaluronan was inversely associated with lesion progression. In addition, both ECM molecules were present in the lipid pool regions in PIT; however, as the lipid pool converted into a necrotic core in the presence of infiltration of macrophages, the existence of versican, biglycan and hyaluronan decreased. Lipid pools contained microvesicular lipid and were devoid of SMCs. The lack of SMCs in

lipid pools appear to be attributed to apoptotic cell death, which is associated with calcification [21]. The focal microcalcification (0.5 μ m in size) represented over 50% of lipid pools. On the other hand, early fibroatheromas show macrophage infiltration within areas of lipid pools that are being converted to necrotic cores, presumably through the digestion of hyaluronan and versican from increasing infiltration by macrophages, followed by apoptosis and accumulating calcium. What was surprising was that calcification was observed in all cases of early and late fibroatheromas, the latter demonstrates more confluent areas of calcification (fragment or sheet calcification). A most important element in the transition of early plaque to late plaque was the increasing presence of macrophages and buildup of apoptotic bodies suggesting defective clearance of apoptotic bodies that help convert the lipid pool to a necrotic core, while simultaneously there is increasing presence of calcification. The lipid deposition also changes in character with lipid pools showing microvesicular appearance and the late plaques containing more globular lipid within necrotic cores with greater contribution from foamy macrophages.

Chemical composition of lipid in early lesion fatty streaks is mostly composed of cholesterol esters (77%) and phospholipids (10%) with free cholesterol being less than 10%, whereas intermediate lesions (AHA Type III) are composed of greater quantities of phospholipids (20%) and free cholesterol (20%) and less cholesterol esters (55%) [22]. However, once gruel is observed, i.e., lesions of fibroatheromas with necrotic core, there is significantly greater free cholesterol and less of cholesterol ester and phospholipids [22, 23]. These changes in chemical composition are also seen as physiochemical properties of lipid [22] when stained by oil red O stains as demonstrated in the character of the lipid droplets observed in lipid pools and necrotic cores.

4.1. Macrophage Infiltration, Apoptosis and Apoptotic Body Clearance

The infiltration of macrophages in PIT lesions is an early and important step towards the progression of atherosclerotic lesions to fibroatheromas. The accumulation of lipid-laden macrophages occurs near the lumen and ultimately has an impact on atherosclerotic lesion progression. With progression to early fibroatheromas, there is an increase in macrophage apoptosis [24, 25]. In normal physiology, many cells throughout the body undergo apoptosis each day, especially during embryogenesis where there is a balance between apoptosis and cell growth. In plaque progression however, eventually most cells present within the atherosclerotic plaques, including endothelial cells, SMCs, lymphocytes, and macrophages, have been shown to undergo apoptotic cell death [26]. This broad apoptotic process may have several implications for the multifactorial processes that are involved in plaque progression.

The apoptosis of macrophages and T-lymphocytes may have a potential beneficial effect because decrease of these cells within the plaque would attenuate the inflammatory response and lower the synthesis of matrix metalloproteinases [25]. However, the loss of macrophages would also decrease the uptake of apoptotic bodies, which were also observed in the present study. Macrophages secrete pro-inflammatory mediators during the ingestion of apoptotic bodies and contribute to the formation of secondary necrosis [27]. Moreover, the cells undergoing secondary necrosis potentially releasing chemokines which could

trigger a renewed cycle of inflammatory response [28]. Rapid phagocytic clearance of the apoptotic bodies (“efferocytosis”) prevents subsequent postapoptotic necrosis. However, an inefficient removal of apoptotic bodies by macrophages, as shown by us and others, leads to the accumulation of apoptotic bodies resulting in the formation of necrotic debris [24].

Indeed, there is evidence in mouse models that macrophage apoptosis occurs in early lesions, but efficient efferocytosis renders this process possibly beneficial with a decrease in lesion progression [28]. Conversely, in advanced lesions, macrophage apoptosis is associated with defective clearance and secondary necrosis, with the formation of necrotic debris from defective efferocytosis resulting in the propagation of the plaque inflammation. Direct support for this theory was provided by Schrijvers et al, who showed that late fibroatheromas in human carotid plaques contained a substantial number of apoptotic bodies within the necrotic core that were not phagocytosed by macrophages [25]. On the other hand, the tonsillar tissue, an example of an efficient system, showed that most of the apoptotic bodies were intracellular with the ability to rid of senescent cells. Our observations in the present study suggest that a similar process to the mouse models also occurs in human coronary lesions and that plaque progression and the formation of necrotic core is initiated from the collection of apoptotic bodies. Increased macrophage apoptotic cell death has been shown previously by us at sites of plaque rupture within the thin fibrous cap and may contribute to its discontinuity [19].

Possible mechanisms accounting for the defective phagocytic clearance of apoptotic bodies by macrophages in advanced atherosclerotic lesions involve competitive inhibition of phagocyte receptors, such as the type A scavenger receptor, CD36, and CD68, between apoptotic bodies and oxidized molecules which exist in necrotic cores [29]. In addition, naturally occurring antibodies to oxidized low-density lipoprotein (LDL) in atherosclerotic lesions can bind phagocyte ligands on apoptotic macrophages, leading to the inhibition of their uptake [30].

4.2. Proteoglycans in Plaque Progression

In addition to the response-to-retention hypothesis, the current study showed the relation of the decrease in proteoglycan versican and hyaluronan to the conversion of the lipid pool to a necrotic core. Versican likely plays a key role in the development and progression of plaque as previously shown in the studies performed in monkeys, and in restenotic and thrombosis prone plaques in man [10]. Versican mainly accumulates in human vessels susceptible to atherosclerosis such as the coronary artery and saphenous veins used for grafting, while only minor accumulation is seen in vessels resistant to atherosclerosis such as internal mammary [31]. Versican interacts with hyaluronan, resulting in the immobilization of hyaluronan and versican through the presence of link protein [32-34]. A number of studies have shown that both ECM molecules are increased in diseased arteries [20, 35-37].

In early human coronary lesions, a family of ECM molecules, including biglycan and decorin, were identified to contribute to the early phases of coronary lesion formation before the stage of PIT [15]. In more advanced human coronary disease, versican is prominent at the edges of the necrotic core, but not in the lipid rich center of the necrotic core [20]. In addition, versican-hyaluronan complexes are also present at the plaque thrombus interfaces

[16]. This suggests that the complex functions as ancillary platelet ligands, together with other known ligands, influence platelet deposition after rupture of the atherosclerotic plaques resulting in the formation of thrombus [13]. In the present study, versican accumulation was observed in all lesions but topographically was observed especially in the lipid pool areas of PIT, while in lesions with necrotic core, it was observed at the edges of the core. The interaction of versican with hyaluronan was confirmed by the similarity of their distribution in early plaques.

ECM molecules interact with apoB-rich lipoproteins causing retention, aggregation and modification of LDL particles in early plaque development [38] as demonstrated by the presence of microvesicular lipid droplets observed only in the lipid pools of PIT. The proteoglycan-LDL complexes are taken up rapidly by macrophages and SMCs. This uptake leads to the formation of foam cells which begins close to the luminal surface with migration of such cells into the lipid pools, which is observed in the transition of such lesions into early necrotic core and the presence of globular lipid droplets seen in the early and late fibroatheromas [15]. Accumulation of versican and hyaluronan may also influence the retention of inflammatory cells contributing to the progression of atherosclerotic lesions. For example, studies have shown that ECM molecules are involved in stabilizing CD44-dependent interactions [39], supporting macrophage adhesion [40, 41], and interacting with inflammatory chemokines [42]. Recently, a study by Nagy et al. showed that the inhibition of hyaluronan synthesis accelerates in the murine model of atherosclerosis thereby facilitating leukocyte adhesion, subsequent inflammation, and progression of atherosclerosis [43].

The gradual decrease in versican in the development of a necrotic core in association with an increased number of macrophages as seen in the present study, suggests that macrophages likely have a role in versican degradation. One of the possible mechanisms responsible for this process is the macrophage-derived interleukin-1, which possibly decreases versican expression while increasing the synthesis of decorin [44, 45].

4.3. Limitations

A retrospective analysis of autopsy tissue cannot identify mechanisms of lesion progression and thrombosis because the study material represents static observations. Because of these limitations, it is difficult to discern whether the observations are a pathogenetic factor or a consequence of sequential alterations. The lack of significant differences in morphometry, apoptosis, and the extent of ECM accumulation between PIT with and without macrophages raises a question if macrophage infiltration truly represents the progression of PIT or not. Although the accumulation of lipid in the extracellular location proceeds the transition of monocytes to foamy macrophages, it is likely that macrophages presence in the intima must occur prior to the eventual infiltration into the lipid pool as the lipid pool itself without macrophages could not convert to necrotic core. *In vivo* studies in animals are limited by nature, for example a versican knockout model is not compatible with life due to its indispensable role in heart and blood vessel development [46]. On the other hand, recent studies have shown reliable animal models that recapitulate several features of vulnerable plaques leading to plaque rupture, including low shear regions, intraplaque hemorrhage,

intramural thrombus, and neovascularization, which could be used to investigate disease progression [47-49]. Nevertheless, animal lesions do not resemble those seen in man. Therefore, we sought to understand the important mechanisms of early stages of plaque progression by careful and detailed study of human coronary lesions. The implications of these findings as a mechanism of plaque progression will help facilitate the development of novel imaging modalities and biomarkers that will be needed to prevent acute vascular complications; nevertheless, this will require *in vivo* clinical studies.

5. Conclusion

Increase in macrophage presence, apoptotic bodies and defective clearance by macrophages, decrease in proteoglycan and hyaluronan accumulation, and increase in calcification observed during plaque progression from PIT to late fibroatheroma, show the importance of these observations. The current study provides detailed histomorphological characteristics during plaque progression from PIT to late fibroatheroma in human coronary arteries, which have only sporadically been reported. Our findings suggest that proteoglycans, hyaluronan and lipid deposition together with macrophages further contribute to the progression of atherosclerosis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Nakashima Y, Wight TN, Sueishi K. Early atherosclerosis in humans: role of diffuse intimal thickening and extracellular matrix proteoglycans. *Cardiovasc Res.* 2008; 79:14–23. [PubMed: 18430750]
2. Velican C, Velican D. Discrepancies between data on atherosclerotic involvement of human coronary arteries furnished by gross inspection and by light microscopy. *Atherosclerosis.* 1982; 43:39–49. [PubMed: 7092981]
3. McGill HC Jr, McMahan CA, Herderick EE, et al. Effects of coronary heart disease risk factors on atherosclerosis of selected regions of the aorta and right coronary artery. PDAY Research Group. Pathobiological Determinants of Atherosclerosis in Youth. *Arterioscler Thromb Vasc Biol.* 2000; 20:836–845. [PubMed: 10712411]
4. Motoyama S, Sarai M, Harigaya H, et al. Computed tomographic angiography characteristics of atherosclerotic plaques subsequently resulting in acute coronary syndrome. *J Am Coll Cardiol.* 2009; 54:49–57. [PubMed: 19555840]
5. Hansson GK. Inflammation, atherosclerosis, and coronary artery disease. *N Engl J Med.* 2005; 352:1685–1695. [PubMed: 15843671]
6. Kockx MM, Herman AG. Apoptosis in atherosclerosis: beneficial or detrimental? *Cardiovasc Res.* 2000; 45:736–746. [PubMed: 10728396]

7. Littlewood TD, Bennett MR. Apoptotic cell death in atherosclerosis. *Curr Opin Lipidol.* 2003; 14:469–475. [PubMed: 14501585]
8. Schrijvers DM, De Meyer GR, Herman AG, et al. Phagocytosis in atherosclerosis: Molecular mechanisms and implications for plaque progression and stability. *Cardiovasc Res.* 2007; 73:470–480. [PubMed: 17084825]
9. Wight, TN. The vascular extracellular matrix. In: Fuster, V.; Ross, R.; Topol, EJ., editors. Chapter 24 Atherosclerosis and coronary artery disease. Philadelphia, PA: Lippincott-Raven Publishers; 1996. p. 421-440.
10. Wight TN, Merrilees MJ. Proteoglycans in atherosclerosis and restenosis: key roles for versican. *Circ Res.* 2004; 94:1158–1167. [PubMed: 15142969]
11. Wight TN. Arterial remodeling in vascular disease: a key role for hyaluronan and versican. *Front Biosci.* 2008; 13:4933–4937. [PubMed: 18508558]
12. Williams KJ, Tabas I. The response-to-retention hypothesis of early atherogenesis. *Arterioscler Thromb Vasc Biol.* 1995; 15:551–561. [PubMed: 7749869]
13. Kolodgie FD, Burke AP, Wight TN, et al. The accumulation of specific types of proteoglycans in eroded plaques: a role in coronary thrombosis in the absence of rupture. *Curr Opin Lipidol.* 2004; 15:575–582. [PubMed: 15361794]
14. Gustafsson M, Boren J. Mechanism of lipoprotein retention by the extracellular matrix. *Curr Opin Lipidol.* 2004; 15:505–514. [PubMed: 15361785]
15. Nakashima Y, Fujii H, Sumiyoshi S, et al. Early human atherosclerosis: accumulation of lipid and proteoglycans in intimal thickenings followed by macrophage infiltration. *Arterioscler Thromb Vasc Biol.* 2007; 27:1159–1165. [PubMed: 17303781]
16. Kolodgie FD, Burke AP, Farb A, et al. Differential accumulation of proteoglycans and hyaluronan in culprit lesions: insights into plaque erosion. *Arterioscler Thromb Vasc Biol.* 2002; 22:1642–1648. [PubMed: 12377743]
17. Virmani R, Kolodgie FD, Burke AP, et al. Lessons from sudden coronary death: a comprehensive morphological classification scheme for atherosclerotic lesions. *Arterioscler Thromb Vasc Biol.* 2000; 20:1262–1275. [PubMed: 10807742]
18. Kolodgie FD, Gold HK, Burke AP, et al. Intraplaque hemorrhage and progression of coronary atheroma. *N Engl J Med.* 2003; 349:2316–2325. [PubMed: 14668457]
19. Kolodgie FD, Narula J, Burke AP, et al. Localization of apoptotic macrophages at the site of plaque rupture in sudden coronary death. *Am J Pathol.* 2000; 157:1259–1268. [PubMed: 11021830]
20. Evanko SP, Raines EW, Ross R, et al. Proteoglycan distribution in lesions of atherosclerosis depends on lesion severity, structural characteristics, and the proximity of platelet-derived growth factor and transforming growth factor-beta. *Am J Pathol.* 1998; 152:533–546. [PubMed: 9466580]
21. Proudfoot D, Skepper JN, Hegyi L, et al. Apoptosis regulates human vascular calcification in vitro: evidence for initiation of vascular calcification by apoptotic bodies. *Circ Res.* 2000; 87:1055–1062. [PubMed: 11090552]
22. Katz SS, Shipley GG, Small DM. Physical chemistry of the lipids of human atherosclerotic lesions. Demonstration of a lesion intermediate between fatty streaks and advanced plaques. *J Clin Invest.* 1976; 58:200–211. [PubMed: 932206]
23. Felton CV, Crook D, Davies MJ, et al. Relation of plaque lipid composition and morphology to the stability of human aortic plaques. *Arterioscler Thromb Vasc Biol.* 1997; 17:1337–1345. [PubMed: 9261265]
24. Gautier EL, Huby T, Witztum JL, et al. Macrophage apoptosis exerts divergent effects on atherogenesis as a function of lesion stage. *Circulation.* 2009; 119:1795–1804. [PubMed: 19307478]
25. Schrijvers DM, De Meyer GR, Kockx MM, et al. Phagocytosis of apoptotic cells by macrophages is impaired in atherosclerosis. *Arterioscler Thromb Vasc Biol.* 2005; 25:1256–1261. [PubMed: 15831805]
26. Stoneman VE, Bennett MR. Role of apoptosis in atherosclerosis and its therapeutic implications. *Clin Sci (Lond).* 2004; 107:343–354. [PubMed: 15230690]

27. Fadok VA, Bratton DL, Konowal A, et al. Macrophages that have ingested apoptotic cells in vitro inhibit proinflammatory cytokine production through autocrine/paracrine mechanisms involving TGF-beta, PGE2, and PAF. *J Clin Invest.* 1998; 101:890–898. [PubMed: 9466984]
28. Tabas I. Consequences and therapeutic implications of macrophage apoptosis in atherosclerosis: the importance of lesion stage and phagocytic efficiency. *Arterioscler Thromb Vasc Biol.* 2005; 25:2255–2264. [PubMed: 16141399]
29. Sambrano GR, Steinberg D. Recognition of oxidatively damaged and apoptotic cells by an oxidized low density lipoprotein receptor on mouse peritoneal macrophages: role of membrane phosphatidylserine. *Proc Natl Acad Sci U S A.* 1995; 92:1396–1400. [PubMed: 7877989]
30. Chang MK, Bergmark C, Laurila A, et al. Monoclonal antibodies against oxidized low-density lipoprotein bind to apoptotic cells and inhibit their phagocytosis by elicited macrophages: evidence that oxidation-specific epitopes mediate macrophage recognition. *Proc Natl Acad Sci U S A.* 1999; 96:6353–6358. [PubMed: 10339591]
31. Merrilees MJ, Beaumont B, Scott LJ. Comparison of deposits of versican, biglycan and decorin in saphenous vein and internal thoracic, radial and coronary arteries: correlation to patency. *Coron Artery Dis.* 2001; 12:7–16. [PubMed: 11211169]
32. Radhakrishnamurthy B, Jeanson N, Tracy RE, et al. A monoclonal antibody that recognizes hyaluronic acid binding region of aorta proteoglycans. *Atherosclerosis.* 1993; 98:179–192. [PubMed: 7681290]
33. Levesque H, Girard N, Maingonnat C, et al. Localization and solubilization of hyaluronan and of the hyaluronan-binding protein hyaluronectin in human normal and arteriosclerotic arterial walls. *Atherosclerosis.* 1994; 105:51–62. [PubMed: 7512338]
34. Binette F, Cravens J, Kahoussi B, et al. Link protein is ubiquitously expressed in non-cartilaginous tissues where it enhances and stabilizes the interaction of proteoglycans with hyaluronic acid. *J Biol Chem.* 1994; 269:19116–19122. [PubMed: 8034670]
35. Halpert I, Sires UI, Roby JD, et al. Matrilysin is expressed by lipid-laden macrophages at sites of potential rupture in atherosclerotic lesions and localizes to areas of versican deposition, a proteoglycan substrate for the enzyme. *Proc Natl Acad Sci U S A.* 1996; 93:9748–9753. [PubMed: 8790402]
36. Lin H, Wilson JE, Roberts CR, et al. Biglycan, decorin, and versican protein expression patterns in coronary arteriopathy of human cardiac allograft: distinctness as compared to native atherosclerosis. *J Heart Lung Transplant.* 1996; 15:1233–1247. [PubMed: 8981209]
37. Riessen R, Isner JM, Blessing E, et al. Regional differences in the distribution of the proteoglycans biglycan and decorin in the extracellular matrix of atherosclerotic and restenotic human coronary arteries. *Am J Pathol.* 1994; 144:962–974. [PubMed: 8178945]
38. Chait A, Wight TN. Interaction of native and modified low-density lipoproteins with extracellular matrix. *Curr Opin Lipidol.* 2000; 11:457–463. [PubMed: 11048888]
39. Kawashima H, Hirose M, Hirose J, et al. Binding of a large chondroitin sulfate/dermatan sulfate proteoglycan, versican, to L-selectin, P-selectin, and CD44. *J Biol Chem.* 2000; 275:35448–35456. [PubMed: 10950950]
40. de la Motte CA, Hascall VC, Drazba J, et al. Mononuclear leukocytes bind to specific hyaluronan structures on colon mucosal smooth muscle cells treated with polyinosinic acid:polycytidylic acid: inter-alpha-trypsin inhibitor is crucial to structure and function. *Am J Pathol.* 2003; 163:121–133. [PubMed: 12819017]
41. de La Motte CA, Hascall VC, Calabro A, et al. Mononuclear leukocytes preferentially bind via CD44 to hyaluronan on human intestinal mucosal smooth muscle cells after virus infection or treatment with poly(I.C). *J Biol Chem.* 1999; 274:30747–30755. [PubMed: 10521464]
42. Hirose J, Kawashima H, Yoshie O, et al. Versican interacts with chemokines and modulates cellular responses. *J Biol Chem.* 2001; 276:5228–5234. [PubMed: 11083865]
43. Nagy N, Freudemberger T, Melchior-Becker A, et al. Inhibition of hyaluronan synthesis accelerates murine atherosclerosis: novel insights into the role of hyaluronan synthesis. *Circulation.* 2010; 122:2313–2322. [PubMed: 21098434]

44. Edwards IJ, Wagner WD, Owens RT. Macrophage secretory products selectively stimulate dermatan sulfate proteoglycan production in cultured arterial smooth muscle cells. *Am J Pathol.* 1990; 136:609–621. [PubMed: 2316626]
45. Ostberg CO, Zhu P, Wight TN, et al. Fibronectin attachment is permissive for IL-1 mediated gene regulation. *FEBS Lett.* 1995; 367:93–97. [PubMed: 7541375]
46. Mjaatvedt CH, Yamamura H, Capehart AA, et al. The *Cspg2* gene, disrupted in the *hdf* mutant, is required for right cardiac chamber and endocardial cushion formation. *Dev Biol.* 1998; 202:56–66. [PubMed: 9758703]
47. Cheng C, Tempel D, van Haperen R, et al. Atherosclerotic lesion size and vulnerability are determined by patterns of fluid shear stress. *Circulation.* 2006; 113:2744–2753. [PubMed: 16754802]
48. Chen YC, Bui AV, Diesch J, et al. A novel mouse model of atherosclerotic plaque instability for drug testing and mechanistic/therapeutic discoveries using gene and microRNA expression profiling. *Circ Res.* 2013; 113:252–265. [PubMed: 23748430]
49. Van der Donckt C, Van Herck JL, Schrijvers DM, et al. Elastin fragmentation in atherosclerotic mice leads to intraplaque neovascularization, plaque rupture, myocardial infarction, stroke, and sudden death. *Eur Heart J.* 2014

Figure 1.

Representative histologic sections showing pathologic intimal thickening (PIT) without macrophage (mac) infiltration, PIT with macrophages, early fibroatheroma (EFA), and late fibroatheroma (LFA). The left two columns show low and high power images of sections stained with Movat pentachrome, and the right three columns show high power images of immunohistochemical stains for macrophages (CD68), T-lymphocytes (CD45RO), and vasa vasorum (CD31/34).

Figure 2.

Bar graphs and box-and-whisker plots showing the results of morphometric analysis and quantitative assessment of macrophage and apoptotic body density along with percentage of engulfed apoptotic bodies in 60 human coronary lesions. Bars represent mean values and T-bars indicate SD. Lines within boxed represent median values; the upper and lower lines of the boxes represent the 75th and 25th percentiles, respectively; and the upper and lower bars outside the boxes represent the 90th and 10th percentiles, respectively. IEL=internal elastic lamina.

Figure 3.

(A) Representative histologic sections (low power images = Movat pentachrome, high power images = immunostaining) showing increase in macrophage infiltration and apoptotic bodies in association with plaque progression. The middle column shows single immunostaining for macrophages (CD68, brown), whereas the right column shows dual staining for macrophages (CD68, blue) and apoptotic bodies (red). (B) Dual immunostaining for macrophages (CD68, blue) and apoptotic bodies (red) demonstrated that most apoptotic bodies co-localized with macrophages and were engulfed in PIT with macrophages (a [$\times 400$] and b [$\times 1000$]), whereas the proportion of free apoptotic bodies increased in late fibroatheroma (c [$\times 400$] and d [$\times 1000$]) where engulfed apoptotic bodies decreased.

Figure 4.

(A) Immunohistochemical identification of extracellular matrix (ECM) molecules hyaluronan and proteoglycan (versican, biglycan and decorin) in human coronary plaques. Movat pentachrome staining show lipid pool (LP) with or without macrophage infiltration in pathologic intimal thickening (PIT) and necrotic core (NC) formation in early (EFA) and late fibroatheromas (LFA). Immunohistochemistry shows intense staining for hyaluronan in LPs of PIT whereas early NC shows partial loss of staining and late NC exhibits almost complete loss of hyaluronan. Gradual decrease in versican was also noted from PIT without macrophages to LFA where the staining was almost absent in late NC. Immunohistochemical reaction to biglycan and decorin were relatively mild as compared with other two ECM molecules; however, the staining for biglycan in LFA was significantly less as compared to PIT and EFA. (B) Quantitative assessment of hyaluronan and proteoglycan (versican, biglycan, and decorin) showed a significant decrease in hyaluronan, versican, and biglycan from PIT to EFA and LFA.

Figure 5.
Representative histologic frozen sections showing lipid accumulation as assessed by oil red O (ORO) stain.

Figure 6. Representative histologic non-decalcified sections showing progression of microcalcification as assessed by alizarin red and von Kossa stains.

Table 1
Patient and Lesion Characteristics

Patient (n=67)	
Age (years)	47.6 ± 11.1
Male gender	60 (90%)
Hypertension	39 (58%)
Hyperlipidemia	14 (21%)
Diabetes mellitus	9 (13%)
Smoking	13 (19%)
Prior myocardial infarction	20 (30%)
Cause of death	
Sudden coronary death	
Plaque rupture	15 (22%)
Plaque erosion	6 (9%)
Severe CAD without acute thrombus	20 (30%)
Non-cardiac death	26 (39%)
Lesions (n=151)	
Paraffin embedded sections for morphometry (n=60)	
PIT without macrophages	10 (16%)
PIT with macrophages	18 (30%)
Early fibroatheroma	19 (32%)
Late fibroatheroma	13 (22%)
Paraffin embedded sections for proteoglycan staining (n=40)	
PIT without macrophages	10 (25%)
PIT with macrophages	10 (25%)
Early fibroatheroma	10 (25%)
Late fibroatheroma	10 (25%)
Paraffin embedded non-decalcified sections for calcium staining (n=28)	
PIT without macrophages	7 (25%)
PIT with macrophages	9 (32%)
Early fibroatheroma	12 (43%)
Frozen sections for oil red O staining (n=23)	
Adaptive intimal thickening	3 (13%)
Fatty streak	3 (13%)
PIT without macrophages	3 (13%)
PIT with macrophages	4 (17%)
Early fibroatheroma	4 (17%)
Late fibroatheroma	6 (26%)

Values are expressed as means ± SD or n (%).

PIT = pathologic intimal thickening.

Table 2

Location of Coronary Lesions for Paraffin-Embedded Sections

	PTT without macrophages	PTT with macrophages	Early fibroatheroma	Late fibroatheroma	P value
All paraffin-embedded lesions (n=128)	(n=27)	(n=37)	(n=41)	(n=23)	
Artery (LAD / LCX / RCA)	14 (52%) / 9 (33%) / 4 (15%)	20 (54%) / 6 (16%) / 11 (30%)	22 (54%) / 9 (22%) / 10 (24%)	6 (26%) / 7 (30%) / 10 (43%)	0.21
Location (proximal / mid / distal)	20 (74%) / 6 (22%) / 1 (4%)	16 (43%) / 17 (46%) / 4 (11%)	13 (32%) / 20 (49%) / 8 (20%)	13 (57%) / 6 (26%) / 4 (17%)	0.15
For the assessment of inflammation, vasa vasorum, and apoptosis (n=60)	(n=10)	(n=18)	(n=19)	(n=13)	
Artery (LAD / LCX / RCA)	5 (50%) / 4 (40%) / 1 (10%)	10 (56%) / 2 (11%) / 6 (33%)	13 (68%) / 2 (11%) / 4 (21%)	4 (31%) / 6 (46%) / 3 (23%)	0.11
Location (proximal / mid / distal)	8 (80%) / 1 (10%) / 1 (10%)	7 (39%) / 9 (50%) / 2 (11%)	8 (42%) / 7 (39%) / 4 (21%)	7 (54%) / 4 (31%) / 2 (15%)	0.38
For proteoglycan staining (n=40)	(n=10)	(n=10)	(n=10)	(n=10)	
Artery (LAD / LCX / RCA)	4 (40%) / 4 (40%) / 2 (20%)	5 (50%) / 2 (20%) / 3 (30%)	4 (40%) / 4 (40%) / 2 (20%)	2 (20%) / 1 (10%) / 7 (70%)	0.20
Location (proximal / mid / distal)	6 (60%) / 4 (40%) / 0 (0%)	4 (40%) / 5 (50%) / 1 (10%)	2 (20%) / 6 (60%) / 2 (20%)	6 (60%) / 2 (20%) / 2 (20%)	0.35
For calcium staining (n=28)	(n=7)	(n=9)	(n=12)	N/A	
Artery (LAD / LCX / RCA)	5 (71%) / 1 (14%) / 1 (14%)	5 (56%) / 2 (22%) / 2 (22%)	5 (42%) / 3 (25%) / 4 (33%)	N/A	0.80
Location (proximal / mid / distal)	6 (86%) / 1 (14%) / 0 (0%)	5 (56%) / 3 (33%) / 1 (11%)	3 (25%) / 7 (58%) / 2 (17%)	N/A	0.15

Values are expressed as n (%).

LAD = left anterior descending artery; LCX = left circumflex artery; N/A=not applicable; PTT = pathologic intimal thickening; RCA = right coronary artery.

Table 3
Prevalence and Extent of Microcalcification as Assessed by von Kossa Staining in 28 Non-decalcified Early Coronary Lesions

	PIT without macrophages (n=7 sections)	PIT with macrophages (n=9 sections)	Early fibroatheroma (n=12 sections)	P value
Prevalence of Microcalcification				0.011
None	3 (43%)	2 (22%)	0 (0%)	
Early microcalcification (0.5µm, <15 µm)	4 (57%)	3 (33%)	2 (17%)	
Punctate microcalcification (15 µm)	0 (0%)	4 (44%)	10 (83%)	
Extent of microcalcification				
Circumferential extent				0.094
None	3 (43%)	2 (22%)	0 (0%)	
1 quadrant	2 (29%)	2 (22%)	3 (25%)	
2 quadrants	2 (29%)	4 (44%)	3 (25%)	
3 quadrants	0 (0%)	1 (11%)	6 (50%)	
Location (depth) within the intima				0.11
None	3 (43%)	2 (22%)	0 (0%)	
Outer 1/3 (deeper intima)	3 (43%)	4 (44%)	3 (25%)	
Outer 2/3 (deeper and mid intima)	1 (14%)	3 (33%)	7 (58%)	
Diffuse	0 (0%)	0 (0%)	2 (17%)	