Dynamics of intracoronary thrombosis in STEMI and sudden death patients
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Natural progression from pathologic intimal thickening to late fibroatheroma in human coronary plaques: a pathology study

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In preparation for submission.
Abstract

**Background:** Macrophage infiltration, cell apoptosis, apoptotic cell uptake, proteoglycans and lipoprotein-associated phospholipase A₂ (Lp-PLA₂) have the potential of significant importance in the dynamic and multifactorial process of plaque progression.

**Objective:** Our objective was to investigate the association of macrophages, cell apoptosis and the uptake of apoptotic bodies by macrophages in the progression of pathologic intimal thickening (PIT) to late fibroatheroma progression in human coronary artery lesions. Furthermore, we also determined the association and expression of proteoglycans and Lp-PLA₂ in plaque progression.

**Methods:** A total of 60 atherosclerotic coronary lesions were collected from 30 sudden coronary death victims. Lesion morphology was classified as pathologic intimal thickening (PIT) macrophage poor, PIT with macrophage rich, and early and late fibroatheromas. The expression of macrophages and Lp-PLA₂ was detected using specific monoclonal antibodies and quantified by computer-assisted image analysis. Apoptosis was identified by in situ end labeling of DNA fragmentation and quantified as density/mm². Apoptotic cell clearance was assessed as the ratio of engulfed to total apoptotic bodies. The most prominent extra cellular proteoglycans matrix molecules, versican, hyaluronan, biglycan and decorin, were identified and assessed both quantitive and topographically.

**Results:** Lesion progression from PIT macrophage poor to late fibroatheroma was associated with increase in macrophage infiltration (p<0.001), and apoptotic bodies (p<0.001), as well as decreased apoptotic cell clearance (p<0.001). Overall, the proteoglycan expression of hyaluronan (p<0.001), versican (p<0.001), and biglycan (p<0.013) declined along with lesion progression within the lipid pool in PIT to the necrotic core in fibroatheroma. Lp-PLA₂ showed a gradual increase in expression as plaques progressed (p<0.003).

**Conclusion:** Increased macrophage infiltration, apoptotic bodies, and defective clearance of apoptotic bodies by macrophages along with increase Lp-PLA₂ expression and decreased in most proteoglycans was associated with plaque progression from PIT to late fibroatheroma. Our findings suggest macrophage infiltration and cell death is of critical importance in plaque progression in human coronary lesions and mechanisms involved need further investigation in appropriate animal models.
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 lesions in coronary arteries starts in industrialized nations in the second decade of their life. However, only a very small minority of these lesions will progress to late stage where they eventually result in catastrophic acute vascular events such as myocardial infarction or sudden cardiac death. Since the mouse models do not mimic the progression of atherosclerotic plaque seen in man it is therefore essential that the lesions of progression be studied in human coronary arteries to advance our knowledge so that new modalities can be developed to further prevent cardiovascular disease.

Plaque progression

The earliest feature of progressive atherosclerosis as described by the modified AHA classification is pathologic intimal thickening (PIT), which is characterized by extracellular lipid accumulation (lipid pools) that are rich of proteoglycans and lack cells. Inflammation plays a pivotal role in the progression and destabilization of atherosclerotic lesions. The infiltration of macrophages is a distinctive characteristic of progression of PIT to fibroatheromas, however the process involved are poorly understood. It has been previously shown by others and us that the fibrous cap atheroma (fibroatheroma) is characterized by a dense fibrous cap overlying a necrotic core. Cell types present in atherosclerotic plaques, including endothelial cells, smooth muscle cells (SMCs), lymphocytes, and macrophages. It has been previously shown in animals and in man that apoptosis of macrophages is an integral process of plaque progression. In general, apoptosis has several implications both negatively and positively that influence plaque progression. Furthermore, a decreased uptake of apoptotic bodies by macrophages is thought to be of utmost importance in necrotic core formation in fibroatheroma. It is believed that eventually, the fibrous cap thinning of a fibroatheroma (thin cap fibroatheroma) due to macrophage infiltration results in rupture of atherosclerotic plaques.

Proteoglycans

Matrix 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-fibrollar components of the extracellular matrix have been identified in atherosclerotic plaques and are synthesized by vascular smooth muscle cells and influenced by growth factors. Studies carried out in the primate model of hypercholesterolemia, cholesterol levels in the range of 650 to 760 mg/dl, develop early and advanced lesions of atherosclerosis and that proteoglycans play a
distinct role during plaque progression. Evanko et al., showed the presence of hyaluronan, and proteoglycans versican and biglycan but weak staining for decorin in intermediate lesions, which were defined as lesions rich in smooth muscle cells and matrix with surface macrophages. Whereas lesion with a necrotic core, the so called fibroatheromas, had strong staining for hyaluroanan in fibrous cap and lipid core, and proteoglycan, versican staining strongly in fibrous cap and around the core but weak within the lipid core. The response-to-retention hypothesis in early atherogenesis states that atherogenic lipoproteins are retained in the intima by binding to extracellular matrix proteoglycans. This hypothesis further stated that lipoprotein–proteoglycan complexes exhibit increased susceptibility to oxidation and lead to uptake by macrophages to form foam cells. Not only do the proteoglycans contribute to early plaque development, but they are also associated with pathogenic processes in late stage plaque progression. Proteoglycans have been reported to play a fundamental role in cellular and extracellular events associated with the pathogenesis of vascular lesions, such as thrombosis, lipid metabolism, and vascular proliferation and migration. In a study by Nakashima of early human coronary atherosclerosis showed that biglycan and decorin were distributed in the outer layer of diffuse intimal thickening when no lipids were detected. However, lesions called as PIT defined as a lesion with extracellular lipid underneath a layer of lipid-laden macrophages, with decorin colocalized with lipid in some instances but much less consistently with biglycan. The authors did not address the presence and localization of hyaluronan and versican in this study. Therefore, in the current study we studied the expression of hyaluronan and other proteoglycans in the progression of plaque from PIT to late fibroatheroma and also how these may relate to the presence of macrophage and apoptosis.

Lipoprotein-associated phospholipase A₂

Inflammation plays a pivotal role in the progression and destabilization of atherosclerotic lesions. Numerous epidemiological studies have shown that inflammatory markers such as C-reactive protein or interleukin-6 are associated with future risk of cardiovascular events, while their causal involvement in the atherosclerotic disease progression remains unknown. Lipoprotein-associated phospholipase A₂ (Lp-PLA₂) is an inflammatory enzyme produced and secreted by monocyte-derived macrophages, T lymphocytes, and mast cells, and is bound predominantly to low-density lipoprotein (LDL). Several lines of evidence have shown that Lp-PLA₂ activity and mass are associated with increased risk of cardiovascular events independently of traditional cardiovascular risk factors. Lp-PLA₂ is involved in the hydrolysis of oxidized phospholipids in LDL in circulation, which results in the production of two proinflammatory and proapoptotic mediators, i.e., lysophosphatidylcholine and oxidized nonesterified fatty acids. In particular, lysophosphatidylcholine may be closely associated with defective clearance of apoptotic cells in atherosclerotic lesions, which can lead to the necrotic core progression. An experimental study showed that Lp-PLA₂ inhibitor
darapladib reduced lesion lysophosphatidylcholine content and necrotic core area in diabetic and hypercholesterolemic swine, indicating a causative role of Lp-PLA2 in the progressive atherosclerosis. We have previously reported that Lp-PLA2 is detected abundantly within the necrotic core and macrophages of vulnerable and ruptured plaques. However, the contribution of Lp-PLA2 to early necrotic core formation has not been fully clarified. Elucidating the role of the pivotal processes of atherosclerotic progression, through which lipid pool convert to the necrotic core, is practically important to prevent the development of more advanced lesions. Along with macrophage infiltration, apoptosis, apoptotic cell uptake, proteoglycans and Lp-PLA2 have the potential to be of great importance in the dynamic and multifactorial process of plaque progression. In the present study we therefore investigated the association of plaque progression with the infiltration of macrophages, apoptosis and the uptake of apoptotic bodies by macrophages. Furthermore, the association of the localization and expression of proteoglycans and Lp-PLA2 with the progression of PIT to late fibroatheroma in human coronary lesions was determined.

Methods

Selection of Cases
The coronary arteries for this study were obtained from the hearts of patients who had died suddenly of coronary causes including acute coronary thrombosis and severe coronary atherosclerotic disease as described previously. Major epicardial arteries were serially sectioned at 3- to 4-mm interval, and coronary segments were processed for histology. A total of 60 coronary plaques with various phenotypes of early atherosclerosis were selected from 36 patients. 10 cases from this patient group, and additional 30 cases from other patients previously reported, were selected for additional proteoglycan staining.

Classification of Lesions
Early coronary plaques were classified using our modified American Heart Association classification, to include PIT and fibroatheromas. PIT is characterized by the presence of smooth muscle cells and mild extracellular matrix towards the lumen with areas of extracellular lipid accumulation (lipid pool) and absence of cells, and was further divided into 2 groups based on the degree of macrophage infiltration; PIT macrophage poor 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 as previously defined. Early fibroatheroma (EFA) was defined as a lesion with necrosis that has macrophage infiltration into the lipid pool, focal loss of proteoglycans, and accumulated free cholesterol. A necrotic core with discrete collections of cellular debris, increased free cholesterol, and complete depletion of extracellular matrix was defined as late fibroatheroma (LFA).
Histological Preparation
Coronary arteries 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 µm, mounted on charged slides, and stained with hematoxylin-eosin (H&E) and the Movat pentachrome method.

Immunohistochemistry
Immunohistochemistry for the identification of macrophages was carried out using a CD68 antibody (KP-1 clone, Dako Carpinteria, CA), and immunohistochemical detection of Lp-PLA2 was performed using a monoclonal antibody clone 4B4 (diaDexus, South San Francisco, CA) as described previously.\(^\text{14}\) 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).

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.\(^\text{15}\) Co-localization of apoptotic nuclei and macrophages, as well as of apoptotic nuclei, was evaluated by combined ISEL and CD68 antibody. Tissue sections were initially stained for DNA fragmentation substituting, followed by immunostaining of macrophages.

Proteoglycan staining
Early and late fibroatheroma cases were stained for proteoglycans. 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 NiCl\(_2\). 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.
Morphometric analyses
Morphometric measurements of coronary sections were performed using image-processing software (IPLabs, Scanalytics, Rockville, MD) on slides stained with Movat pentachrome. Quantitative planimetry included areas 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, Lp-PLA2 and proteoglycans within regions of interest, and the percentage of positive staining in regions of interest was determined. Apoptotic cell density was evaluated as the ratio of ISLE-positive nuclei to total cell number in 2 different high power fields (x400 magnification). In addition, the number of free and engulfed apoptotic cells was counted in 2 different high power fields (x400 magnification) on slides with dual immunostaining for macrophages and apoptotic cells, and the degree of apoptotic cell clearance was assessed as the ratio of engulfed to total (free and engulfed) apoptotic cells.

Statistical Analysis
Continuous variables with normal distribution were expressed as mean ± SD. Variables with non-normal distribution were expressed as median and interquartile range (IQR). Comparisons of continuous variables with normal distribution were tested by the one-way 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. For the evaluation of the correlation among proteoglycans, macrophages, Lp-PLA2 and apoptotic cell density, we used logarithm-transformed values to account for skewed distribution of these variables. Normality of distribution was tested with the Wilk-Shapiro test. A value of \( p < 0.05 \) was considered statistically significant.

Results
Patient and Lesion Characteristics
The sudden coronary death cases studied included 36 cases of sudden coronary dead with acute thrombosis observed in XX secondary to plaque rupture (n=X) or erosion (n=X) and XX cases with severe luminal narrowing. Selected coronary lesions consist of 10 PIT macrophage poor, 18 PIT with macrophages, 19 early fibroatheroma, and 13 late fibroatheroma. Patient and lesion characteristics are summarized in Table 1.
Table 1. Patient and lesion characteristics.

<table>
<thead>
<tr>
<th></th>
<th>Patient (n=36)</th>
<th>Lesion (n=60)</th>
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<tbody>
<tr>
<td>Age (years)</td>
<td>50 ± 12</td>
<td></td>
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<tr>
<td>Male gender</td>
<td>31 (86)</td>
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<tr>
<td>Hypertension</td>
<td>22 (61)</td>
<td></td>
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<tr>
<td>Hyperlipidemia</td>
<td>4 (11)</td>
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<tr>
<td>Diabetes mellitus</td>
<td>4 (11)</td>
<td></td>
</tr>
<tr>
<td>Smoking</td>
<td>7 (19)</td>
<td></td>
</tr>
<tr>
<td>Prior myocardial infarction</td>
<td>12 (33)</td>
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<tr>
<td>Multi-vessel disease</td>
<td>24 (67)</td>
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Lesion Type

- PIT macrophage poor: 10 (16)
- PIT with macrophages: 18 (30)
- Early fibroatheroma: 19 (32)
- Late fibroatheroma: 13 (22)

Lesion Location

- Left anterior descending: 32 (54)
- Left circumflex: 14 (23)
- Right coronary artery: 14 (23)

Values are expressed as means ± SD or n (%). PIT = pathologic intimal thickening.

Morphometric Analysis

A summary of morphometric analyses including IEL, plaque area, percent stenosis, and necrotic core size in various lesion morphologies is shown in Figure 1. PIT with macrophages had smaller plaque area as compared to early and late fibroatheromas. Stenosis severity increased in concordance with lesion progression from PIT to fibroatheroma. Necrotic core size did not differ significantly between early and late fibroatheromas. Representative images of lesion types, lymphocytes and vaso vasorum in association with lesion progression are illustrated in Figure 2.

Figure 2: Lesion types, lymphocytes, and vaso vasorum in association with lesion progression.

A-E, Pathological intimal thickening macrophage poor; F-J, Pathological intimal thickening with macrophages; K-O, early fibroatheroma; P-T, late fibroatheroma. A,B,F,G,K,L,P,Q - Lesion types by stained by movat pentachrome method with high magnification images (x400) of lesion core. C,H,M,R - High magnification images of immunohistochemistry staining for the identification of macrophages (CD68 antibody) with an increasing number in the progression of the lesions. D,I,N,S - High magnification images of immunohistochemistry staining for the identification of T-lymphocytes with an increasing number in the progression of the lesions. E,J,O,T - High magnification images of immunohistochemistry staining for the identification of vaso vasorum with an increasing number in the progression of the lesions.
Figure 1: Morphometric characteristics of the different lesion morphologies. Bars are expressed as means ± SD. P-value is overall significance among the groups by ANOVA. * Significant difference between PIT macrophage poor vs. PIT with macrophages (p=0.008). † Significant difference between PIT with macrophages vs. EFA (p<0.001), and PIT with macrophages vs. LFA (p<0.001). ‡ Significant difference between PIT macrophage poor vs. EFA (p=0.002), PIT macrophage poor vs. LFA (p<0.001), PIT with macrophages vs. EFA (p<0.001), and PIT with macrophages vs. LFA (p<0.001). § non significant differences between the groups. EFA=early fibroatheroma, IEL = internal elastic lamina, LFA=late fibroatheroma, PIT = pathologic intimal thickening.
Macrophage, Apoptotic Cell Density and Apoptotic Cell Clearance

Macrophage infiltration increased significantly in association with lesion progression from PIT macrophage poor to late fibroatheroma (PIT macrophage poor; median 0.37% [IQR 0.15-1.00], PIT with macrophages; 1.29% [1.01-1.75], early fibroatheroma; 1.51% [0.83-2.68], and late fibroatheroma 2.99% [2.15-5.36], p<0.001) (Table 2). Apoptotic cell density was the least in PIT with a gradual increase in early and late fibroatheromas (PIT macrophage poor; 6.98% [4.05-12.62], PIT with macrophages; 6.22% [2.76-8.08], early fibroatheroma; 10.65% [7.50-14.39], and late fibroatheroma 18.70% [10.86-29.03], p<0.001). Representative images of macrophages and apoptotic cell expression in association with lesion progression are illustrated in Figure 3.

Most apoptotic cells co-localize with macrophages and are engulfed in PIT lesions, whereas the proportion of free apoptotic bodies increases through the process of lesion progression from PIT to early and late fibroatheromas (apoptotic cell engulfment: PIT macrophage poor: NA; PIT with macrophages: 67.5%±19.1; early fibroatheroma: 41.5%±15.7; and late fibroatheroma: 32.1%±10.5, p<0.001). Dual immunostaining for macrophages (CD68) and apoptotic cells show co-localization of these cells in various stages of human early coronary plaques (Figure 4).

Lp-PLA2 expression

The expression of Lp-PLA2 increased in concordance with lesion progression (PIT macrophage poor: 0.31% [0.09-0.36]; PIT with macrophages: 0.38% [0.20-2.21]; early fibroatheroma: 0.66% [0.41-1.36]; and late fibroatheroma: 1.13% [0.73-1.91], p=0.003) (Table 2). Lp-PLA2 expression was significantly lower in PIT macrophage poor lesions as compared to early (p=0.006) and late fibroatheroma (p<0.001). Lp-PLA2 was predominantly detected in macrophages and regions bordering the necrotic core where the level of apoptosis increased with plaque progression from early to late necrosis (Figure 3). Further dual immunostaining confirmed the co-expression of Lp-PLA2 and apoptotic cells. Moreover, Lp-PLA2 expression correlated positively with macrophage (r²=0.26, p<0.001) and apoptotic cell density (r²=0.10, p=0.026). Macrophage and apoptotic cell density showed a positive correlation as well (r²=0.12, p=0.016). In addition, there was a weak but significant inverse relationship between Lp-PLA2 expression and apoptotic cell clearance (r²=0.10, p=0.031).
Table 2. Quantitative analysis of macrophage and apoptotic cell density, percentage of engulfed apoptotic cells, and lipoprotein-associated phospholipase A2 (Lp-PLA2) expression in 60 coronary lesions.

<table>
<thead>
<tr>
<th></th>
<th>PIT macrophage poor (n=10)</th>
<th>PIT with macrophages (n=18)</th>
<th>Early fibroatheroma (n=19)</th>
<th>Late fibroatheroma (n=13)</th>
<th>p value</th>
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<tr>
<td>Macrophages (%)</td>
<td>0.37 (0.15, 1.00)</td>
<td>1.29 (1.01, 1.75)</td>
<td>1.51 (0.83, 3.68)</td>
<td>2.99 (2.15, 5.36)</td>
<td>&lt;0.001†</td>
</tr>
<tr>
<td>Apoptotic cells (%)</td>
<td>6.98 (4.05, 12.62)</td>
<td>6.22 (2.76, 8.08)</td>
<td>10.65 (7.50, 14.39)</td>
<td>18.70 (10.86, 29.03)</td>
<td>&lt;0.001‡</td>
</tr>
<tr>
<td>Percentage of engulfed apoptotic cells (%)*</td>
<td>84.5 ± 10.9</td>
<td>84.5 ± 13.6</td>
<td>60.8 ± 18.8</td>
<td>55.5 ± 16.0</td>
<td>&lt;0.001§</td>
</tr>
<tr>
<td>Lp-PLA2 (%)</td>
<td>0.31 (0.09, 0.36)</td>
<td>0.36 (0.11, 1.92)</td>
<td>0.66 (0.37, 1.27)</td>
<td>1.09 (0.74, 1.75)</td>
<td>0.003‖</td>
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</table>

Values are expressed as means ± SD or medians (interquartile range). * Engulfed apoptotic cell divided by total (free and engulfed) apoptotic cells. † Significant difference between PIT macrophage poor vs. PIT with macrophages (p=0.008), PIT macrophage poor vs. EFA (p=0.005), PIT macrophage poor vs. LFA (p<0.001), PIT with macrophages vs. LFA (p=0.005), and EFA vs. LFA (p=0.030). ‡ Significant difference between PIT macrophage poor vs. LFA (p=0.022), PIT with macrophages vs. EFA (p=0.005), PIT with macrophages vs. LFA (p<0.001), and EFA vs. LFA (p=0.018). § Significant difference between PIT macrophage poor vs. EFA (p=0.001), PIT macrophage poor vs. LFA (p<0.001), PIT with macrophages vs. EFA (p<0.001), and PIT with macrophages vs. LFA (p<0.001). ‖ Significant difference between PIT macrophage poor vs. EFA (p=0.010), PIT macrophage poor vs. LFA (p<0.001), and PIT with macrophages vs. LFA (p=0.037). EFA=early fibroatheroma, LFA=late fibroatheroma, PIT=pathologic intimal thickening.
Figure 3: Macrophages and apoptotic cell expression in association with lesion progression.

A-D, Pathological intimal thickening macrophage poor (PIT Mφ poor); E-H, Pathological intimal thickening with macrophages (PIT with Mφ); I-L, Early fibroatheroma; M-P, Late fibroatheroma. A,E,I,M - Lesion types by stained by Movat pentachrome method. B,F,J,N - High magnification (x400) images of immunohistochemistry staining for the identification of macrophages (CD68 antibody) with an increasing number in the progression of the lesions. C,G,K,O - High magnification images of dual immunostaining for macrophages (CD68) (blue) and apoptotic cells (red) showing co-localization of these cells in various stages of human early coronary plaques. Combined immunohistochemistry and in situ end labeling (ISEL) DNA fragmentation staining show that most apoptotic cells co-localize with macrophages and are engulfed in pathologic intimal thickening (PIT) lesions, whereas the proportion of free apoptotic bodies increases through the process of lesion progression from PIT to early and late fibroatheromas. D,H,L,P – High magnification images of immunohistochemistry staining for the identification of lipoprotein-associated phospholipase A2 (Lp-PLA2) expression. Note that the expression of Lp-PLA2 is increasing along progression of the plaque and predominantly identified in macrophages and regions bordering the necrotic core in association with increased apoptotic cells.
Figure 4: Co-localization of macrophages and apoptotic cells in association with lesion progression. High magnification images (x400) of dual immunostaining for macrophages (CD68) (blue) and apoptotic cells (red) showing colocalization of these cells in various stages of human early coronary plaques. Combined immunohistochemistry and in situ end labeling (ISEL) DNA fragmentation staining show that most apoptotic cells co-localize with macrophages and are engulfed in pathologic intimal thickening (PIT) lesions, whereas the proportion of free apoptotic bodies increases through the process of lesion progression from PIT to early and late fibroatheromas.
Proteoglycan expression

The expression of proteoglycans in the lipid pool/necrotic core declines in concordance with progression of lesions from PIT to early and late fibroatheroma. Hyaluronan is highly present in PIT macrophage poor (33.4% [21.2-50.1]) and PIT with macrophages (54.4% [30.6-77.8]), and a gradual decline to early fibroatheroma (28.1% [10.5-49.9]) and little expression in late fibroatheroma (3.0% [2.0-9.7]) (p<0.001). Versican show a similar pattern to hyaluronan with high expression in PIT macrophage poor (59.4% [36.5-64.8]) and PIT with macrophages (41.1% [19.5-74.0]), and a gradual decline to early fibroatheroma (21.3% [5.8-38.0]) and little expression in late fibroatheroma (1.0% [0.02-6.1]) (p<0.001). Although the expression of biglycan and decorin are lower compared to hyaluronan and versican similar patterns are observed. Biglycan expression has a similar significant decline with almost no expression in late fibroatheroma (0.1% [0.01-1.3]) (p=0.013). The expression of decorin is low compared to other proteoglycans, however among the lesion types a non-significant (p=0.921) but a similar trend is observed (Table 3, Figure 5).

The localization of the proteoglycans in PIT is dispersed throughout the lesions, while in early and late fibroatheroma the decline in expression is mainly observed in the region of the necrotic core. In late fibroatheroma the expression is very low and proteoglycans are effectively non-existent in the necrotic core (Figure 6).

Figure 5: Proteoglycan expression in the lipid pool (LP) / necrotic core (NC) in pathological intimal thickening macrophage poor (PIT mac poor), pathological intimal thickening with macrophages (PIT with mac), early fibroatheroma (EFA) and late fibroatheroma (LFA).

Table 3. Quantitative analysis of proteoglycans hyaluronan, versican, biglycan and decorin in lipid pool / necrotic core in 40 human coronary lesions.

<table>
<thead>
<tr>
<th></th>
<th>PIT macrophage poor (n=10)</th>
<th>PIT with macrophages (n=10)</th>
<th>Early fibroatheroma (n=10)</th>
<th>Late fibroatheroma (n=10)</th>
<th>p value</th>
</tr>
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<tbody>
<tr>
<td>Hyaluronan (%)</td>
<td>33.4 (21.2, 50.1)</td>
<td>54.4 (30.6, 77.8)</td>
<td>28.1 (10.5, 49.9)</td>
<td>3.5 (2.0, 9.7)</td>
<td>&lt;0.001†</td>
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<tr>
<td>Versican (%)</td>
<td>59.4 (36.5, 64.8)</td>
<td>41.1 (18.5, 74.0)</td>
<td>21.3 (5.8, 38.0)</td>
<td>1.0 (0.02, 6.1)</td>
<td>&lt;0.001†</td>
</tr>
<tr>
<td>Biglycan (%)</td>
<td>15.7 (8.4, 48.3)</td>
<td>11.5 (9.4, 19.2)</td>
<td>13.7 (9.8, 15.8)</td>
<td>0.1 (0.01, 1.3)</td>
<td>0.013†</td>
</tr>
<tr>
<td>Decorin (%)</td>
<td>4.4 (0.9, 28.1)</td>
<td>4.8 (0.5, 19.1)</td>
<td>7.3 (0.2, 16.0)</td>
<td>1.1 (0.6, 5.0)</td>
<td>0.921</td>
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</table>

Values are expressed as medians (interquartile range). * Significant difference between PIT macrophage poor vs. LFA (p<0.001), PIT with macrophages vs. LFA (p<0.001), and EFA vs. LFA (p=0.003). † Significant difference between PIT macrophage poor vs. EFA (p=0.009), PIT macrophage poor vs. LFA (p<0.001), PIT with macrophages vs. LFA (p<0.001), and EFA vs. LFA (p=0.007). ‡ Significant difference between PIT macrophage poor vs LFA (p=0.009), PIT with macrophages vs. LFA (p=0.009), and EFA vs. LFA (p=0.014). EFA=early fibroatheroma, LFA=late fibroatheroma, PIT = pathologic intimal thickening.
Figure 6: 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 LP 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 mac poor to LFA where the staining was almost absent in late NC. Immunohistochemical reaction to biglycan and decorin were relatively mild as compared to other two ECM molecules; however, the staining for biglycan in LFA was significantly less as compared to PIT and EFA.
Discussion

The current study demonstrates the natural progression of human coronary plaques from PIT macrophage poor to late fibroatheroma that is associated with increasing macrophage and apoptotic cell density, as well as decreased apoptotic cell clearance. The expression of ECM molecules versican and hyaluronan was inversely associated with lesion progression. In addition, both ECM molecules were present in PIT but, as the lipid pool converted into a necrotic core, through the infiltration of of macrophages the presence of versican and hyaluronan decreased. Lp-PLA2 was predominantly identified in macrophages and regions bordering the necrotic core in association with increasing apoptotic cells. Furthermore, Lp-PLA2 expression correlated positively with macrophages and apoptotic cell density, and inversely with apoptotic cell clearance. These observations suggest that ECM molecules and Lp-PLA2 may contribute to the conversion of the lipid pool to early necrotic core and finally to late necrotic core formation and may be determined by the increasing macrophages and cell death as well as defective clearance of apoptotic bodies.

Macrophage infiltration, apoptosis and apoptotic cell clearance

The infiltration of macrophages in PIT lesions is as a step towards the progression of atherosclerotic lesions to fibroatheroma. The accumulation of lipid-laden macrophages occurs near the lumen and has a tremendous impact on atherosclerotic lesion progression. As atherosclerotic lesions further progress to early fibroatheroma, macrophage apoptosis begin to occur. In normal physiology, many cells throughout the body undergo apoptosis each day, balanced by an almost equal cell growth. In plaque progression however, eventually most cells present within the atherosclerotic plaques, including endothelial cells, smooth muscle cells (SMCs), lymphocytes, and macrophages have been shown to undergo apoptotic cell death. 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. However, the loss of macrophages could also decrease the uptake of apoptotic bodies, which was 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. Moreover, the cells undergoing secondary necrosis release potentially toxic components which may trigger a renewed cycle of inflammatory responses. Rapid phagocytic clearance of the apoptotic cell corpses (“efferocytosis”) prevents subsequent cell leakage, known as postapoptotic necrosis, and activates anti-inflammatory cell-signaling pathways. An inefficient removal of apoptotic cells by macrophages may lead to the collection of postapoptotic necrosis and the accumulation of necrotic debris.
Indeed, there is evidence that macrophage apoptosis occurs in early lesions, but efficient efferocytosis renders this process not only harmless but possibly beneficial. In advanced lesions, however, macrophage apoptosis is associated with defective clearance and secondary necrosis, suggesting defective efferocytosis. Direct support for this theory was provided by Schrijvers et al, who showed that human carotid late fibroatheroma contained a substantial number of apoptotic cells within the necrotic core that were not engulfed by phagocytes. As a control, most of the apoptotic cells in tonsillar tissue, which is an efficient system, were intracellular, suggestive of an intact phagocytosis process with the ability to rid of senescent cells. To our knowledge, the observations in the present study show for the first time a similar process in human coronary lesions with the exception that such a system is defective and allows the collection of apoptotic bodies that lead to early and late necrotic core formation. Possible mechanisms accounting for the defective phagocytic clearance of apoptotic macrophages in advanced atherosclerotic lesions involve competitive inhibition of phagocyte receptors, such as the type A scavenger receptor, CD36, and CD68, between apoptotic cells and oxidized molecules which exist in necrotic cores. In addition, naturally occurring antibodies to oxidized LDL in atherosclerotic lesions can bind phagocyte ligands on apoptotic macrophages, leading to the inhibition of their uptake. Furthermore, lysophosphatidylcholine, which is produced from oxidized phospholipids by Lp-PLA2 enzymatic activity, may also inhibit apoptotic cell clearance, thereby perpetuating vascular inflammation and promoting necrotic core formation.

Proteoglycans in plaque progression
In addition to the response-to-retention hypothesis the current study shows the importance of the decrease in proteoglycan versican and hyaluronan as important steps towards in plaque progression. Versican is likely to play a key role in the development and progression of plaque. 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 and radial arteries. Versican interacts with hyaluronan, resulting in association of versican rich areas with hyaluronan rich areas. A number of studies have shown that both ECM molecules are increased in diseased arteries. In early human coronary lesions a family of ECM molecules, including versican an hyaluronan, were identified to contribute to the early phases of coronary lesion formation before the stage of the PIT. 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. In addition, versican-hyaluronan complexes are also present at the plaque thrombus interfaces. This suggests that complex functions as ancillary platelet ligands, together with other known ligands, influencing platelet deposition after rupture of the atherosclerotic plaques resulting
in the formation of thrombus. In the present study versican accumulation was observed in all lesions, topographically versican was observed throughout the lesional area of PIT, and especially at the edges of the necrotic core in fibroatheroma. The interaction of versican with hyaluronan was confirmed by the similarity of its distribution within the plaque regions were the ECM molecules were expressed in all lesion studied.

ECM molecules interact with apoB-rich lipoproteins causing retention, aggregation and modification of LDL particles in early plaque development. The proteoglycan-LDL complexes are taken up rapidly by macrophages and smooth muscle cells (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 lesion into early necrotic core and the conversion into fibroatheromas. Accumulation of versican and hyaluronan may also influence the retention of inflammatory cells contributing to the development of atherosclerotic lesions. For example, studies have shown that ECM molecules are involved in stabilizing CD44-dependent interactions, supporting macrophage adhesion and interacting with inflammatory chemokines. 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.

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 could have a role in versican degradation. One of the possible mechanisms responsible this process is the macrophage-derived interleukin-1, which possibly decreases versican expression while increasing the synthesis of decorin.

Lipoprotein-associated phospholipase A2
Lp-PLA2 is an enzyme produced by inflammatory cells and is bound mainly to LDL, in particular small, dense LDL particles. While we have previously reported that Lp-PLA2 is strongly expressed in the necrotic core of vulnerable and ruptured plaques, the current study showed that Lp-PLA2 expression increased significantly in concordance with the plaque progression from PIT macrophage poor to late fibroatheroma, indicating an important role of Lp-PLA2 in the development of early necrotic core formation. Lp-PLA2 was closely associated with macrophages, which agrees with previous reports showing a significant positive correlation between coronary expression of the genes encoding Lp-PLA2 and CD 68 (r=0.81; p<0.0001) in diabetic and hypercholesterolemic swine.

In this regard, Lp-PLA2 potentially contributes to the defective phagocytic clearance of apoptotic macrophages and subsequent atherosclerotic plaque progression. Our findings showing the co-expression of Lp-PLA2 and apoptotic cells as well as an inverse relationship between Lp-PLA2 expression and apoptotic cell clearance may partially support this theory. Recently, specific Lp-PLA2 inhibitor darapladib has been developed, and several studies
support the possible efficacy of Lp-PLA2 inhibition, suggestive of causal involvement of Lp-PLA2, in preventing atherosclerotic disease progression. Wilensky et al. showed that darapladib reduced plasma and lesion Lp-PLA2 activity, lesion lysophosphatidylcholine content, and coronary atherosclerotic plaque size as well as necrotic core area in a model of diabetic and hypercholesterolemic swine. In humans, the IBIS-2 (the Integrated Biomarker and Imaging Study-2) trial, which included 330 patients with angiographically documented coronary artery disease, demonstrated that 12 months of treatment with darapladib (160 mg/day) resulted in 59% inhibition of Lp-PLA2 activity as compared to placebo (p<0.001), whereas high-sensitivity C-reactive protein levels and coronary atheroma deformability as assessed by intravascular ultrasound palpography did not differ significantly between the groups. The change in total atheroma volume did not differ between the groups; however, the placebo group showed a significant increase in necrotic core volume as assessed by intravascular ultrasound radiofrequency despite standard-of-care treatment with average LDL-cholesterol levels <90 mg, whereas darapladib treatment halted this increase in necrotic core volume. The expansion of the necrotic core in the placebo group may reflect the dynamic process of atherosclerotic lesion progression, while preventing expansion of necrotic core in the darapladib group suggesting the efficacy of Lp-PLA2 inhibition in preventing the natural progression of atherosclerosis. Our results complement these findings; however further studies are required to clarify whether Lp-PLA2 inhibition contribute to the regression and stabilization of atherosclerotic lesions and eventual prevention of cardiovascular events.

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 non observed alterations. 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. Therefore, we sought to understand which mechanisms may be important by careful and detailed study of human coronary lesions in the various early stages of plaque progression. The implications of these findings as a mechanism of plaque progression require further study.

Conclusion
Increase in macrophage presence, apoptotic bodies and defective clearance by macrophages, Lp-PLA2 expression and decrease in proteoglycans and hyaluronan expression observed with plaque progression of atherosclerosis from PIT macrophage poor to late fibroatheroma, shows the importance of these molecules in early atherosclerotic processes. The current findings suggest that proteoglycans, hyaluronan and Lp-PLA2 and their interplay with macrophages contribute to the progression of atherosclerosis even in its early phases.
References


