Gelatinolytic Activity in Atherosclerotic Plaques Is Highly Localized and Is Associated With Both Macrophages and Smooth Muscle Cells In Vivo

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Background—Atherosclerosis is considered an inflammatory disease. Recent studies provided evidence for a predominant upstream location of plaque inflammation. The present study introduces a novel technique that evaluates the underlying mechanism of this spatial organization.

Methods and Results—In hypercholesterolemic rabbits, atherosclerosis of the infrarenal aorta was induced by a combination of endothelial denudation and a high-cholesterol diet (2% cholesterol for 2 months). At the time of death, aortic vessel segments were dissected and reconstructed with a new technique that preserved the original intravascular ultrasound–derived lumen geometry. This enabled us to study the spatial relation of histological markers like macrophages, smooth muscle cells, lipids, gelatinolytic activity, and oxidized low-density lipoprotein. Results showed a predominant upstream localization of macrophages and gelatinase activity. Colocalization studies indicated that gelatinase activity was associated with macrophages and smooth muscle cells. Further analysis revealed that this was caused by subsets of smooth muscle cells and macrophages, which were associated with oxidized low-density lipoprotein accumulation.

Conclusions—Upstream localization of a vulnerable plaque phenotype is probably due to an accumulation of oxidized low-density lipoprotein, which activates/induces subsets of smooth muscle cells and macrophages to gelatinase production. (Circulation. 2007;115:609-616.)

Key Words: atherosclerosis • gelatinases • histology • inflammation • metalloproteinases • oxidized low-density lipoprotein

Nowadays, atherosclerosis is considered a lipid-driven inflammatory disease. Inflammation has been associated with plaque progression, plaque rupture, thrombosis, and subsequent myocardial infarction.1–4 Several studies indicated that plaque inflammation is unevenly distributed over its length, with a predominant upstream presence of inflammatory cells and/or a location in the plaque shoulders.5–9 These observations indicate a spatially oriented mechanism, which to date has not received much attention. To study the underlying mechanism of such a highly spatially localizing mechanism, there is a need for a precise, quantitative technique enabling the study of plaque heterogeneity in experimental atherosclerosis. The first aim of the present study was to present a 3-dimensional histological technique that permits the study of plaque heterogeneity in more detail than before.

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Both plaque progression and plaque rupture have been associated with a larger infiltration of macrophages regardless of the underlying plaque morphology.10,11 Activated macrophages produce numerous factors, including matrix metalloproteinases (MMPs).12 MMPs belong to a family of zinc-activated proteases modulating the extracellular matrix in the vascular wall.13–15 The activity of some family members induces weak spots in the extracellular matrix, thereby introducing a condition sensitive to plaque rupture.12,16–18 Only a relatively small fraction of macrophages can be measured in the entire plaque19; therefore, either the process of plaque weakening may be very localized, or other cell types may be involved in the process. Indeed, several studies

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have indicated that, besides macrophages, endothelial cells and smooth muscle cells (SMCs) may produce MMPs when brought into an inflammatory, atherogenic environment.\[^{20-22}\] Recently, a new technique has been introduced to measure gelatinolytic activity in histological cross sections with a high spatial resolution.\[^{21,24}\] We have adopted this method for vascular tissue and incorporated it into 3-dimensional histological reconstructions. Our second aim was to evaluate, using a combination of the above techniques, whether MMPs are active very locally or multiple cell types are involved in experimental atherosclerosis in vivo.

Low-density lipoprotein (LDL) accumulates in the vessel wall where it may become oxidized (oxLDL). OxLDL is known to be involved in many processes related to atherosclerosis, including stimulation of macrophage infiltration and foam cell formation, stimulation of vascular SMC migration and proliferation, and endothelial cell apoptosis.\[^{25-29}\] Recent studies indicated that oxLDL is associated with plaque instability.\[^{29}\] This observation might be explained by the modulation of activation of some MMP family members by oxLDL.\[^{15}\] Most of the oxLDL-related studies, however, have been conducted on isolated cells in vitro, which are devoid of the complex environment of the atherosclerotic vessel wall, thereby identifying the need to study the role of oxLDL in vivo.

It is presently unknown whether oxLDL is distributed heterogeneously within the plaque or if it is associated locally with gelatinolytic activity in plaques in vivo. Therefore, the third aim of the present study was to evaluate whether oxLDL might be the cause of the spatially restricted accumulation of activated macrophages in plaques in vivo.

### Methods

#### Instrumentation

Three days before baseline measurements, male New Zealand white rabbits (n=8; weight, 3.6±0.2 kg; Harlan Netherlands BV, Horst, the Netherlands) were fed a high-cholesterol (2%) diet for a 2-month period. At experimentation, rabbits were anesthetized with an intramuscular injection of ketamine (Sanaket 10%, 25 mg/kg, Anisane Ore). Three days before baseline measurements, male New Zealand white rabbits (n=8; weight, 3.6±0.2 kg; Harlan Netherlands BV, Horst, the Netherlands) were fed a high-cholesterol (2%) diet for a 2-month period. At experimentation, rabbits were anesthetized with an intramuscular injection of ketamine (Sanaket 10%, 25 mg/kg, Anisane BV, Raamsondkveer, Holland) and a subcutaneous injection of medetomidine (Domitor, 0.5 mg/kg, Orion, Espoo, Finland). The marginal ear artery was cannulated for arterial pressure measurement with a fluid-filled catheter (Amatek, US Gauge, Feesterville, Pa) and for arterial blood withdrawal. A 4F guiding catheter was advanced from the left femoral artery up to the level of the renal artery ostium. After angiography, a 40-MHz intravascular ultrasound catheter (CVUS, Boston Scientific, Maastricht, the Netherlands) was advanced through the guiding catheter and located 1 cm upstream of the lower of the 2 renal arteries. Subsequently, a motorized pullback was performed at a speed of 0.5 mm/s spanning an arterial segment of 7 cm, which was recorded on high-resolution videotape. Endothelial denudation was performed within this predefined segment by twisting and pulling back an inflated 3F Fogarty balloon (Applied Cardiac Systems, Inc. Laguna Hills, Calif) over a length of 5 cm.

#### Follow-Up

After 8 weeks of follow-up, rabbits were anesthetized as described above. Next, the right femoral artery was dissected for the introduction of a 4F sheath. An angiographic overview of the infrarenal aorta was performed, and radiopaque markers were located subcutaneously to indicate the previously denuded region. Subsequently, the intravascular ultrasound pullback was repeated at the location of the previously denuded segment. Then, the abdomen was opened; a longitudinal marker and 2 transverse markers were placed externally on the aortic vessel wall; the lumen was filled with OCT (optimal cutting temperature) compound (Tissue-Tek, Sakura Finetek Inc, Torrance, Calif); and the arterial segment of interest was dissected, collected, and snap-frozen in liquid nitrogen. The distance between both transverse markers was measured before and after excision and was used to calculate a correction factor for shrinkage resulting from arterial elasticity.

All experiments were performed in accordance with institutional regulations and the Guide for the Care and Use of Laboratory Animals as approved by the Council of the American Physiological Society.

#### Plasma Lipids

Lipid profiles were measured according to well-established enzymatic calorimetric methods (Roche Diagnostics, Pleasanton, Calif). Cholesterol levels were determined at the 8-week follow-up in the present hypercholesterolemic group and in a normocholesterolemic control group, which consisted of sex- and age-matched rabbits.

#### Intravascular Ultrasound

The high-resolution videotaped intravascular ultrasound data were digitized at intervals of 0.5 mm with a resolution of 800×600 pixels and stored on a standard personal computer. Next, the lumen and the acoustic interface between media and the adventitial layer were traced semiautomatically by a well-validated software package\[^{30}\]; then the lumen area and media bounded area were calculated from these contours. The difference between these 2 was defined as the wall area.

#### Tissue Harvesting and Histological Analysis

From the excised 5-cm aortic segment, tissue blocks were prepared every 2 mm with a cutting device developed in-house. This resulted in ~20 to 25 blocks per blood vessel, depending on the extent of shrinkage. Immunohistochemistry was performed on 5-μm cryosections obtained from the middle of the 2-mm tissue blocks. Sections were stained for macrophages (RAM-11, Dako Diagnostics BV, Glostrup, Denmark), SMCs (α-actin, Dako Diagnostics BV, Glostrup, Denmark), oxLDL (epitope against apolipoprotein B100,\[^{31}\] a kind gift from P. Holvoet, PhD), and lipids (Oil Red O, Sigma, Rotterdam, the Netherlands). After staining, sections were digitized with a high-resolution charge-coupled camera (Axiocam HR, Zeiss, Jena, Germany) and quantitatively analyzed with commercial image-analysis software (Clemex Technologies Inc, Longueuil, Canada).

#### In Situ Zymography

Gelatinolytic activity was demonstrated in unfixed cryosections with DQ-gelatin as a substrate (EnzChek, Molecular Probes, Eugene, Ore). Sections were air-dried for 60 minutes; during that period, DQ-gelatin was dissolved in a concentration of 1 mg/mL in Milli-Q and then diluted 1:10 in 1% (wt/vol) low-gelling-temperature agarose (Sigma) in phosphate-buffered saline. Subsequently, 25 μL of this mixture was placed on each cryosection and incubated for 2 hours at room temperature after placement of a coverslip. Fluorescence was detected with a confocal microscope (LSM 510 Meta, Zeiss) using an argon laser for excitation at 488 nm and emission collection at 512 to 542 nm with appropriate filters, background, and autofluorescence correction. Detailed testing for specificity of the in situ zymography was described previously\[^{32}\] and is explained in the online Data Supplement.

#### Three-Dimensional Histology

The 3-dimensional reconstruction of histological cross sections consisted of the following steps: (1) acquisition of quantitative image analysis data from histological sections; (2) rotation of sections on the basis of an externally placed longitudinal marker, which was still present after histological processing; (3) stacking of image data and correction for shrinkage in the longitudinal and radial directions; and (4) rotation of the entire stack of data with respect to the renal artery and mapping of histology on 3-dimensional intravascular ultrasound.
reconstructions. Details of the 3-dimensional reconstruction, including several tests, are given in the online Data Supplement.

**Data and Statistical Analyses**

As a first approach, we averaged each histological variable (plaque area, macrophages, SMCs, lipids, gelatinolytic activity, and oxLDL) per cross section and displayed the longitudinal heterogeneity per blood vessel. Subsequently, plaque areas of each blood vessel were shifted so that maximal plaque areas of all vessels were aligned. The shift for each individual blood vessel was applied to all its histological variables, which eventually were spatially averaged. To study spatial differences in relation to the histological variables described above and in relation to plaque area, spatial averages were calculated upstream and downstream of the maximum plaque area for each individual animal. Differences between these averages were then evaluated by an exact Wilcoxon signed rank test.

To further explore underlying mechanisms, we performed colocalization studies of the reordered data and linear regression analyses. Colocalization was defined as the existence of 2 variables in the same radial segment. The colocalized pixels per histological variable were counted and divided by the number of total elements in the segment of interest. Differences between colocalization of macrophage-gelatinolytic activity and SMC-gelatinolytic activity and of macrophage-oxLDL and SMC-oxLDL were tested. In addition, percentage macrophages (area/area) and percentage SMC (area/area) were calculated, and differences in colocalization were tested as described above. Detailed explanation of the image segmentation is given in the online Data Supplement.

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

**Results**

**Animal Characteristics**

Systolic, diastolic, and mean arterial blood pressures were 88±1, 60±3, and 69±1 mm Hg, respectively. These values remained unchanged during the experimental procedures. The 2% cholesterol–rich diet significantly increased total plasma cholesterol (from 1.4±0.2 to 33.4±15.7 mmol/L), high-density lipoprotein (from 0.7±0.1 to 14.5±2.7 mmol/L), and LDL (from 0.2±0.1 to 30.9±10.8 mmol/L). Triglyceride levels remained unchanged.

**Longitudinal Plaque Heterogeneity Displays an Upstream Location of Inflammatory Cells, Gelatinolytic Activity, and a Vulnerable Phenotype**

In plaques generated in the aorta of a representative hypercholesterolemic rabbit, longitudinal plaque heterogeneity of lipid particles, macrophages, and SMCs was clearly present (Figure 1). Surprisingly, a similar upstream accumulation of gelatinolytic activity and oxLDL was measured (Figure 2).
When the data were shifted and spatially averaged (see above), the predominant accumulation of macrophages was demonstrated for all animals (Figure 3). Distribution of each variable with plaque area revealed a higher accumulation of macrophages upstream (11.6%) compared with downstream (7.9%; $P=0.016$) of the plaque. In contrast, a more diffuse distribution of vascular SMCs (26.6% versus 27.1%) and lipids (2.9% versus 2.5%) was measured. As a consequence, a local vulnerability index (modified from Shiomi et al)$^{32}$ displayed a maximum upstream of maximal plaque area.

**Figure 2.** Reconstructions (3-dimensional) of the same aorta as presented in Figure 1. Presented are histological markers projected on a flat plane only. From left to right are displayed oxLDL distribution, macrophage distribution, distribution of metalloproteinase gelatinolytic activity, and SMC distribution. Flow direction is from bottom to top. Note the predominant upstream location of all components, similar to that in Figure 1.

**Figure 3.** Longitudinal heterogeneity of plaque area (A) and macrophage (B), SMC (C), and lipid (D) distribution. Displayed in each figure is the longitudinal plaque area distribution as averaged for all rabbits. Note that all variables are located predominantly upstream of the plaque.
compared with downstream (3.2 versus 2.8; \(P = 0.039\)) (Figure 4). This higher vulnerability upstream is similar to observations in the carotid arteries of patients.

**In Vivo Gelatinolytic Activity Is Colocalized With Both SMCs and Macrophages**

Inspection of histological cross sections revealed that both SMCs and macrophages were associated with gelatinolytic activity (Figure 5). Quantitative analysis confirmed these findings because the total amount of gelatinolytic activity associated with SMCs and macrophages was 85±10%, distributed into an equal SMC-gelatinase fraction of 42±7% and macrophage-gelatinase fraction of 43±9% (Figure 6). Although this pointed to a similar contribution of SMC and macrophages to overall gelatinolytic activity, these fractions represent only a minor fraction of overall macrophage (23±7%) and SMC (22±7%) content, which were similar for both cell types. When we reexamined the cross section containing gelatinase-producing vascular SMCs and macrophages, we identified foamy SMCs and foamy macrophages (Figure 5, right).

**OxLDL Identifies Subsets of Gelatinase-Producing SMCs and Macrophages**

Inspection of histological cross sections revealed that both SMCs and macrophages were associated with oxLDL accumulation (Figure 5). These single observations were confirmed by quantitative analysis; OxLDL almost entirely (98±19%) colocalizes with both macrophages and SMCs, with an even distribution between both cell types (SMC: 54±9% and macrophages: 44±7%) (Figure 6). These colocalization studies identified similar subsets of SMCs (28±4%) and macrophages (28±8%) that were spatially associated with oxLDL.

**Discussion**

Atherosclerosis is considered a lipid-driven inflammatory disease. Several studies indicated a strong heterogeneity of

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**Figure 4.** Local distribution of the vulnerability index (VI-index) adapted from Shiomi et al. displayed as a function of longitudinal location in a series of atherosclerotic rabbit aortas (n=10). A high value signifies a more vulnerable phenotype; a low value, a more stable phenotype. The highest values are displayed upstream of the plaque.

**Figure 5.** Nearly consecutive cross sections of a single atherosclerotic rabbit aorta at low magnification showing macrophage accumulation (A), gelatinolytic activity (B; dashed white line indicates the tunica media), and SMC distribution (C). Note that near the cap the gelatinolytic activity is high. At this location, both SMCs and macrophages are identified. A ×100 magnification of nearly consecutive cross sections indicates that foam cells (Oil Red O staining; E) belong to the macrophage fraction (RAM-11; D) and to the SMC fraction (\(\alpha\)-actin; F). Dashed black lines indicate an area where foamy SMCs are present.
the inflammatory process,\textsuperscript{11,33,34} consisting of an accumulation of inflammatory cells in the shoulders and/or upstream of the plaque.\textsuperscript{7} Not much attention has been paid to this heterogeneity in experimental atherosclerosis, probably because of the lack of a suitable technique to study such phenomena in animals.

Using a new 3-dimensional reconstruction technique for histology, we were able to show that the inflammatory component in the atherosclerotic plaque was spatially located upstream of the maximal cross-sectional plaque area, similar to that reported for human conditions.\textsuperscript{7} In addition, we demonstrated that gelatinolytic activities also were spatially confined to the same region and were associated with macrophages and SMCs. This tight spatial localization of gelatinases enabled us to study colocalization with other cell types. We found that not only macrophages but also SMCs contributed significantly to gelatinolytic activity in vivo. Previous studies indicated that SMCs in culture produce pro–MMP-2 after stimulation and, at the same time, reduce their production of tissue inhibitor of metalloproteinase, leading to a higher MMP-2 activity.\textsuperscript{35} Furthermore, macrophages stimulated with oxLDL decrease their tissue inhibitor of metalloproteinase-1 release and increase pro–MMP-9 release, leading to MMP-9 activation.\textsuperscript{22} The imbalance between tissue inhibitor of metalloproteinase and pro-MMP release may explain the high gelatinase activity found in the present study. Thus, from the present findings, one may postulate that local weak spots in the extracellular matrix occur upstream of the plaque because of a highly localized gelatinolytic concentration produced by both (foamy) macrophages and (foamy) SMCs.

The reason that such a localized accumulation of cells in atherosclerotic plaques occurs is currently unknown, but several lines of evidence indicate that oxLDL is involved. OxLDL has been measured in (vulnerable) plaques, where it modulates macrophage accumulation and foam cell formation through the expression of adhesion factors, secretion of monocyte chemotactic protein-1, migration of SMCs, and apoptosis of SMCs and endothelial cells.\textsuperscript{27–29,36} Furthermore, several studies identified a modulating effect of oxLDL on MMP activation by macrophages and SMCs in vitro.\textsuperscript{37–41} Therefore, we tested whether oxLDL colocalized with macrophages and SMCs. Only 25% of either cell type was associated with oxLDL, but these 2 cell types accounted for all oxLDL colocalization and all gelatinolytic activity in our plaques. This indicates that the total oxLDL is taken up by SMCs and macrophages in approximately similar amounts but that this uptake is performed by subsets of both cell types.

Highly evolutionary preserved subsets of monocytes have been identified in the blood of humans and mice,\textsuperscript{42,43} which results in different macrophage phenotypes as maturation occurs.\textsuperscript{43,44} These circulating subsets express different chemokine receptors\textsuperscript{43,45} and different scavenger receptors.\textsuperscript{43,44} These studies may offer an explanation for the finding of a subset of macrophages with a preferential location upstream of the plaque, combined with a particular predominance of foam cell differentiation.

Because subsets of SMCs may change into foam cells, their role in atherogenesis may have been underestimated.\textsuperscript{46,47} Recently, it was demonstrated that discrete clones of SMCs exist in human vessels that differentially accumulate cholesterol esters when exposed to oxidized lipoproteins.\textsuperscript{47,48} Once turned into foamy SMC, they start to produce cytokines and express chemokine receptors,\textsuperscript{49} providing an explanation for their tight colocalization within the plaque. The fact that foamy SMCs and foamy macrophages are located in similar vessel segments might be explained by the evidence that macrophages secrete factors enhancing uptake of cholesterol ester by vascular SMCs.\textsuperscript{50} To the best of our knowledge, the association of foamy SMC subsets, foamy macrophage subsets, and gelatinolytic activity in atherosclerotic plaques in vivo has not been described before.

Limitations of the methods are described in the online Data Supplement.

Conclusions
A specific spatial colocalization of macrophages, lipids, and SMCs was demonstrated upstream of the plaque, similar to that found in patients with proven upstream plaque ruptures. This upstream plaque composition is characterized by an accumulation of (subsets of) macrophages and SMCs, oxLDL, and gelatinolytic activity. We hypothesize that activation of these subsets by oxLDL induces gelatinolytic activity,
followed by breakdown of the extracellular matrix and subsequent weakening of the plaque. The unexpected, important role of a subset of SMCs in this process warrants further study.

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Disclosures

None.

References


CLINICAL PERSPECTIVE

Vulnerable plaques have raised great clinical interest because they are prone to rupture, leading to massive clotting and causing 70% of sudden cardiac deaths in humans. Heterogeneity in single plaques is of great importance because plaques usually do not rupture at random over their entire length. Instead, they rupture more locally and usually upstream of maximal plaque location. The latter result might be of importance for interventional procedures. The results of our present study show that such a localized vulnerability is due in part to the localized gelatinolytic activity in this area. In addition, we demonstrated that this plaque-weakening activity is not restricted to the macrophage but also occurs in a subset of vascular smooth muscle cells. As a consequence, smooth muscle cells should be placed in a wider perspective in which they are not solely the producing cells of stabilizing collagen fibers. The 2 faces of the smooth muscle cell are of significance in the concept of plaque weakening and thus in the development of new pharmaceutical and interventional procedures.
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