Evidence for Increased Collagenolysis by Interstitial Collagenases-1 and -3 in Vulnerable Human Atheromatous Plaques

Galina K. Sukhova, PhD; Uwe Schönbeck, PhD; Elena Rabkin, MD, PhD; Frederick J. Schoen, MD, PhD; A. Robin Poole, PhD, DSc; R. Clark Billinghurst, DVM, PhD; Peter Libby, MD

Background—Several recent studies attempted to classify plaques as those prone to cause clinical manifestations (vulnerable, atheromatous plaques) or those less frequently associated with acute thrombotic complication (stable, fibrous plaques). Defining the cellular and molecular mechanisms that underlie these morphological features remains a challenge. Because interstitial forms of collagen determine the biomechanical strength of the atherosclerotic lesion, this study investigated expression of the collagen-degrading matrix metalloproteinase (MMP) interstitial collagenase-3 (MMP-13) and the previously studied MMP-1 in human atheroma and used a novel technique to test the hypothesis that collagenolysis in atheromatous lesions exceeds that in fibrous human atherosclerotic lesions.

Methods and Results—Human carotid atherosclerotic plaques, similar in size, were separated by conventional morphological characteristics into fibrous (n=10) and atheromatous (n=10) lesions. Immunohistochemical and Western blot analysis demonstrated increased levels of MMP-1 and MMP-13 in atheromatous versus fibrous plaques. In addition, collagenase-cleaved type I collagen, demonstrated by a novel cleavage-specific antibody, colocalized with MMP-1– and MMP-13–positive macrophages. Macrophages, rather than endothelial or smooth muscle cells, expressed MMP-13 and MMP-1 on stimulation in vitro. Furthermore, Western blot analysis demonstrated loss of interstitial collagen type I and increased collagenolysis in atheromatous versus fibrous lesions. Finally, atheromatous plaques contained higher levels of proinflammatory cytokines, activators of MMPs.

Conclusions—This report demonstrates that atheromatous rather than fibrous plaques might be prone to rupture due to increased collagenolysis associated with macrophages, probably mediated by the interstitial collagenases MMP-1 and MMP-13. (Circulation. 1999;99:2503-2509.)

Key Words: atherosclerosis ▪ collagen ▪ cells ▪ enzymes

Pathoanatomic studies showed that human atheroma that rupture share certain common characteristics. So-called vulnerable plaques, referred to here as atheromatous, characteristically have a thin fibrous cap (FC), a prominent collection of lipid, and abundant macrophages (Mφ).1–4 In contrast, plaques with more vascular smooth muscle cells (SMCs), a well-developed FC, and lower Mφ and lipid content, referred to here as fibrous, appear less prone to disruption.

The integrity of the FC of the plaque and hence its resistance to rupture depend greatly on its content of fibrillar interstitial collagen.5,6 Hence, much effort has focused on the study of proteinases that may degrade interstitial collagen.

Specialized pathways of degrading macromolecules of the extracellular matrix involve a group of proteolytic enzymes known as matrix metalloproteinases (MMP), including 3 interstitial collagenases, any of which could catalyze the initial proteolytic attack on fibrillar interstitial collagen. We and others have shown expression of interstitial collagenase-1 (MMP-1),7–9 the prototypical MMP, in human atheroma.7,10

At least 2 other interstitial collagenases exist in mammals. Collagenase-2, or MMP-8, is expressed by neutrophils, cells not commonly found in human atheroma. However, collagenase-3, or MMP-13, a major interstitial collagenase in rodents, can occur in human tissues characterized by chronic inflammation and extensive matrix remodeling.11–15 Because atherosclerotic lesions and particularly vulnerable plaques also exhibit signs of chronic inflammation and matrix remodeling, we hypothesized that MMP-13 might also localize in atheroma.
Morphological Characteristics of Fibrous and Atheromatous Plaques

<table>
<thead>
<tr>
<th>Type of Lesion</th>
<th>Fibrous (n=10)</th>
<th>Atheromatous (n=10)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plaque size, mm²</td>
<td>17.1±5.3</td>
<td>18.5±9.0</td>
<td>NS</td>
</tr>
<tr>
<td>FC thickness, mm</td>
<td>1.2±0.4</td>
<td>0.2±0.1</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>SMC, %</td>
<td>21.5±8.1</td>
<td>2.7±2.5</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Mϕ, %</td>
<td>4.1±2.5</td>
<td>25.2±4.4</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Lipids (oil red O), %</td>
<td>3.3±2.5</td>
<td>46.8±11.5</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

Values are mean±SD.

Interstitial collagenases mediate the initial step of collagen degradation by cleaving triple-helical fibrils of interstitial collagen types I, II, and III at a single site (Gly775-Leu/Ile776), resulting in the generation of three-quarter- and one-quarter–length fragments. Previous studies have provided evidence for augmented gelatinolytic and caseinolytic activity in unselected human atheroma. However, to date, no direct experimental evidence demonstrates collagenolytic activity in the atherosclerotic plaque.

Therefore, we sought direct evidence for collagenolysis in human atheroma of various morphologies using a novel and more biochemically rigorous in situ technique than those previously used.

Methods

Human Tissue

Surgical specimens of human carotid plaques from endarterectomies (n=20) as well as nonatherosclerotic arteries (carotids from autopsies [n=6] and aortas from cardiac transplantation donors [n=5]) were obtained by protocols approved by the Human Investigation Review Committee at the Brigham and Women’s Hospital. We used well-established criteria of plaque vulnerability. Plaques with a minimal FC thickness ≥0.8 mm and an immunohistochemically determined positive area ≥10% for SMCs and ≤10% for Mϕ and lipid content were designated as fibrous. In contrast, lesions with minimal FC thickness ≤0.3 mm, ≤10% positive area for SMC, and ≥20% positive area for Mϕ and lipid content were designated as atheromatous (Table 1).

The tissue samples were divided into 2 macroscopically identical portions. One half was embedded in OCT compound for morphological analysis, and the other half was snap-frozen for biochemical study.

Immunocytochemistry

Serial cryostat sections (6 μm) were fixed in acetone (~−20°C, 5 minutes), air-dried, and stained by the avidin-biotin-peroxidase method. Tissue sections were treated with 3% hydrogen peroxide to inhibit endogenous peroxidase activity and incubated with primary antibodies diluted in PBS supplemented with 4% of the species-appropriate normal serum. The subsequent processing was performed according to the manufacturer’s recommendations (Universal Dako LSAB kit, peroxidase; Dako Co). The reaction was visualized with 3-amino-9-ethyl carbazole as substrate (AEC; Sigma Chemical Co). Sections were counterstained with Gill’s hematoxylin solution (Sigma).

Cell types were identified with monoclonal anti-muscle actin HHF-35 (Enzo Diagnostics), monoclonal anti-human CD68 (Mϕ), or CD31 (endothelial cells [ECs]) (DAKO Corp) antibodies.

Figure 1. Dichotomization criteria of fibrous versus atheromatous plaques. Representative photomicrographs (n=10/group) used for dichotomization of fibrous (left) versus atheromatous (right) plaques by well-established characteristics, namely, FC thickness as well as SMC and Mϕ content. The Table presents the quantitative analysis of immunohistochemistry and Picrosirius red staining in the entire series (n=10/group).

Polycyclonal rabbit anti-human MMP-1 (a gift of Dr M.W. Lark and Merck Research Laboratories) and MMP-13 (Triple Point Biologics) antibodies were used for immunohistochemistry and Western blot analysis. Adjacent sections treated with nonimmune IgG served as controls for antibody specificity.

Sites of collagenase-cleaved interstitial type I collagen were detected by staining with a polyclonal rabbit antibody reactive to the carboxy-terminal COL2–3/4Cneo neoepitope generated by cleavage of native human collagen by either human collagenases MMP-1, MMP-8, or MMP-13. The antibody showed no immunoreactivity to native or denatured human type I or III collagens.

For double immunofluorescent staining for collagenase-cleaved and intact type I collagen, frozen sections were incubated for 90 minutes with rabbit anti-human COL2–3/4Cneo antibody, followed by biotinylated secondary goat anti-rabbit antibody (45 minutes; Vector Laboratories), and finally FITC-conjugated streptavidin (30 minutes; Amersham Corp). Subsequently, specimens were treated with an avidin-biotin blocking kit (Vector Laboratories) and washed and stained with mouse anti-human type I collagen antibody overnight at 4°C, biotinylated secondary horse anti-mouse antibody, and streptavidin conjugated with Texas red (Amersham Corp). Nuclei were stained with bisbenzimide (Calbiochem).

Staining of Collagens Type I and III by Picrosirius Red

Frozen sections were incubated for 90 minutes in 0.1% Sirius red F3BA (Polyscience Inc) in saturated picric acid. After rinsing twice in 0.01N HCl and in distilled water, sections were briefly dehydrated with 70% ethanol and put under coverslips. Staining with Sirius red was analyzed by polarization microscopy.

Quantitative Analysis for Histology and Immunohistochemistry

Analysis of immunohistochemistry for Mϕ, SMCs, MMP-1, MMP-13, and cleaved collagen and Sirius red and oil red O staining were
performed with a personal computer–based quantitative 24-bit Optimas 5.2 color image analysis system. The percentage of the total area with positive color for each section was recorded.

**Cell Culture**

Human saphenous vein SMCs and ECs were isolated and cultured in DMEM or M199 (BioWhitaker), respectively, supplemented with 10% fetal bovine serum (Hyclone). Cells were rendered quiescent by culture in serum-free insulin/transferrin medium (SMCs) or M199 supplemented with 0.1% BSA (ECs) 24 hours before stimulation.

Monocytes were isolated from leukopacs of healthy donors by counterflow centrifugation and plated into 6-well culture plates (Nunc, Inc), as described previously.

**Western Blot Analysis**

Frozen tissue samples from 5 nonatherosclerotic arteries and 6 fibrous and 7 atheromatous carotid plaques were homogenized (Ultra-turrax T 25, IKA-Labortechnik) lysed (0.3 mg of tissue per 1 mL of lysis buffer), and clarified by centrifugation (16 000g, 15 minutes) as described previously. Fifty micrograms of total protein of tissue extracts, 10× ECs and SMCs or 2.5× Mφ culture supernatants (Centricron 3 devices, Amicon), were separated by 12% SDS-PAGE under reducing conditions and blotted onto polyvinylidene difluoride membranes (Millipore) with a semidy blotting apparatus (3.0 mA/cm², 30 minutes; Bio-Rad). Blots were blocked overnight, and primary antibodies (1:1000 polyclonal rabbit anti-human MMP-1 and COL2–3/4Cshort; 1:5000 polyclonal rabbit anti-human MMP-13; 1:1000 monoclonal mouse anti-human collagen type I, interleukin [IL]-1α [Endogen], and interferon [IFN]-γ [Genzyme]) and secondary antibodies (goat anti-rabbit or anti-mouse [1:10,000]) were diluted in 5% nonfat milk/PBS/0.1% Tween 20. Specificity of the rabbit anti-human MMP-1 and -13 antibodies was confirmed by use of antibodies preincubated (1 hour, 37°C) with the respective recombinant antigen (Chemicon and Triple Point Biological). Blots were developed by chemiluminescence (NEN). Densitometric analysis was performed with NIH Image software.

**In Situ Hybridization**

In situ hybridization was performed according to the instructions of the manufacturer (Hyb-Probe, Shandon/Lipshaw) with a mixture of FITC-labeled MMP-13-specific (5′-TAC GTA GTA CCC CAG GAC CGA CCG AAG GAG GAC-3′; 5′-AAC AAC GAC GCG TAC TCA ACG CCG TGA GGA-3′; 5′-TGC TAC CGT AAC GAC GCT ACT AGT-3′; 5′-TAC TAT TAT GAT TGG TAT TAT AAC ACC TAT-3′) or random oligomers. In situ hybridization analysis with the respective negative control yielded no signal (data not shown). Western blot analysis of tissue extracts for MMP-1 and MMP-13 provided biochemical corroboration of the results obtained by immunohistochemistry (Figure 4). Densitometric analysis showed a 2.7±1.3-fold (P<0.001) greater level of MMP-1 and a 5.4±2.6-fold higher level of MMP-13 in extracts of atheromatous (n=7) compared with fibrous (n=6) plaques. Nonatherosclerotic arteries (control) contained little or no immunodetectable MMP-1 or MMP-13.

**Statistical Analysis**

Data are presented as mean±SD and were compared between fibrous and atheromatous plaques by an unpaired Student t test. A value of P≤0.05 was considered significant.

**Results**

Collagenase-3 (MMP-13) colocalized with MMP-1 in human atheroma (Figure 2A), appeared most abundant in Mφ, and localized mostly in the shoulder region of the plaques as well as in areas surrounding the lipid core. By quantitative image analysis, levels of MMP-1 as well as MMP-13 in atheromatous plaques (21.4±3.4% and 20.4±2.7%, respectively) significantly (P<0.001; n=10/group) exceeded those in fibrous plaques (5.9±2.1% and 2.8±0.9%, respectively), corresponding to the content of Mφ (Figure 2B). Nonatherosclerotic arteries contained neither MMP-1 or MMP-13 protein (data not shown). In accordance with these findings, normal arteries did not contain MMP-13 mRNA in contrast with human atherosclerotic plaques (Figure 3), as detected by in situ hybridization. Within the lesions, MMP-13 gene transcripts localized most prominently in areas described above as positive for the immunoreactive protein. MMP-13 mRNA was detected in luminal ECs, SMCs of the fibrous cap, and clusters of Mφ. In situ hybridization analysis with the respective negative control yielded no signal (data not shown).

Western blot analysis of tissue extracts for MMP-1 and MMP-13 provided biochemical corroboration of the results obtained by immunohistochemistry (Figure 4). Densitometric analysis showed a 2.7±1.3-fold (P<0.001) greater level of MMP-1 and a 5.4±2.6-fold higher level of MMP-13 in extracts of atheromatous (n=7) compared with fibrous (n=6) plaques. Nonatherosclerotic arteries (control) contained little or no immunodetectable MMP-1 or MMP-13.

Although MMP-13 protein appeared most abundant in Mφ, ECs and SMCs within atheroma also contained MMP-13 protein and mRNA (Figures 2A and 3). To determine the relative capacities of these atheroma-associated cell types to express MMP-13 (as well as MMP-1), we analyzed the media of homogeneous cell cultures by Western blotting (Figure 5). Unstimulated SMCs and ECs barely expressed MMP-13 (data not shown), but after stimulation with PMA 50 ng/mL (Figure 5) as well as IL-1β 10 ng/mL or TNF-α 50 ng/mL...
(data not shown), these cells elaborated MMP-13 that migrated with bands seen in extracts of atheromatous plaques. In vitro, as in situ, Mφ expressed more MMP-13 (as well as MMP-1) than did SMCs or ECs. Indeed, 4 times less protein load in the Mφ lanes yielded much stronger signals (≈20-fold by densitometric analysis) than those derived from vascular cells (Figure 5). Even unstimulated Mφ cultured for 10 days released MMP-13 constitutively (data not shown).

Because the presence of immunoreactive MMPs does not necessarily correspond to the active enzyme, we sought direct evidence for collagenase activity by immunostaining for the neoepitope of type I collagen fibrils generated uniquely by collagenase cleavage.17 Nonatherosclerotic human arteries do not express this epitope (data not shown). However, human atherosclerotic plaques contained cleaved interstitial type I collagen, as demonstrated by immunostaining for this neoepitope (Figure 6A). Cleaved collagen characteristically localized on the margins of intact interstitial type I collagen, as demonstrated by double immunofluorescent labeling. Impor-
tantly, areas of cleaved collagen colocalize with MMP-1– and MMP-13–positive Mφ. Color image analysis showed a substantial increase of neoepitope in atheromatous (22.8 ± 7.9%; n = 7) versus fibrous (3.3 ± 2.2%; n = 6; P < 0.05) plaques (Figure 6B). This finding corresponded to a reduced type I collagen–positive area in atheromatous compared with fibrous lesions (35.3 ± 10.3% versus 67.9 ± 11.9%; P < 0.001). Accordingly, Western blot experiments of tissue extracts showed increased amounts of cleaved collagen in atheromatous versus fibrous plaques (Figure 6C). Densitometry of immunoreactive bands showed that fibrous lesions (n = 6) contained 2.4 ± 1.1-fold more cleaved collagen than normal control tissue (n = 5). Some samples of normal arteries and fibrous plaques, however, contained no immunodetectable cleaved collagen (Figure 6C), which accounts for the large standard error. In contrast to fibrous lesions, all atheromatous plaques analyzed (n = 7) showed increased cleaved collagen, averaging 4.9 ± 1.8-fold greater amount than in normal tissue. Interestingly, measurement of type I collagen by Western blotting showed a reciprocal decrease. The amount of intact collagen type I in fibrous plaques (86.9 ± 4.7%; n = 6) did not differ significantly from that in normal arteries, whereas atheromatous plaques showed a 52.2 ± 8.9% (P < 0.001; n = 7) decrease of immunoreactive intact collagen type I, corroborating the results obtained by Sirius red birefringence in situ (Figure 6B).

Finally, IL-1β, TNF-α, and IFN-γ, potential mediators of MMP expression, were not detected in normal arteries but
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were expressed in atherosclerotic lesions (Fig. 7). Interestingly, atheromatous plaques contained not only more pro–IL-1β but even more importantly, more of the biologically active 17-kDa form versus fibrous lesions.

Discussion

The integrity of the collagenous skeleton critically determines the lability of an atheromatous plaque to rupture. The collagen content of plaques depends on the balance between synthesis and breakdown. Collagen biosynthesis is difficult to assess in intact humans; however, SMCs, the major source of arterial interstitial collagen, are less frequent in atheromatous than fibrous plaques (Table). Moreover, vulnerable regions of human atherosclerotic plaques exhibit reduced procollagen gene expression.

With regard to collagen catabolism, interstitial collagenases catalyze the critical initial rate-limiting step in collagen breakdown, which yields trypsin-like or gelatinolytic activities. The tightly wound triple helix of interstitial collagen fibrils resists degradation by most proteinases. The actions of interstitial collagenases yield characteristic fragments 75% and 25% of the span of the intact collagen molecule, which are further degraded by collagenases and other members of the MMP family.

Using an antibody raised against the C-terminal peptide neopeptidome of the three-quarter fragment that selectively recognizes cleaved forms of type I collagen (as well as type II), we provide direct evidence for increased collagenolysis in situ in atheromatous plaques. Specificity of the antibody, as established previously, was further supported by our Western blot analysis, which yielded a single immunoreactive band of the expected molecular weight for the three-quarter piece of collagenase-cleaved type I collagen within extracts of human atheroma (Figure 6).

The present study also provides new information regarding the spectrum of proteinases potentially involved in collagen catabolism in atheroma. MMP-13, an interstitial collagenase recently described in certain carcinomas and extravascular sites of chronic inflammation, colocalizes with the “classic” interstitial collagenase MMP-1 that was described previously by us and others in human atheroma. Western blot analysis of tissue extracts from atheromatous plaques, as well as stimulated cultured human Mφ and vascular cells (ECs and SMCs), showed immunoreactive bands corresponding in molecular weight to the zymogen and active forms of the collagenases MMP-1 and MMP-13. Immunohistochemistry and Western blot analysis pointed to Mφ as the major source of these collagenases. This observation agrees well with the finding that cultured human Mφ express collagenolytic activity and our previous demonstration of de novo synthesis and release of MMP-1 by lipid-laden Mφ isolated from experimental rabbit atheroma. Expression of MMP-13 in addition to MMP-1 and the presence of nonmetalloenzymes capable of degrading extracellular matrix components suggest that strategies to control proteolysis therapeutically may require a broad rather than narrow spectrum of inhibitors.

Careful clinical-pathological correlations have led to the concept that plaque morphology determines its propensity to provoke acute manifestations. The dichotomy of plaques into “stable” versus “vulnerable,” although doubtless oversimplifed, has proved a useful framework for a better understanding of the pathophysiology of the thrombotic complications of atheroma. The present observations provide support for the established morphological, anatomic classification at the molecular level. Our biochemical studies on plaque extracts (although limited to extremes of a broad spectrum) show that classification of plaques a priori on the basis of morphology alone can predict their content of relevant proteinases and their potential activators. The cytokines that we found to be elevated in atheromatous plaques (IL-1β, TNF-α, and IFN-γ) have been shown to induce expression of MMPs. Therefore, one strategy to limit matrix degradation would involve targeting inflammatory stimuli proximal to protease gene expression rather than the multiple enzymes themselves.

Vulnerable plaques characteristically contain relatively few SMCs in relation to Mφ (Table). The low content of SMCs in atheromatous plaques can result from impaired growth or even death of SMCs and may contribute to plaque vulnerability, because SMCs synthesize interstitial collagen. One of the cytokines (IFN-γ) found to be overexpressed in atheromatous plaques in the present study can cause cytostasis of SMCs. A combination of 3 of the proinflammatory cytokines we found overexpressed in atheromatous lesions (IL-1β plus TNF-α plus IFN-γ; Figure 6) can promote death of SMCs by apoptosis.

The notion that augmented collagenolysis can precipitate rupture of atherosclerotic plaques still requires direct proof. However, several recent in vivo studies support the concept that unchecked proteolytic activity can lead to dilatation of the aorta or rupture of aortic aneurysms in experimental animals. In the case of human atherosclerosis, we lack the type of direct evidence provided by gene transfer or germ-line mutation that is currently possible in experimental animals. However, the present unambiguous demonstration of enhanced cleavage of collagen by collagenases associated with an increase in the presence of Mφ-derived active forms of the interstitial collagenases MMP-1 and MMP-13 in human atherosclerotic plaques provides strong support for the role of Mφ-mediated collagenolysis in matrix.
degradation, remodeling, and plaque rupture and hence in clinical complications of human atherosclerotic plaques.

Acknowledgments
This work was supported by a grant from the National Heart, Lung, and Blood Institute to Dr Libby (HL-34636) and was performed during the tenure of a Paul Dudley White fellowship (Dr Schönbeck) from the American Heart Association, Massachusetts Affiliate. We thank our colleagues Dr M.W. Muszynski, E. Shvartz, and M. Herman for their skillful assistance.

References
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_Circulation._ 1999;99:2503-2509
doi: 10.1161/01.CIR.99.19.2503

_Circulation_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
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