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

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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.

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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.14 These fragments then become accessible to other proteases, such as gelatinases and stromelysins, which together with collagenases further catabolize collagen.7,10,16 Previous studies have provided evidence for augmented gelatinolytic and caseinolytic activity in unselected human atheroma.9,16 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.6,18 Plaques with a 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).

Polycystic 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 COL2–3/4Cshort neoepitope generated by cleavage of native human collagen by either human collagenases MMP-1, MMP-8, or MMP-13.17 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 COL2–3/4Cshort 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.19

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-Labornetchnik) lysed (0.3 mg of tissue per 1 mL of lysis buffer), and clarified by centrifugation (16,000 g, 15 minutes) as described previously. Fifty micrograms of total protein of tissue extracts, 10× ECs and SMCs or 2.5× Mφ culture supernatants (Centrican 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 GAG-3′); 5′-AAG AAC GAC GCG TAC TCA ACG CGG TGA GGA-3′; 5′-TGC TAC CGT AAC GAC GCT ACT AGA-3′; 5′-TAC TAT TAC GTG TAT TAT ACC AAT TAT-3′) or random oligomers.

**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 nor 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 characteristicly 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

![Figure 6. Increased collagenolysis in human atheromatous compared with fibrous plaques. A, Immunofluorescence for collagenase-cleaved type I collagen neoepitope (FITC, green; top left) and collagen type I (Texas red; middle) detected cleaved collagen in shoulder area. Top right, Double immunolabeling for both antigens (nuclei were counterstained with bisbenzimide [blue]). Adjacent sections were stained immunohistochemically for interstitial collagenases MMP-1 and -13, as well as for human Mφ (bottom). B, Photomicrographs of these studies were quantified by color image analysis of percent positively stained area. Values represent mean±SD (n=6). C, Tissue extracts (50 μg of total protein/lane) from 2 nonatherosclerotic arteries (Control) and 3 fibrous and 4 atheromatous plaques were analyzed by Western blotting for expression of collagenase-cleaved type I collagen as well as for collagen type I. Molecular weight markers are indicated. Analyses were repeated at least twice, yielding similar results.](http://circ.ahajournals.org/)

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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.5 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 plaques22,23 (Table). Moreover, vulnerable regions of human atherosclerotic plaques exhibit reduced procollagen gene expression.24

With regard to collagen catabolism, interstitial collagenases catalyze the critical initial rate-limiting step in collagen breakdown.7,10,16,17 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,14 which are further degraded by collagenases and other members of the MMP family.7,10,16,17

Using an antibody raised against the C-terminal peptide neoepitope of the three-quarter fragment that selectively recognizes cleaved forms of type I collagen (as well as type II),17 we provide direct evidence for increased collagenolysis in situ in atheromatous plaques. Specificity of the antibody, as established previously,17 was further supported by our Western blot analysis, which yielded a single immunoreactive protein 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,11-15 colocalizes with the “classic” interstitial collagenase MMP-1 that was described previously by us and others7-9 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 thezymogen and active forms of the collagenases MMP-1 and MMP-13.25-27 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 activity28 and our previous demonstration of de novo synthesis and release of MMP-1 by lipid-laden Mφ isolated from experimental rabbit atheroma.29 Expression of MMP-13 in addition to MMP-1 and the presence of nonmetalloenzymes30 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 dichotomization of plaques into “stable” versus “vulnerable,” although doubtless oversim-

Figure 7. Increased expression of potential modulators of MMP expression and activation in human atheromatous plaques. Tissue extracts (50 μg of protein/lane) from 2 nonatherosclerotic arteries (Control) and 3 fibrous and 4 atheromatous plaques were analyzed by Western blotting for expression of the proinflammatory cytokines IL-1β, TNF-α, and IFN-γ. Molecular weight markers are indicated. Analyses were repeated at least twice, and all tested samples showed similar results.
degradation, remodeling, and plaque rupture and hence in clinical complications of human atherosclerotic plaques.

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