Expression of Neutrophil Collagenase (Matrix Metalloproteinase-8) in Human Atheroma
A Novel Collagenolytic Pathway Suggested by Transcriptional Profiling

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Background—Loss of interstitial collagen, particularly type I collagen, the major load-bearing molecule of atherosclerotic plaques, renders atheroma prone to rupture. Initiation of collagen breakdown requires interstitial collagenases, a matrix metalloproteinase (MMP) subfamily consisting of MMP-1, MMP-8, and MMP-13. Previous work demonstrated the overexpression of MMP-1 and MMP-13 in human atheroma. However, no study has yet evaluated the expression of MMP-8, known as “neutrophil collagenase,” the enzyme that preferentially degrades type I collagen, because granulocytes do not localize in plaques.

Methods and Results—Transcriptional profiling and reverse transcription–polymerase chain reaction analysis revealed inducible expression of MMP-8 transcripts in CD40 ligand–stimulated mononuclear phagocytes. Western blot analysis demonstrated that 3 atheroma-associated cell types, namely, endothelial cells, smooth muscle cells, and mononuclear phagocytes, expressed MMP-8 in vitro upon stimulation with proinflammatory cytokines such as interleukin-1β, tumor necrosis factor-α, or CD40 ligand. MMP-8 protein elaborated from these atheroma-associated cell types migrated as 2 immunoreactive bands, corresponding to the molecular weights of the zymogen and the active molecule. Extracts from atherosclerotic, but not nondiseased arterial tissue, contained similar immunoreactive bands. Moreover, all 3 cell types expressed MMP-8 mRNA and protein in human atheroma in situ. Notably, MMP-8 colocalized with cleaved but not intact type I collagen within the shoulder region of the plaque, a frequent site of rupture.

Conclusions—These data point to MMP-8 as a previously unsuspected participant in collagen breakdown, an important determinant of the vulnerability of human atheroma. (Circulation. 2001;104:1899-1904.)

Key Words: atherosclerosis ■ collagen ■ metalloproteinases ■ inflammation

Rupture of atherosclerotic lesions triggers most acute clinical manifestations of atherosclerosis such as myocardial infarction or stroke.1 Previous studies established thinning and weakening of the fibrous cap as the mechanism that renders atheroma prone to rupture.2,3 Stability of the fibrous cap depends primarily on the content of intact interstitial type I collagen, the major load-bearing molecule.4 Indeed, plaques with histopathological hallmarks of vulnerability exhibit enhanced collagenolysis, a process associated with matrix metalloproteinases (MMPs).

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Interstitial collagen fibrils resist degradation by most proteinases. Only interstitial collagenases I (MMP-1), II (MMP-8), and III (MMP-13) can initiate the breakdown of intact, triple-helical collagen, degrading types I, II, and III collagen into one-quarter and three-quarter fragments. Although collagenases have overlapping substrate specificities, MMP-1 and MMP-13 preferentially cleave type III and II collagen,5–10 respectively. MMP-8, however, degrades type I collagen 3 times more potently than MMP-1 or MMP-13.11–14 After this initial cleavage, fibrillar collagen fragments become susceptible to further degradation by various MMPs overexpressed in atheroma, eg, MMP-2, MMP-3, and MMP-9.5–10

Many cell types, including endothelial cells (ECs), smooth muscle cells (SMCs), and macrophages (MØs), can express both MMP-1 and MMP-13.5–7,10 However, only polymorphonuclear granulocytes (PMNs) have been considered capable of expressing MMP-8. Originally cloned from mRNA extracted from peripheral blood leukocytes of a patient with chronic granulocytic leukemia, and later described in the postpartum murine uterus, this member of the MMP family was dubbed “neutrophil collagenase.”15,16 In contrast with

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most MMP family members, PMN precursors synthesize MMP-8 early during differentiation and store thezymogen within special granules, which are released on PMN activation.17,18 Numerous studies have reported a role for MMP-8 in connective tissue turnover in acute inflammatory reactions involving neutrophils.17,19–21

We and others previously reported expression of the interstitial collagensases MMP-1 and MMP-13 in ECs, SMCs, and MØs in human and experimental atherosclerosis.5,7,10 We further provided direct evidence for collagenolysis in human atherosclerotic lesions and demonstrated that degraded type I collagen colocalizes with MMP-1 and MMP-13.5 Despite the preference of MMP-8 for type I collagen,11 we and others had neglected a potential role for this MMP in atherogenesis, because atheroma contain few if any neutrophils.22 However, as we report here, transcriptional profiling analysis of in vitro–differentiated peripheral blood monocyte-derived MØs stimulated with CD40 ligand (CD40L), a potent inducer of MMP expression,23–25 demonstrated the capacity of these cells to express transcripts for MMP-8. The present study reports the surprising finding that atheroma-associated ECs, SMCs, and mononuclear phagocytes express the “neutrophil” collagenase MMP-8 in vitro and in situ.

Methods

Materials

Human recombinant interleukin (IL)-1β and tumor necrosis factor (TNF)-α were obtained from Endogen, Escherichia coli endotoxin (LPS) from Sigma, and recombinant human MMP-8 and CD40L from Chemicon and Leinco Technologies, respectively.

Cell Isolation and Culture

Human vascular ECs and SMCs were isolated from saphenous veins by collagenase treatment (1 mg/mL; Worthington Biochemicals) and explant outgrowth, respectively, and were cultured as described previously.5,25 Mononuclear phagocytes were isolated from freshly prepared human peripheral blood mononuclear cells by density gradient centrifugation with lymphocyte separation medium (Organon-Teknika) and subsequent adherence to plastic culture flasks. Mononuclear phagocytes were used directly (monocytes) for the experiments or cultured for 1, 3, or 11 days (MØs) in RPMI 1640 containing 2% human serum (Sigma). The purity of monocytes/MØs was ≥92%, as determined by fluorescence-activated cell sorter analysis (anti-human CD68 mAb FITC, PharMingen). Before (24 hours) and during stimulation, all 3 cell types were cultured in medium lacking serum, as described previously.5,25

PMNs were obtained from peripheral blood by venipuncture into 0.1 vol of sodium citrate anticoagulant (Sigma) with neutrophil isolation media (Cardinal Assoc) and were kindly provided by Dr. M. Glogauer (Brigham and Women’s Hospital, Boston, Mass). The preparation contained ≥95% neutrophils as determined by hematology and eosin staining.

RNA Isolation, Transcriptional Profiling, and Reverse Transcription–Polymerase Chain Reaction

Total RNA was isolated from ECs, SMCs, or in vitro–differentiated peripheral blood monocyte-derived MØs with RNazol (Tel-Test) and was reverse transcribed (SuperScript Reverse Transcriptase; Gibco-BRL) to obtain either the oligo-dT30 primed, [α-32P]dCTP-labeled first-strand cDNA probe for microarray analysis or the cDNA templates for reverse transcription–polymerase chain reaction (RT-PCR). Hybridization experiments were performed on a custom DNA array, MPG version 4.1, composed of 6144 human cDNA clones. Quadruplicate filters per probe were prehybridized (65°C, 1 hour) in 10% formamide-Church buffer containing salmon sperm DNA (10 mg/mL) and subsequently hybridized (18 hours) with the respective probe. Filters were washed twice (65°C, 15 minutes) with 2× SSC/1% SDS and 0.1× SSC/0.5% SDS, respectively, rinsed in 2× SSC, and baked (2 hours, 85°C). Finally, dried filters were exposed on phosphoimaging plates (Fuji-Film), and median intensity ±SD for each probe in quadruplicate was calculated. Treatment with CD40L was compared with the respective time point of untreated control.

For RT-PCR analysis, cDNA templates (1 μL) were mixed with the respective primer pair (sense: 5'–GGAACCCCGAG-GTGAAAGC-3'; antisense: 5'–CCTGAGACATATTGG-GATACATAGG-3'; 0.2 μmol/L each) in 50 μL of total reaction mixture (MgCl2 1.5 mmol/L, dNTPs 0.2 mmol/L, platinum Taq DNA polymerase 2.5 U, and 5 μL of PCR buffer). The PCR reaction mix was applied to 35 cycles at 94°C (1 minute), 55°C (1 minute), and 72°C (1.5 minutes). Aliquots of the PCR product (expected size 417 bp) were run on 1.5% agarose gels and visualized by ultraviolet transillumination. RT reaction products obtained in the absence of RT, as well as H2O, were used as mock controls.

Western Blot Analysis

Tissue extracts (50 μg of total protein per lane) obtained from frozen nonatherosclerotic (n = 3) and atheromatous human carotid arteries and aortas (n = 6), dichotomized a priori into fibrous (stable; n = 3) and atheromatous (unstable; n = 3) plaques by morphological criteria,5 as well as culture lysates (50 μg of total protein per lane) and supernatants (50 μL), were separated by SDS-PAGE under reducing conditions and applied to Western blot analysis as described previously4,23 with the respective primary (rabbit anti-human MMP-8; Chemicon) and secondary antibody. Immunoreactive proteins were visualized by the Western Blot chemiluminescence system (NEN). Data were validated in additional experiments that used antibodies of different origin (mouse anti-human MMP-8; Calbiochem) and antibodies preincubated (18 hours, 4°C) with trypsin-activated recombinant human MMP-8 (5 μg/mL; Chemicon).

In Situ Hybridization

In situ hybridization was performed according to the instructions of the manufacturer (Biogenex). Frozen tissue sections of nonatherosclerotic tissue (n = 3) and atheromatous plaque (n = 3) specimens were fixed in cold acetone, air dried, and incubated (10 minutes, 65°C; subsequently 2 hours, 37°C) with a mixture of FITC-labeled MMP-8 (5'–TCAAGCTTCCGCACTTCTTCTTCGTAT-3'; 5'–CGGAACCGACAGG GTTGAACGAAAGTCC-3'; 5'–TGGATATAGAAACATTTCTGTTAAAGAC-3'; 5'–TCTGTAC TAAGAATAATCTTCCATCTA-3') or random (control) oligomers in hybridization buffer (30% formamide, 0.6 mol/L NaCl, 10% dextran sulfate, 50 mM/L Tris, pH 7.5; 0.1% sodium pyrophosphate, 0.2% Ficoll, and 5 mmol/L EDTA). Finally, slides were washed 3 times and stained with alkaline phosphatase–conjugated rabbit Fab′ anti-FITC (30 minutes) and NBT/BCIP chromogen solution (1 hour).

Immunohistochemistry

Serial cryostat sections (5 μm) of surgical specimens of 3 nonatherosclerotic aortas and carotid arteries and 6 atheromatous carotid plaques, dichotomized into stable (n = 3) and vulnerable (n = 3) plaques (all obtained from different donors) by morphological criteria as described previously,5 were cut, air dried onto microscope slides, fixed in acetone (−20°C, 5 minutes), and preincubated with PBS containing 0.3% hydrogen peroxide. Subsequently, sections were incubated (30 minutes) with primary (rabbit anti-human MMP-8; Chemicon) or control (rabbit Ig, Jackson Immunoresearch) antibody and processed according to the suppliers’ recommendations (LSAB Kit, Dako Co). For control purposes, staining was validated in additional experiments with an anti-MMP-8 antibody of different origin (mouse anti-human-MMP-8; Calbiochem; and mouse myeloma protein MOPC-21, Sigma).
For colocalization of MMP-8 with the respective cell type, anti-human MMP-8 antibody (1:400) was applied (90 minutes), followed by biotinylated secondary antibody (45 minutes) and Texas red–conjugated streptavidin (Amersham; 20 minutes). After application of the avidin/biotin blocking kit (Vector Laboratories), anti-muscle actin monoclonal antibody (mAb) for SMCs (1:200; Enzo Diagnostics), anti-CD31 mAb for ECs (1:35, Dako), or anti-CD68 mAb for MØs (1:500, Dako) was added and sections were incubated overnight (4°C). Subsequently, biotinylated horse anti-mouse secondary antibodies were applied (45 minutes), followed by streptavidin-FITC (Amersham; 20 minutes). Staining of type I and type III collagen used Picrosirius red, as described previously.5 Cleaved interstitial type I collagen was detected by staining with a polyclonal rabbit antibody reactive with the COL3/4Cshort neoepitope, kindly provided by Dr Robin Poole (McGill University, Montreal, Quebec, Canada).5

For immunofluorescence double labeling for MMP-8 with cleaved or intact type I collagen, frozen sections were treated as described above, with rabbit anti-human COL3/4C short or mouse anti-human type I collagen antibody (90 minutes) as the first antibody and mouse anti-human MMP-8 antibody (overnight, 4°C) as the second antibody. Nuclei were stained with bisbenzimide (Calbiochem).

Results
Expression of MMP-8 in Human Atheroma-Associated Cells In Vitro
Transcriptional profiling demonstrated that stimulation of mononuclear phagocytes with CD40L enhanced the expression of MMP-8 transcript (Figure 1). RT-PCR revealed expression of MMP-8 transcripts in MØs, vascular SMCs (both Figure 1), and ECs (data not shown) only after stimulation, eg, via CD40L. In accord with the mRNA data, unstimulated cultures of ECs, SMCs, and mononuclear phagocytes expressed minimal or no MMP-8 protein constitutively (Figure 2). However, stimulation with proinflammatory cytokines, eg, IL-1β or CD40L (Figure 2), as well as TNF-α or LPS (data not shown), induced expression and release of immunoreactive MMP-8 in all 3 cell types. Atheroma-associated cells released 2 major MMP-8 protein species that migrated at ≈75 kDa, corresponding to the latent and active forms of this enzyme, respectively. EC culture supernatants expressed only a single band at ≈75 kDa. In contrast, PMNs constitutively expressed cell-associated MMP-8, the release of which required stimulation. In addition to previously described inducers of secretion, we demonstrate here that ligation of CD40 on PMN triggers release of this collagenase.
Because MØs constitute a major source of matrix-degrading proteinases, particularly interstitial collagenases within human atheroma, we further analyzed whether differentiation of freshly isolated peripheral blood mononuclear phagocytes into monocyte-derived MØs affected the expression of MMP-8. Freshly isolated mononuclear phagocytes did not release MMP-8, even when stimulated with IL-1α/H9252 or CD40L (Figure 3). However, culture for 11 days yielded low basal expression of MMP-8, which increased substantially on stimulation with either IL-1β or CD40L.

Expression of MMP-8 in Human Atheroma-Associated Cells In Situ

Given the inducibility of MMP-8 expression in atheroma-associated cells in vitro, we tested whether ECs, SMCs, and MØs within human atherosclerotic lesions express MMP-8 transcripts and protein in situ. In contrast to unaffected arteries, human atheroma expressed MMP-8 mRNA abundantly (Figure 4). MMP-8 localized in the MØ-enriched shoulder, the SMC-enriched fibrous cap, and the overlying endothelium. MMP-8 transcript expression corresponded to MMP-8 protein localization in atherosclerotic but not in nondiseased arteries (Figure 5). Like its mRNA, MMP-8 protein accumulated predominantly within the atheromatous shoulder region, a frequent site of plaque rupture. Immunofluorescence double labeling formally demonstrated colocalization of the enzyme with all 3 atheroma-associated cell types, namely, ECs, SMCs, and MØs (Figure 6).

Interestingly, advanced atherosclerotic lesions characterized by features associated with rupture-prone plaques, eg, a large lipid core and a thin fibrous cap, expressed more immunoreactive MMP-8 than did plaques with more stable morphology or nonatherosclerotic tissue, as determined by Western blot analysis of protein extracts (Figure 7). Analysis of these samples with anti-MMP-8 antibody preincubated with the recombinant protein substantially diminished band intensities, which supports the specificity of the antibody. Semiquantitative analysis with recombinant human MMP-8 used as a standard revealed an approximate concentration of 350 ng of total immunoreactive MMP-8 per milligram of tissue in atherosclerotic lesions, levels similar to those ob-

Figure 4. Transcripts for MMP-8 localize in human atherosclerotic lesions. Serial cryostat sections from nonatherosclerotic aortas (Normal; top left) and atherosclerotic carotid atheroma (top middle) were analyzed for MMP-8 transcript expression by in situ hybridization. Higher magnifications demonstrated localization of MMP-8 transcripts within luminal endothelium (bottom right), SMC-enriched fibrous cap (bottom middle), and MØ-enriched shoulder region (bottom left). Scrambled oligomers of identical size were used as negative control (top right). Analysis of nondiseased arteries and surgical specimens of atheroma from 3 different donors showed similar results.

Figure 5. Enhanced expression of MMP-8 protein in human atherosclerotic lesions. Serial cryostat sections from nonatherosclerotic aortas (Normal; left) and atherosclerotic carotid atheroma, dichotomized by features associated with either stable (middle) or vulnerable (right) lesions, were analyzed for expression of MMP-8 (top), as well as smooth muscle α-actin (SMC; middle) or CD68 (MØs; bottom). Analysis of 3 nondiseased arteries, as well as 3 stable and vulnerable surgical specimens of atheroma obtained from different donors, showed similar results.

Figure 6. MMP-8 colocalizes with human vascular ECs, SMCs, and MØs in human atherosclerotic lesions. Double-immunofluorescence staining was used to colocalize MMP-8 (red) with ECs (anti-CD31), SMCs (anti-α-actin), or macrophages (MØ, anti-CD68) within atherosclerotic plaques. Analysis of surgical specimens of atheroma from 3 different donors showed similar results.
tained for MMP-1 and MMP-13. These comparisons, however, account for neither varying antibody affinities nor local accumulation of the enzyme within distinct microenvironments of the plaque, and thus their interpretations require caution. MMP-8 colocalized with the three-quarter-length type I collagen breakdown products and demonstrated an inverse correlation between the enzyme and intact type I collagen (Figure 8), which implicates MMP-8 in the processes underlying collagenolysis within the atheromatous plaque.

**Discussion**

Degradation of extracellular matrix macromolecules, particularly interstitial type I collagen, the major load-bearing molecule within the fibrous cap of the plaque, promotes the evolution of atherosclerotic lesions toward vulnerable, rupture-prone plaques. Previous studies implicated MMPs in these degradative processes. Indeed, our group recently provided direct evidence for MMP-mediated cleavage of type I collagen within the shoulder region of human atheroma, a frequent site of rupture characterized by elevated expression of the interstitial collagenases I (MMP-1) and III (MMP-13). MMP-8 exhibits 3-fold greater enzymatic activity against type I collagen than the other interstitial collagenases, which probably makes it the most efficient type I collag enolytic enzyme in humans.

We and others previously neglected the possible role of MMP-8 in atherogenesis in light of its traditional attribution as a product of neutrophils, a cell type not commonly observed in atheroma. The unbiased survey afforded by transcriptional profiling pointed to a potential role of this enzyme in atherogenesis, despite its nomenclature. Our surprising finding that ECs, SMCs, and MØs within human atherosclerotic lesions express MMP-8 affirms that the expression of this interstitial collagenase extends beyond a single cell type. Recent reports suggesting expression of MMP-8 by rheumatoid synovial fibroblasts and ECs, as well as articular chondrocytes, and the observation that murine tissue not typically associated with PMN infiltration, such as kidney and muscle tissue, also expressed this enzyme support this finding. The cytokine-induced expression of MMP-8 in ECs, SMCs, and MØs differs from the release pattern in the traditional source, the neutrophil, which stores MMP-8 zymogen in granules and releases the collagenase almost immediately on stimulation. Thus, whereas MMP-8 release can occur immediately in acute inflammation associated with PMN infiltration, such as kidney and muscle tissue, also expressed this enzyme support this finding. The cytokine-induced expression of MMP-8 in ECs, SMCs, and MØs differs from the release pattern in the traditional source, the neutrophil, which stores MMP-8 zymogen in granules and releases the collagenase almost immediately on stimulation. Thus, whereas MMP-8 release can occur immediately in acute inflammation associated with PMN infiltration, MMP-8 synthesis and release by ECs, SMCs, and MØs at sites of chronic inflammation, such as atheroma, requires prolonged exposure to proinflammatory cytokines. In view of the role of hypochlorous acid in MMP-8 activation, it is noteworthy that a subpopulation of MØs in advanced (but not early) atherosclerotic lesions contain myeloperoxidase, the enzyme responsible for hypochlorous acid production. Thus, expression of MMP-8 and its activator myeloperoxidase likely occurs contemporaneously during the differentiation of monocyte-derived MØs in vitro and in atherosclerotic lesion development in situ. Highlighted expression of MMP-8 by MØs compared with monocytes agrees with previous reports on other MMPs.

The localization of the 55-kDa form of MMP-8 in atheroma corresponding to the active form of the enzyme and its...
colocalization with cleaved rather than intact type I collagen underscore the relevance of our findings to atherosclerosis and its acute clinical sequelae, such as plaque rupture and thrombosis. Degradation of type I collagen likely leads to thinning of the fibrous cap of plaques, a characteristic of the vulnerable, rupture-prone plaque.6 Accordingly, MMP-8 concentrations in lesions prone to rupture exceed those in lesions with a more stable phenotype. Previous studies suggesting that the collagenolytic activity found at some sites of chronic inflammation, such as periodontitis, derives from MMP-8 rather than the other interstitial collagenses further support the potential relevance of this enzyme in plaque destabilization.23

The surprising finding that human vascular ECs, SMCs, and MØs express the interstitial collagensase MMP-8 in vitro on stimulation and in situ in atherosclerotic lesions not only broadens knowledge of the expression pattern of this “neutrophil collagensase” but further suggests a novel pathological role of MMP-8. Designing MMP inhibitors of restricted specificity may obviate some of the toxicity encountered in clinical trials of broad-spectrum agents. The present identification of a likely role for MMP-8 in atherogenesis thus has practical therapeutic and theoretic implications.

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