Increased Expression of Membrane Type 3–Matrix Metalloproteinase in Human Atherosclerotic Plaque
Role of Activated Macrophages and Inflammatory Cytokines

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Background—Matrix metalloproteinases (MMPs) are thought to play a prominent role in atherogenesis and destabilization of plaque. Pericellularly localized membrane-type (MT)–MMPs activate secreted MMPs. We investigated the hypothesis that MT3-MMP is expressed in human atherosclerotic plaques and is regulated by locally produced inflammatory cytokines and oxidized low-density lipoprotein (Ox-LDL).

Methods and Results—Expression and cellular localization of MT3-MMP in normal and atherosclerotic human coronary arteries were examined using specific antibodies. Abundant MT3-MMP expression was noted in medial smooth muscle cells (SMCs) of normal arteries. In atherosclerotic arteries, MT3-MMP expression was observed within complex plaques and colocalized with SMCs and macrophages (Mφ). Cultured human monocyte-derived Mφ constitutively expressed MT3-MMP mRNA and proteolytically active protein, as demonstrated by mRNA analyses, immunoblotting, and gelatin zymography, respectively. Ox-LDL, tumor necrosis factor-α, or macrophage colony–stimulating factor caused dose- and time-dependent increases in steady-state levels of MT3-MMP mRNA in cultured Mφ. This correlated with a 2- to 4-fold increase in levels of MT3-MMP immunoreactive protein and enzymatic activity in Mφ membranes. Confocal microscopy and flow cytometry confirmed induction and spatial distribution of MT3-MMP protein from intracellular domains to the Mφ plasma membrane by Ox-LDL, tumor necrosis factor-α, or macrophage colony–stimulating factor.

Conclusions—MT3-MMP is expressed by SMCs and Mφ in human atherosclerotic plaques. Proinflammatory molecules cause a progressive increase in the expression of MT3-MMP in cultured Mφ. Our results suggest a mechanism by which inflammatory molecules could promote Mφ-mediated degradation of extracellular matrix and thereby contribute to plaque destabilization. (Circulation. 2002;106:3024-3030.)

Key Words: metalloproteinases ■ inflammation ■ plaque

Atherogenesis involves two key events: migration of circulating monocytes and other inflammatory cells into the subendothelium and migration of smooth muscle cells (SMCs) from the media to the intima.1 Eventually, plaque erosion and rupture may directly precipitate thrombosis and its frequently devastating sequelae.2 All of these pathobiological processes share a common requirement, focal matrix degradation, which is predominantly accomplished by the proteolytic action of locally expressed and activated matrix metalloproteinases (MMPs).3

MMPs comprise a family of proteases that are capable of degrading virtually all components of the extracellular milieu. MMPs perform a variety of tasks necessary for normal homeostasis, including maintenance of the dynamic integrity of the extracellular structure within arteries, but a growing body of evidence indicates that dysregulation of MMPs underlies pathobiological alterations associated with diverse diseases.3 Within the MMP family is a subfamily that is not diffusible because it contains a transmembrane domain or a glycosylphosphatidylinositol anchor that localizes it to the cell membrane. Six membrane-type (MT)-MMPs have been identified.3 For several reasons, MT-MMPs are of particular interest. First, MT-MMPs are not synthesized in zymogen form but are instead constitutively active in situ. Second, MT-MMPs may function in a “privileged” microenvironment, where they are relatively protected from inactivation by TIMPs and diffusible nonspecific protease inhibitors. Third, MT-MMPs catalytically activate soluble MMPs from their zymogen to active forms and thus not only exert control over MMP activation but, more importantly, are also capable of...
initiating an amplified, cascade-like biological effect. This last feature in particular makes MT-MMPs attractive candidates for potential therapeutic targets.

A variety of extracellular stimuli, including cytokines, cell-cell, and cell-matrix interactions, can induce MMP expression. Of particular relevance to atherosclerotic pathology, increased expression and activity of MMPs have been noted in vulnerable plaque regions. Serum MMPs are elevated in patients with acute coronary syndromes but not in those with stable angina. In addition, studies have shown that polymorphisms in MMP promoters are linked to susceptibility to coronary artery disease. We have shown that oxidized low-density lipoprotein (Ox-LDL) but not native LDL increased MMP-9 and MT1-MMP mRNA and protein expression and decreased TIMP-1 expression in cultured macrophages (Mφ). We have also reported that inflammatory cytokines increase expression and activity of MT1-MMP by cultured vascular endothelial cells, SMCs, and Mφ. Additionally, we have demonstrated that MT1-MMP is expressed in human atherosclerotic plaques and colocalizes with SMCs and Mφ.

In this study, we sought to determine whether MT3-MMP, another member of the MT-MMP family, is expressed in human atherosclerotic plaques. To understand the nature and regulation of the factors that promote expression of MT3-MMP, this study investigated whether inflammatory molecules regulate expression and function of MT3-MMP in cultured human Mφ.

Methods

Tissues and Immunocytochemical Staining

Specimens of normal (n=5) and atherosclerotic human coronary arteries (n=20) were obtained at the time of autopsy and fixed in 10% formalin. Atherosclerotic lesions were defined according to the histological classification of atherosclerosis of the American Heart Association. Plaques were categorized into predominantly fibrous (n=10) versus lipid and macrophage-rich atheromatous lesion (n=10) using previously published criteria of plaque vulnerability.

Serial sections (5 µm thick) of paraffin-embedded arterial tissues were subjected to immunohistochemical staining for localization of MT3-MMP, MMP-2, SMCs, and Mφ, essentially as described using a commercially available kit from Dako Co. Antibodies included MT3-MMP (Oncogene Research Products, San Diego, Calif) and MMP-2 (Chemicon International, Inc, Temecula, Calif) to identify SMC and Mφ, and a mouse nonspecific IgG (Dako Co.) as a negative control. Vascular SMCs were used as a positive control for MT3-MMP. Immunolocalization of SMC, Mφ, and MT3-MMP in fibrous and atheromatous plaques was quantitatively analyzed using Image Pro (MediaCybernetics).

Isolation and Culture of Monocyte-Derived Mφ

Human peripheral blood monocytes were isolated as described. Monocyte-derived Mφ were cultured in RPMI 1640 ( Gibco-Invitrogen) containing 10% FCS (HyClone Laboratories), 100 U/mL penicillin, 100 µg/mL streptomycin, and 0.25 µg/mL amphotericin B for 7 days and then starved in the culture medium without FCS but with 0.1% low endotoxin BSA (Sigma Chemical). Experiments were performed in the starvation medium with or without varying concentration of Ox-LDL (obtained from Dr Judith Berliner, UCLA, Los Angeles, Calif), tumor necrosis factor (TNF-α) (PeproTech, Inc), and macrophage colony-stimulating factor (M-CSF) (R&D Systems).

Amplification of Human MT3-MMP-Specific cDNA Sequence

Two primers (MT-3 upper, TGGATGAAGAAGCCCAGGAT [nucleotides 283 to 301]; MT-3 lower, GATGGCAGTGGGGTATG [nucleotides 771 to 789]) were synthesized corresponding to the published cDNA sequence of human MT3-MMP. Reverse transcriptase-polymerase chain reaction (RT-PCR) was performed to amplify a 507-nucleotide-long cDNA sequence using total RNA prepared from SMCs and Mφ. The identity of the amplified product as MT3-MMP was established by DNA sequencing.

Northern Blot Analyses

RNA blots containing total cellular RNA were hybridized with a [32P]-labeled MT3-MMP-specific cDNA probe. Blots were washed, autoradiographed, and, after stripping, rehybridized with a β-actin cDNA probe as an internal control. Quantitative results of the assays were obtained by performing densitometry of autoradiograms.

Immunoblot Analysis

Extracts of partially purified plasma membrane fractions of Mφ grown in absence or presence of Ox-LDL, TNF-α, or M-CSF were added to culture media harvested from human SMC containing pro-MMP-2 and assayed for gelatinolytic activity as described. Equal amounts of culture media obtained from Mφ grown with or without Ox-LDL, TNF-α, or M-CSF were used to detect MMP-2 and MMP-9 activity. Proteins were electrophoresed using discontinuous sodium dodecyl sulfate (SDS)-polyacrylamide gels containing gelatin and visualized as described.

Gelatin Zymography

Equal amounts of extracts of plasma membrane fractions of Mφ grown in absence or presence of Ox-LDL, TNF-α, or M-CSF were added to culture media harvested from human Mφ containing pro-MMP-2 and assayed for gelatinolytic activity as described. Equal amounts of culture media obtained from Mφ grown with or without Ox-LDL, TNF-α, or M-CSF were used for secretion MMP-2 and MMP-9 activity. Proteins were electrophoresed using discontinuous sodium dodecyl sulfate (SDS)-polyacrylamide gels containing gelatin and visualized as described.

Immunofluorescent Localization of MT3-MMP and MMP-2

Human monocytes (10⁶/well) were seeded on poly-DL-lysine–coated glass cover slips in serum-containing media for 16 hours, and the nonadherent cells were removed. The adherent monocytes were cultured in serum-containing medium for 7 days and then treated in serum-free medium with M-CSF, TNF-α or Ox-LDL for 24 hours. Cells were washed with PBS, fixed in 4% paraformaldehyde, permeabilized with 0.05% Triton X-100, blocked with 5% normal goat serum, and then incubated with anti-MT3-MMP mAb (1:250 dilution) together with MMP-2 pAb (1: 500 dilution) or treated with PE-conjugated goat anti-mouse (1:500; CalTag Laboratories, Burlingame, Calif), Alexa 568-conjugated goat anti-rabbit (1:500; Molecular Probes, Eugene, Ore), and nuclear stain TOTO-3 (1:15,000; Molecular Probes). After wash, the cover slips were mounted in Immuno Fluore (ICN) and visualized using a Leica scanning confocal microscope (inverted) equipped with Argon (488 nm) and Krypton (568 nm) lasers. PE-stained MT3-MMP and Alexa 568 signal for MMP-2 were obtained by sequential scans. To establish possible colocalization of MT3-MMP (green channel) and MMP-2 (red channel), corresponding 2D sections from both channels were merged using Leica software, and viewed as maximum intensity projections as well as xz and yz projections of selected cellular regions.

Flow Cytometric Analysis

Human Mφ cultured in the absence or presence of Ox-LDL, TNF-α, or M-CSF were stained with anti-MT3-MMP antibody (0.5 µg diluted to a total volume of 50 µL), followed by staining with PE-conjugated goat anti-mouse IgG as described. The cells were analyzed by FACScan flow cytometer (Becton Dickinson).
Data Analysis
Intensities of experimental bands from RNA and protein blots were measured by computer-assisted densitometry. Results are expressed as mean±SEM. Statistical analyses were performed by Student’s t test to determine the significance of change in the measurements. A significant difference was considered for P≤0.05.

Results
Localization of MT3-MMP in Normal and Atherosclerotic Human Arteries
Sections of normal human coronary arteries showed MT3-MMP immunoreactivity that colocalized with MMP-2 staining within the medial SMC (Figure 1A, a through c). Antibodies used to identify MT3-MMP and MMP-2 in these sections did not distinguish between active and inactive enzymes. Human atherosclerotic plaques contained MT3-MMP proteins in the media underlying fibrous and lipid-rich regions (Figure 1A, d through f). Mouse nonspecific IgG used in place of primary antibody showed no background or nonspecific staining in atherosclerotic plaque (Figure 1A, g). Immunoreactive MT3-MMP proteins were abundantly detectable in macrophages of the atheromatous plaques (Figure 1A, h through l). Quantitative image analyses showed that the levels of MT3-MMP antigen staining were significantly (P<0.001; n=10/group) increased and colocalized with MΦ in lipid-rich atheromatous (27.7±7%) compared with predominantly fibrous (13.8±6.9%) plaques (Figure 1B).

Inflammatory Molecules Increase the Levels of MT3-MMP mRNA in Cultured Human MΦ
Expression of MT3-MMP mRNA was determined by Northern blotting of total RNA prepared from cultured MΦ, as well as RT-PCR (Figures 2 and 3). An RT-PCR product of the expected size (507 bp) was identified (Figure 3). The DNA sequence of the amplified fragment revealed a complete identity to the human MT3-MMP cDNA sequence and showed homologies to other published MT-MMP cDNA sequences. RNA blotting indicated that cultured MΦ express a major MT3-MMP mRNA species of 12 kb (Figure 2), a size similar to MT3-MMP mRNA observed in vascular SMCs. Exposure of cultured MΦ to increasing concentrations of Ox-LDL, TNF-α, or M-CSF resulted in a dose- and time-dependent accumulation of MT3-MMP mRNA. These inductive effects were first apparent within 2 to 4 hours of treatment and peaked 3- to 5-fold above control by 6 hours (Figures 2 and 3).

Activated MΦ Produce Increased Levels of MT3-MMP Protein
Immunoblots of plasma membrane extracts derived from MΦ that had been stimulated for 24 hours with Ox-LDL, TNF-α, or M-CSF exhibited increased levels of MT3-MMP protein compared with unstimulated MΦ (Figure 4A). The observed increases in MT3-MMP mRNA levels (Figure 2) correlated well with corresponding increments in MT3-MMP protein levels in MΦ stimulated with Ox-LDL (1.7-fold), TNF-α (3.5-fold), and M-CSF (3.4-fold) for 24 hours (Figure 4B).

Activated MΦ Exhibit Increased MT3-MMP Catalytic Activity
To determine whether increased levels of MT3-MMP protein corresponded with increased enzymatic activity, we per-
formed gelatin zymography on plasma membrane preparations of MΦ stimulated with Ox-LDL, TNF-α, or M-CSF. Incubation of medium conditioned by human SMCs that contained pro-MMP-2 with plasma membrane extracts from variously activated MΦ increased proteolytic conversion of 72-kDa pro-MMP-2 to new gelatinolytic bands of 70- and 68-kDa, corresponding to the processed, active MMP-2 (Figure 5A). We also examined conditioned medium obtained from cultured MΦ that were grown in presence or absence of Ox-LDL, TNF-α, or M-CSF, redistribution of both MT3-MMP and MMP-2 protein to focal cytoplasmic or plasma membrane regions was observed. Furthermore, colocalization of MT3-MMP and MMP-2 in discrete areas was revealed in the merged images (Figure 6). Flow cytometric studies showed an ~2.5-fold increase in the number of MT3-MMP-positive MΦ after 24 hours of treatment with as little as 100 ng/mL of M-CSF (Figure 7).

**Discussion**

We report here that MT3-MMP is expressed in human atherosclerotic plaque. Double immunostaining demonstrated that MT3-MMP colocalized with MΦ. Cell culture studies showed that MΦ express MT3-MMP mRNA and protein with enzymatic activity. To our knowledge, this is the first demonstration of MT3-MMP expression in MΦ. Inflammatory molecules (Ox-LDL, TNF-α, and M-CSF) significantly increased MT3-MMP expression in cultured human MΦ in a dose- and time-dependent manner. Confocal microscopy and flow cytometry confirmed induction and spatial redistribution of MT3-MMP protein and MMP-2 protein from intracellular domains to the MΦ plasma membrane by Ox-LDL, TNF-α,
or M-CSF. Within the context of the known ability of MT3-MMP to activate arterial extracellular matrix (ECM) degradation via a proteolytic cascade, our results suggest that MT3-MMP may impact plaque development and structural stability. Because inflammatory molecules increased MT3-MMP in Mφ, our findings identify a mechanism by which inflammatory activation of Mφ might directly affect the formation, development, and complications of atherosclerosis. We did not directly observe collagenolytic activity in vivo; however, we found that pro-MMP-2 was converted to active MMP-2 by plasma membrane extracts prepared from Mφ treated with Ox-LDL, TNF-α, or M-CSF in vitro. Our studies are therefore consistent with the suggestion that inflammatory molecules upregulate Mφ expression of MT3-MMP, which in turn might activate downstream matrix-degrading components such as MMP-2 and other MMP substrates of MT3-MMP and thereby contribute to structural weakening of the plaque.

Increased expression of MT3-MMP has been reported to occur in several pathological conditions involving tissue invasion or inflammation, including primary and metastatic tumor growth,3,16 infection,17 rheumatoid arthritis,3,18 and vascular SMCs after injury.19 Expression of MT3-MMP in SMCs increases after treatment with platelet-derived growth factor or fibronectin.19 Although our results suggest a general role for increased MT3-MMP expression in inflammation and

Figure 4. A, Immunoblotting of Mφ membrane extracts (50 μg) using MT3-MMP antibody from untreated cells (control) and cells treated with Ox-LDL (100 μg/mL), TNF-α (10 ng/mL), and M-CSF (250 ng/mL) for 24 hours. Blots are representative of 3 separate experiments. B, Histogram representing quantitative results obtained by densitometry of the MT3-MMP bands shown in panel A.

Figure 5. A, Increased proteolytic processing of 72-kDa pro-MMP-2 from media conditioned by unstimulated human SMCs by plasma membrane preparations of Mφ stimulated with Ox-LDL (100 μg/mL), TNF-α (10 ng/mL), and M-CSF (250 ng/mL) for 24 hours. Positions of molecular weight (MW) markers indicated in kDa. B, Gelatinolytic activity of MMP-2 and -9 in the culture medium conditioned by human Mφ grown in absence or presence of Ox-LDL (100 μg/mL), TNF-α (10 ng/mL), or M-CSF (250 ng/mL) for 24 hours. C, Control gel without gelatin after Coomassie Blue staining. Comparable protein bands including BSA used in the extraction buffer ensured equal loading under different conditions.
tissue invasion or destruction, the possibility that MT3-MMP induction is a result of these conditions rather than a cause cannot be excluded.

Our findings here support and extend previous studies showing that MT1-MMP is similarly expressed in human coronary artery plaques and is associated with Mϕ accumulation in the shoulder regions of plaque, and that its expression is augmented by inflammatory molecules.9,10 Taken together, these data are consistent with a growing body of evidence suggesting that dysregulation of ECM degradation and remodeling by proteases is a key feature of all phases of atherosclerosis and that Mϕ are an important source of these MMPs. Increased expression of several MMPs and MT-MMPs by Mϕ in plaque has been reported, and expression of these seems to be restricted to specific plaque regions, particularly in shoulder regions of plaque and areas bordering acellular lipid cores.20–23 Whatever the specific activation mechanism, once activated by MT-MMPs, soluble MMPs can then degrade components of the matrix in plaque areas spatially remote from the site of their activation. Some of the MMPs activated by MT3-MMP can then in turn activate other distinct MMPs with different substrate specificities.3,24 Directly or indirectly then, MT3-MMP has the ability to degrade virtually all components of the arterial ECM. The capacity of MT3-MMP to initiate a signal that can be amplified in a matrix-degrading cascade may have far-reaching consequences for plaque development, composition, and stability.

Figure 6. Confocal microscopy of serum-starved (-serum), M-CSF–treated, TNF-α–treated, or Ox-LDL–treated Mϕ. Optical sections from z-series corresponding to MT3-MMP (green), MMP-2 (red), and TOTO-3 stain for nuclei (blue) were visualized as maximal intensity projections. Signals from the green, red, and blue channels were merged in maximum intensity projections to visualize colocalization of MT3-MMP and MMP-2 (yellow to orange) due to induction and translocation from diffused distribution pattern in serum-starved controls (top) to discrete, cytoplasmic compartments or plasma membrane domains when treated with M-CSF (second panel from top), TNF-α (third panel from top), or Ox-LDL (bottom). Orthogonal slices with cutting planes indicated by the cross hair are displayed toward bottom (yz) or right side (xz) of each merged image (section) of untreated and treated cells shown in the extreme right column. Scale bar=5 μm in length.

Figure 7. A, Flow cytometric analysis of human Mϕ with mAb to human MT3-MMP. In these experiments, control staining was done with an irrelevant PE-conjugated IgG1. All analyses were performed with FACScan using PE-conjugated goat anti-mouse IgG. Plots represent distribution of arbitrary fluorescence units (on abscissas, on a logarithmic scale) vs cell number (on ordinates). B, Histogram indicates the positive events of the cell population. The data were presented as a percentage without M-CSF treatment and represent the mean±SEM for 3 separate experiments performed in duplicate. *P<0.05 (versus Mϕ without M-CSF treatment).
The topographical relationships between Mφ accumulation, augmented MMP and MT-MMP expression, and location of sites of predilection to cap erosion, tearing, or rupture have led to the notion that ECM degradation in the context of inflammation might be a general mechanism of plaque destabilization.25 Our findings here and previously9,10 identify and implicate MT-MMPs as novel and important mediators of plaque structure and stability; based on these findings, it is likely that MT-MMPs will have significant therapeutic implications to stabilize atherosclerotic plaque and thus minimize clinical events resulting from plaque destabilization.

Acknowledgments

This work was supported by grants from the National Heart, Lung, and Blood Institute (HL51980 and HL58555 to Dr Rajavashisth). The authors thank the Mirisch Foundation, United Hostesses Charities, the Eisner Foundation, the Grand Foundation, Ornstein Family Foundation, the Entertainment Industry Foundation, and the Heart Fund at Cedars-Sinai Medical Center, Los Angeles, Calif, for their generous support.

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_Circulation_. 2002;106:3024-3030; originally published online November 18, 2002; doi: 10.1161/01.CIR.0000041433.94868.12

_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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