Matrilysin is expressed by lipid-laden macrophages at sites of potential rupture in atherosclerotic lesions and localizes to areas of versican deposition, a proteoglycan substrate for the enzyme

(matilloelastase/metalloproteinase/chondroitin sulfate)

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Communicated by David M. Kipnis, Washington University School of Medicine, St. Louis, MO, June 7, 1996 (received for review December 1995)

ABSTRACT Certain matrix metalloproteinases (MMP) are expressed within the fibrous areas surrounding acellular lipid cores of atherosclerotic plaques, suggesting that these proteinases degrade matrix proteins within these areas and weaken the structural integrity of the lesion. We report that matrilysin and macrophage metalloelastase, two broad-acting MMPs, were expressed in human atherosclerotic lesions in carotid endarterectomy samples (n = 18) but were not expressed in normal arteries (n = 7). In situ hybridization and immunohistochemistry revealed prominent expression of matrilysin in cells confined to the border between acellular lipid cores and overlying fibrous areas, a distribution distinct from other MMPs found in similar lesions. Metalloelastase was expressed in these same border areas. Matrilysin was present in lipid-laden macrophages, identified by staining with anti-CD-68 antibody. Furthermore, endarterectomy tissue in organ culture released matrilysin. Staining for versican demonstrated that the vascular proteoglycan was present at sites of matrilysin expression. Biochemical studies showed that matrilysin degraded versican much more efficiently than other MMPs present in atherosclerotic lesions. Our findings suggest that matrilysin, specifically expressed in atherosclerotic lesions, could cleave structural proteoglycans and other matrix components, potentially leading to separation of caps and shoulders from lipid cores.

Coronary artery disease is the leading cause of mortality in developed countries. In more than 90% of affected patients, atherosclerosis is the underlying disease process, resulting from exuberant inflammation, smooth muscle cell proliferation, and fibrosis in response to vascular injury (1–3). Advanced atherosclerotic lesions are characterized by a lipid-filled, acellular core surrounded by a neointimal-derived cap on its luminal side and shoulders at its lateral extents (4). During lesion formation, the number of macrophages infiltrating into the intima increases along with the intracellular accumulation of lipid droplets in these cells. This influx of macrophages is associated with intimal disorganization and thickening, deformity of the arterial wall, and often severe complications, such as fissure, hematomata, and thrombosis (5). Most of the sudden deaths due to acute myocardial infarction are caused by rupture or fissure of lesions, particularly in the margins of the overlying fibrous cap, resulting in hemorrhage into the plaque, thrombosis, and rapid occlusion of the artery (6, 7). Because these vulnerable areas contain numerous macrophages (8), it is generally thought that the presence of these cells and their local release of matrix-degrading proteinases cause weakening, fissure, or rupture of the plaque structure (4, 9). Plaque rupture, however, does not result solely from a weakened cap. Hemodynamic stresses also contribute to plaque disruption.

Supporting this hypothesis are the observations that several members of the matrix metalloproteinase (MMP) family are produced by macrophages in human atherosclerotic plaques. Henney et al. (10), Gais et al. (11), Brown et al. (12), and Nikkari et al. (13) reported that stromelysin-1 (MMP-3), collagenase-1 (MMP-1), and 92-kDa gelatinase (MMP-9) are expressed by lipid-laden macrophages present throughout the fibrous areas surrounding lipid cores. Together, the proteolytic capacity of these MMPs is able to degrade many, if not all, extracellular components present within the fibrous areas of an atherosclerotic plaque. Collagenase-1 cleaves type I and III collagens in their triple helices, and 92-kDa gelatinase effectively and completely degrades collagenase-cleaved gelatin fragments. In addition, 92-kDa gelatinase and stromelysin-1 degrade basal lamina proteins, and stromelysin-1 is a potent proteoglycanase (14). Thus, by acting on numerous and diverse matrix proteins and because they are expressed within and throughout the fibrous areas of atherosclerotic lesions, these MMPs could contribute to plaque rupture by weakening the structural integrity of the overlying cap.

Human macrophages align within regions where plaques split, and other MMPs may cause damage in these areas that leads to rupture of the fibrous cap. This idea implies a proteolytic process that may be mechanistically distinct from the progressive destruction of fibrous components within the cap. In addition to the enzymes discussed above, macrophages produce matrilysin (MMP-7) and human metalloelastase (MMP-12) (15–17); these MMPs have a broad and potent capacity to degrade numerous matrix components, including proteoglycans, insoluble elastin, and fibronectin. In this report, we demonstrate that lipid-laden macrophages confined to the border between acellular lipid cores and fibrous areas prominently express these two MMPs, especially matrilysin. Furthermore, we show that matrilysin degrades versican, an abundant chondroitin sulfate proteoglycan in atherosclerotic lesions, suggesting that this structural matrix component is an important target of macrophage-mediated tissue destruction.

METHODS

Tissues. Specimens of carotid endarterectomies (n = 18) were obtained at surgery, and similar size, nonatherosclerotic arteries (n = 7) were obtained from donor kidney or liver at the time of transplant. Vessels were cut into two equal pieces; one piece was immediately frozen in liquid nitrogen for RNA

Abbreviation: MMP, metalloproteinase.

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isolation, and the other was fixed in 10% buffered formalin and processed for routine paraffin embedding. For organ culture, whole specimens were collected in serum-free DMEM containing penicillin and streptomycin. These studies were approved by the Barnes Hospital Human Rights Committee.

**RNA Isolation and Analysis.** Total RNA was isolated from frozen tissue using RNAzol-B (Test-Test, Friendswood, TX), and 10 μg was resolved through a formaldehyde-agarose gel, and then transferred and cross-linked to nylon membrane. An 800-bp human matrilysin cDNA, a 1800-bp human metalloelastase cDNA (17), and GAPDH cDNA were labeled with [32P]dCTP by random priming. Northern blot hybridization was done under stringent conditions as described (18).

**Immunohistochemistry.** An antibody raised against a synthetic peptide corresponding to amino acids 93–108 of human matrilysin and that specifically recognizes both the 29-kDa zymogen and the 19-kDa activated forms of matrilysin (15) was affinity purified as described (19). An antibody raised against a fusion protein corresponding to full-length versican was affinity purified using a versican peptide column (20). Deparaffinized 5-μm sections were processed for immunohistochemistry as described (21). Endogenous peroxidase activity was blocked by incubation in 0.3% H2O2 for 30 min at room temperature. Affinity-purified anti-matrilysin antibody was diluted 1:1000, and anti-versican antibody was diluted 1:400. Bound antibody was detected using a Vectastain ABC Elite kit (Vector Laboratories) following the manufacturer’s instructions and 3,3-diaminobenzidine tetrahydrochloride as the chromogenic substrate. Sections were counterstained with Harris hematoxylin. For negative controls, we processed sections with preimmune serum at a comparable dilution, or we coincubated affinity-purified antibodies with excess peptide antigen (5 μg/ml) to inhibit specific interactions with matrilysin in the tissue. Tissue macrophages were identified on serial sections using a monoclonal antibody (KP-1, Dako no. M814) that reacts with CD-68, a specific macrophage marker (22).

**In Situ Hybridization.** In vitro transcribed antisense and sense RNA probes were labeled with [α-35S]UTP as described (21). Sections were hybridized with 35S-labeled RNA probes (4 × 106 cpm/μl of hybridization buffer) and were washed under stringent conditions, including treatment with RNase-A (21, 23). After autoradiography for 14 to 21 days, the photographic emulsion was developed, and the slides were stained with hematoxylin and eosin.

**Organ Culture and Zymography.** Endarterectomy specimens were minced into 2 mm2 pieces, and 120 μg of tissue was placed into individual wells of a 24-well tissue culture dish containing 1.5 ml DMEM and antibiotics. After a 72-h incubation at 37°C and 5% CO2/95% air, conditioned medium was collected, and secreted matrilysin was isolated by immunoprecipitation of 0.5 ml of conditioned medium using a specific polyclonal antibody (15). Immunoprecipitated matrilysin was detected by substrate zymography after electrophoresis through a nondenaturing SDS/12% polyacrylamide gel comoyoanized with 1 mg/ml casein (24). Purified native matrilysin (about 50% in its proform and 50% in its activated form) was included as an internal control. SDS was neutralized by treatment with Triton X-100, and gels were incubated for 24 h at 37°C in 50 mM Tris (pH 8.0), 5 mM CaCl2, and 0.5 μM ZnCl2, and stained with Coomassie blue for 30 min.

**Versican Degradation Assay.** 35SO42- labeled versican was purified from monkey arterial smooth muscle cell conditioned medium by ion-exchange chromatography over DEAE cellulose and molecular sieve chromatography on Sepharose CL-2B (Pharmacia) as described (25). The final product was >98% pure as defined by sensitivity to chondroitin ABC lyase and had a specific activity of 3 × 108 dpm/μg chondroitin sulfate proteoglycan. The radiolabeled versican was extensively dialyzed against 50 mM Tris-HCl (pH 7.5), 10 mM CaCl2, and 0.15 M NaCl before incubation with MMPs. Highly purified human collagenase-1, stromelysin-1, and 92-kDa gelatinase, and recombinant human matrilysin were activated with trypsin or p-aminophenylmercuric acetate as described (26), and equimolar concentrations of these MMPs (see Fig. 5 legend) were incubated with 8000 dpm of versican for 1 h at 37°C. The concentration of activated MMP was determined by inhibitor titration (26). Reaction products were denatured by boiling in electrophoresis sample buffer and resolved through 3% stacking-5% separating SDS/polyacrylamide gels and visualized by autoradiography.

**RESULTS**

**Matrilysin and Metalloelastase Are Expressed in Atherosclerotic Lesions.** As demonstrated by Northern blot hybridization, matrilysin and macrophage metalloelastase mRNAs were detected in all endarterectomy specimens examined, although the level of expression varied among samples (Fig. 1). As discussed below, because only a subset of cells express matrilysin and metalloelastase in these lesions, a relatively weak and variable hybridization signal for their mRNAs would be predicted in an analysis of total RNA. No signal for matrilysin or metalloelastase transcripts was detected in RNA from normal human arteries (Fig. 1). These findings demonstrate that matrilysin and metalloelastase are selectively expressed in atherosclerotic lesions and are not produced in normal arteries. In addition, we typically detected a stronger signal for these mRNAs in samples with prominent lipid cores relative to the signals detected in specimens with less advanced plaques.

**Location of Matrilysin Expression.** We used in situ hybridization to determine the sites of matrilysin and metalloelastase expression in atherosclerotic lesions. In agreement with the Northern blot hybridization data, matrilysin mRNA-positive cells were detected in all samples examined. Many matrilysin mRNA-positive cells were seen in endarterectomy samples with large lipid cores (7 out of 18), whereas fewer matrilysin mRNA-positive cells were detected in specimens with small cores (11 out of 18). In all samples, however, signal for matrilysin mRNA was seen in and confined to cells along the border between cellular fibrous areas and acellular lipid cores (Fig. 2A, B, and E). Immunohistochemistry with an affinity-purified antibody confirmed that matrilysin protein was also present at and confined to sites of mRNA expression (Fig. 2G). Neither matrilysin mRNA nor protein was detected in the intact areas of the vessel (Fig. 2B) or elsewhere in the lesion, particularly within the body of the fibrous cap where other MMPs are expressed (10, 11).

**Localization of Metalloelastase and TIMP-1.** Signal for metalloelastase mRNA was also seen in cells along the border between cores and cellular compartments, but the expression of this MMP was typically confined to only a few cells (Fig. 2H).

![Fig. 1.](image-url)
(Fig. 2. Legend appears on the opposite page.)
(The relatively stronger signal for metalloelastase mRNA in the Northern blots is due both to the larger size of the cDNA probe and to longer autoradiographic exposure.) As reported by others (11), TIMP-1 mRNA is expressed in atherosclerotic lesions, but only by cells that were some distance from those expressing matrilysin (compare Fig. 2 D with B).

**Macrophages Express Matrilysin.** As assessed by *in situ* hybridization and immunostaining of serial sections, cells that expressed matrilysin also stained with anti-CD-68, thus demonstrating that this MMP is produced by macrophages in atherosclerotic plaques (Fig. 3). Because these CD-68-positive cells have an overtly vacuolated cytoplasm (Fig. 3C), we conclude that matrilysin is expressed by lipid-laden macrophages. No signal was seen in samples incubated with sense probes or in those processed with preimmune antibody or with excess antigenic peptide (data not shown).

**Secretion of Matrilysin.** To determine if macrophages actively release matrilysin in atherosclerotic plaques, we incubated equivalent amounts of freshly obtained endarterectomy specimens in serum-free medium, isolated secreted protein by immunoprecipitation, and detected it by casein zymography. Consistent with our *in situ* hybridization and immunohistochemistry findings, no matrilysin protein was detected in medium conditioned by cultured samples of normal artery (Fig. 4). Caseinolytic activity migrating at the same mass as recombinant promatrilysin was detected in all atherosclerosis specimens examined (Fig. 4), and in some specimens we detected evidence of activated matrilysin. Although the zymographic signal is somewhat low, this weak signal is predictable for two reasons. (i) Matrilysin is expressed only by a subset of macrophages (see Figs. 2 and 3), and, although the concentration of this proteinase may be high at sites of secretion within the tissue, the amount released into the medium would be relatively low. (ii) Because activated MMPs bind their matrix substrates with high affinity and do not readily diffuse from tissue, the matrilysin activity detected likely reflects the excessive amount of this MMP released from macrophages within the tissue samples. In addition, we immunoprecipitated matrilysin from only one-third of the culture volume.

**Matrilysin Degrades Versican.** To determine which matrix components are present at sites of matrilysin expression, we stained endarterectomy sections with Pentachrome to identify areas of elastin, collagen, proteoglycan, and fibrin deposition. Green-to-pale green staining, indicative of proteoglycan, was consistently seen in the areas where matrilysin-positive macrophages were detected (Fig. 2C). Immunostaining for versican, an abundant component of atherosclerotic lesions, confirmed that this chondroitin sulfate proteoglycan was present in these same areas (Fig. 2F). In fact, staining for versican was evident throughout the cellular areas of atherosclerotic plaques.

The close spatial correlation of matrilysin expression and versican in areas potentially vulnerable to rupture suggest that plaque destabilization may result upon degradation of versican by matrilysin. Because other MMPs, namely 92-kDa gelatinase, stromelysin-1, and collagenase-1, are expressed at sites of potential damage in atherosclerotic plaques, we compared the ability of these MMPs to matrilysin to degrade purified versican. Because of variations in the number and length of the chondroitin sulfate side chains, intact versican has an approximate Mr = 1.2 x 10^6, and, thus, it resolves as a broadband within the 3% polyacrylamide stacking gel (Fig. 5) (25). With digestion by matrilysin, radiolabeled versican migrated much faster and was detected as two bands that entered the resolving gel (Fig. 5). Furthermore, matrilysin degraded versican much more effectively than equimolar concentrations of activated 92-kDa gelatinase, stromelysin-1, or collagenase-1, which had little to no effect on the substrate. In fact, partial degradation of versican was detected in samples incubated with 0.048 x 10^-7 M or 0.24 x 10^-7 M matrilysin. Comparable degradation was seen in samples incubated with 6.0 x 10^-7 M collagenase-1 or stromelysin-1, indicating that matrilysin is at least 25-fold more effective on a molar basis at degrading versican than these other MMPs.

**Fig. 2.** Location of matrilysin and metalloelastase expression in atherosclerotic plaques. Carotid endarterectomy specimens were obtained during surgery, and fixed and processed for paraffin embedding. (A) *In situ* hybridization using an 35S-labeled antisense RNA probe showed prominent signal for matrilysin mRNA in cells (small arrows) at the border of the fibrous cap and the acellular core of an atherosclerotic plaque. Signal was not detected elsewhere in the vessel wall. (B) Higher magnification view of the shoulder area at the large arrow in A. (C) Pentachrome staining demonstrated that the fibrous cap is rich in proteoglycans (p), which stain pale green. Collagen (c, yellow) and elastin (e, black) are present in the intact fibrous lesion. (D) *In situ* hybridization for TIMP-1 mRNA revealed that this MMP inhibitor is expressed by cells (arrows) away from those expressing matrilysin. (E) In another lesion, matrilysin-positive cells (arrows) are confined to the border between a cellular area to the right and an acellular, area to the left. (F) Immunostaining for versican reveals an abundance of deposited proteoglycan in the cellular areas surrounding lipid cores. Arrows mark the perimeter where matrilysin-positive cells were seen. (G) Immunostaining for matrilysin demonstrated colocalization of protein and mRNA. (H) *In situ* hybridization for HME mRNA revealed that this MMP is also produced along this border. Autoradiographic exposure for all *in situ* hybridization results shown was 14 days. (A, E, and G, bar = 240 μm; B–D and F, bar = 48 μm; H, bar = 24 μm.)
with determined resolved nase, of participate cap degrades thoroughly matrix. Principal proteinases MMPs. Purified was I participated expression collagenase-1, stromelysin-1, and 92-kDa gelatinase was detected in conditioned medium and detected by casein zymography. Native human matrilysin (about 50% in its proform and 50% in its activated form) was included as an internal size marker. The proform migrates at 28 kDa, and the activated form migrates at 19 kDa. No caseinolytic activity was detected in medium conditioned by normal arteries, whereas activity migrating at 28 kDa and a minor band at about 19 kDa was seen in medium conditioned by endarterectomy specimens.

**DISCUSSION**

The findings we present here, along with earlier reports by others (10–13), suggest a role for macrophage-derived MMPs in initiating rupture of susceptible atherosclerotic lesions. Based on the location of where the various MMPs are expressed in vascular lesions, we propose that the different proteinases contribute to distinct tissue remodeling events. Stromelysin-1, 92-kDa gelatinase, and collagenase-1 are primarily expressed by macrophages within the fibrous caps and shoulders (10, 11). Because these cellular regions are rich in type I and III collagens, the activity of collagenase-1, the principal human proteinase that can cleave native fibrillar collagens, would be needed to initiate breakdown of this matrix. Stromelysin-1, which can lyse the globular ends of fibrillar collagens, and 92-kDa gelatinase, which potently and thoroughly degrades collagenase-cleaved collagen, could participate in the further breakdown of the collagen-rich matrix of the cap and shoulder regions. In addition, stromelysin-1 and 92-kDa gelatinase have the combined or individual ability to degrade insoluble elastin, basal lamina proteins, nonfibrillar collagens, and proteoglycan core proteins (27). Thus, the coexpression of collagenase-1, stromelysin-1, and 92-kDa gelatinase provides infiltrating macrophages with the enzymatic activity to degrade essentially all extracellular matrix components of the fibrous areas of atherosclerotic lesions. As suggested by Libby (4), sustained proteolysis by these MMPs, coupled with altered matrix production, could weaken the integrity of the cellular regions surrounding the lipid core, thereby creating an area vulnerable to plaque rupture.

In contrast to the diffuse expression of the MMPs discussed above within the fibrous regions, we found that matrilysin and macrophage metalloelastase were prominently expressed at sites susceptible to rupture, particularly where cellular and acellular areas are juxtaposed. Thus, the proteolytic activity of matrilysin, and possibly that of metalloelastase, may cause excision of the cap by degrading structural macromolecules between the lipid core and cellular fibrous regions. Plaque rupture is generally assumed to represent the proximate event leading to local and potentially lethal thrombus formation in at least 50% of cases of acute infarction (1). Once started, excision of the neointimal areas surrounding the plaque may progress relatively rapidly because separation of the cap from the lipid core would require degradation of matrix components only at the junction of the two structures. In contrast, damage caused by other MMPs within the cap and shoulder regions may occur over extended periods leading to less catastrophic lesion fissuring.

We also demonstrated that matrilysin effectively degrades versican much more so than other MMPs, namely 92-kDa gelatinase, collagenase-1, and stromelysin-1, produced in atherosclerotic lesions. Versican, a high molecular weight chondroitin sulfate proteoglycan, is a component of normal blood vessels (28) but is particularly abundant in atherosclerotic lesions (T.N.W., unpublished observations). Thus, cleavage of this structural proteoglycan at the periphery of the lipid cores could conceivably lead to cap destabilization and rupture. The ability of matrilysin to degrade versican is consistent with its strong activity against the core protein of aggrecan, a cartilage-specific chondroitin sulfate proteoglycan (14, 29). By analogy to its action on aggrecan, it is likely that matrilysin cleaves the core protein of versican as the enzyme possesses no chondroitinase activity.

Versican is likely not the only matrix component that maintains the structural integrity of plaques at the junction of the lipid core and cellular fibrous regions. This possibility, however, does not diminish the potential importance of matrilysin in mediating significant tissue damage in atherosclerotic plaques. Matrilysin, the smallest of the known MMPs, lacks the C-terminal hemopexin-like domain that is common to all other MMPs and that directs and restricts substrate specificity (30). Consequently, matrilysin possesses a very broad substrate specificity and efficiently degrades many extracellular matrix components, with the notable exception of helical collagens. In addition to proteoglycans, elastin, laminin, fibronectin, entactin, and basement membranes are all highly susceptible substrates (14, 26). Matrilysin expression is limited in vivo to selected cell types, namely monocytes and macrophages (15) and essentially all glanular epithelia (19, 31). Furthermore, because the C-terminal domain of MMPs participates in the binding of TIMPs, the major physiologic inhibitors of MMPs, the lack of this region makes matrilysin substantially less susceptible to inhibition by these tissue-based inhibitors (32). Because matrilysin degrades a wide variety of matrix components and is somewhat constrained by TIMPs, it would be able to cleave many matrix constituents present at sites of plaque fissure with little impedence from natural inhibitors. Metalloelastase is also a potent, broad spectrum MMP, degrading several matrix substrates including elastin, laminin, fibronectin, type IV collagen,
and basement membranes (S.D.S., unpublished observations). Thus, together these two MMPs would be able to cause extensive and potentially harmful tissue destruction at the cellular border of lipid cores.

Our observations also indicate that matrilysin and metalloelastase are expressed by a subpopulation of lipid-laden macrophages distinct from those cells that express 92-kDa gelatinase, stromelysin-1, and collagenase-1. As discussed, others have reported that these MMPs are produced by macrophages within the fibrous regions of ath erosclerotic plaques, whereas we report here that matrilysin and metalloelastase are produced only by foam cells that reside along the perimeter of lipid cores. Our in situ hybridization results on the same specimens used here confirmed that these other MMPs (92-kDa gelatinase, stromelysin-1, and collagenase-1) are expressed diffusely in fibrous regions of atherosclerotic lesions (data not shown) where matrilysin and metalloelastase were not seen.

The distinct localization of these enzymes within atherosclerotic lesions indicates that the expression of matrilysin and metalloelastase in macrophages may be controlled separately from other MMPs. Indeed, this concept has been demonstrated by comparing matrilysin expression to that of other MMPs in differentiating monocytes (15). The selective expression of matrilysin in certain macrophages may be controlled by soluble factors. In other cell types, matrilysin production is stimulated by tumor necrosis factor α, interleukin 1, and epidermal growth factor (33, 34), but these agents are potent inducers of other MMPs as well. In mononuclear phagocytes, matrilysin expression is potently induced by lipopolysaccharide and phagocytized material and is coordinately inhibited with collagenase-1 and 92-kDa gelatinase by the lymphokines interleukin 4, interleukin 10, and interferon-γ (16, 35), and hence, these effectors may not be likely candidates that regulate the site-specific expression of matrilysin in atherosclerotic plaques.

Although cytokines may potentially influence matrilysin expression in atherosclerotic plaques, other events, particularly cell-matrix interactions and cholesterol uptake by macrophages during foam cell formation, may be relevant mechanisms that control MMP expression in vascular tissue. Contact with specific extracellular matrix components potently induces MMP expression in many cell types, and an emerging paradigm suggests that the substrate regulates production of the proteinase. For example, contact with native type I collagen induces collagenase-1 expression in macrophages (36), keratinocytes (21), and fibroblasts (37), and laminin-1 stimulates 72-kDa gelatinase in metastatic cells (38). These factors are likely to modulate the participation, or with associated matrix components, may regulate production of this MMP in macrophages. Interestingly, these factors bind to proteoglycan-hyaluronan complexes via the receptor CD44; this interaction potently induces expression of cytokines and other genes (39) and may modulate the production of additional bioactive molecules, such as matrilysin.

In addition, Rouis et al. (40) showed that cultured human macrophages fed acetylated LDL markedly up-regulate both cell-associated and soluble elastase activity. These data conform nicely to our observations that lipid-laden macrophages at sites of potential plaque rupture express matrilysin and metalloelastase. Thus, we speculate that the matrix composition of the local tissue environment and proximity to the lipid core influences neo-intimal macrophages to express matrilysin. However, additional studies are needed to identify the in vivo mechanisms controlling MMPs expression in vascular disease.

We thank Drs. Gregorio Sicard and Robert Thompson for their assistance in obtaining endarterectomy specimens; Dr. Lynn Matrisian (Vanderbilt University, Nashville) for the human matrilysin cDNA; Dr. Richard LeBaron (University of Texas, San Antonio) for the versican antibody; Drs. Michael Shipley and Robert M. Senior for recombinant matrilysin; and Catherine Fliszar, Margaret E. Kolodziej, Theresa Tolley, Stephanie Lara, Clement Chung, and Ronald Mc-