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### Research Article

Dysregulated extracellular matrix (ECM) metabolism may contribute to vascular remodeling during the development and complication of human atherosclerotic lesions. We investigated the expression of matrix metalloproteinases (MMPs), a family of enzymes that degrade ECM components in human atherosclerotic plaques (n = 30) and in uninvolved arterial specimens (n = 11). We studied members of all three MMP classes (interstitial collagenase, MMP-1; gelatinases, MMP-2 and MMP-9; and stromelysin, MMP-3) and their endogenous inhibitors (TIMPs 1 and 2) by immunocytochemistry, zymography, and immunoprecipitation. Normal arteries stained uniformly for 72-kD gelatinase and TIMPs. In contrast, plaques' shoulders and regions of foam cell accumulation displayed locally increased expression of 92-kD gelatinase, stromelysin, and interstitial collagenase. However, the mere presence of MMP does not establish their catalytic capacity, as the zymogens lack activity, and TIMPs may block activated MMPs. All plaque extracts contained activated forms of gelatinases determined zymographically and by degradation of 3H-collagen type IV. To test directly whether atheromata actually contain active matrix-degrading enzymes in situ, we devised a method which allows the detection and microscopic localization of MMP enzymatic activity directly in tissue sections. In situ zymography revealed gelatinolytic and caseinolytic activity in frozen sections of atherosclerotic but not of uninvolved arterial tissues. The MMP inhibitors, EDTA and 1,10-phenanthroline, as well as recombinant TIMP-1, reduced these activities which colocalized with regions of [...]

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# Increased Expression of Matrix Metalloproteinases and Matrix Degrading Activity in Vulnerable Regions of Human Atherosclerotic Plaques

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## Abstract

Dysregulated extracellular matrix (ECM) metabolism may contribute to vascular remodeling during the development and complication of human atherosclerotic lesions. We investigated the expression of matrix metalloproteinases (MMPs), a family of enzymes that degrade ECM components in human atherosclerotic plaques ( $n=30$ ) and in uninvolved arterial specimens ( $n=11$ ). We studied members of all three MMP classes (interstitial collagenase, MMP-1; gelatinases, MMP-2 and MMP-9; and stromelysin, MMP-3) and their endogenous inhibitors (TIMPs 1 and 2) by immunocytochemistry, zymography, and immunoprecipitation. Normal arteries stained uniformly for 72-kD gelatinase and TIMPs. In contrast, plaques' shoulders and regions of foam cell accumulation displayed locally increased expression of 92-kD gelatinase, stromelysin, and interstitial collagenase. However, the mere presence of MMP does not establish their catalytic capacity, as the zymogens lack activity, and TIMPs may block activated MMPs. All plaque extracts contained activated forms of gelatinases determined zymographically and by degradation of  $^3\text{H}$ -collagen type IV. To test directly whether atheromata actually contain active matrix-degrading enzymes in situ, we devised a method which allows the detection and microscopic localization of MMP enzymatic activity directly in tissue sections. In situ zymography revealed gelatinolytic and caseinolytic activity in frozen sections of atherosclerotic but not of uninvolved arterial tissues. The MMP inhibitors, EDTA and 1,10-phenanthroline, as well as recombinant TIMP-1, reduced these activities which colocalized with regions of increased immunoreactive MMP expression, i.e., the shoulders, core, and microvasculature of the plaques. Focal overexpression of activated MMP may promote destabilization and complication of atherosclerotic plaques and provide novel targets for therapeutic intervention. (*J. Clin. Invest.* 1994. 94:2493-2503.) Key words:

vascular remodeling • atherosclerosis • plaque rupture • collagenase • in situ zymography

## Introduction

Remodeling of the arterial extracellular matrix (ECM)<sup>1</sup> occurs during all phases of human atherosclerosis, including acute disruptions of the plaque that commonly cause acute myocardial infarction. Lesion development involves migration of leukocytes and smooth muscle cells (SMCs), accumulation of ECM components, and creation of new capillary beds. Compensatory enlargement of arteries during plaque evolution involves major changes in vessel wall architecture (1, 2). At later stages, as growth of the atherosclerotic plaque outstrips this compensatory ectasia, the lesion causes arterial stenosis, and rupture and thrombosis may occur. Each of these important steps in plaque evolution involves modification of the ECM, a process regulated at the levels of both synthesis and degradation of matrix components. We and others (3-6) have previously reported that human vascular cells can produce in vitro members of the matrix metalloproteinase (MMP) family, enzymes that may play crucial roles in vascular remodeling during development, growth, and pathology (7). After proteolytic activation of their secreted zymogen forms (8), MMPs can degrade various ECM components at physiological pH. Henney et al. (9) detected mRNA encoding stromelysin in atheroma. We investigated here in normal and atherosclerotic human arteries the expression and state of activation of members of all three known MMP families: gelatinases specialized in the digestion of collagen fragments, basement membrane-type collagens, or elastin; interstitial collagenase (MMP-1) which digests fibrillar collagens; and stromelysin (MMP-3), which degrades proteoglycans, fibronectin, laminin, elastin, and can activate the zymogen forms of the other MMPs as well (10). We also evaluated the expression in these arterial specimens of the endogenous inhibitors of MMPs, the tissue inhibitors of metalloproteinases (TIMPs) 1 and 2.

## Methods

### Reagents

Cells were identified using monoclonal anti-muscle actin HHH-35 (Enzo Diagnostics, Syosset, NY); monoclonal anti-human macrophage HAM-56; monoclonal anti-human leukocyte common antigen (LCA); mono-

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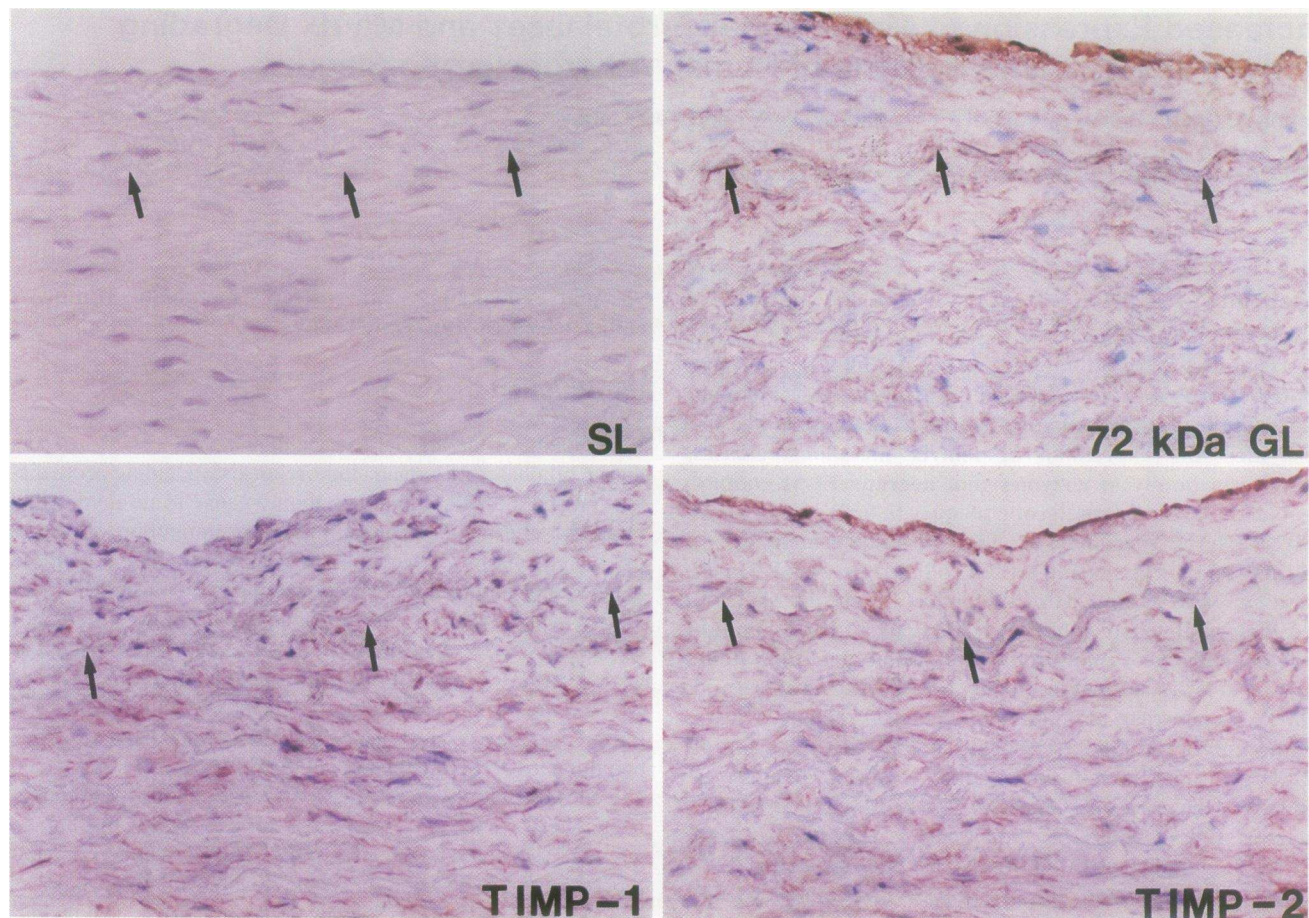
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1. Abbreviations used in this paper: CL, collagenase; GL, gelatinase; ECM, extracellular matrix; MMP-1, interstitial collagenase; MMP-2, the 72-kD gelatinase; MMP-3, stromelysin; MMP-9, the 92-kD gelatinase; MMP, matrix metalloproteinase; o.m., original magnification; pro-MMP-2, zymogen form of the 72-kD gelatinase; pro-MMP-9, zymogen form of the 92-kD gelatinase; SL, stromelysin; SMC, smooth muscle cell; TIMP, tissue inhibitor of MMPs.



**Figure 1.** Non-atherosclerotic human coronary arteries contain immunoreactive 72-kD GL and TIMPs. Smooth muscle cells in both intima and media contain immunoreactive 72-kD GL, TIMP-1, and TIMP-2. Arrows point to the internal elastic lamina. Staining for SL in non-atherosclerotic arteries is weak or absent. Original magnification (o.m.) X400. These results are typical of five specimens studied.

clonal anti-human T cell CD3; monoclonal anti-human von Willebrand Factor (vWF) (Dako Corporation, Carpinteria, CA). Rabbit polyclonal antibodies against recombinant human collagenase (MMP-1), stromelysin (MMP-3), and TIMP-1 were obtained as previously described (11). Monoclonal anti-72-kD-gelatinase (hybridoma CA 719E3C) was purchased from Molecular Oncology (Gaithersburg, MD). Rabbit polyclonal antisera against 72-kD gelatinase (MMP-2) and against TIMP-2 purified from human melanoma cells (12, 13) were generously provided by Dr. William Stetler-Stevenson (National Institutes of Health, Bethesda, MD). Anti-92-kD-gelatinase polyclonal antibodies were generously provided by Dr. Howard Welgus (Washington University, St. Louis, MO) and monoclonal anti-92-kD-gelatinase antibodies (14) by Dr. Deborah French (State University of NY at Stony Brook, NY).

#### Human tissue

Atherosclerotic specimens ( $n=30$ ) and non-atherosclerotic arteries ( $n=11$ ) were obtained at surgery or autopsy. Surgical specimens included excess human arterial tissue from transplant donors and endarterectomy specimens. These tissues were studied by both histological and biochemical methods.

**Tissue sections.** Sections were cut from both tissue embedded in O.C.T. (Miles, Elkhart, IN) and frozen in 2-methylbutane cooled with liquid nitrogen, and from tissue fixed in 10% formaldehyde and embedded in paraffin.

**Arterial extracts.** Tissue was stored at  $-70^{\circ}\text{C}$  until extraction. Specimens of atherosclerotic plaques ( $n=16$ ) or non-atherosclerotic arteries ( $n=9$ ) were weighed, minced with scissors, and extracted using

chilled glass homogenizers in ice-cold 10 mM Na phosphate, pH 7.2, containing 150 mM Na chloride (PBS), and 1 % Triton X-100, 0.1% SDS, 0.5% Na deoxycholate and 0.2% Na azide (PBSTS).

#### Immunoprecipitation

Rabbit polyclonal antibodies raised against the 72-kD gelatinase were first incubated with protein A-Sepharose (Sigma Chemical Co., St. Louis, MO) at room temperature for 1 h. The excess anti-serum was washed off and the tissue extracts were added to the primary antibodies bound to protein A-Sepharose. To immunoprecipitate the 92-kD gelatinase, the monoclonal antibody was added to protein A-Sepharose at the same time as the tissue extracts. After 2 h of incubation the Sepharose was washed with PBSTS, the immunoprecipitates were eluted by heating at  $65^{\circ}\text{C}$  in nonreducing SDS-PAGE sample buffer and loaded on gelatin-containing gels.

#### SDS-PAGE zymography

SDS polyacrylamide gels containing gelatin or casein (1 mg/ml) were used to identify proteins with gelatinolytic or caseinolytic activity (4) extracted from the tissue. This method detects both activated and zymogen forms of MMPs. In the presence of SDS otherwise inactive forms can lyse the substrate contained in the gel due to detergent-induced conformational change. After electrophoresis, gels were incubated in 2.5% Triton X-100 (2 x 15 min), then overnight at  $37^{\circ}\text{C}$  in 50 mM Tris-HCl, pH 7.4, containing 10 mM Ca chloride and 0.05% Brij 35 (Sigma Chemical Co.). Identical gels were incubated in the above buffer containing either 20 mM EDTA, an inhibitor of MMPs, or 1 mM PMSF,

Table I. Semiquantitative Analysis of MMPs and their Endogenous Inhibitors Detected by Immunocytochemistry in Nonatherosclerotic Arteries (N, n = 11) and in Atherosclerotic Arteries (A, n = 30): Interstitial CL, 72-kD GL, SL, 92-kD GL, TIMP-1 and TIMP-2

		Intimal SMCs	Medial SMCs	Luminal EC	Microvasc EC	Macrophages
CL (MMP-1)	N (n = 5)	0.80±0.40	0.80±0.40	0.00±0.00	N.P.	N.P.
	A (n = 15)	1.93±0.25	1.60±0.49	1.33±0.60	1.71±0.45	1.87±0.34
72 kDa GL (MMP-2)	N (n = 4)	1.50±0.50	1.50±0.50	0.50±0.87	N.P.	N.P.
	A (n = 16)	1.94±0.24	1.25±0.43	0.87±0.34	1.00±0.00	1.69±0.46
SL (MMP-3)	N (n = 5)	0.80±0.40	0.60±0.44	0.20±0.40	N.P.	N.P.
	A (n = 14)	2.00±0.00	1.25±0.43	0.75±0.43	1.67±0.37	1.69±0.46
92 kDa GL (MMP-9)	N (n = 5)	0.80±0.40	0.60±0.80	0.20±0.40	N.P.	N.P.
	A (n = 14)	1.86±0.35	1.75±0.43	1.14±0.64	1.20±0.75	1.79±0.41
TIMP-1	N (n = 5)	1.60±0.49	1.60±0.49	0.25±0.43	N.P.	N.P.
	A (n = 15)	1.87±0.34	2.00±0.0	0.93±0.46	1.00±0.00	1.93±0.25
TIMP-2	N (n = 4)	1.75±0.43	1.75±0.43	2.00±0.00	N.P.	N.P.
	A (n = 17)	1.76±0.42	2.00±0.00	1.44±0.61	1.73±0.45	1.64±0.48

Staining was evaluated in SMCs, luminal endothelial cells and in microvascular endothelium of the plaques (microvasc EC), and in macrophages. Consistent positive staining was recorded as (2), variable or weak staining (1), no staining as (0). N.P. = cells not typically present in the specimens. Results are reported as mean ± standard deviation of the average score. Variation of the score assigned by two independent observers was in the range of standard deviation. These data are not continuously distributed and are intended to convey the degree of consistency of the results obtained by immunocytochemistry in the entire sample, rather than to give a quantitative representation (i.e., in non-atherosclerotic tissue a score of 1.6 for TIMP-1 vs. 0.8 for stromelysin does not imply twice as much TIMP-1 protein).

an inhibitor of serine proteases. Gels were then fixed and stained with Colloidal Brilliant Blue G (Sigma Chemical Co.).

### Immunocytochemistry

Staining was performed on frozen sections after fixation in cold acetone (−20°C) or on paraffin sections after deparaffinization with xylene and ethanol, using the same panel of antibodies and the same immunostaining protocol. The specificity of the anti-MMP and anti-TIMP antibodies used for immunocytochemistry was previously confirmed in our laboratory by immunoprecipitation and Western blotting of respective antigens secreted in vitro by cultured human vascular smooth muscle cells (6). Endogenous peroxidase activity was reduced by preincubation of sections with 0.3% hydrogen peroxide in Dulbecco's PBS. The sections were then incubated with primary antibodies, diluted in PBS supplemented with 10% horse serum (HyClone, Logan, UT), at room temperature for 60 min. After washing in PBS and then in 100 mM Tris-HCl, 150 mM Na chloride containing 2% horse serum, species-appropriate biotinylated secondary antibodies were applied followed by avidin-peroxidase complexes (Vector Laboratories, Burlingame, CA) and the reaction was visualized with 3-amino-9-ethyl carbazole as substrate (AEC; Sigma Chemical Co.). In the case of double immunolabeling, the specimens were treated for 15 min with an avidin/biotin blocking kit (Vector Laboratories) after the first staining, then processed for detection of the second antigen using streptavidin coupled to alkaline phosphatase. This second reaction was developed using a Fast Blue substrate. Sections were counterstained with Gill's Hematoxylin (Sigma Diagnostics). Omission of primary antibodies and staining with type- and class-matched irrelevant immunoglobulin served as negative controls.

### Detection of enzymatic activity in vitro

**Enzymatic activity in tissue extracts.** Type IV collagenase activity in human arterial tissue extracts was investigated using human <sup>3</sup>H-collagen type IV (DuPont-NEN, Boston, MA) as a substrate (10), according to the protocol suggested by the manufacturer. Radiolabeled collagen (0.7 μg, specific activity 1.4 μCi/μg) was added over tissue extracts of non-

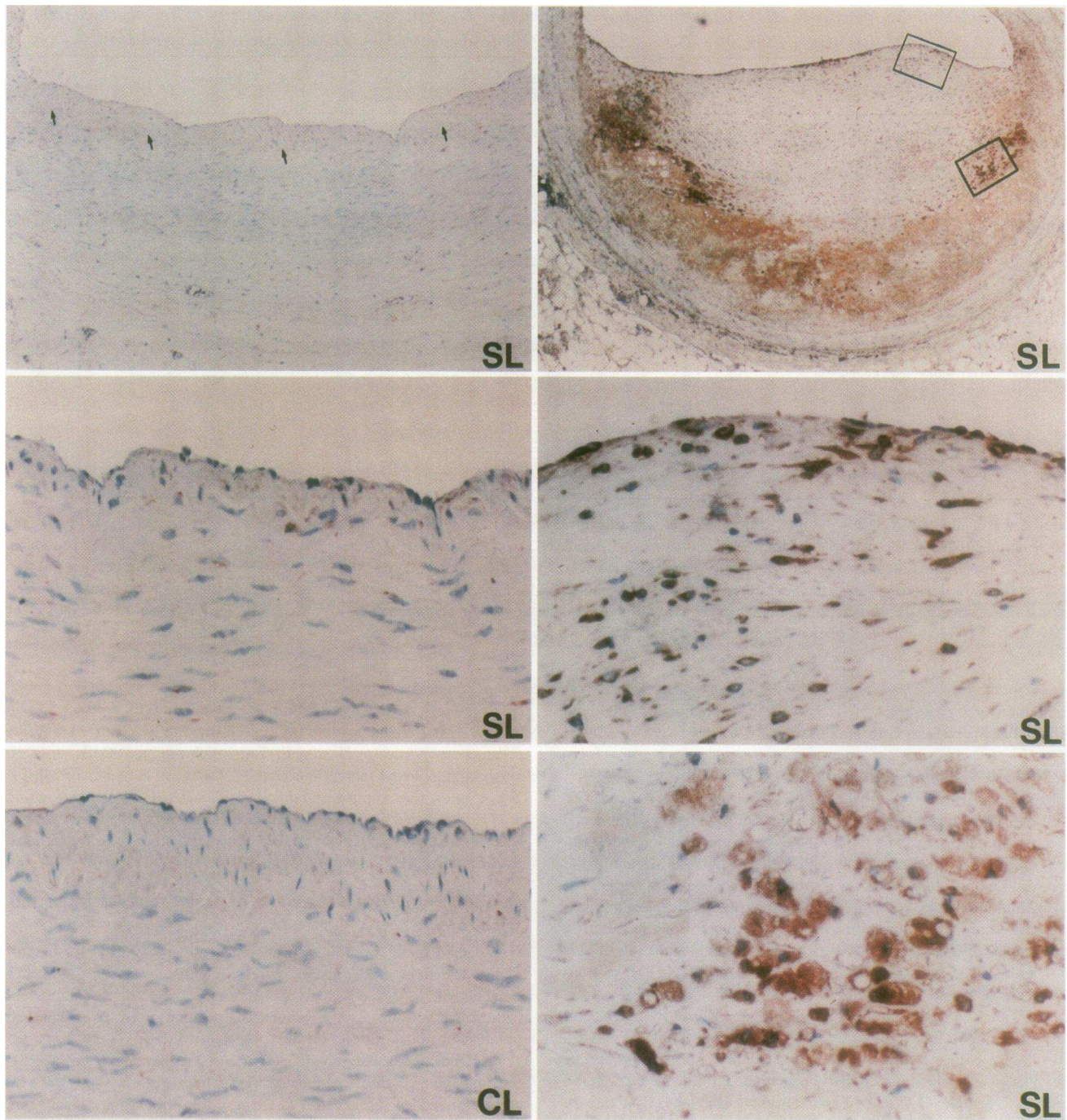
atherosclerotic or atherosclerotic tissue containing 50 μg total protein in PBSTs buffer. Incubations were performed at 37°C for 24 h in the presence or absence of 20 mM EDTA, which inhibits MMPs, or of 10 mM *p*-aminophenyl mercuric acetate (APMA), an activator of MMPs. The reaction was stopped by boiling in SDS-PAGE sample buffer and products analyzed by SDS-PAGE. Degradation of radiolabeled collagen was detected by fluorography of dried polyacrylamide gels impregnated with EN<sup>3</sup>HANCE (DuPont-NEN).

### Detection of enzymatic activity in tissue sections by in situ zymography

We adapted for this purpose an approach previously used for macroscopic detection of collagenase activity released by tissue slices (15). Detection of MMP activity in thin tissue sections required substrates with sensitivity and resolution suitable for microscopy. We identified suitable substrates for two different types of MMPs.

(a) Detection of caseinolytic activity used casein coupled to fluorescent molecules. Frozen sections were applied to glass slides covered with a film made from 1 mg/ml casein-resorufin (Boehringer Mannheim, Indianapolis, IN) or casein-FITC (Sigma Chemical Co.) mixed (1:1) with 1% agarose melted in 50 mM Tris-HCl, pH 7.4, containing 10 mM Ca chloride and 0.05% Brij 35. After incubation of tissue specimens (vide infra), examination by epifluorescence permitted detection of areas of caseinolytic activity as zones lacking fluorescence, and therefore appearing as black holes in the fluorescent substrate films.

(b) To detect gelatinolytic activity, glass slides holding frozen tissue sections were dipped into NTB 2 photographic emulsion (Eastman Kodak Co., Rochester, NY), normally used for autoradiography and whose main component is gelatin. Slides were incubated in both cases into humidified chambers for various lengths of time. Subsequently, glass slides covered with emulsion were dried, processed by photographic development, and examined with a Nikon Optiphot-2 microscope in transmitted light. The background of these specimens is black due to exposure of emulsion to ambient light during processing, while lysis of gelatin in the emulsion produces transparent spots on the slides.

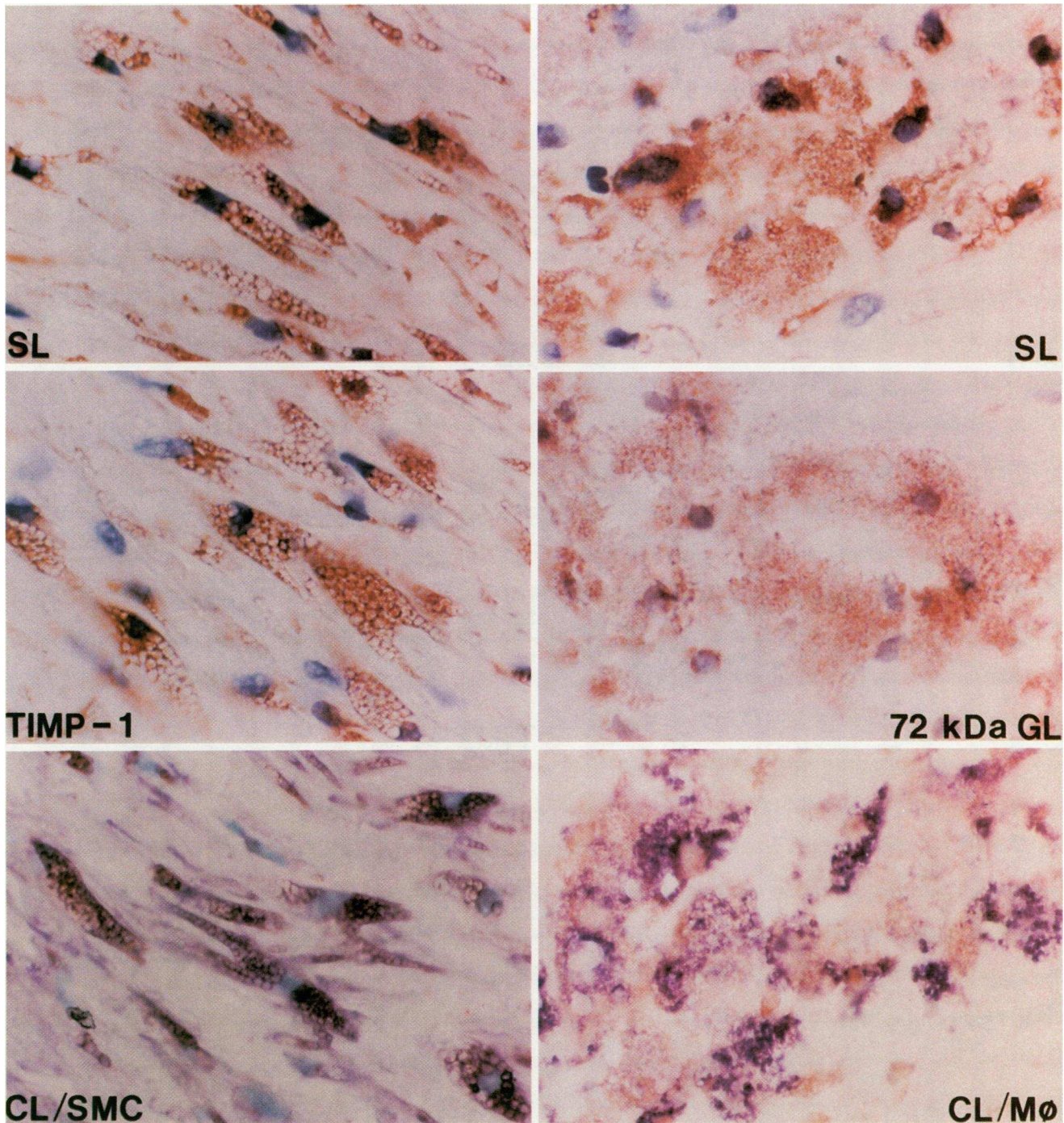


**Figure 2.** Increased regional expression of immunoreactive MMPs in atherosclerotic plaques. Left panels show representative results obtained on non-atherosclerotic coronary arteries; (*upper left*) SL (arrows point to the internal elastic lamina, o.m. X40); (*center and bottom*) details of SL and CL staining (o.m. X400). Right panels show staining of an atherosclerotic plaque in a coronary artery. Areas of increased immunoreactivity, enclosed in boxes in the low magnification view of the plaque (*upper right*) (o.m. X40), are shown at higher power (o.m. X400) in the two panels beneath; (*middle right*) the plaque's fibrous cap; (*lower right*) a portion of one shoulder of the atheroma.

During three independent experiments using *in situ* zymography, 5–15 serial sections of each lesion were processed using the three different substrates. Test incubations were performed at ambient temperature. Controls for detection of enzymatic activity included incubation of sections at 4°C or in the presence of chemical inhibitors of MMPs, such as EDTA and 1,10-phenanthroline (Sigma Chemical Co.), or of recombinant human TIMP-1, a physiological MMP inhibitor.

## Results

*Non-atherosclerotic arteries contain ubiquitous immunoreactive 72-kD gelatinase (MMP-2) and TIMPs 1 and 2.* SMCs in all layers of non-atherosclerotic arteries contained 72-kD gelatinase (MMP-2), an MMP specialized in the degradation of



**Figure 3.** In human atherosclerotic plaques, foam cells stain with antibodies against all MMPs studied and their inhibitors. Staining of serial sections for SL, interstitial CL, 72-kD gelatinase, and TIMP-1 is shown (o.m. X1,000). These results are typical of 30 plaques studied. The three panels on the left show staining of an area rich in SMC-derived foam cells, formally identified using anti-actin antibodies (*bottom left*), while the right-hand panels show staining of foam cells derived from macrophages, identified with the monoclonal antibody HAM-56 (*bottom right*). Double immunostaining with anti-MMPs antibodies (*reddish-brown reaction*) and cell type-specific antibodies (*blue reaction product*) for identification of SMC or macrophage (M $\phi$ ) derived foam cells showed colocalization (*purple*) of MMPs with both cells types (o.m. X1,000).

type IV collagen. The endogenous inhibitors TIMP-1 and TIMP-2 showed similar immunolocalization (Fig. 1 and Table I). Non-atherosclerotic arteries stained weakly or not at all for the other three MMPs studied, the 92-kD gelatinase (MMP-9), interstitial collagenase (MMP-1), or stromelysin (MMP-3) (Fig.

2). Luminal endothelium of these arteries usually stained with anti-72-kD gelatinase and anti-TIMP-2 (Fig. 1 and Table I).

*Atheromata contain in addition immunoreactive interstitial collagenase (MMP-1), 92-kD gelatinase (MMP-9), and stromelysin (MMP-3) in macrophages, smooth muscle cells, lympho-*

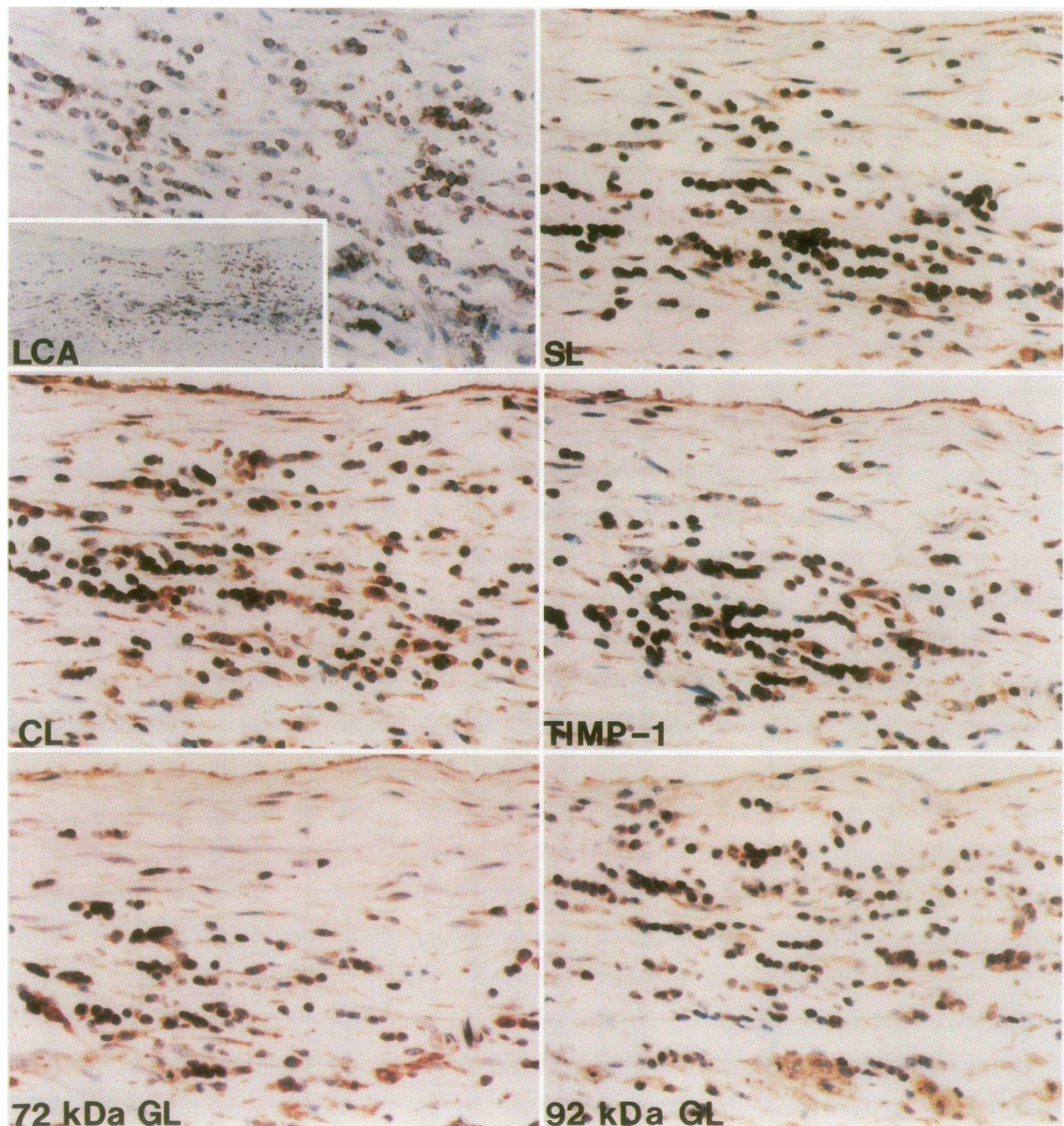
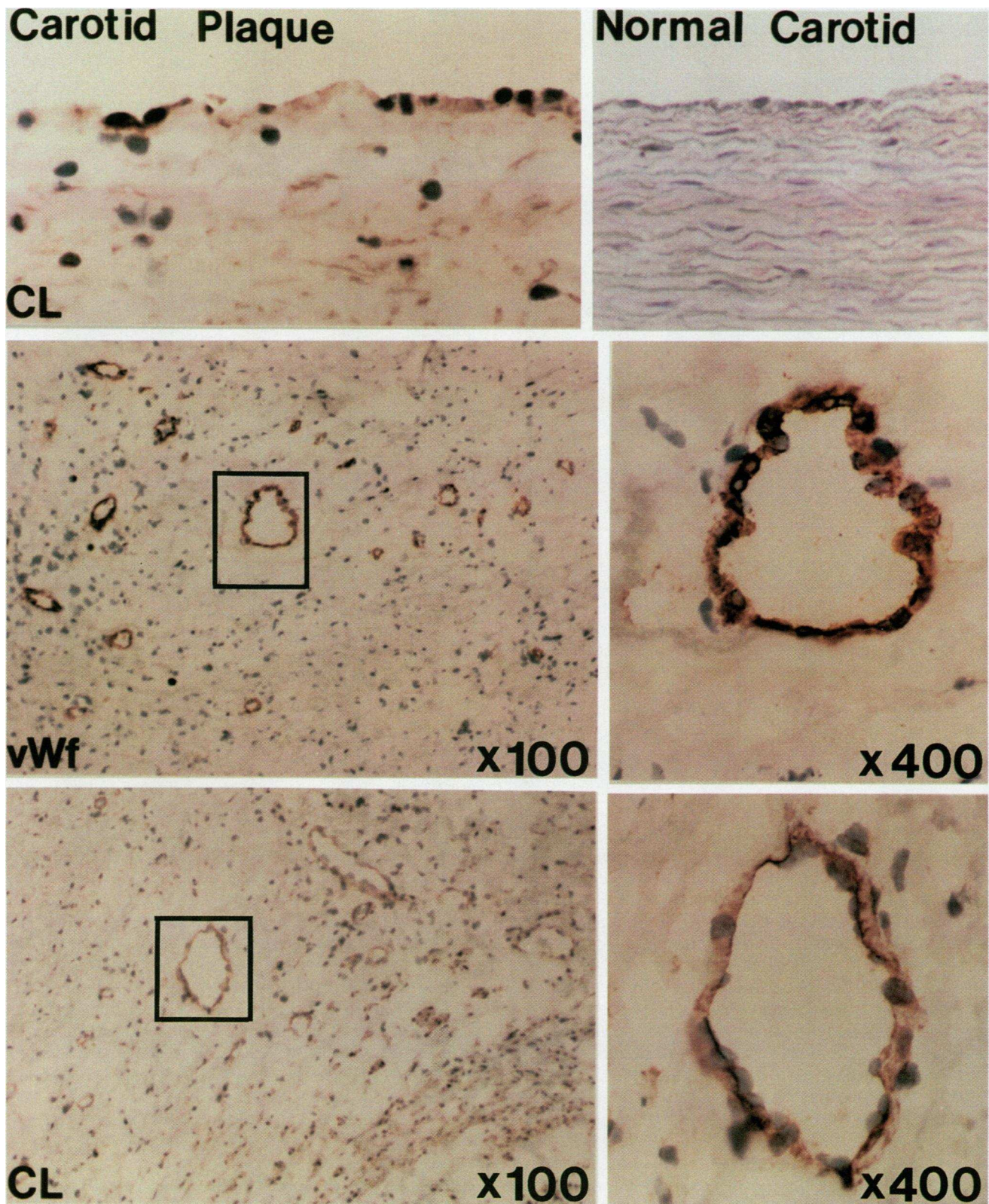


Figure 4. Leukocyte infiltrates, identified with an antibody raised against the common leukocyte antigen (LCA) stain for all MMPs studied (o.m. X100). Note the colocalization of MMPs and TIMP-1 with small lymphocytes in this example.

cytes, and endothelium. In contrast to normal arteries, atherosclerotic plaques stained for all MMPs tested. Increased MMP immunoreactivity localized particularly with the fibrous cap, the shoulders of the lesions, and the base of the lesions' lipid core (Fig. 2). Staining of serial sections, as well as double immunostaining, indicated that both macrophages and SMCs contained immunoreactive MMPs and TIMPs (Fig. 3, Table I). Areas rich in lymphocytes also colocalized with all MMPs examined (Fig. 4). Interstitial collagenase, undetectable in the

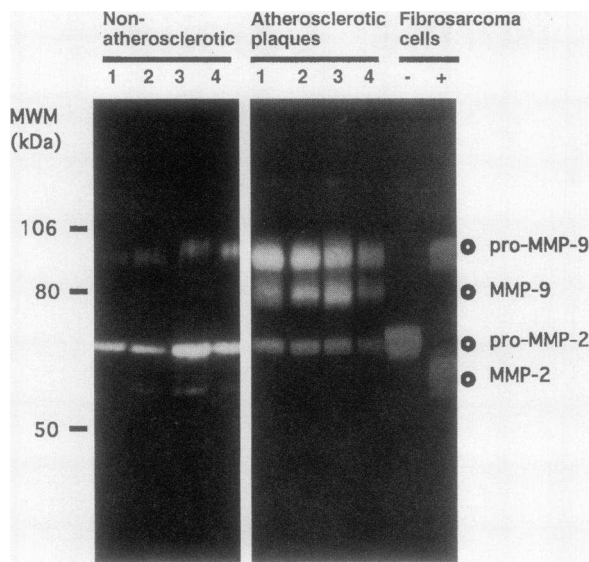
luminal endothelium of non-atherosclerotic arteries, immunolocalized to the luminal endothelium covering plaques, suggesting a novel marker of activation of endothelial cells overlying atherosclerotic lesions (Fig. 5). The microvascular endothelium of the plaque neovessels also contained immunoreactive interstitial collagenase (Fig. 5).

*Atherosclerotic plaques contain activated MMPs.* The increased MMP immunoreactivity documented in atherosclerotic lesions (Table I) does not necessarily correspond to augmented



**Figure 5.** Endothelial cells in atherosclerotic plaques stain for interstitial collagenase. (*Upper two panels*) Luminal endothelium covering a carotid plaque stains for CL, while endothelium of a non-atherosclerotic carotid artery does not (o.m. X400). (*Lower panels*) Plaque microvessels (o.m. X100) identified with antibodies against von Willebrand Factor (vWF) contain interstitial CL. Some of the capillaries are shown at higher magnification in the right panels (o.m. X400). These results are typical of 8 non-atherosclerotic arteries and 22 plaques stained for CL.





**Figure 6.** Identification of gelatinolytic proteins in extracts of human arterial tissue by SDS-PAGE zymography. Equal amounts of total protein were loaded on each lane of a gelatin-containing gel. On this gel, extracts obtained from four non-atherosclerotic arteries and four atherosclerotic plaques were run in parallel with positive controls of culture media conditioned by fibrosarcoma cells. Unstimulated (-) fibrosarcoma cells are known to secrete the zymogen form of the 72-kD gelatinase (pro-MMP-2), while cells stimulated (+) with phorbol ester activate MMP-2 and release the pro-92-kD gelatinase (pro-MMP-9). The results displayed here represent those obtained in multiple experiments. All atherosclerotic tissue extracts tested ( $n = 16$ ) displayed gelatinolytic activity comigrating with the zymogen forms of the two gelatinases, pro-MMP-2 and pro-MMP-9. Non-atherosclerotic extracts ( $n = 9$ ) typically contained pro-MMP-2 and variable amounts of pro-MMP-9. Only the plaques contained the activated forms of both MMP-2 and MMP-9.

enzymatic activity since all MMPs require activation before they can digest their substrates (16). Moreover, areas that contained MMPs in the plaques also contained TIMPs, molecules that can prevent the matrix-degrading action and activation of MMPs (17). Since immunocytochemistry with available antibodies did not distinguish between the zymogen and activated forms of MMPs, we sought other approaches to determine whether the arterial tissue contained activated forms of MMPs. SDS-PAGE zymography, a method that allows identification of proteins with gelatinolytic activity, demonstrated that all extracts of uninvolved tissues ( $n=9$ ) and all plaque extracts investigated ( $n=16$ ) contained the zymogen form of the 72-kD gelatinase (pro-MMP-2) (Fig. 6 and data not shown). By comparison with this gelatinase, non-atherosclerotic tissue extracts contained much smaller amounts of the zymogen form of the 92-kD gelatinase (pro-MMP-9). All endarterectomy extracts contained pro-MMP-9 and the activated forms of both gelatinases, MMP-2 and MMP-9. In addition to their pattern of migration, immunoprecipitation formally established the identity of these gelatinases (data not shown).

The presence of activated gelatinases in the tissue extracts was further documented by enzymatic assay using radiolabeled collagen type IV, a typical substrate for gelatinases (also known as type IV or type V collagenases). Plaque extracts contained

type IV collagenolytic activity as demonstrated by fluorography of radiolabeled collagen incubated with the extracts (data not shown). This enzymatic degradation did not require chemical activation of the extracts, indicating the presence of endogenously activated gelatinases, and was inhibited by EDTA, consistent with the metal-dependence characteristic of MMPs. Extracts of non-atherosclerotic tissue assayed in the same manner did not contain type IV collagenolytic activity (not shown).

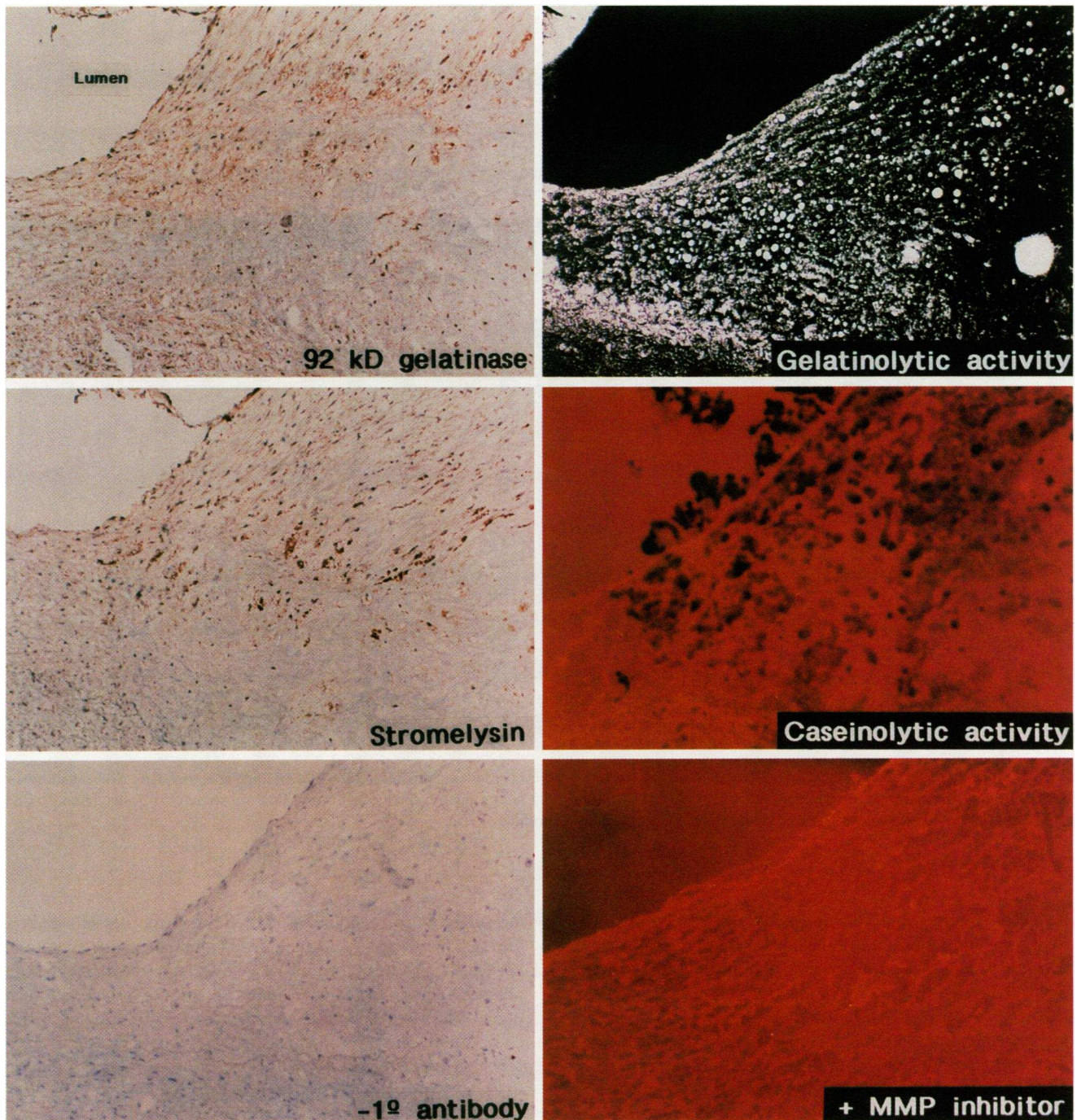
*Regional overexpression of matrix degrading activities in situ.* To localize MMP activity microscopically within atherosclerotic plaques, serial frozen sections of some of the freshly excised surgical specimens were analyzed by both in situ zymography and immunocytochemistry (Fig. 7). Areas of both gelatinolytic and caseinolytic activity localized in the same regions in all the plaques examined ( $n=5$ ). A major area of substrate lysis localized in the shoulders of plaques, the same areas of increased expression of immunoreactive MMPs 1,3, and 9. The core of the plaques also displayed gelatin or casein lysis. In situ zymographic signals diminished in sections incubated at 4°C (instead of ambient temperature), or in the presence of EDTA or 1,10-phenanthroline (Fig. 7 and data not shown). Addition of recombinant human TIMP-1 to the incubation buffer also reduced the extent of the fluorescent substrates lysis by atherosclerotic specimens (data not shown). The persistence of some signal in the presence of these inhibitors could indicate incomplete inhibition of MMPs, or the participation of non-metalloenzymes (e.g., serine proteases) in substrate lysis. The non-atherosclerotic arteries ( $n=3$ ) and the uninvolved areas of atherosclerotic specimens did not exhibit enzymatic activity determined by this method (Fig. 8).

## Discussion

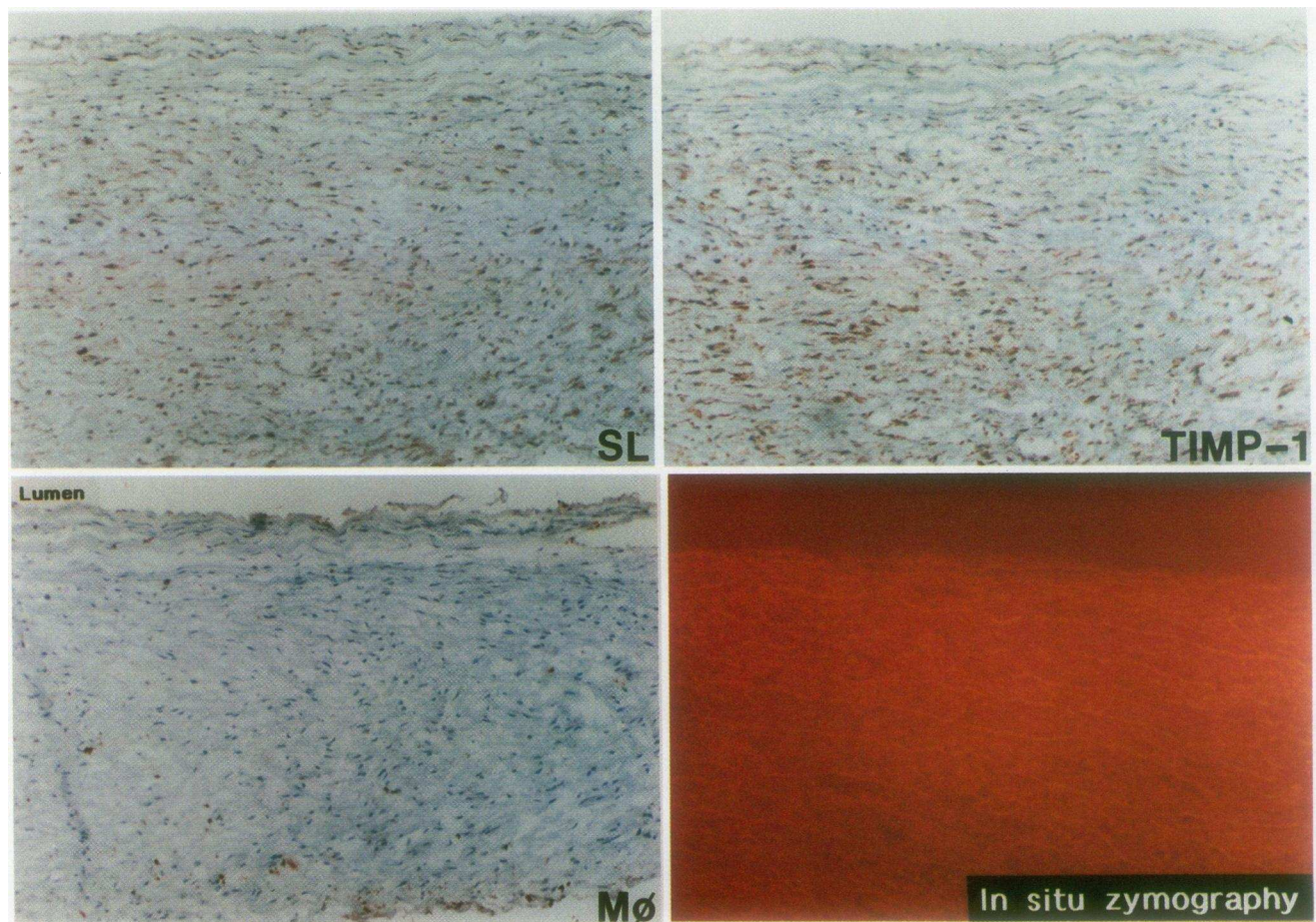
In both uninvolved and atherosclerotic arteries SMCs stained for MMP-2, TIMP-1, and TIMP-2. Gelatin zymography of arterial tissue extracts also showed the presence of MMP-2. This finding agrees well with the constitutive expression of these molecules in vitro by human vascular SMCs previously reported (5, 6). However, unlike atherosclerotic tissue, uninvolved arteries did not appear to contain active MMPs. MMP-2 associates tightly with its selective endogenous inhibitor TIMP-2, also uniformly present in normal arteries. Indeed, vascular SMCs (6) and other cells (13, 18) usually secrete MMP-2 in a complex with its inhibitor, indicating at least stoichiometric equivalence of the enzyme and inhibitor. Thus, TIMPs probably limit the matrix-degrading potential of the constitutively expressed MMP-2 in normal vessels, consistent with our in situ zymographic results.

In addition to the 72-kD gelatinase (MMP-2), SMCs within atherosclerotic plaques also contained stromelysin, interstitial collagenase, and 92-kD gelatinase. In cultured human SMCs (6), cytokines such as IL-1 or TNF- $\alpha$  induce the expression of the same three MMP we found augmented in plaques in situ. The same specimens of human atherosclerotic plaques contained regions of immunoreactive IL-1 and TNF- $\alpha$  associated with macrophages or SMCs (data not shown), as previously described (19–21). In addition to increasing MMP gene expression, cytokines may regulate activation of MMPs (6) through a cell-mediated mechanism (22), or regulate plasmin activity (23), a putative in vivo activator of MMPs.

Macrophages accumulate in rupture-prone regions of plaques (24), and in our specimens colocalized with all MMPs



**Figure 7.** Detection of MMP immunoreactivity and enzymatic activity in frozen sections of human atherosclerotic plaques. Serial sections were analyzed by immunocytochemistry, (*left panels*) and in situ zymography (*right panels*). Five atherosclerotic plaques were stained for several MMPs and TIMPs and processed during three independent experiments for detection of gelatinolytic or caseinolytic activity by in situ zymography. Areas of intense caseinolytic activity, detected as the black spots on the bright background of the fluorescent substrate, typically localized to the shoulders of the plaques. The lytic activity seemed to diffuse into the portions of the overlay in periluminal areas. Gelatinolytic activity was detected using a gelatin-containing photographic emulsion. Due to exposure to ambient light and subsequent photographic processing, the background of this substrate is black, gelatin lysis produces transparent spots. The main areas of immunoreactivity for MMPs in the tissue colocalized with the degradation of MMP substrates in contact with serial sections, suggesting the presence of activated forms of the enzymes in the shoulders of the plaques. (*Lower right*) Incubation of a serial section in the presence of a MMP inhibitor (1,10-phenanthroline) substantially reduced the extent of casein substrate lysis, indicating that most of the caseinolytic activity was due to MMPs. (*Lower left*) Control for immunocytochemistry, sections processed in the absence of primary antibodies ( $-1^{\circ}$  antibodies).



**Figure 8.** Normal artery analyzed by immunocytochemistry for stromelysin, its endogenous inhibitor, TIMP-1, and macrophages (M $\phi$ ), and by in situ zymography (lower right panel). Non-atherosclerotic arteries ( $n=3$ ) did not produce lysis of any of the MMP substrates ( $n=3$ ), as demonstrated in this case by the homogeneously bright fluorescent layer of casein-resorufin.

we studied. Henney et al. (9) detected by in situ hybridization stromelysin mRNA in some of the human atherosclerotic lesions that they investigated, mostly in macrophages and in a few cases in scattered SMCs. By immunocytochemistry, we found even more widespread stromelysin expression, as all plaques contained numerous immunoreactive macrophages and SMCs within the cap and shoulders. This difference may result from a higher expression of stromelysin protein than its mRNA, the relative stability or concentrations of the protein and RNA, or the sensitivity of the methods used for their detection. In addition to stromelysin, we found that macrophages also stained for interstitial collagenase and the two gelatinases. Therefore, macrophages, infrequent in normal arterial tissue, seem indeed to provide an additional source of matrix-degrading enzymes in the atherosclerotic plaques. Lymphocytes, another important component of the leukocytic infiltrate of plaques (25) also expressed MMPs.

Both the endothelium overlying plaques and that of the lesion's microvasculature expressed interstitial collagenase, an enzyme not detected in normal endothelium. Cultured endothelial cells can express collagenase after stimulation by phorbol ester or certain cytokines (4). Expression of interstitial collagenase by the endothelium of microvessels forming in plaques may

aid migration of endothelial cells through the vessel's matrix during angiogenesis, a characteristic of human plaque evolution.

ECM constitutes the bulk of many advanced atherosclerotic plaques. Therefore, ECM metabolism in atherosclerosis must favor overall net accumulation rather than degradation of matrix components. However, focal accumulation of cells that overexpress activated forms of MMPs may promote local destruction of ECM. Our immunohistochemical data demonstrated this degradative potential in plaque regions particularly vulnerable to rupture (26, 27).

However, TIMPs can interfere with the proteolytic processing of MMP zymogens, as well as block substrate binding to activated MMPs. MMPs can therefore perform their biological function only after activation, and where and when a local stoichiometric excess over endogenous inhibitors prevails (28). Immunocytochemistry cannot distinguish quantitatively such imbalances between MMPs and TIMPs. To explore whether MMPs expressed in key locations in the plaques may actually exert their enzymatic activity, we developed a method for the microscopic localization of MMP enzymatic activity directly in sections of arterial tissue. This approach revealed MMP activity in the plaques and allowed the microscopic localization of these sites. Zones of gelatin or casein lysis colocalized respectively

with immunoreactive gelatinases or stromelysin on serial sections obtained from the same lesion. These findings indicate that increased extracellular matrix degradation may occur in regions of increased MMP expression in atheroma, such as the plaque's shoulders and core. Moreover, such regions colocalize with those independently shown to be sites of maximal circumferential stress (27) and prone to clinical rupture (26). Our results thus suggest a biochemical mechanism for destabilization of human atheroma that commonly causes acute clinical manifestation, including many strokes and myocardial infarctions.

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