Cytokine-Stimulated Human Vascular Smooth Muscle Cells Synthesize a Complement of Enzymes Required for Extracellular Matrix Digestion

Zorina S. Galis, Maria Muszynski, Galina K. Sukhova, Elissa Simon-Morrissey, Elaine N. Unemori, Michael W. Lark, Edward Amento, Peter Libby

Abstract Vascular matrix remodeling occurs during development, growth, and several pathological conditions that affect blood vessels. We investigated the capacity of human smooth muscle cells (SMCs) to express matrix metalloproteinases (MMPs), enzymes that selectively digest components of the extracellular matrix (ECM), in the basal state or after stimulation with certain cytokines implicated in vascular homeostasis and pathology. Enzymatic activity associated with various proteins secreted in the culture media was detected by gelatin or casein sodium dodecyl sulfate-polyacrylamide gel electrophoresis zymography. Proteins were identified by immunoprecipitation and mRNA by Northern blotting. SMCs constitutively secreted a 72-kD gelatinase and the tissue inhibitors of MMPs (TIMPs) types 1 and 2. SMCs stimulated with interleukin-1 or tumor necrosis factor-α-synthesized de novo 92-kD gelatinase, interstitial collagenase, and stromelysin. Several lines of evidence suggest that when stimulated by cytokines, SMCs produce activated forms of MMPs. Together, the constitutive and the cytokine-induced enzymes can digest all of the major components of the vascular ECM. Moreover, since these mediators augment the production of MMPs without appreciably affecting the synthesis of TIMPs, locally secreted cytokines may tip the regional balance of MMP activity in favor of vascular matrix degradation. (Circ Res. 1994;75:181-189.)

Key Words • matrix metalloproteinases • extracellular matrix • smooth muscle cells • cytokines • collagenase

Smooth muscle cells (SMCs) are the major cell type of medium- and large-sized blood vessels. These cells synthesize important components of the extracellular matrix (ECM), including collagens, elastin, and proteoglycans. SMCs produce the bulk of the ECM of the vessels. However, knowledge of the capacity of SMCs to catabolize this matrix remains incomplete. Cell migration and proliferation, angiogenesis, and accumulation or resorption of ECM all depend on altered metabolism of its constituents. These cellular events occur during physiological processes such as morphogenesis, development, and growth, and in several pathological conditions.1-2 SMCs might contribute to remodeling of the blood vessel matrix, especially in situations in which these cells accumulate, including atheroma or lesions that form after vascular interventions.

Enzymes that selectively digest the individual components of ECM are collectively called matrix metalloproteinases (MMPs) and belong to three main groups: the interstitial collagenases, the type IV collagenases or gelatinases, and the stromelysins.3 Reshaping of connective tissues occurring in inflammation, wound healing, or tumor invasion likely involves the action of various MMPs secreted by immune cells, fibroblasts, and tumor cells.4-5 The enzymatic activity of MMP is regulated at several levels. Many growth factors, cytokines, hormones, or tumor promoters regulate MMP expression at the transcriptional level.6 All secreted MMPzymogens require proteolytic processing to exert their matrix-degrading potential,7 a posttranslational control mechanism. The balance between MMPs and their natural inhibitors, called the tissue inhibitors of MMPs (TIMPs), further modulates the enzymatic activity of MMPs.

Previous studies of MMP production by vascular cells have often dealt with microvascular endothelial cells, whose capacity to produce matrix-degrading enzymes appears crucial in angiogenesis.8-9 In normal and diseased aortic tissue, the 92-kD gelatinase (also called MMP-9, type V gelatinase, or gelatinase B) and TIMP-1 localize preferentially within the adventitia.10 Explants of the tunica media of rabbit aorta secrete the 72- and 92-kD gelatinases but reportedly not interstitial collagenase or stromelysin.11 Others12 have found that human SMCs can produce interstitial collagenase (or MMP-1) when stimulated by platelet-derived growth factor (PDGF), interleukin-1 (IL-1), or the tumor promoter tetradecanoylphorbol acetate (TPA), in addition to a constitutive secretion of 72-kD gelatinase (also called MMP-2, type IV gelatinase, or gelatinase A). As in the case of rabbit aortic explants,13 the latter group also failed to detect production of stromelysin (MMP-3) by human SMCs.14 Although such reports have estab-
lished that SMCs can express certain members of the MMP family, the ability of these cells to secrete the whole spectrum of MMP and to produce active forms required for vascular matrix remodeling remains uncertain. Knowledge of the expression of endogenous inhibitors of MMP (TIMPs 1 and 2) by SMCs is also lacking. Therefore, we investigated the capacity of SMCs to produce MMPs and TIMPs under basal conditions and examined their regulation by certain cytokines potentially relevant to vascular pathobiology.

Materials and Methods

Antibodies

We used rabbit polyclonal antibodies raised against recombinant human collagenase, stromelysin (or MMP-3), and TIMP-1.13 The rabbit polyclonal antiserum against the 72-kD gelatinase and the TIMP-2 purified from human melanoma cells14,15 were generously provided by Dr William Stetler-Stevenson (National Institutes of Health, Bethesda, Md), and the monoclonal anti-92-kD gelatinase antibodies16 were provided by Dr Deborah French (State University of New York at Stony Brook). Dr French also provided a purified preparation of the pro–92-kD gelatinase secreted by fibrolar 12-myristate 15-acetate-stimulated HT 1080 cells.

Nucleic Acid Probes

Two partially overlapping oligonucleotides were synthesized according to the sequence of stromelysin.17 The 700-bp EcoRI fragment of TIMP-1 cDNA18 was a gift from Dr Steven Clark (Genetics Institute, Cambridge, Mass). A 3.1-kb cDNA insert of the 72-kD gelatinase in pBluescript KS was kindly provided by Dr Gregory Goldberg (Washington University, St Louis, Mo).

Cell Culture

SMCs obtained from human saphenous veins were subcultured at passages 2 through 5 into 90-mm Petri dishes and grown to confluence in Dulbecco’s modified Eagle medium (DMEM, BioWhittaker) containing penicillin B (100 U/mL), streptomycin (100 µg/mL), and amphotericin (1.25 µg/mL) and supplemented with 5% fetal calf serum. Human fibrosarcoma HT 1080 cells passage 21 (American Type Culture Collection) were grown to confluence in DMEM supplemented with 10% fetal calf serum. Human synovial fibroblasts were isolated and grown as reported.4

Stimulation Experiments

Confluent cells were washed twice with Hanks’ balanced salt solution and transferred into serum-free medium prepared from DMEM/F12 (1:1) supplemented with 1 mmol/L insulin and 5 mg/mL transferrin19 and kept in this medium for 24 hours. SMCs were stimulated by incubation in fresh serum-free medium containing one of the following cytokines: recombinant tumor necrosis factor-α (TNF-α) (Genzyme), transforming growth factor-β (TGF-β) (Genzyme), IL-1α (Hoffmann-La Roche), recombinant IL-1β (Genzyme), recombinant basic fibroblast growth factor (bFGF, R & D Systems), and bFGF+TGF-β or TPA (Sigma Chemical Co). Cytokines were added in the presence of 0.1% pyrogen-free human serum albumin, used as a carrier. Both time-course and concentration-dependent stimulation experiments on SMCs were performed for up to 48 hours. HT 1080 cells were stimulated for 24 hours with 100 ng/mL TPA or 10 ng/mL IL-1α in serum-free DMEM containing 0.1% human serum albumin. Unstimulated SMCs or fibrosarcoma cells were kept in the same serum-free medium and handled in the same manner as the stimulated cells. At the end of the stimulation period, the cells were metabolically labeled. Culture media were used for identification of MMPs. RNA was extracted from the cell layer and processed for Northern blots.

Metabolic Labeling

Cytokine-containing media were collected, and cells were rinsed and incubated for 30 minutes in DMEM lacking methionine (Met) and cysteine (Cys). Expressed35S35S protein labeling mix (DuPont-NEN) containing [35S]Met and [35S]Cys in Met- and Cys-free DMEM (50 µCi/mL) was then added (5 µL per plate). After 24 hours, 35S-labeled media were collected and used to analyze the newly synthesized proteins.

Zymography

Proteins with gelatinolytic or caseinolytic activity were identified by electrophoresis in the presence of sodium dodecyl sulfate (SDS) in 10% or 12% discontinuous polyacrylamide gels containing 1 mg/mL gelatin or casein.8 Culture media were loaded on gels either directly or after immunoprecipitation with specific antibodies. After electrophoresis, the proteins in the gels were renatured by exchanging SDS with Triton X-100 (two 15-minute incubations in 2.5% Triton X-100). Gels were subsequently incubated overnight at 37°C in 50 mmol/L Tris-HCl, pH 7.4, containing 10 mmol/L CaCl2 and 0.05% Brij 35 (Sigma). To verify the MMP nature of the lytic activity detectable by zymography, identical gels were incubated in the above buffer containing either 20 mmol/L EDTA, an inhibitor of MMP, or 1 mmol/L phenylmethylsulfonyl fluoride (PMSF), an inhibitor of serine proteases. At the end of incubation, gels were stained with colloidal Brilliant Blue G (Sigma). Newly synthesized proteins were detected by fluorography of the same gels.

Immunoprecipitation

Rabbit polyclonal antibodies raised against 72-kD gelatinase, stromelysin, collagenase, TIMP-1, and TIMP-2 were isolated from antisera by use of protein A (PrA)-Sepharose (Sigma) and used to immunoprecipitate 35S-labeled proteins secreted in culture by SMCs. Culture media were incubated with the primary antibodies bound to the PrA-Sepharose gel for 2 hours at room temperature. Nonspecifically bound proteins were removed by four 5-minute washes of the gel with 10 mmol/L sodium phosphate, pH 7.2, containing 150 mmol/L NaCl (PBS) and supplemented with 1% Triton X-100, 0.1% SDS, 0.5% sodium deoxycholate, and 0.2% sodium azide. Controls for nonspecific binding included incubation of culture media with normal rabbit serum, with irrelevant monoclonal antibodies, or directly with PrA-Sepharose. Sequential immunoprecipitation was used to check for the possible crossreaction of the anti-TIMP-1 and –TIMP-2 antibodies. In this case, paired aliquots of culture media from metabolically labeled SMCs were incubated with antibodies raised against one of the TIMPs. In this case, proteins not bound during the first immunoprecipitation were incubated with antibodies raised against the other inhibitor. Immunoprecipitated proteins were eluted by heating of the Sepharose gel at 65°C for 10 minutes in nonreducing SDS–polyacrylamide gel electrophoresis (PAGE) sample buffer and separated by SDS-PAGE. The polyacrylamide gels were fixed, impregnated with ENHANCE (DuPont-NEN), dried, and exposed to x-ray film at −70°C for detection of immunoprecipitated proteins.

N-Glycosidase Treatment

Metabolically labeled immunoprecipitated stromelysin and TIMP-1 were treated with N-glycosidase F (New England BioLabs) according to the manufacturer’s instructions. Duplicates, incubated in the absence of the enzyme, were compared by fluorography with the treated samples.

Immunoblotting

Proteins were transferred from minigels onto nitrocellulose with a semidry blotting system (Bio-Rad Laboratories). An alkaline phosphatase kit (Vector Laboratories) or a chemiluminescent detection system (Amersham Corp) was used for
antigen detection according to the manufacturer’s instructions. Blocking of nonspecific binding and dilution of the primary and secondary antibodies were made with a 5% solution of dry defatted milk in PBS containing 0.1% Tween 20.

Northern Blot Analysis

RNA, extracted after the metabolic labeling of SMCs, was separated in formamide/agarose gels and transferred to Hybond nylon membranes (Amersham Corp). The cDNA and oligonucleotide probes were labeled with [32P]CTP (DuPont-NEN) by use of a random-priming kit (Pharmacia LKB Biotechnology). Probes were hybridized overnight at 42°C and then the nylon membranes were successively washed at 55°C with three changes of 2× standard saline citrate (SSC) and 1× SDS (15, 15, and 45 minutes), 1× SSC and 0.1% SDS (1 hour), and 0.5× SSC and 0.05% SDS (20 minutes) and exposed overnight to x-ray film at −70°C.

Data Analysis

Gels and blots were analyzed by densitometry using the National Institutes of Health IMAGE program version 1.49. Data were compared by one-way ANOVA.

Immunocytochemistry

The presence of various pro-MMPs and TIMPs in confluent SMCs grown on glass coverslips was detected by simple or dual immunofluorescence. After stimulation with cytokines, cells were incubated for 3 hours in the presence of 1 mmol/L monensin to inhibit MMP secretion21 and then fixed with cold acetone. Slides were incubated with 10% normal goat serum in PBS and then with the primary antibodies diluted in 2% goat serum in PBS for 1 to 2 hours. After washing with PBS (three times for 5 minutes), the cells were layered with biotinylated goat anti-rabbit IgG (Vector Laboratories) for 30 minutes. Immunolabeling was detected with streptavidin conjugated with Texas red or fluorescein isothiocyanate (Amersham Corp). Double immunofluorescence was performed by a procedure based on the successive immunostaining of each antigen.22 Controls for double immunofluorescence included omission of each of the primary antibodies, reversal of the staining order, or of the use of fluorochromes. Nuclei were stained for 2 minutes with 0.5 mg/mL bisbenzimide H 33258 fluorochrome (Calbiochem) in PBS.

Results

Vascular SMCs Constitutively Express 72-kD Gelatinase and May Activate This Enzyme When Exposed to Cytokines

The 72-kD gelatinase (MMP-2) specifically digests nonfibrillar collagens, such as those typically found in the cellular basement membrane, as well as denatured collagen fragments. Invasive behavior of transformed cells correlates with high levels of 72-kD gelatinase expression.23 Unstimulated SMCs constitutively secrete gelatinolytic activity, as revealed by zymography (Fig 1). This activity comigrated in SDS-PAGE with that released by synovial fibroblasts (Fig 1) and HT 1080 fibrosarcoma cells (data not shown). Lysis of gelatin was completely inhibited by incubation of gels in the presence of EDTA but not with the serine proteinase inhibitor PMSF, consistent with the MMP nature of the gelatinolytic proteins detected by zymography.

The major zone of gelatinolytic activity in the media harvested from SMCs exposed to various stimuli (Fig 2) comigrated with that from unstimulated cells. This activity has been identified as associated with the 72-kD gelatinase by its immunoprecipitation with specific antibodies (Fig 3, left panel). The zone of gelatin lysis (Fig

![Fig 1. Gelatin zymography of proteins constitutively secreted by cultured human synovial fibroblasts (FB) or vascular smooth muscle cells (SMC). Areas of lysis appear as light on the darker background of gelatin-containing gels stained with Coomassie Blue (arrows), indicating the presence of proteins with gelatinolytic activity. Triplicate sections of a gel were incubated overnight in the absence of protease inhibitors (−) or in the presence (+) of EDTA or phenylmethylsulfonyl fluoride (PMSF). Migration of molecular mass markers is indicated in kilodaltons.](http://circres.ahajournals.org/lookup/doi/10.1161/01.RES.88.2.183)

![Fig 2. Proteins with gelatinolytic activity secreted by cultured human smooth muscle cells (SMCs) under basal conditions (0) or after 48 hours of stimulation with the following cytokines: tumor necrosis factor-α (TNFα, 10 ng/mL), transforming growth factor-β (TGFβ, 10 ng/mL), interleukin-1β (IL1β, 10 ng/mL), interleukin-1α (IL1α, 10 ng/mL), basic fibroblast growth factor (bFGF, 10 ng/mL), bFGF + TGFβ (10 ng/mL each), and tetradecanoylphorbol acetate (TPA, 25 nmol/L). Left, Gelatin zymography. Right, Fluorography of the same gel showing the total biosynthetically labeled proteins secreted during the incubation. Note that bands comigrating with the major gelatinolytic activity (arrowhead) appear to constitute major secretory products of SMCs. Migration of molecular mass markers (MWM) is indicated.](http://circres.ahajournals.org/lookup/doi/10.1161/01.RES.88.2.183)
cytokine-treated cells (Fig 3, right gel of left panel) showed bands of apparent molecular mass (MW) of −50 kD in addition to the 72-kD band. Activation of the 72-kD gelatinase proenzyme by fibroblast membranes yields similar lower MW forms.24 Chemical treatment of the recombinant pro−72-kD gelatinase25 or of purified TIMP-2−free enzyme26 also yields activated forms of this enzyme in the range of 40 to 50 kD. Thus, the 50-kD bands in the supernates of cytokine-stimulated SMCs could represent proteolytically processed 72-kD gelatinase. However, the possibility that these bands represent an inactive form of 72-kD gelatinase or some cross-reaction of the anti−72-kD gelatinase antibodies with another cytokine-induced MMPs (eg, collagenase) cannot be excluded. The level of mRNA encoding the 72-kD gelatinase was high in confluent SMCs under basal conditions and did not appear to vary substantially as a result of cytokine stimulation (Fig 4), possibly because of the saturation of signal on the Northern blots.

### Cytokine Stimulation Induces De Novo Synthesis and Secretion of the 92-kD Gelatinase by SMCs

The 92-kD gelatinase (MMP-9) shares some sequence homology and substrate specificity with the 72-kD gelatinase. However, different genes encode these two gelatinases, and they have distinct patterns of regulation.27 In addition to the nonfibrillar collagens, the 92-kD gelatinase can also digest proteoglycans and elastin, important constituents of the arterial ECM. Human SMCs treated with TNF-α or IL-1 released additional gelatinolytic activity, which migrated in SDS-PAGE zymography (Fig 2, left gel) more slowly than the constitutively secreted 72-kD gelatinase. Fluorography of the same gel (Fig 2, right gel) showed de novo synthesis of metabolically labeled proteins that comigrated with the gelatinolytic bands contained in the media of stimulated cells. IL-1 induced time-dependent release of this activity (Fig 5). The cytokine-inducible gelatinolytic band comigrated with a purified preparation of the pro−92-kD gelatinase secreted by TPA-stimulated HT 1080 cells (Fig 6). Anti−92-kD gelatinase antibodies immunoprecipitated this gelatinolytic activity from the media of cytokine-stimulated but not of unstimulated SMCs (Fig 6).

**SMCs Stimulated With IL-1 Synthesize Stromelysins**

The stromelysins (MMP-3s) are probably the most versatile MMPs. They can degrade a broad range of...
ECM components\textsuperscript{28,29} and also activate the zymogen forms of other MMPs,\textsuperscript{20,30} Culture media of IL-1-stimulated SMCs but not those of unstimulated SMCs produced areas of lysis at MW consistent with that of stromelysin in casein zymography, a method that allows detection of this MMP (Fig 7). IL-1 treatment also produced a concentration-dependent increase in the caseinolytic activity of another protein secreted by SMCs. This activity comigrated with the fastest band of gelatinolytic activity, as shown by running the same samples on a gelatin-containing gel (Fig 7, rightmost lane). This protein could represent the active form of the 72-kD gelatinase, which is detectable by both gelatin and casein zymography.\textsuperscript{10,28} In contrast to the IL-1 effect, \( \gamma \)-interferon (IFN\( \gamma \)), which can modulate collagen gene expression by SMCs,\textsuperscript{31} did not affect the caseinolytic activity secreted by SMCs.

Anti-stromelysin polyclonal antibodies immunoprecipitated two proteins synthesized de novo and secreted by IL-1-stimulated cells (Fig 8, left panel). Differences of protein chain length or extent of glycosylation might

---

**Fig 5.** Time-dependent release of gelatinolytic activity (arrow) by interleukin-1 (IL1)-stimulated smooth muscle cells (SMCs). Proteins secreted by confluent SMCs during 24 hours of incubation without stimulation (\(-\)) or in the presence (\(+\)) of IL1 (10 ng/mL) for 4, 24, or 48 hours were analyzed by gelatin zymography in 10% sodium dodecyl sulfate–polyacrylamide gel. The position of molecular mass markers is indicated.

**Fig 6.** Immunoprecipitation of the 92-kD gelatinase (MMP9) revealed by gelatin zymography. Culture media of unstimulated smooth muscle cells (SMCs) or cells stimulated with 10 ng/mL interleukin-1 (IL1) or tumor necrosis factor-\( \alpha \) (TNF\( \alpha \)) for 24 hours were incubated with three different monoclonal antibodies (7-11C, 8-3H, or 6-6B). Antibody 7-11C recognizes only pro-MMP9; the other two recognize both the latent and the activated forms of this matrix metalloproteinase. Controls for the immunoprecipitation included incubation of the culture media in the absence of primary antibodies (0) or with irrelevant monoclonal antibodies (not shown in the figure). Preparations of MMP9 purified from the culture media of HT 1080 cells were loaded as such or after chemical activation with \( \beta \)-aminophenylmercuric acetate. The last three lanes contain unfractionated culture media (CM) from unstimulated SMCs and IL1- and TNF\( \alpha \)-stimulated SMCs used for immunoprecipitation. The position of migration for immunoprecipitated gelatinolytic proteins (arrowheads on left) and for molecular mass markers (right) is indicated. The lower molecular mass band in the immunoprecipitate could represent nonspecific binding of 72-kD gelatinase to protein A-Sepharose.

**Fig 7.** Casein zymography of the culture media conditioned by unstimulated smooth muscle cells (SMCs) (0) or SMCs incubated with increasing concentrations of interleukin-1\( \alpha \) (IL1\( \alpha \)), ng/mg) or \( \gamma \)-interferon (IFN\( \gamma \), mU/mL). IL1\( \alpha \) stimulation induced caseinolytic activity with an apparent molecular mass of \(-50\) kD (arrow). Caseinolytic activity migrating with higher apparent molecular mass and detectable in all samples (\(*\)) increased with IL1\( \alpha \) concentration. Culture medium from endothelial cells (ECs) was also applied for comparison. The rightmost lane shows gelatin zymography of supernate from unstimulated SMCs run in parallel. The positions of prestained molecular mass markers (MWM) are indicated.

**Fig 8.** Immunoprecipitation of de novo synthesized proteins by anti-stromelysin (\( \alpha \)-SL), anti-collagenase (\( \alpha \)-CL), and anti-tissue inhibitor of matrix metalloproteinase-1 (\( \alpha \)-TIMP1) antibodies. Left, Fluorography of 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were immunoprecipitated from conditioned media (CM) of unstimulated smooth muscle cells (SMCs) (\(-\)) or SMCs stimulated (\(+\)) with 10 ng/mL interleukin-1 (IL1) for 24 hours. CM incubated directly with protein A-Sepharose (no primary antibodies) were loaded for identification of nonspecific (NS) binding. No precipitates were obtained by incubation of culture media with normal rabbit serum (data not shown). These results are representative of results obtained in six independent experiments. Right, Immunoprecipitated stromelysin was incubated in the absence (\(-\)) or in the presence (\(+\)) of N-glycosidase (PNG-ase). Fluorography of 10% SDS-PAGE.
account for different MW values of the two forms. However, treatment of the immunoprecipitate with N-glycosidase F did not modify the apparent MW of either band (Fig 8, right panel). In the same conditions the N-glycosidase treatment decreased the apparent MW of immunoprecipitated TIMP-1 (data not shown), confirming the efficacy of the deglycosylation treatment. Therefore, the more rapidly migrating species immunoprecipitated by the anti-stromelysin antibodies may represent activated stromelysin. IL-1 stimulation induced accumulation of stromelysin mRNA as well (Fig 9).

Cytokine-Stimulated SMCs Produce Intersitial Collagenses

Mature interstitial collagens exist in triple helical coils, highly resistant to protease degradation. The interstitial collagenses (MMP-1) can cleave these helical collagen fibrils, producing fragments susceptible to subsequent digestion by the gelatinases. Cytokine-stimulated SMCs released activities compatible with interstitial collagenses, barely detectable by gelatin zymography. Cytokine stimulation of SMCs caused release of collagenase protein, identified by Western blotting (Fig 10). TNF-α or IL-1 induced the de novo synthesis and secretion of collagenase, as shown by the immunoprecipitation of radiolabeled protein from the supernates of stimulated SMCs (Fig 8).

SMCs Produce TIMPs

Understanding the matrix-degrading potential of cells also requires consideration of their capacity to produce inhibitors for MMP. The two inhibitors TIMP-1,33,34 and TIMP-2,215 share 38% sequence homology35 and appear to interact preferentially with various MMPs. TIMPs can block both activation of MMP precursors and the ability of activated MMP to degrade their substrates.36,37

The media of unstimulated SMCs contained both TIMP-1 (Fig 8) and TIMP-2 (Fig 11). The antibody raised against TIMP-2, the specific inhibitor of the 72-kD gelatinase,15,38 coprecipitated the major gelatinolytic protein from SMC-conditioned media. The use of reducing conditions for SDS-PAGE abolished the gelatinolytic activity of the immunoprecipitate and resolved it into two 35S-labeled proteins (Fig 11B) that migrated at positions described for the 72-kD gelatinase and TIMP-2.15,39 Therefore, as described for fibroblasts, most of the TIMP-2 protein secreted by SMCs appeared complexed with the 72-kD gelatinase. Various cytokines did not appear to modify substantially the constitutive synthesis of TIMPs (Fig 8) or the mRNA level of TIMP-1 (Fig 4).
Intracellular Detection of MMPs and Their Inhibitors

In vivo and in vitro, SMCs exhibit heterogeneity. To visualize the response of individual cells to cytokine stimulation and to determine whether the same SMC can produce both MMPs and their inhibitors, we used double immunofluorescence. The foregoing biochemical experiments showed that cytokines stimulate the secretion of MMPs by SMCs. To enhance the intracellular accumulation of MMPs after cytokine treatment, SMCs were incubated for 3 hours before staining with monensin. The immunocytochemical detection of 72-kD gelatinase, stromelysin, collagenase, TIMP-1, and TIMP-2 correlated with the biochemical data shown above. Both unstimulated and IL-1-stimulated SMCs contained 72-kD gelatinase (Fig 12). SMCs uniformly contained TIMP-1 and TIMP-2. Only cells previously incubated with IL-1 stained for stromelysin (Fig 12) or collagenase (data not shown). We found that during the period analyzed, the same SMCs synthesized both MMPs and their inhibitors.

Discussion

Homeostasis of the normal vascular ECM requires balanced synthesis and catabolism by the resident cells. Programmed remodeling of this matrix occurs during normal development and growth, whereas several vascular diseases involve unscheduled reshaping of the tissue. The enzymes that digest ECM components can influence the quantity and quality of the matrix and untether cells restrained by the highly structured ECM of arteries. Resident SMCs surrounded by a basal lamina and anchored to the ECM must modulate this matrix to migrate and multiply. Migration of SMCs from the tunica media contributes to intima formation during the development of human arteries and may underlie some forms of vascular pathology. This migration requires SMCs to traverse major extracellular barriers, including the internal elastic lamina and a dense mesh of interstitial proteoglycans and collagens. Digestion of the vascular ECM may also contribute to remodeling of arteries associated with compensatory enlargement.
during atherogenesis and aneurysm formation. Rupture of coronary atheroma, the mechanism that accounts for many acute myocardial infarctions, may also result from weakening of the fibrous cap of plaques that is due to excessive matrix degradation; hence, it is important to understand the capacity of vascular SMCs to produce the range of enzymes required for local matrix degradation and the factors that regulate their activity.

Rat aortic SMCs cultured on ECM previously laid down by other cultured SMCs can partially degrade this substrate. This study suggested both plasminogen-dependent and -independent pathways but did not define specific enzymes responsible for the digestion of ECM. Interestingly, cytokine activation of vascular cells can regulate net plasmin activity, a suggestion that these mediators may modulate ECM degradation by an indirect plasmin-mediated mechanism. Factors such as cytokines can also influence the expression of MMPs directly. For example, unstimulated human SMCs produce the 72-kD gelatinase and, when exposed to IL-1 or PDGF, can produce interstitial collagenase. We report here that cytokine-activated human SMCs can also synthesize the 92-kD gelatinase and stromelysin. Together with the 72-kD gelatinase and interstitial collagenase, stromelysin and the 92-kD gelatinase complete a group of MMPs capable of degrading all major ECM components.

Cytokine regulation of MMP activity may bear in vivo relevance because vascular lesions can exhibit enhanced expression of TNF-α or IL-1. These cytokines may arise either from blood-borne cells that interact with the vessel or from resident vascular endothelial cells or SMCs. The present results show that MMPs represent major secretory products of cultured SMCs and that stimulation by cytokines increases the range of MMPs secreted by SMCs. Induction of 92-kD gelatinase, stromelysin, and interstitial collagenase in response to IL-1 or TNF-α was demonstrated by mRNA analysis, zymography, immunoprecipitation, and Western blotting. TGF-β stimulates interstitial collagen synthesis and may alter the balance of MMPs and their inhibitors in some cell types. However, TGF-β had little or no effect on MMP or TIMP-1 expression in human SMCs under the conditions studied here.

Our data also raise the possibility that cytokine treatment caused activation of MMPs. Exposure to IL-1 or TNF-α did not appreciably alter the level of MMP-2 mRNA (Fig 4). However, our zymographic results indicate that IL-1 or TNF-α treatment enhances formation of active forms of this enzyme. Likewise, fluorography of immunoprecipitates showed lower MW forms, consistent with proteolytic processing of MMP-2. Similarly, the anti-stromelysin antibodies immunoprecipitated two different MW forms of this enzyme from the culture media of stimulated SMCs, one of which may represent the activated form of MMP-3.

Because of their enzymatic nature, changes in MMP mRNA or protein levels under the influence of various factors do not necessarily imply parallel variations of their activity. The net result between opposing actions of MMP activators and inhibitors determines the biological action of the matrix-degrading enzymes. Our experiments showed that unstimulated SMCs contained high levels of mRNA and protein of TIMPs 1 and 2, which appeared unaffected by treatment with IL-1, TNF-α, or TGF-β. On the other hand, IL-1 or TNF-α induced the expression of several MMPs by cultured SMCs and the apparent elaboration of active forms of MMP by stimulated cells. These data further support the concept that cytokines alter the balance between molecules that promote or inhibit matrix degradation.

If SMCs exhibit such properties in vivo as we show here in vitro, the local secretion of cytokines could sway the balance between the production of MMPs andTIMPs to favor remodeling of vascular ECM by SMCs. More knowledge of MMP regulation and action within the vessel wall should enhance our understanding of the mechanisms of vascular development and pathology. Furthermore, such advances could yield novel strategies for experimental or therapeutic manipulation of vascular remodeling and provide new avenues of intervention in situations involving vascular cell migration, proliferation, and invasion.

Acknowledgments

This study was supported by National Heart, Lung, and Blood Institute grant HL-34636-10. Dr Galis is a Fellow of the American Heart Association, Massachusetts Affiliate, Inc. We thank Drs William Stetler-Stevenson, Deborah French, Stan Zucker, and Zena Werb for discussions and the generous gifts of the antibodies, Dr Peter LoMedico for providing the recombinant IL-1α, and Dr Gregory Goldberg for the 72-kD gelatinase cDNA probe.

References

13. Walakoviats LA, Moore Vi, Bhardwaj N, Gallick GS, Lark MW. Detection of stromelysin and collagenase in synovial fluid from...


15. Stetler-Stevenson WG, Krutzsch HC, Liotta LA. Tissue inhibitor of metalloproteinase (TIMP-2): a new member of the metallopro- 


22. Murphy Z, Harms RM, Murphy G, Werb Z. Commitment to expression of the metalloendopeptidase, collagenase and stromelysin: relationship of inducing events to changes in cyto
d 


38. Goldberg GI, Marbler BL, Grant GA, Eisen AZ, Wilhelm S, He 

39. Overall CM, Wanla J, Sodek J. Independent regulation of collage-


Cytokine-stimulated human vascular smooth muscle cells synthesize a complement of enzymes required for extracellular matrix digestion.

Z S Galis, M Muszynski, G K Sukhova, E Simon-Morrissey, E N Unemori, M W Lark, E Amento and P Libby

Circ Res. 1994;75:181-189
doi: 10.1161/01.RES.75.1.181

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 1994 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/75/1/181

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation Research can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation Research is online at:
http://circres.ahajournals.org//subscriptions/