Effects of Cytokines and Monocytes on Matrix Metalloproteinases in Human Vascular Smooth Muscle Cell Cultures

by

Elaine Lee

S.M., Massachusetts Institute of Technology, 1986
S.B., Massachusetts Institute of Technology, 1984

Submitted to the Harvard-MIT Division of Health Sciences and Technology in Partial Fulfillment of the Requirements for the Degree of

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Abstract

In coronary arteries, smooth muscle cells (SMCs) normally regulate the metabolism of matrix molecules in atherosclerotic plaques. Excess matrix degradation may render the plaque vulnerable to rupture, possibly triggering a myocardial infarction. This research investigated how cytokines affect the secretion and activation of matrix metalloproteinases (MMPs) and the secretion of tissue inhibitors of MMPs (TIMPs) in SMC cultures modeling plaque tissue.

The cytokines interleukin-1, tumor necrosis factor alpha (TNF-α), and platelet-derived growth factor induced secretion of collagenase and stromelysin by SMCs, but in serum-free conditions, they did not increase degradation of collagen or proteoglycans. This was consistent with the observation that little of the induced MMPs were fully activated.

To evaluate conditions that promote MMP activation, physiological amounts of plasminogen were added to cultures treated with TNF-α. In SMC cultures, plasminogen was converted to plasmin, which activated MMPs. TNF-α, however, induced the secretion of plasminogen activator inhibitor type 1 (PAI-1) in a concentration-dependent manner. High concentrations of TNF-α inhibited MMP activation by blocking plasmin formation. Plasminogen alone also induced secretion and activation of collagenase and stromelysin, as well as secretion of PAI-1. Even when levels of activated MMPs were greatly increased by plasminogen and TNF-α, TIMP-1 levels also increased, and matrix degradation remained unchanged.

Interactions between monocytes and SMCs were also studied. Monocytes induced collagenase and stromelysin secretion by SMCs through an interleukin-1-dependent paracrine pathway. Activation of these MMPs occurred in the absence of exogenous plasminogen, but the extent of activation depended on several factors, including the phenotypes of both SMCs and monocytes. Monocytes did not consistently affect TIMP-1 in SMC cultures. When monocytes caused the secretion and activation of MMPs and simultaneously decreased TIMP-1 secretion, matrix degradation did increase, but lysosomal enzymes released by cell death may have played a role.
The main determinant of my happiness at a workplace is the people I interact with day to day. For both their technical help and their contributions to making the lab a friendly place, I also thank my other labmates over the years, including Bob Sah, Young-Jo Kim, Tom Quinn, Minerva Garcia, Eliot Frank, Larry Bonassar, and Jane Murray. Minerva receives special appreciation for her support and her empathy in the agonies of long-distance relationships. Others who also contributed to the enjoyable lab atmosphere include Martha, Ann, Scott, Claribel, Sophia, Claudia, Sandip, Arthur, Gretchen, Greg, Dave, Steve, Michelle, Jen, Marc, Andy, Colleen, Patty, and Julianne. Special recognition goes to Linda Bragman, who, in her unique way and beyond the call of duty, shows that she cares about the people in the lab.

Thanks also go to my friend and housemate of 9 years, Sue Klasky, who tolerated the fact that I was rarely around, and who compensated for the fact that I was practically an absentee cat-parent to Mandarin and Diamond. I am also grateful to her family for being my "family away from family" and including me at their gatherings. I will miss that delicious matzo-ball soup at Passover!

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Finally, I thank my family for their love and support. Most of all, I am deeply indebted to my parents for raising me with a combination of care, nurturing, discipline, encouragement, and trust that has helped me reach my goals and become what I am today: a very happy human being.

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This work highlighted the potential role of vascular smooth muscle cells and cytokines in the weakening of atherosclerotic fibrous caps and demonstrated the complexity of regulatory mechanisms involved in the secretion, activation, and inhibition of metalloproteinases. Future strategies to prevent acute vascular events by stabilizing plaques should consider these mechanisms.

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Chapter 1. Introduction and Overview

1.1 BACKGROUND AND SIGNIFICANCE

Atherosclerotic plaque rupture. Atherosclerotic plaques are raised lesions in the intimal layer of blood vessel walls. They are characterized by an accumulation of lipids in extracellular pools and within foam cells, occasional calcific components, and fibrosis of the intima. The fibrous tissue contains primarily vascular smooth muscle cells (SMCs) and extracellular matrix. In normal vessels, the SMCs are found only in the medial and adventitial layers of the walls, but in atherosclerotic vessels, they have accumulated in the intima. SMCs produce the extracellular matrix, which comprises primarily collagen and proteoglycans (PGs). The major collagens in vascular matrix are type I and type III, and the major PGs, which contain the glycosaminoglycans chondroitin sulfate and dermatan sulfate, are versican, decorin, and biglycan.

Rupture of atherosclerotic plaques is followed by formation of thrombi, which cause acute stenoses of varying severity. Severe stenoses in critical arteries can cause acute vascular syndromes, including unstable angina, myocardial infarction, stroke, and sudden death. At least 95% of fatal heart attacks result from plaque disruption, and most non-fatal heart attacks are probably also caused by plaque rupture.

The likelihood that a particular plaque will rupture depends on the imposed stresses, the plaque geometry, and the strength of the tissue in the plaque. The imposed stresses may be affected by turbulence resulting from vessel geometry or by changes in blood pressure. The particular plaque geometry (proportions and arrangement of the fibrous tissue, intra- and extracellular lipids, and calcium) can render it more or less prone to rupture. Plaques with thin fibrous caps covering a lipid pool are particularly vulnerable, especially at the stress concentrations at the shoulder regions of the fibrous cap. The strength of the fibrous tissue depends on the composition of the extracellular matrix. In vascular matrix, collagen and the PG versican are the major tension- and compression-bearing elements, respectively. A decrease in collagen and PG content has been associated with rupture sites in plaques.
The extracellular matrix in plaques probably undergoes constant remodeling by SMCs. It has been shown in situ that vascular SMCs produce not only collagen, PGs, and other matrix molecules, but also interstitial collagenase (MMP-1), stromelysin (MMP-3), 72-kD gelatinase (MMP-2), 92-kD gelatinase (MMP-9), and tissue inhibitors of metalloproteinases types 1 and 2 (TIMP-1 and TIMP-2). Collagen type I can be cleaved by collagenase and 72-kD gelatinase. PGs can be cleaved by stromelysin, collagenase, 72- and 92-kD gelatinases, and other SMC proteinases. In blood vessels as well as in other tissues, a balance between matrix synthesis and matrix degradation is normally maintained. Certain physiologic or disease states disrupt this balance. Atherogenesis itself may be an example of such a disruption.

Regulation of matrix remodeling by cytokines. The process of matrix accumulation or loss may be regulated by cytokines. For example, there is strong evidence that the destruction of cartilage in osteoarthritis results from cytokine-induced changes in PG metabolism. There is also considerable evidence from numerous studies that cytokines can affect the metabolism of collagen and PGs by vascular SMCs. Many of these studies were aimed toward understanding the mechanisms of accumulation of lipids and fibrous tissue in atherogenesis, but they may be equally relevant to understanding the loss or weakening of fibrous tissue in the development of unstable plaques.

Table 1.1 lists some cytokines and their in vitro effects that may be relevant to remodeling of extracellular matrix. Note that interleukin-1 (IL-1) can increase the synthesis of collagen and PGs as well as the synthesis of collagenase, stromelysin, and other proteinases by SMCs. The net effect on the accumulation or degradation of extracellular matrix may depend on the relative magnitudes of the opposing effects, as well as on the amount of TIMPs present. Cytokines can also change TIMP metabolism, but effects on TIMPs were not measured in many of the studies listed here. In addition, there is evidence of more complex levels of cytokine regulation, since cytokines can increase the synthesis of other cytokines. For example, IL-1 can induce IL-1 synthesis, creating a positive feedback loop. Finally, dose response is also important to consider, since in some cases different concentrations of the same cytokine can have different or even opposing effects on the same cell type; for
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**Abbreviations:** IL-1=interleukin-1; PDGF=platelet-derived growth factor; TGF-β=transforming growth factor beta; TNF-α=tumor necrosis factor alpha; SMC=smooth muscle cell; MMP-1=interstitial collagenase; MMP-2=gelatinase; MMP-3=stromelysin; TIMP-1=tissue inhibitor of metalloproteinases type 1; PG=proteoglycan.
example, TNF-α at 0.005 ng/mL increases TIMP-1 synthesis by fibroblasts, but has no effect on TIMP-1 synthesis at 0.5 to 50 ng/mL.  

There are several possible sources of cytokines in vascular tissue. *In vitro* studies have shown that SMC-regulating cytokines can be produced by endothelial cells, which line the vascular wall, by macrophages, which are found in increased numbers in atherosclerotic plaques; by T-cells and mast cells in the fibrous cap, and by SMCs themselves. Cytokine production may be a response to humoral signals, which might include other cytokines, or a response to mechanical stimuli.

The localization of cytokines in plaques provides further evidence that cytokines may play a major role in atherosclerosis and potentially in the development of unstable plaques. For example, platelet-derived growth factor (PDGF) transcription is higher in plaque tissue than in normal vessels. TNF-α has been detected in SMCs, macrophages, and endothelial cells in atherosclerotic vessels but not in normal vessels.

Thus, plaques contain cytokines that can stimulate SMCs to secrete matrix metalloproteinases (MMPs). Furthermore, increased expression of MMPs and matrix-degrading activity has been demonstrated in rupture-prone sites in plaques. These observations suggest that in atherosclerotic plaques, cytokines induce SMC secretion of MMPs, which degrade matrix and render the fibrous cap more vulnerable to rupture. This hypothesis still lacks supporting evidence in two key areas. One is the demonstration that net degradation of matrix can be a direct consequence of cytokine stimulation in plaques. The other is proof that cytokine-induced matrix degradation results from MMP-specific cleavage, rather than from the activity of other proteinases.

### 1.2 OVERVIEW OF THESIS RESEARCH

The objective of these studies was to investigate the possible role of cytokine-stimulated SMCs in the regulation of metalloproteinase secretion leading to matrix degradation in fibrous caps. Fibrous-cap tissue was modeled by cultures of SMCs, either in monolayers or in three-dimensional collagen gels. (Characterization experiments and discussion of SMC-collagen gel cultures are found in Appendix A.)
Metalloproteinases and TIMPs were detected by Western analysis or zymography. Collagen and PG degradation was monitored by pulse-chase studies.

In the studies of Chapter 2, IL-1 and TNF-α induced secretion of collagenase and stromelysin by SMCs, but collagen and PG degradation did not increase. This was consistent with the observation that the induced metalloproteinases remained mostly unactivated.

In the studies of Chapter 3, exogenous plasminogen caused the activation of collagenase and stromelysin induced by TNF-α, but increasing concentrations of both plasminogen and TNF-α stimulated increased secretion of plasminogen activator inhibitor type 1, inhibiting the formation of plasmin and thus inhibiting metalloproteinase activation. When concentrations of plasminogen and TNF-α were selected to maximize the amounts of activated collagenase and stromelysin, the level of TIMP-1 also increased, and collagen and PG degradation did not increase.

In the studies of Chapter 4, monocytes and monocyte-conditioned medium induced collagenase and stromelysin secretion by SMCs through an IL-1-dependent mechanism. Furthermore, these metalloproteinases could be activated in the absence of exogenous plasminogen. Metalloproteinase activation did not occur consistently, and appeared to depend on the phenotypes of both SMCs and monocytes. Monocytes also did not affect TIMP-1 levels consistently. When levels of activated collagenase and stromelysin increased and TIMP-1 levels decreased in one experiment, matrix degradation did increase, but the role of lysosomal enzymes released by cell death was not ruled out.

This thesis highlights the potential role of SMCs and cytokines in the weakening of fibrous caps and demonstrated the complexity of regulatory mechanisms involved in the secretion, activation, and inhibition of metalloproteinases.
Chapter 2. Effects of Cytokines on Metalloproteinases and Matrix Degradation

2.1 ABSTRACT

Smooth muscle cells (SMCs), endothelial cells, macrophages, and T-cells are all potential sources of cytokines in a fibrous cap. Some cytokines present in fibrous caps are known to stimulate metalloproteinase secretion by SMCs in culture. The purpose of this study was to determine if the addition of these cytokines to SMC-collagen gel cultures would accelerate degradation of proteoglycans and collagen. Results of pulse-chase studies and Western analysis of culture media indicated that while interleukin-1, tumor necrosis factor alpha, and platelet-derived growth factor increased levels of collagenase and stromelysin, these matrix metalloproteinases remained primarily in zymogen forms, levels of tissue inhibitor of metalloproteinase type 1 also increased, and there was no increase in matrix degradation.

2.2 INTRODUCTION

Various cytokines can affect the metabolism of extracellular matrix by regulating the secretion of matrix molecules, matrix metalloproteinases (MMPs), and tissue inhibitors of MMPs (TIMPs). Possible sources of cytokines in atherosclerotic plaques include T-cells, endothelial cells, macrophages, and SMCs. The upregulation of platelet-derived growth factor (PDGF), interleukin-1 (IL-1), and tumor necrosis factor alpha (TNF-α) in plaque tissue provides further evidence that cytokines may play a major role in atherosclerosis and potentially in the development of unstable plaques.

PDGF, IL-1, and TNF-α can stimulate secretion of MMPs by cultured vascular SMCs. The purpose of this study was to determine if the addition of these cytokines to SMC-collagen gel cultures would increase the loss of proteoglycans (PGs) and collagen from the matrix. Experimental results showed that while all three cytokines increased collagenase and stromelysin levels, there was little or no activation of the zymogens, and TIMP-1 levels also increased. IL-1 and TNF-α had no effect on collagen or PG degradation, and PDGF slightly decreased collagen loss.
2.3 MATERIALS AND METHODS

Cell culture and pulse-chase experiments. Cell harvesting from explants, establishment of SMC-collagen gel cultures, pulse-chase protocols, and analysis of samples were performed as described in Appendix A. Briefly, SMCs from human saphenous veins were grown in monolayers for several passages before being suspended in gels of bovine dermal collagen. Gel cultures were maintained for 12 days in media containing 10% fetal calf serum, after which the cultures were serum-free. On the 14th day, PGs and collagen were radiolabeled with 65 μCi/mL ^35^S-sulfate and 170 μCi/mL ^3^H-proline for 24 hrs. After unincorporated radiolabel was washed out, the cultures were treated with 10 ng/mL recombinant human cytokines: IL-1α (gift of C. Dinarello), TNF-α (Genzyme), or PDGF-BB (Genzyme). This concentration of these cytokines had been shown to stimulate strong synthetic responses in human vascular SMCs.23,50

Every 24 hrs for up to 8 days of chase, media were collected and replaced with fresh media containing cytokines. The media for some control gels contained carrier for IL-1α and TNF-α (5 μL of 0.5% human serum albumin added to each milliliter of medium), and the media for other control gels contained carrier for PDGF-BB (5 μL of 0.5% human serum albumin in 1 M acetic acid added to each milliliter of medium). On the last day, the gels were also collected. In one experiment, IL-1α was inadvertently omitted from the media during the first 3 chase days.

Analysis of SMC-collagen gels and media. Radiolabeled collagen and PGs in gels and media, as well as total GAG and DNA contents of gels were measured as described in Appendix A. The media from some chase days of the same gel were pooled before analysis. Western analyses for interstitial collagenase, stromelysin, and TIMP-1; and gelatin zymography were performed on media samples as described in Appendix B. Rabbit antibodies against human collagenase, stromelysin, and TIMP-1 were the kind gift of Dr. Michael W. Lark.

2.4 RESULTS

Effects of cytokines on loss of matrix molecules from SMC-collagen gels. None of the three cytokines increased the loss of radiolabeled proteoglycans or
collagen over 8 days of treatment (Figure 2.1). PDGF slightly decreased the loss of newly synthesized collagen (P = 0.02; t-test).

The DNA content of cytokine-treated SMC-collagen gels was not significantly different from that of controls (data not shown). The average DNA content of all the gels was 3.0 ± 0.5 μg/gel (mean ± SD). PDGF, but not IL-1 or TNF-α (Figure 2.2), caused a 30% increase in total GAG content of gels (P = 0.04; t-test).

Effects of cytokines on MMP and TIMP-1 secretion. IL-1, TNF-α, and PDGF all increased collagenase, stromelysin, and TIMP-1 levels compared to controls, as determined by Western analysis (Figure 2.3). Very little of the induced MMPs were in fully activated forms. In the experiment represented in Figure 2.3, collagenase and stromelysin induction by PDGF at 10 ng/mL was less than that of IL-1 or TNF-α at the same concentration, and TIMP-1 induction by PDGF was greater than that of IL-1 or TNF-α. However, the relative potencies of the cytokines varied among experiments using different sources of SMCs.

2.5 DISCUSSION

The results of this study demonstrated that PDGF, IL-1, and TNF-α, which are upregulated in atherosclerotic plaques, can stimulate secretion of collagenase and stromelysin by cultured vascular SMCs. The increased presence of these MMPs, however, did not accelerate the degradation of collagen or PGs as determined by pulse-chase studies. Very little of the induced collagenase and stromelysin was activated, and the small quantity that was activated may have been inhibited by increased TIMP-1 levels.

Activation of MMPs is not well understood, but may involve autolysis, plasmin, membrane-type metalloproteinases, and other pathways. The required pathways are available in atherosclerotic lesions, where MMP activity has been detected in situ. The addition of a single cytokine to the serum-free SMC-collagen gel culture system may not provide conditions conducive to MMP activation. The concentration of MMPs induced by a single cytokine may not suffice for efficient autolytic activation. Plasminogen, the source of plasmin in vivo, is not available in
Figure 2.1.A. Effects of cytokines on loss of collagen. Collagen was radiolabeled in smooth muscle cell-collagen gels, which were then treated with 10 ng/mL IL-1, TNF-α, or PDGF in serum-free media. Culture media were collected and replaced with fresh media and cytokines each day. Radiolabeled collagen was measured in chase media and in gels at the end of the chase period. Labeled collagen was measured as tritiated hydroxyproline. For each control, n = 2; for each experimental condition, n = 4. Error bars are standard deviations.
Figure 2.1.B. Effects of cytokines on loss of proteoglycans. Proteoglycans (PG) were radiolabeled in smooth muscle cell-collagen gels, which were then treated with 10 ng/mL IL-1, TNF-α, or PDGF in serum-free media. Culture media were collected and replaced with fresh media and cytokines each day. Radiolabeled PG was measured in chase media and in gels at the end of the chase period. Labeled proteoglycans were measured as macromolecular $^{35}$S-sulfate. For each control, n = 2; for each experimental condition, n = 4. Error bars are standard deviations.
Figure 2.2. *Effects of cytokines on glycosaminoglycan (GAG) content of gels.* Smooth muscle cell-collagen gels were spectrophotometrically assayed for GAG after 8 days of treatment with 10 ng/mL IL-1, TNF-α, or PDGF in serum-free media. For each control, n = 2; for each experimental condition, n = 4. Error bars are standard deviations.
Figure 2.3. Effects of cytokines on metalloproteinase and tissue inhibitor of metalloproteinases type 1 (TIMP-1) secretion. Western analysis was performed on culture media from the eighth day of treatment of smooth muscle cell-collagen gels with 10 ng/mL IL-1, TNF-α, or PDGF in serum-free media. Blots were probed with antibodies against human (A) collagenase, (B) stromelysin, or (C) TIMP-1. In (A) and (B), each lane contained 21 μL of medium; in (C), each lane contained 6 μL of medium. The antibodies against collagenase and stromelysin cross-reacted with human serum albumin (HSA). Locations of bands corresponding to the unglycosylated zymogen (z) and fully activated (a) forms of collagenase and stromelysin are indicated.
this culture system. Other stimuli that are present in a fibrous cap, such as other cell types and the soluble mediators that they secrete, may be required for MMP activation.

TIMP-1 secretion may also play a physiologically important role in regulating collagenase and stromelysin activity. TIMP-1 inhibits the activity of these MMPs by binding in a 1:1 molar ratio. The parallel increase in TIMP-1 with MMPs in response to cytokines may be a mechanism of SMCs to limit degradation.

In the case of PDGF, the increase in TIMP-1 may have exceeded the increase in collagenase, such that TIMP-1 inhibited some of the baseline collagenase activity. Another possible explanation for decreased collagen loss in the presence of PDGF may be that a 30% increase of PG in the matrix had the physical effect of slowing the diffusion of collagen fragments out of the gel. Increased PG synthesis is an expected response of SMCs to PDGF.44

Increased MMP activity in atherosclerotic plaques may require the presence of specific facilitators of MMP activation as well as multiple stimuli that override the homeostatic mechanisms of SMCs in favor of increased matrix degradation.
Chapter 3. Interactions of TNF-α with the Plasminogen and Metalloproteinase Systems

3.1 ABSTRACT

Tumor necrosis factor alpha (TNF-α) induces matrix metalloproteinase (MMP) secretion in cultures of human vascular smooth muscle cells (SMCs), but in serum-free conditions the MMPs remain largely in latent forms. Plasmin has been shown in vitro to activate MMPs. The purpose of this study was to determine if physiological levels of plasminogen added to SMC cultures would activate TNF-α-induced MMPs and cause an increase in matrix degradation. Initial results indicated that (1) the addition of TNF-α and plasminogen each caused an increase in MMP secretion, (2) MMP activation correlated with increasing plasminogen, (3) increasing TNF-α could inhibit plasminogen-induced activation, and (4) TNF-α increased levels of tissue inhibitor of metalloproteinases type 1 (TIMP-1). TNF-α increases plasminogen activator inhibitor type 1 (PAI-1) secretion, as determined by enzyme-linked immunosorbent assay of culture media, and inhibits conversion of plasminogen to plasmin. Plasminogen also increases PAI-1 levels. However, even in cases in which concentrations of TNF-α and plasminogen are chosen to maximize the amount of activated MMPs in the culture, TIMP-1 levels also increase, and consequently matrix degradation does not usually increase. These results demonstrate coordination and negative feedback mechanisms of cytokines and serine proteases in regulating MMP secretion, activation, and inhibition.

3.2 INTRODUCTION

Vascular smooth muscle cells secrete matrix metalloproteinases (MMPs) as zymogens that require extracellular activation. The serine protease plasmin can activate latent collagenase and stromelysin in vitro and may perform this role in vivo in some tissues. Plasmin, in turn, must be generated from plasminogen by plasminogen activators, and plasmin may directly degrade several components of extracellular matrix. Vascular SMCs can synthesize plasminogen activators as well as
Plasminogen activator inhibitors. Plasmin may also regulate remodeling beyond its role in activating latent MMPs; Werb and Aggeler reported that cultured fibroblasts treated with plasmin increase collagenase secretion. Therefore, regulation of vascular extracellular matrix degradation can occur at multiple levels involving the secretion and activation of MMPs. Furthermore, tissue inhibitor of metalloproteinases (TIMPs) may inhibit the activity of the activated enzymes.

Other investigators have demonstrated that interleukin-1 (IL-1), platelet-derived growth factor (PDGF), and tumor necrosis factor alpha (TNF-α) induce interstitial collagenase and stromelysin synthesis by cultured vascular SMCs. The results of Chapter 2 showed that these cytokine-induced MMPs remained mostly unactivated. Not surprisingly, collagen and proteoglycan degradation did not increase when SMCs were treated with IL-1, PDGF, or TNF-α.

The study described in the present chapter investigated the role of plasminogen, with and without TNF-α, on the synthesis and activation of MMPs by human vascular SMCs in monolayer cultures. Experimental results show that both plasminogen and TNF-α induce the synthesis of collagenase and stromelysin, while plasminogen also induces cleavage of the latent MMPs to the active forms. In addition, both plasminogen and TNF-α induce plasminogen activator inhibitor-1 (PAI-1), providing a negative feedback mechanism for the matrix degradation response. These results demonstrate coordination of cytokines and serine proteases in regulating matrix remodeling by MMPs. Treatment of SMC-collagen gel cultures with a combination of plasminogen and TNF-α that strongly augments activated MMP levels does not increase collagen and proteoglycan degradation. This indicates the presence of an additional regulatory mechanism, probably involving TIMP-1, the secretion of which also increased when SMC cultures were treated with plasminogen and TNF-α.

3.3 MATERIALS AND METHODS

Materials. Dulbecco's modified Eagle's medium (DMEM), Ham's F-12 medium, and Limulus amebocyte lysate test kit were obtained from BioWhittaker (Walkersville, MD); fetal calf serum from Hyclone Laboratories (Logan, UT); tissue culture dishes from Costar (Cambridge, MA); ascorbate-2-phosphate from Wako Pure...
Chemical Industries (Osaka, Japan); non-essential amino acids, human lys-
plasminogen, kringle 1-3 and kringle 4 from human plasminogen, aprotinin, and
endotoxin from *E. coli* 0111:B4 from Sigma Chemical Co. (St. Louis, MO); insulin
and transferrin from Collaborative Research (Bedford, MA); TNF-α from Genzyme
(Cambridge, MA); plasmin and goat anti-human plasminogen IgG from Enzyme
Research Laboratories (South Bend, IN); Centricon-10 microconcentrators from
Amicon (Beverly, MA); enhanced chemiluminescence detection system from
Amersham (Arlington Heights, IL); goat anti-rabbit IgG antibody with horseradish
peroxidase from Bio-Rad Laboratories (Richmond, CA); and enzyme-linked
immunosorbertent assay (ELISA) kits from Biopool AB (Umea, Sweden). Rabbit anti-
human antibodies to human collagenase and stromelysin were prepared as previously
described.  

**Cell culture.** Medial-layer explants were cultured from unused portions of
human saphenous veins from coronary bypass surgery. The culture medium was
DMEM with 10% fetal calf serum, 25 mmol/L Hepes, 100 U/mL penicillin, 100
µg/mL streptomycin, 1.25 µg/mL amphotericin B, and 2 mmol/L L-glutamine. This
medium is selective for SMCs over endothelial cells. Cells in some experiments
were stained for alpha actin. Unlike cultured rat vascular SMCs, cultured human
vascular SMCs do not uniformly stain for alpha actin. Approximately 50% of cells in
this study stained positively; this level is similar to other human vascular SMC
preparations in our laboratories. Human dermal fibroblasts used as a control did not
stain for alpha actin (<5%).

SMCs from the explants were grown in tissue-culture flasks. At confluence,
the cells were split 1:3 using 0.25% trypsin and EDTA. The cells were cultured in
monolayer for 2 to 6 passages before use in experiments.

*Treatment with plasminogen and TNF-α.* Cells were plated in 22-mm or 35-
mm culture wells at 1-2.5 × 10⁴ cells/cm² and 0.26 mL/cm² of medium. The medium
was the same as that described above, further supplemented with 0.07 mmol/L
ascorbate-2-phosphate, 0.1 mmol/L non-essential amino acids, and 0.75 mmol/L
sodium sulfate. After 2 days, the medium was removed and replaced with 0.13
mL/cm² of fresh medium. After 2 more days, serum was removed from the wells by
washing 4 times with 0.26 mL/cm\(^2\) of IT medium (equal volumes of DMEM and Ham's F-12, with 12.5 mmol/L Hepes, 100 U/mL penicillin, 100 μg/mL streptomycin, 1.25 μg/mL amphotericin B, 1.5 mmol/L L-glutamine, 0.07 mmol/L ascorbate-2-phosphate, 0.1 mmol/L non-essential amino acids, 0.75 mmol/L sodium sulfate, 1 μmol/L insulin, and 5 μg/mL transferrin). After the last wash, 0.13 mL/cm\(^2\) of IT medium was added to each well. The IT medium was changed at 24 hrs. At 48 hrs, the IT medium was changed again, and human plasminogen and/or recombinant human TNF-α was added to the wells. Cells were also treated with human plasminogen kringles, human plasmin, or aprotinin in some experiments. The media were collected after 24 hrs of treatment. Some wells were counted for total and viable cells using Trypan Blue exclusion following collagenase and trypsin digestion of the cell layer; none of the treatments significantly changed cell numbers or cell viability. Each treatment was performed at least twice in independent experiments.

Pulse-chase experiments were performed using SMC-collagen gel cultures to determine the effects of plasminogen and TNF-α on degradation of radiolabeled endogenous collagen and proteoglycans. Establishment of gel cultures, pulse-chase protocols, and analysis of radiolabeled molecules are described in Appendix A. Briefly, collagen and proteoglycans were labeled for 24 hrs with tritiated proline and \(^{35}\)S-sulfate, respectively. Various concentrations of plasminogen and TNF-α were then added to the cultures. The gels and media were collected after 3 days. Radiolabeled collagen and proteoglycans in the gels and media were then measured. Total GAG and DNA contents of the gels were also assayed as described in Appendix A.

Because bacterial endotoxin can induce a variety of products, including MMPs and PAI-1,\(^{60-62}\) fresh media containing 100 μg/mL of plasminogen or plasmin were assayed for endotoxin using the chromogenic Limulus amebocyte lysate test.\(^{63}\) The plasminogen sample contained 8 pg/mL and the plasmin sample contained 56 pg/mL endotoxin. Fresh medium containing 2 trypsin inhibitor units/mL of aprotinin was similarly assayed and contained 150 pg/mL endotoxin. TNF-α is tested by the manufacturer for endotoxin level by gel clot test, and a concentration of 10 ng/mL TNF-α contains 0.02 pg/mL endotoxin. To test for possible effects due to the contaminating endotoxin, some cells were treated for 24 hrs with up to 10 ng/mL of
endotoxin from E. coli; no significant effect on PAI-1 or MMP synthesis was seen at these concentrations of endotoxin.

**Electrophoresis and immunoblotting (Western analysis).** Because the levels of response varied with the cell source in these primary cultures of SMCs, media samples from a TNF-α concentration-response experiment were concentrated up to 20-fold in Centricon-10 centrifugal microconcentrators. All other samples were analyzed without concentration. Samples underwent electrophoresis on sodium dodecyl sulfate/10% polyacrylamide gels under reducing conditions (SDS-PAGE). The proteins were transferred to nitrocellulose membranes, and an enhanced chemiluminescence detection system was used to detect collagenase or stromelysin. A 5% solution of non-fat dried milk in phosphate-buffered saline containing 0.1% Tween-20 was used to block non-specific binding and to dilute the primary and secondary antibodies. The primary antibodies were rabbit anti-human collagenase IgG (0.97 μg/μl; diluted 1:800 or 1:600), rabbit anti-human stromelysin antiserum (diluted 1:400), rabbit anti-human TIMP-1 (diluted 1:1000), and rabbit anti-human TIMP-2 (Amgen) (diluted 1:600). The secondary antibody was goat anti-rabbit IgG conjugated with horseradish peroxidase. Details of the Western protocol are found in Appendix B.

**Enzyme-linked immunosorbent assay (ELISA) for PAI-1.** Media samples were assayed for PAI-1 as previously described. In brief, samples were incubated in microtiter plates coated with monoclonal antibodies against PAI-1, unbound antigen was washed off, and bound antigen was detected by addition of a second specific antibody conjugated to horseradish peroxidase. Standard curves were constructed from dilutions of purified PAI-1.

**Zymography.** Gelatin zymography was performed to assess levels of 72-kD gelatinase and other gelatinolytic proteins in culture media. Details of the protocol are found in Appendix B.

### 3.4 RESULTS

**Effects of plasminogen and plasmin on MMP secretion and activation.** Western-blot analysis demonstrated that plasminogen induced secretion of both collagenase and stromelysin in a concentration-dependent manner in vascular SMCs.
In the absence of plasminogen, SMCs secreted little collagenase in its latent glycosylated and unglycosylated forms, while the lower-molecular-weight, activated forms of the enzymes were not detectable (Figure 3.1.A). Total collagenase secretion increased with plasminogen concentration, although the absolute amounts varied among experiments using SMCs from different sources. These differences are explained by the variability in the magnitude of response to stimuli in primary cultures of SMCs. Plasminogen levels of 50 or 100 μg/mL, depending on the particular experiment, were sufficient to cause activation of the collagenase to the smaller forms. The response of stromelysin secretion to plasminogen was similar (Figure 3.1.B). Total stromelysin levels increased with plasminogen concentration. The unglycosylated and glycosylated activated forms appeared with 50 or 100 μg/mL plasminogen, but only the latent forms appeared at lower concentrations. Some cross-reactivity of the stromelysin antiserum with plasmin is evident near 60 kD.

To explore further the induction of MMPs, vascular SMCs were cultured with plasmin (Figure 3.2). As in the experiments with plasminogen, Western-blot analysis of the culture media showed that both collagenase and stromelysin were induced in a concentration-dependent manner by plasmin; furthermore, activation of the proenzymes to the active forms was also concentration-dependent. Some cross-reactivity of the collagenase antiserum with plasmin is evident near 60 kD.

Treatment of the SMCs with plasminogen lysine-binding-site fragments was performed to examine the possibility of a direct effect of plasminogen on MMP secretion. Kringle 4 did not affect MMP secretion, even at 10 times the molar concentration of plasminogen that increased MMP production (data not shown). Kringles 1-3 cross-reacted with the anti-collagenase and anti-stromelysin antisera, and co-migrated as a smear in the same molecular weight range as activated MMPs, such that detection of activated collagenase and stromelysin was not possible.

Effect of aprotinin on induction of MMPs by plasminogen and plasmin. The activation of collagenase and stromelysin by 100 μg/mL plasminogen or plasmin was completely blocked by treatment with 2 trypsin inhibitor units/mL of the serine protease inhibitor aprotinin (Figure 3.3). Unexpectedly, aprotinin itself caused the induction of latent MMPs in the absence of cytokines, plasminogen, or plasmin.
Figure 3.1. Concentration dependency of induction and activation of matrix metalloproteinases by plasminogen. Cells were treated for 24 hrs with the indicated concentrations of plasminogen (Plgn). Media samples underwent sodium dodecyl sulfate/polyacrylamide gel electrophoresis and immunoblotting with antibody to (A) collagenase or (B) stromelysin. The larger-molecular-weight doublets represent the glycosylated and unglycosylated zymogens. The smaller-molecular-weight doublets represent their activated forms. The lanes marked Pg and Pm contain media with 100 μg/mL plasminogen and plasmin, respectively, that had not been exposed to cells. (The identity of the higher molecular weight species recognized by the antiserum in the third lane is unknown.)
Figure 3.2. *Concentration dependency of induction and activation of matrix metalloproteinases by plasmin.* Cells were treated for 24 hrs with the indicated concentrations of plasmin (Plmn). Media samples underwent sodium dodecyl sulfate/polyacrylamide gel electrophoresis and immunoblotting with antibody to (A) collagenase or (B) stromelysin. The lanes marked Pm contain media with 100 µg/mL plasmin that had not been exposed to cells.
Figure 3.3. *Effect of aprotinin on induction of matrix metalloproteinases by plasminogen and plasmin.* Cells were treated for 24 hrs with 100 µg/mL plasminogen (Pg) or 100 µg/mL plasmin (Pm), with or without 2 trypsin inhibitor units/mL aprotinin (Aprot.). Media samples underwent sodium dodecyl sulfate/polyacrylamide gel electrophoresis and immunoblotting with antibody to collagenase.
Plasminogen or plasmin combined with aprotinin did not increase collagenase levels above those induced by aprotinin alone. The results for aprotinin's effects on stromelysin activation were similar (data not shown).

**Effect of TNF-α on MMP secretion and activation.** Vascular SMCs responded to 0, 0.1, 1, or 10 ng/mL TNF-α with a concentration-dependent increase of collagenase and stromelysin (data not shown). Most of the induced MMPs were in the latent forms, but a small amount appeared in the activated forms. These results are consistent with those reported by Galis et al., in which TNF-α induced *de novo* synthesis of these two enzymes by vascular SMC cultures.48 In the presence of 100 μg/mL plasminogen, the concentration of TNF-α had varying effects on total collagenase secretion in experiments using SMCs from different sources. However, the trend in activation state of collagenase was consistent among independent experiments. While the collagenase was primarily in the active forms with 0, 0.1, and 1 ng/mL TNF-α, most of the collagenase appeared in the latent forms with 10 ng/mL TNF-α (Figure 3.4.A), suggesting that TNF-α induced an inhibitor of MMP activation. The addition of TNF-α to 100 μg/mL plasminogen also had varying effects on total stromelysin secretion by SMCs in independent experiments. However, as with collagenase, 10 ng/mL TNF-α consistently inhibited plasminogen-induced activation of stromelysin, while lower concentrations of TNF-α allowed most of the stromelysin to be activated (Figure 3.4.B).

**Effects of TNF-α and plasminogen on PAI-1 secretion.** The TNF-α inhibition of MMP activation by plasminogen suggested that TNF-α increased serine protease inhibitory activity. TNF-α has been shown to induce PAI-1 secretion in endothelial cells.62 Therefore, measurements of PAI-1 in the culture media were performed. As expected, TNF-α induced PAI-1 in a concentration-dependent manner; at 10 ng/mL, PAI-1 levels were 4.2 fold over control levels (Figure 3.5.A). Surprisingly, plasminogen (Figure 3.5.B) also induced PAI-1 in a concentration-dependent manner (3.6 fold over control at 100 μg/mL). The combination of TNF-α and plasminogen led to significantly higher levels of PAI-1 than either one alone (Student's t-test; p<0.05), but less than would be expected if their effects were simply additive (Figure 3.5.C).
Figure 3.4. *Effect of tumor necrosis factor-α (TNF-α) on activation of plasminogen-induced metalloproteinases.* Cells were treated for 24 hrs with 100 μg/mL plasminogen plus the indicated concentrations of TNF-α. Media samples underwent sodium dodecyl sulfate/polyacrylamide gel electrophoresis and immunoblotting with antibody to (A) collagenase or (B) stromelysin.
Figure 3.5. Individual and combined effects of tumor necrosis factor-α (TNF-α) and plasminogen on induction of plasminogen activator inhibitor (PAI-1). A, TNF-α; B, plasminogen; C, plasminogen with 10 ng/mL TNF-α. Cells were treated for 24 hrs with the indicated concentrations of TNF-α and plasminogen (Plgn). PAI-1 levels were measured by enzyme-linked immunosorbent assay.
3.5.C). In a separate experiment, plasmin (100 μg/mL) induced PAI-1 levels more than 9 fold over control.

*Activity of PAI-1 induced by TNF-α.* To test the activity of the TNF-α-induced PAI-1, the conversion of plasminogen to plasmin in the absence or presence of TNF-α in SMC cultures was determined by Western analysis, using an antibody that recognizes both plasminogen and plasmin (Figure 3.6). In the absence of TNF-α, plasminogen was cleaved to plasmin, although the efficiency of the conversion varied among experiments using different sources of SMCs. In the presence of 10 ng/mL TNF-α, and therefore in the presence of PAI-1, plasminogen activation was almost completely blocked.

*Pulse-chase of collagen and proteoglycans.* Western analysis of media from SMC-collagen gel cultures treated with 100 μg/mL plasminogen and 0, 0.1, or 1 ng/mL TNF-α indicated that the combination of plasminogen and 1 ng/mL TNF-α resulted in the highest levels of activated stromelysin and collagenase (Figure 3.7). This treatment, however, did not increase the loss of radiolabeled collagen or proteoglycans compared to controls treated with only plasminogen (Table 3.1). (Note that because proteoglycans are substrates of plasmin, 3 days of treatment with 100 μg/mL plasminogen alone caused a 2.4-fold increase in loss of radiolabeled proteoglycans compared to untreated controls; data not shown.) Western analysis of TIMP-1 levels in the same media indicated that 1 ng/mL TNF-α caused a slight increase in TIMP-1 (Figure 3.7). Total GAG and DNA contents of the SMC-collagen gels were not affected by plasminogen or different levels of TNF-α (data not shown).

*Zymography of culture media from duplicate experiments with SMC-collagen gels.* indicated that untreated controls contained 72-kD gelatinase, mostly in activated forms (Figure 3.8.A). Increasing concentrations of TNF-α in the presence of 100 μg/mL plasminogen caused decreasing activation of 72-kD gelatinase. This decrease in activation occurred concomitantly with a decrease in TIMP-2 secretion as assessed by Western analysis of the same media (Figure 3.8.B). TNF-α did not affect 72-kD gelatinase activation and TIMP-2 secretion in the absence of plasminogen (not shown). Weak gelatinolytic activity was also detected in bands corresponding to the molecular
Figure 3.6. *Effect of tumor necrosis factor-α (TNF-α) on conversion of plasminogen to plasmin.* Cells were treated for 24 hrs with 100 μg/mL plasminogen in the absence (Pg) or presence (Pg+T) of 10 ng/mL TNF-α. The lanes marked Pg* and Pm* contain media with 100 μg/mL plasminogen and plasmin, respectively, that had not been exposed to cells.
Figure 3.7. *Effect of tumor necrosis factor-α (TNF-α) on metalloproteinase secretion in smooth muscle cell-collagen gel cultures.* Cells were treated with 100 μg/mL plasminogen and the indicated concentrations of TNF-α for 3 days. Media samples underwent sodium dodecyl sulfate/polyacrylamide gel electrophoresis and immunoblotting with antibody to (A) collagenase, (B) stromelysin, or (C) tissue inhibitor of metalloproteinases type 1 (TIMP-1). Locations of bands corresponding to the unglycosylated zymogen (z) and fully activated (a) forms of collagenase and stromelysin are indicated.
Table 3.1. *Effect of TNF-α on loss of matrix molecules.* Percent of radiolabeled matrix molecules lost from smooth muscle cell-collagen gels after 3 days of treatment with 100 μg/mL plasminogen and 0 or 1 ng/mL TNF-α. Labeled collagen was measured as tritiated hydroxyproline. Labeled proteoglycans were measured as macromolecular 35S-sulfate. Values are mean ± SD (n = 4).

<table>
<thead>
<tr>
<th></th>
<th>0 ng/mL TNF-α</th>
<th>1 ng/mL TNF-α</th>
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<tbody>
<tr>
<td>^3H-Collagen</td>
<td>42.2 ± 2.9</td>
<td>39.6 ± 3.8</td>
</tr>
<tr>
<td>^35S-Proteoglycans</td>
<td>68.7 ± 2.3</td>
<td>66.0 ± 1.9</td>
</tr>
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Figure 3.8  *Effects of TNF-α and plasminogen on 72-kD gelatinase and TIMP-2 in smooth muscle cell-collagen gel cultures.* Cells were treated with the indicated concentrations of TNF-α and plasminogen (Plgn) for 3 days. Media samples underwent sodium dodecyl sulfate/polyacrylamide gel electrophoresis and (A) gelatin zymography or (B) immunoblotting with antibody to tissue inhibitor of metalloproteinases type 2 (TIMP-2). Zymogen (Gz) and activated (Ga) forms of 72-kD gelatinase are indicated on the zymogram.
weights of forms of collagenase in samples containing plasminogen and 0 to 10 ng/mL TNF-α.

3.5 DISCUSSION

The matrix degradation process is regulated at several levels. The MMPs are secreted in latent forms and require activation, possibly by plasmin. Once the MMPs are activated, their activity may be inhibited by TIMPs. Vascular SMCs can produce not only matrix molecules and MMP zymogens, but also plasminogen activators, plasminogen activator inhibitors, and TIMPs. Furthermore, vascular cells can vary the expression of plasminogen activator receptors to facilitate or decrease activation of plasminogen. SMCs may also influence the degradation of plasminogen activators.

In this study, we found that plasminogen not only increases MMP secretion and activation, but also induces PAI-1 secretion in SMCs. This concentration-dependent induction of PAI-1 synthesis by plasminogen suggests a negative feedback mechanism for downregulating plasmin's effects (Figure 3.9). If plasmin and MMPs were not tightly regulated, matrix degradation could proceed too rapidly for appropriate remodeling responses by the SMCs. The secretion of PAI-1 could have the dual negative feedback effect of both downregulating plasmin-mediated degradation and downregulating MMP activation. This may provide the cell with a mechanism to control MMP activity even after the MMPs have been synthesized and secreted into the extracellular space. The cell-specificity of this negative feedback mechanism warrants further study, since Etingin et al. found no effect of plasminogen on PAI-1 secretion by cultured vascular endothelial cells.

Although TNF-α increased MMP secretion in SMCs, the MMPs were largely in the latent zymogen forms. We also found that TNF-α at 1 ng/mL and higher concentrations can partially inhibit the activation of MMPs, even in the presence of plasminogen levels sufficient to activate MMPs in the absence of TNF-α. These results agree with the finding that TNF-α increases PAI-1, decreasing the amount of plasmin available to activate MMPs. Analysis by immunoblotting confirmed that PAI-1 induced by TNF-α almost completely inhibited the conversion of plasminogen to plasmin in SMC cultures. Consequently, at higher concentrations of TNF-α, there was
Figure 3.9. *Interactions of TNF-α with the plasminogen and metalloproteinase systems in matrix degradation by vascular smooth muscle cells.* Tumor necrosis factor-α (TNF-α) and plasmin induce the production of latent matrix metalloproteinases (collagenase and stromelysin). Plasmin, which must be generated from plasminogen by plasminogen activator (PA), can activate the metalloproteinases. TNF-α and plasmin also induce plasminogen activator inhibitor (PAI-1). Induction of PAI-1 may represent a negative feedback mechanism to downregulate matrix degradation both by limiting plasmin generation and by reducing activation of matrix metalloproteinases. TNF-α also increases secretion of tissue inhibitor of metalloproteinases type 1 (TIMP-1), which blocks collagenase and stromelysin activity.
much less plasmin available to induce MMP secretion, such that TNF-α played a
greater role than plasmin in increasing MMP levels. On the other hand, PAI-1
induced by plasminogen (or by plasminogen-derived plasmin) did not inhibit
plasminogen-induced MMP activation, probably because sufficient plasmin was
already generated to activate MMPs.

The level of matrix-bound PAI-1 may respond to stimuli differently from PAI-1
in the media. Functionally, matrix-bound PAI-1 is more stable and active. In this
study, only the free forms were assayed. A comparison of the levels of free and
bound forms might provide more insight into their relative importance in matrix
remodeling. Furthermore, changes in plasminogen activator levels may be occurring in
parallel with changes in PAI-1 levels. Thus, although circumstantial evidence
indicates that PAI-1 is inhibiting plasminogen activation, other plasminogen activator
inhibitors synthesized by vascular SMCs such as PAI-2 and protease-nexin I may
play significant roles as well.

The data of the present study agree with the hypothesis that induction and
activation of MMPs by plasminogen are consequences of plasmin activity. The ability
of aprotinin to inhibit plasminogen-induced MMP activation provides further evidence
for the role of plasmin. However, aprotinin inhibits a wide variety of serine proteases,
and therefore it is possible that its effects on MMP activation are via a different
pathway. Similarly, the unexpected induction of latent MMPs by aprotinin in control
experiments may be an indirect consequence of its inhibition of endogenous active
serine proteases.

A direct plasmin-independent effect of plasminogen has not been excluded by
these studies. Treatment of SMC cultures with kringle 4, a lysine-binding elastase
fragment of plasminogen, did not affect MMP levels, but the results of treatment
with kringles 1-3, another lysine-binding fragment, were inconclusive. (The fragment
including kringles 1-3, which is nearly identical to angiostatin, has antimetastatic
properties, at least in part because it inhibits endothelial cell proliferation, a process
required for angiogenesis. Induction of PAI-1 secretion by kringles 1-3, if true,
might provide an additional mechanism against angiogenesis by inhibiting the
activation of metalloproteinases needed for degradation of basement membrane,
another process required for angiogenesis.) Kringle 5, which has a non-lysine binding site, is the main site of interaction of plasminogen with cultured human umbilical vein endothelial cells\textsuperscript{72} and may play a role in interactions of plasminogen with receptors on SMCs. Effects of kringle 5 were not explored in the present study.

Plasmin can directly degrade many extracellular matrix molecules, including fibronectin, laminin, and proteoglycans.\textsuperscript{73} In addition, plasmin can regulate matrix degradation by increasing the secretion and activation of MMPs. Degradation of matrix may cause changes in cell-matrix interactions, changes in the local stresses on cells, and changes in cell shape. These changes could then signal the cell to express proteins that are necessary for remodeling, including MMPs and PAI-1. For example, fibroblasts express collagenase and stromelysin in response to agents that cause loss of stress fibers.\textsuperscript{74} In addition, Werb et al. found that fibronectin fragments but not native fibronectin induced collagenase and stromelysin gene expression in the absence of obvious changes in cell shape, indicating that fibronectin degradation stimulates remodeling activity.\textsuperscript{75} Thus, it possible that both matrix-receptor mechanisms and cytoskeletal changes may explain the induction of PAI-1 by plasminogen.

Secretion of PAI-1 simultaneously with the secretion of MMPs is only one of several mechanisms that SMCs may use to control matrix degradation. Treating cells with a combination of plasminogen and TNF-\(\alpha\) that strongly augmented levels of activated collagenase and stromelysin did not necessarily lead to an increase in collagen and proteoglycan loss from SMC-collagen gel cultures. This may have been due to the presence of TIMP-1. TIMP-1 secretion increased with the addition of TNF-\(\alpha\), although unstimulated levels of TIMP-1 might already be sufficient to block the induced collagenase and stromelysin activity. The addition of TIMP inhibitors would be useful to determine if TIMPs are responsible for blocking the action of activated MMPs in these cultures.

An alternative explanation for the apparent lack of matrix loss due to increased levels of activated collagenase and stromelysin is that constitutive rates of degradation may be relatively high compared to the increased degradation caused by the induced MMPs. Matrix turnover under basal conditions may be mediated by SMC-produced enzymes other than interstitial collagenase and stromelysin. Fibrillar collagen type I
can be digested by 72-kD gelatinase, although the conditions required for collagenolytic activity are not understood. The collagenolytic activity of 72-kD gelatinase was first detected only in the absence of its inhibitor TIMP-2;\textsuperscript{20,76} and TIMP-2 prevents the autocatalytic activation of 72-kD gelatinase;\textsuperscript{42} yet TIMP-2 can also participate in the activation of 72-kD gelatinase by a membrane-type matrix metalloproteinase.\textsuperscript{77} As for proteoglycans, 72-kD gelatinase can also digest their components, as can matrilysin, hyaluronidase, and acid hydrolases such as cathepsins. Zymographic detection of activated 72-kD gelatinase in SMC-collagen gel cultures provides circumstantial evidence for the role of this metalloproteinase in turnover of collagen and proteoglycans. Increasing the concentration of TNF-\(\alpha\) in the presence of 100 \(\mu\)g/mL plasminogen caused a decrease in activation of 72-kD gelatinase. This decreased activity may have been offset by the increased collagenase and stromelysin activity induced by TNF-\(\alpha\).

It is interesting to note that TIMP-2 levels correlated with levels of activated 72-kD gelatinase, though this relationship is difficult to interpret. These results are consistent with the role of TIMP-2 in the activation of 72-kD gelatinase.\textsuperscript{77} However, the decrease in 72-kD gelatinase activation with increased TNF-\(\alpha\) may also result from increased PAI-1 preventing the formation of plasmin, which can activate 72-kD gelatinase.

The degradation of matrix molecules is regulated at every step, from secretion of enzymes to activation of zymogens to inhibition of proteinase activity. This study demonstrated the complex effects of a single cytokine on all of these levels of regulation.
Chapter 4. Effects of Monocytes and Macrophages on Metalloproteinases and Matrix Degradation

4.1 ABSTRACT

In atherosclerotic plaques, macrophages, which are potent sources of cytokines such as interleukin-1 (IL-1) and tumor necrosis factor alpha (TNF-α), cluster at rupture-prone sites and co-localize with increased cytokine expression and metalloproteinase activity. Vascular smooth muscle cells (SMCs) secrete matrix metalloproteinases (MMPs) in response to IL-1 and TNF-α, suggesting that macrophages have the potential to stimulate SMC-mediated matrix degradation. This study explored interactions between human vascular SMCs and human monocytes or macrophages that result in the secretion of interstitial collagenase (MMP-1) and stromelysin (MMP-3). Monocytes and macrophages alone, or those treated with SMC-conditioned media did not secrete these MMPs as detectable by Western analysis. SMCs increased secretion of MMPs when co-cultured with monocytes, or when treated with monocyte-conditioned media. Co-culture with monocyte-derived macrophages or treatment with macrophage-conditioned media induced SMC secretion of MMPs in one experiment, but not in a second experiment. The extent of activation of monocyte-induced collagenase and stromelysin varied among experiments using different sources of monocytes and SMCs. Degradation of radiolabeled collagen and proteoglycans increased with the presence of fully activated monocyte-induced MMPs. Addition of macrophage colony stimulating factor (< 1000 U/mL) to co-cultures of monocytes and SMCs did not affect MMP secretion or activation. Recombinant IL-1 receptor antagonist inhibited collagenase and stromelysin induction in SMC cultures treated with monocyte-conditioned media, while a neutralizing antibody to TNF-α had no significant effect on MMP secretion. In contrast to the induction by monocyte-conditioned media of collagenase and stromelysin secretion by SMCs, monocyte-conditioned media did not alter 72-kD gelatinase (MMP-2) levels as measured by gelatin zymography. Thus, monocytes selectively induce collagenase and stromelysin by an IL-1-dependent mechanism. This response of SMCs to products of activated...
macrophages or foam cells may contribute to plaque destabilization and acute coronary events.

4.2 INTRODUCTION

Recent studies have demonstrated that greater densities of macrophages appear at rupture-prone sites in plaques and correlate with weaker fibrous cap tissue; and increased matrix metalloproteinase (MMP) activity co-localizes with macrophages in plaques. Thus, macrophages may play an important role in promoting matrix degradation that leads to rupture.

While macrophages are potentially a major source of MMPs in a plaque, MMP overexpression by smooth muscle cells (SMCs) may also be important. Cytokines such as IL-1 and TNF-α can stimulate secretion of MMPs by SMCs, and monocyte-derived macrophages in atherosclerotic lesions can produce these cytokines. Thus macrophages might, through the release of cytokines, increase the secretion of MMPs by neighboring SMCs by a paracrine pathway. This study examined the role of interactions between SMCs and monocytes in MMP synthesis. The results demonstrate that cultured human vascular SMCs can increase collagenase and stromelysin secretion in response to non-cell-contact stimulation by human monocytes via an IL-1-dependent pathway, leading to increased matrix loss.

4.3 MATERIALS AND METHODS

Culture of smooth muscle cells. Cell harvesting from explants, establishment of SMC-collagen gel cultures, pulse-chase protocols, and analysis of samples were performed as described in Appendix A. Briefly, SMCs from human saphenous veins were grown in monolayers for several passages before being suspended in gels of bovine dermal collagen. Each gel was formed in a well of a 24-well plate (16 mm) and contained 250,000 to 300,000 SMCs. Gel cultures were maintained for 12 days in media (1 mL/gel) containing 10% fetal calf serum, after which the cultures were serum-free. On the 14th day, PGs and collagen were radiolabeled with 35S-sulfate and 3H-proline for 24 hrs. After unincorporated radiolabel was washed out, the cultures were treated, and then gels and media were collected after 3 days. In some
experiments, the radiolabel was omitted, but the gels underwent the same washing steps. Radiolabeled collagen and PGs in gels and media, as well as total GAG and DNA contents of gels were measured as described in Appendix A.

Experiments were also performed on monolayer cultures of SMCs. Cells were plated at an initial density of 250,000 SMCs per well in 6-well (35 mm) plates with 2.5 mL of medium containing 10% fetal calf serum. The medium was replaced after 2 days with 1.25 mL fresh medium. After an additional 2 days, serum was removed by washing 4 times with 2.5 mL of serum-free IT medium (see Appendix A). Cells were maintained with 1.25 mL of IT medium for 2 days, with a change of fresh medium after 24 hrs, before undergoing experimental treatments for 3 days.

Monocyte and macrophage preparation. Human monocytes were isolated from the peripheral blood of healthy donors undergoing platelepheresis procedures. Each experiment was repeated with cells from at least two different donors. The blood was layered over Histopaque 1077 (Sigma), diluted with RPMI (RPMI-1640 medium [BioWhittaker] containing 10% fetal calf serum, 100 U/mL penicillin, 100 μg/mL streptomycin, 1.25 μg/mL amphotericin B, and 1.5 mmol/L L-glutamine), and centrifuged at 1200g at room temperature. The mononuclear cell layer above the Histopaque was collected and pelleted with RPMI, decanted, and dispersed in 12 to 18 mL of RPMI. Aliquots of 1 mL were placed in 12 to 18 150-cm² polystyrene flasks containing 19 mL of RPMI. The flasks were incubated for 2 hours (37°C, 5% CO₂, 100% RH) to allow monocytes to adhere. Non-adherent lymphocytes were then removed, and the flasks were washed 3 times with 15 mL of Dulbecco's phosphate-buffered saline (PBS) (Gibco). After a wash with 5 mL of Hanks' balanced salt solution without calcium or magnesium (Gibco), 4 mL of 0.25% trypsin and EDTA were added to each flask, followed by 15 minutes of incubation. Monocytes were then harvested with a cell scraper. Flasks were washed with RPMI, and the washes were pooled with the monocytes, which were then pelleted, decanted, and washed two times with IT medium to remove serum. Ninety to 95% of isolated cells were identified as monocytes by Wright's staining. Details of the monocyte isolation procedure are found in Appendix B.
To allow differentiation of monocytes to macrophages, some flasks were treated differently after the PBS washes. The cells were cultured in the flasks for 6 more days in IT medium supplemented with 10% autologous serum, with a medium change on the third day. The cells after 6 days were noticeably different in phenotype, with 90% to 95% of the cells exhibiting pseudopods. These macrophages were trypsinized and harvested as described above.

**Conditioning of media.** Each aliquot of SMC-conditioned medium was prepared by incubating 1 mL of IT medium with a SMC-collagen gel culture for 24 hrs. Monocyte- or macrophage-conditioned medium was prepared by placing $10^5$ freshly trypsin-harvested monocytes or macrophages in plastic tissue-culture wells (16 mm or 35 mm). In some experiments, a film of Vitrogen 100 bovine dermal collagen (0.24 mg/cm²) was cast onto the bottom of the wells before adding monocytes. One milliliter of IT medium containing 1000 U/mL of macrophage colony-stimulating factor (M-CSF) (Genetics Institute) was incubated with the cells for 24 hrs. All conditioned media were filtered in 0.2 μm Spin-X tubes (Costar).

**Experimental treatment of cells.** The media in SMC-collagen gel culture wells were replaced with media conditioned by SMCs, monocytes, or macrophages. The desired combination of recombinant human cytokines, antagonists, and neutralizing antibodies was added to the conditioned media. These included IL-1 receptor antagonist (Synergen), TNF-α neutralizing antibody (R&D Systems), IL-1β (Genzyme), and TNF-α (Genzyme). M-CSF was added to wells containing SMC-conditioned media for a final concentration of 1000 U/mL (concentrations of 0 to 1000 U/mL were used in dose-response experiments). Freshly harvested monocytes or macrophages were added in a small volume to some of these wells ($10^5$/well). Polymyxin B sulfate (Sigma), an inhibitor of endotoxin, was added to some of the wells containing monocyte-conditioned media. The media were collected after 3 days of incubation.

Some monocytes and macrophages were treated with SMC-conditioned media. In these experiments, $10^5$ freshly harvested monocytes or macrophages in a small volume were placed in a well with an aliquot of SMC-conditioned medium and 1000 U/mL of M-CSF. Control wells of $10^5$ monocytes or macrophages were treated with
IT medium containing 1000 U/mL of M-CSF. After 3 days of incubation, the media were collected and filtered through 0.2 μm Spin-X tubes.

Each treatment on cultures of SMCs, monocytes, or macrophages was performed at least twice in independent experiments.

*Western analysis and zymography.* Media samples underwent electrophoresis (21 μL per lane) for collagenase, stromelysin, and tissue inhibitor of metalloproteinases type 1 (TIMP-1); and gelatin zymography (42 μL per lane) as described in Appendix B.

### 4.4 RESULTS

*Effect of monocytes on collagenase and stromelysin secretion by smooth muscle cells.* Little or no collagenase or stromelysin was detected in media from 1-day (conditioned media) or 3-day cultures of SMCs alone (Figure 4.1). No collagenase or stromelysin was detected in media from 1-day or 3-day cultures of monocytes alone. Increasing the cell number tenfold to 10⁶/well did not induce detectable amounts of these MMPs in monocyte-conditioned media. In contrast, co-cultures of SMCs with monocytes consistently contained increased collagenase and stromelysin levels compared to controls, as determined by Western analysis of the culture media (Figure 4.1). These MMPs were present in proenzyme, partially activated, and fully activated forms; the extent of activation varied among experiments using SMCs and monocytes from different sources.

These results indicated an interaction between SMCs and monocytes leading to increased MMP synthesis. To determine if cell contact was required, monocytes were co-cultured on a porous membrane over SMC monolayers. Again, collagenase and stromelysin levels were elevated in co-cultures compared to cultures of either cell type alone (data not shown).

Conditioned media was next used to investigate the possible role of soluble mediators (Figure 4.1). SMCs treated with monocyte-conditioned media increased their secretion of collagenase and stromelysin more than 20 fold, as determined by scanning densitometry of Western blots. Polymyxin B did not block this effect (not shown), suggesting that MMP induction was not caused by endotoxin contamination.
Figure 4.1. Effects of monocytes on induction of smooth muscle cell (SMC) collagenase and stromelysin secretion. Western analysis using antibodies against (A) human collagenase or (B) human stromelysin was performed on 24-hr conditioned media and on media from 3-day cultures of SMCs in collagen gels. The largest-molecular-weight doublets represent the glycosylated and unglycosylated forms of the metalloproteinase zymogens; doublets of decreasing size represent partially and fully activated enzymes. The extent of activation varied among experiments. Unless otherwise noted, monocyte cultures contained $10^5$ cells. Lane 1 (SMC/SMCCM): SMCs alone. Lane 2 (SMC+Mono): Co-culture of SMCs and monocytes. Lane 3 (Mono/MonoCM): Monocytes alone. Lane 4 (SMCCM): SMC-conditioned medium. Lane 5 (Mono/SMCCM): Monocytes cultured with SMC-conditioned medium. Lane 6 (SMC/MonoCM): SMCs cultured with monocyte-conditioned medium. Lane 7 (MonoCM): Monocyte-conditioned medium. Lane 8: (10xMonoCM) Monocyte-conditioned medium, $10^6$ cells.
Monocytes treated with SMC-conditioned media produced no more collagenase or stromelysin than was already present in the SMC-conditioned media. Thus, a soluble mediator secreted by monocytes could stimulate MMP secretion by SMCs, but SMCs did not secrete a soluble mediator that could induce MMP secretion by monocytes.

The substrate on which the monocytes were cultured, either bare plastic or collagen films, did not affect their ability to produce collagenase or stromelysin, or their ability to induce secretion of these MMPs by SMCs.

**Effects of cytokine inhibitors on induction of MMP secretion.** To identify the potential soluble mediators inducing SMC MMPs, IL-1 receptor antagonist (IL-1ra) or TNF-α neutralizing antibody (anti-TNF) were added to co-cultures and to SMC cultures treated with monocyte-conditioned media (Figure 4.2). Scanning densitometry of Western blots of culture media showed that IL-1ra at a concentration of 10 μg/mL blocked 94% of the collagenase and 96% of the stromelysin secretion that was induced by monocyte-conditioned media. Induction by monocyte co-culture was inhibited by IL-1ra to similar levels. Anti-TNF at a concentration of 10 μg/mL did not decrease the levels of these induced MMPs.

Control experiments on SMC-collagen gel cultures treated with SMC-conditioned media showed that IL-1ra alone did not affect collagenase and stromelysin levels, while anti-TNF alone increased these levels slightly (Figure 4.2). Positive controls showed that 10 ng/mL IL-1β increased collagenase and stromelysin levels more than 20 fold, an effect that was blocked 100% and 99%, respectively, by 10 μg/mL IL-1ra. Similarly, 10 μg/mL anti-TNF blocked most of the collagenase and stromelysin secretion induced by 10 ng/mL TNF-α in SMC cultures treated with SMC-conditioned media.

**Effect of M-CSF on monocyte-induced secretion of collagenase and stromelysin.** To determine if M-CSF was responsible for monocyte-induced MMP secretion, SMCs were co-cultured with monocytes in the presence of 0 to 1000 U/mL M-CSF. This hematopoietic growth factor did not affect the total quantity or level of activation of monocyte-induced collagenase (Figure 4.3) as determined by Western analysis. M-CSF also lacked an effect on monocyte-induced stromelysin (not shown).
Figure 4.2. Effects of cytokine inhibitors on induction of smooth muscle cell (SMC) collagenase and stromelysin secretion. Western analysis using antibodies against (A) human collagenase or (B) human stromelysin was performed on media. The largest-molecular-weight doublets represent the glycosylated and unglycosylated forms of the metalloproteinase zymogens; doublets of decreasing size represent partially and fully activated enzymes. All cultures contained 0.025% human serum albumin (HSA). The antibodies cross-reacted with HSA, which appears at the top of each lane. Media samples were from SMC-collagen gel cultures treated for 3 days as indicated. Mono = monocytes; MonoCM = monocyte-conditioned medium; IL-1ra = 10 µg/mL IL-1 receptor antagonist; IL-1β = 10 ng/mL IL-1β; TNFna = 10 µg/mL TNF-α neutralizing antibody; TNF = 10 ng/mL TNF-α.
Figure 4.3. Effect of macrophage colony-stimulating factor (M-CSF) on monocyte-induced secretion of collagenase by smooth muscle cells (SMCs). Western analysis using antibodies against human collagenase was performed on media from 3-day cultures. The largest-molecular-weight doublets represent the glycosylated and unglycosylated forms of the collagenase zymogen; doublets of decreasing size represent partially and fully activated collagenase. All cultures contained 0.025% human serum albumin (HSA). The antibodies cross-reacted with HSA, which appears at the top of each lane. Media samples were from co-cultures of SMCs in collagen gels and monocytes, treated with the indicated concentrations of M-CSF. The SMCs were from a single source. The monocytes in the cultures represented by the indicated groups were from two different donors.
Effect of monocytes on gelatinase secretion by smooth muscle cells. Gelatin zymography of culture media (Figure 4.4) indicated that SMCs in collagen-gel cultures constitutively secreted 72-kD gelatinase, appearing mostly in the activated form (62 kD). Monocytes alone did not secrete detectable quantities of 72-kD gelatinase. Treatment of SMCs with monocytes or monocyte-conditioned medium did not affect the quantity or activation of 72-kD gelatinase, but caused the appearance of gelatinolytic bands at molecular weights corresponding to forms of collagenase.

Effect of cell phenotypes on MMP induction and activation. Comparing similar numbers of SMCs in three-dimensional gels versus conventional monolayers, the gel configuration consistently resulted in greater activation of collagenase and stromelysin induced by either monocyte co-culture or monocyte-conditioned media as determined by immunoblotting (Figure 4.5). Comparing the effects of monocytes versus monocyte-derived macrophages, the results of one experiment showed that macrophages and macrophage-conditioned media induced less secretion of collagenase and stromelysin by SMCs in both gels and monolayers (Figure 4.5). In a second experiment, macrophages and macrophage-conditioned media did not induce any secretion of these MMPs by SMCs (not shown). Macrophages alone or those treated with SMC-conditioned media did not produce collagenase or stromelysin in either experiment.

Effect of monocytes on matrix loss in SMC-collagen gel cultures. Activation of monocyte-induced MMPs varied among experiments and within experiments using monocytes from different donors (Figure 4.3). In most cases, very little or none of the induced collagenase and stromelysin was detectable in intermediate or fully activated forms. Pulse-chase experiments in SMC-collagen gel cultures did not indicate a correlation between increased loss of radiolabeled matrix molecules and increases in the amount of fully activated MMPs present in the media or decreases in TIMP-1 levels (not shown). In one experiment, co-culture of monocytes with SMCs in gels strongly induced collagenase and stromelysin synthesis and caused complete activation of the MMPs (Figure 4.6.A and B). The level of TIMP-1 in the media from this experiment decreased in the presence of monocytes (Figure 4.6.C), though this effect
Figure 4.4. Effect of monocytes on gelatinase secretion by smooth muscle cells (SMCs). Gelatin zymography was performed on 24-hr conditioned media and on media from 3-day cultures. SMCs were in collagen-gel cultures. Positions of the zymogen (Gz) and activated (Ga) forms of 72-kD gelatinase are indicated. Lane 1 (SMC/SMCCM): SMCs alone. Lane 2 (SMC+Mono): Co-culture of SMCs and monocytes. Lane 3 (SMC/MonoCM): SMCs cultured with monocyte-conditioned medium. Lane 4 (Mono/MonoCM): Monocytes alone. Lane 5 (Mono/SMCCM): Monocytes cultured with SMC-conditioned medium. Lane 6 (MonoCM): Monocyte-conditioned medium. Lane 7 (SMCCM): SMC-conditioned medium.
Figure 4.5. Effects of smooth muscle cell (SMC) culture configuration and monocyte differentiation on secretion and activation of collagenase and stromelysin. Western analysis using antibodies against (A) human collagenase or (B) human stromelysin was performed on 24-hr conditioned media and media from cultures treated as indicated for 3 days. Macrophages (Mφs) were derived from monocytes maintained in culture for 6 days in media with autologous serum. Locations of bands corresponding to the unglycosylated zymogen (z) and fully activated (a) forms of collagenase and stromelysin are indicated. SMCs of lanes 1 to 5, 7, 9, 10, and 19 were in collagen-gel cultures. SMCs of lanes 11 to 16 were in monolayer cultures. Lanes 1 and 11 (SMC/SMCCM): SMCs alone. Lanes 2 and 12 (SMC/MonoCM): SMCs cultured with monocyte-conditioned medium. Lanes 3 and 13 (SMC/MφCM): SMCs cultured with Mφ-conditioned medium. Lanes 4 and 14 (SMC+Mono): Co-culture of SMCs and monocytes. Lanes 5 and 15 (SMC+Mφ): Co-culture of SMCs and Mφs. Lane 6 (Mono/MonoCM): Monocytes alone. Lane 7 (Mono/SMCCM): Monocytes cultured with SMC-conditioned medium. Lane 8 (Mφ/MφCM): Mφs alone. Lane 9 (Mφ/SMCCM): Mφs cultured with SMC-conditioned medium. Lanes 10 and 16 (SMCCM): SMC-conditioned medium. Lane 17 (MonoCM): Monocyte-conditioned medium. Lane 18 (MφCM): Mφ-conditioned medium. Lane 19 (SMC+Mφ): Co-culture of SMCs and Mφs.
Figure 4.6. Effect of monocyte co-culture on levels of collagenase, stromelysin, and tissue inhibitor of metalloproteinases type 1 (TIMP-1) in one smooth muscle cell (SMC)-collagen gel culture. Western analysis using antibodies against (A) human collagenase, (B) human stromelysin, or (C) human TIMP-1 was performed on media from SMC-collagen gels cultured with or without monocytes for 3 days. Locations of bands representing (z) zymogen and (a) fully activated forms of unglycosylated collagenase and stromelysin are indicated. Results are from a single experiment and do not represent general monocyte effects. Middle lanes have been masked for clarity.
was not consistent among multiple experiments. Loss of radiolabeled collagen and PGs from these monocyte-treated gels increased 3.5 fold and 3.2 fold, respectively, compared to controls (Table 4.1). The total GAG content of these monocyte-treated gels was 35% less than that of controls, and their DNA content was 17% less (Table 4.1).

4.5 DISCUSSION

Several recent studies of processes that may lead to atherosclerotic plaque rupture have implicated macrophages as a potential critical cell in compromising the integrity of the extracellular matrix in the fibrous cap. Vascular SMCs and macrophages are both capable of MMP secretion. This study investigated the possible role of macrophage-stimulated SMCs as a source of activated MMPs. Our results indicate that monocytes can stimulate secretion of collagenase and stromelysin by cultured SMCs through signaling pathways that do not require cell contact and are inhibited almost completely by an antagonist of IL-1 receptors. TNF-α did not appear to play a critical role in this regard. The monocyte-induced production of collagenase and stromelysin was selective, as levels of 72-kD gelatinase did not change. Increased levels of fully activated collagenase and stromelysin led to increased loss of collagen and proteoglycans from some SMC-collagen gel cultures.

The simplest interpretation of the data would be that monocytes release IL-1, which directly stimulates SMCs to secrete metalloproteinases. Macrophage-rich foam-cell lesions show an enhanced capacity to produce IL-1 in experimental models. Our observations, however, do not exclude more complex pathways. For example, the effects of monocyte-derived IL-1 or other cytokines may be amplified by inducing autocrine secretion of IL-1 or other MMP-inducing cytokines by SMCs.

Monocytes and macrophages in various states of differentiation and activation have been demonstrated to produce MMPs. In particular, macrophages within atherosclerotic plaques have been associated with MMP secretion. In contrast, under the experimental conditions and cell numbers of the present study, monocytes did not secrete detectable quantities of collagenase or stromelysin; yet under the same
Table 4.1. Effect of monocyte co-culture on smooth muscle cell (SMC)-collagen gel cultures in a single experiment in which monocytes caused secretion and full activation of collagenase and stromelysin (see Figure 4.6). SMCs were co-cultured with monocytes for 3 days. Percent loss of radiolabeled collagen and proteoglycans, total glycosaminoglycan (GAG) content, and DNA content are listed as mean ± SD (n = 2). Individual contributions of SMCs and monocytes to DNA content are not known. Results are from a single experiment and do not represent general monocyte effects.

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<th>Control</th>
<th>Monocyte Co-Culture</th>
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<tr>
<td>Percent of $^3$H-collagen lost from gel</td>
<td>17.0 ± 0.4</td>
<td>58.7 ± 2.6</td>
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<tr>
<td>Percent of $^{35}$S-proteoglycans lost from gel</td>
<td>22.5 ± 1.2</td>
<td>73.1 ± 0.7</td>
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<td>Total GAG content of gel (μg/gel)</td>
<td>10.9 ± 0.7</td>
<td>7.1 ± 0.05</td>
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<td>DNA content of gel (μg/gel)</td>
<td>2.43 ± 0.22</td>
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conditions, they stimulated significant increases in collagenase and stromelysin secretion by SMCs. Macrophages also did not secrete detectable quantities of these MMPs, but stimulated their production by SMCs. Secretion of MMPs by macrophages may require an activated state as exhibited by foam cells in atherosclerotic plaques.81

In vitro differentiation of monocytes to macrophages reduced their ability to induce SMC secretion of collagenase and stromelysin. This is consistent with observations that monocytes lose their ability to secrete IL-1 in response to lipopolysaccharide (LPS) stimulation after more than 2 days in culture.87 More puzzling is what stimulus might have caused IL-1 secretion by monocytes and macrophages, since studies have shown that blood monocytes do not constitutively secrete IL-1.88 LPS stimulation was ruled out by the inability of polymyxin B to block MMP induction by monocytes. Contaminating lymphocytes may have secreted interferon-gamma, which enhances stimulated IL-1 secretion by monocytes and macrophages, though this shifts the question to what stimulated the lymphocytes to secrete interferon-gamma.

M-CSF might play a role in the monocyte-SMC interactions. M-CSF, a factor required by monocytes for survival,89,90 is produced by vascular endothelial and smooth muscle cells in vitro and has been detected in atheromatous plaques in humans and experimental animals.91 M-CSF stimulates differentiation of monocytes to macrophages,90 activates or enhances monocyte and macrophage functions such as phagocytosis of tumor cells,92 and induces IL-1 production by macrophages.93 Although elimination of exogenous M-CSF from the culture media did not decrease the secretion of collagenase and stromelysin in co-cultures of monocytes with SMCs, endogenous M-CSF may have been produced by the monocytes.88

An increase in MMP synthesis does not necessarily lead to matrix degradation, because the MMPs are secreted in zymogen form and require activation. In the present study, the degree of activation of collagenase and stromelysin varied among repeated experiments using different sources of SMCs and using the same or different donors of monocytes. It is not clear whether the variation in activation arose from differences among SMCs, differences among monocytes, or both. Monocyte
differences may result not only from differences among donors, but also from the health status and diet of each individual donor.

MMP activation also appeared to depend on the culture configuration of the SMCs. In experiments comparing similar numbers of SMCs in collagen gel cultures versus monolayer cultures, more activation of collagenase and stromelysin occurred in gel cultures than in monolayers. The three-dimensional culture configuration may cause phenotypic changes in the SMCs that are more conducive to MMP activation. Mechanisms of activation are thought to involve plasmin-dependent and plasmin-independent pathways, including autolytic reactions, but regulation of these processes is not well understood.

Even an increase in levels of activated MMPs may not result in matrix degradation if TIMPs are also upregulated. Western analysis of culture media from multiple experiments did not reveal a consistent effect of monocytes or monocyte-conditioned media on TIMP-1 secretion by SMCs. Furthermore, increased matrix degradation may not lead to matrix weakening if synthesis rates of matrix molecules also increase.

When co-culture of monocytes with SMC-collagen gels led to increased levels of fully activated MMPs and decreased levels of TIMP-1 in one experiment, matrix loss increased. However, DNA content of these gels decreased, suggesting that monocytes caused some cell death, which may have released lysosomal enzymes that digested collagen and PGs. Treatment with inhibitors of metalloproteinase activity could determine if the increased matrix degradation induced by monocyte co-culture resulted from an increase in MMPs.

Davies and co-workers found that the ratio of macrophages to SMCs was higher in ruptured plaques than in intact plaques. It is possible, therefore, that progressive increases in macrophage density near SMCs in the plaque may tip the balance of extracellular matrix regulation by SMCs toward increased degradation. This study indicates possible importance of local cell-cell interactions and paracrine signalling in the regulation of SMC functions that may critically regulate plaque stability.
Chapter 5. Discussion

5.1 SUMMARY

The acute event triggering most fatal myocardial infarctions is the rupture of an atherosclerotic plaque at a critical location in a coronary artery. One factor influencing the likelihood that a particular plaque will rupture is the mechanical stability of its fibrous cap, which depends on the state of the extracellular matrix molecules, especially collagen and proteoglycans (PGs). Vascular smooth muscle cells (SMCs) normally regulate the metabolism of these matrix molecules, and various cytokines can modulate synthesis and degradation of matrix by SMCs. The research of this thesis investigated how cytokines affect the secretion and activation of matrix metalloproteinases (MMPs) and the secretion of tissue inhibitors of MMPs (TIMPs) in SMC cultures modeling fibrous-cap tissue. These studies have shown that cytokines can play a role in increasing matrix degradation, but that the process is regulated at multiple levels.

Initial experiments demonstrated that the cytokines interleukin-1 (IL-1), tumor necrosis factor alpha (TNF-α), and platelet-derived growth factor (PDGF) induced secretion of collagenase and stromelysin by SMCs, but in serum-free conditions, they did not increase degradation of collagen or PGs. The reason appeared to be that very little of the induced MMPs were in fully activated forms. PDGF even decreased collagen degradation, an effect consistent with the observation that PDGF also strongly increased TIMP-1 levels.

To evaluate conditions that promote MMP activation, physiological amounts of plasminogen were added to cultures treated with TNF-α. In SMC cultures, plasminogen was converted to plasmin, which activated MMPs. TNF-α, however, induced the secretion of plasminogen activator inhibitor type 1 (PAI-1) in a concentration-dependent manner. High concentrations of TNF-α inhibited MMP activation by blocking plasmin formation. Plasminogen alone also induced secretion and activation of collagenase and stromelysin, as well as secretion of PAI-1. Even when levels of activated MMPs were greatly increased by plasminogen and TNF-α, TIMP-1 levels also increased, and matrix degradation remained unchanged.
Mounting evidence implicates macrophages in the destabilization of atherosclerotic plaques. Macrophages may have the means to disrupt the metabolic balance that SMCs maintain so well in the presence of IL-1, TNF-α, and plasminogen. To investigate this possibility, interactions between monocytes/macrophages and SMCs were studied. Monocytes induced collagenase and stromelysin secretion by SMCs through an IL-1-dependent paracrine pathway. Activation of these MMPs occurred in the absence of exogenous plasminogen or plasmin, but the extent of activation depended on a variety of factors, including the phenotypes of both SMCs and monocytes/macrophages. Monocytes did not consistently affect TIMP-1 in SMC cultures, at times increasing, decreasing, or having no effect on TIMP-1 levels. When monocytes caused the secretion and activation of MMPs and simultaneously decreased TIMP-1 secretion by SMCs, matrix degradation did increase, but lysosomal enzymes released by cell death may have played a role. Much remains to be learned about the conditions and stimuli required for monocyte/macrophage-induced matrix loss.

5.2 IMPLICATIONS

A better understanding of the processes influencing plaque destabilization will lead to potential therapies for reducing the incidence of myocardial infarctions and other acute coronary events related to plaque rupture. This thesis highlights the role of cytokine-stimulated SMCs as a source of matrix-degrading enzymes. Because many steps are required between cytokine secretion and matrix degradation, the possible pharmacological targets are numerous. These include cytokines, cytokine receptors, MMP activators, activators of MMP activators, receptors involved in activation, MMPs themselves, and MMP binding sites.

While the immediate goal of new therapies would be to stabilize rupture-prone plaques by reducing matrix degradation, such treatments might also cause growth of plaques by accelerating matrix synthesis. If the lesion grows slowly enough, even to the point of total occlusion of the vascular lumen, such a result may still be acceptable, as long as angiogenesis occurs to provide adequate collateral blood flow. However, if the therapy causes relatively rapid stenosis, then the lesion may become
the site of acute coronary events related to vasospasm. Plaque-stabilizing drugs must
avoid this possible consequence.

An understanding of the coordinated effects of cytokines on SMC matrix
metabolism also pertains to studies of atherogenesis and plaque restenosis following
balloon angioplasty. These processes involve disturbances in the normal turnover of
vascular matrix, but in a direction opposite that of plaque rupture. As demonstrated in
Chapter 2, platelet-derived growth factor decreased collagen degradation and increased
proteoglycan accumulation, and therefore may play an important role in atherogenesis
and restenosis.

While this thesis focused on SMCs as a source of matrix-degrading enzymes in
atherosclerotic lesions, macrophages can also produce an array of proteinases capable
of digesting collagen and proteoglycans. Proteinases from these two sources may
serve different functions. For example, MMPs of SMCs may participate primarily in
the accelerated remodeling processes of atherogenesis and restenosis, while
macrophage proteinases may play a more significant part in acute matrix changes
leading to plaque rupture. Until more is known about these processes, however, the
roles of both SMCs and macrophages should be considered.

This research may have implications for inflammation and wound healing as
well. The hallmarks of chronic inflammation parallel those of atherosclerosis:
presence of macrophages, proliferation of fibroblasts (rather than SMCs), formation of
fibrous tissue, and tissue destruction. While the stimuli precipitating inflammation and
atherosclerosis may differ, the ongoing processes share many similarities. For
example, the cytokines IL-1 and TNF-α are implicated in both as signals for increased
proteinase secretion. Likewise, the formation of granulation tissue in wound repair
also involves macrophages, fibroblast proliferation, and fibrosis. The negative
feedback mechanisms of SMCs and the effects of monocytes/macrophages, elucidated
in this thesis in the context of atherosclerosis and plaque rupture, may provide insight
into the roles of fibroblasts and macrophages in inflammation and wound healing.
5.3 FURTHER STUDY

*Monocyte/macrophage activation.* Secretion of IL-1 by monocytes and macrophages reflects activation of those cells. Identification of the activating stimuli *in vivo* would provide another potential target for drug treatment. Possible candidates include SMC- or T-cell-derived cytokines, and lipoproteins that are taken up by macrophages.

*Activation of MMP zymogens.* MMP activation can occur via plasminogen-dependent or plasminogen-independent pathways. Both merit further study, as one may serve as a back-up for the other. A membrane-type matrix metalloproteinase (MT-MMP) can participate in the activation of 72-kD gelatinase, and may play a role in activating interstitial collagenase and stromelysin. Although monocytes sometimes induced the secretion and activation of MMPs in the absence of plasminogen in these experiments, exposure of SMCs to both monocytes and plasminogen may provide conditions for consistent MMP activation.

*Other matrix-degrading enzymes.* This thesis focused on interstitial collagenase and stromelysin as key suspects in matrix degradation. Other SMC and monocyte/macrophage enzymes, such as gelatinases, elastase, hyaluronidase, and cathepsins, are also capable of digesting collagen type I and proteoglycans. The use of recombinant or synthetic MMP inhibitors in culture and *in situ* could confirm the role of MMPs. Identification of specific cleavage fragments in both culture media and cell-associated matrix, both *in vitro* or *in situ*, is another strategy for determining which proteinases may be important in plaque destabilization.

*Arterial versus venous cells.* The experiments of this thesis used venous SMCs because of their availability. Plaque rupture, however, is an issue of arteries, and venous and arterial SMCs are phenotypically different. The major findings of this thesis were duplicated in a pilot experiment using aortic cells (data not shown), with one important exception. While IL-1 alone caused no significant increase in activated collagenase or stromelysin levels and caused an increase in TIMP-1 levels in cultures of both cell types, IL-1 did increase degradation of radiolabeled collagen and proteoglycans in aortic cell cultures, but not in venous cell cultures. This suggests that in aortic cells but not venous cells, IL-1 induces secretion of proteinases other than...
collagenase and stromelysin that digest collagen and proteoglycans. Further experiments with SMCs to study atherosclerotic plaque rupture would likely have more physiological relevance if performed using arterial rather than venous cells.

**Mechanical effects.** Stress concentrations, macrophages, and MMPs all colocalize in the shoulder region of the fibrous cap, which is the most common rupture site in atherosclerotic plaques. Stresses may provide not only the mechanical stimulus initiating the rupture, but also a stimulus for SMCs to produce MMPs, to express cell-surface receptors for MMP activation or for cytokines, or to secrete autocrine and paracrine cytokines, including chemoattractants such as monocyte chemoattractant protein type 1 (MCP-1). Macrophages may respond to mechanical stimuli as well. Stress-induced tissue remodeling is an essential property of other tissues, including bone and cartilage.

**Matrix synthesis.** Effects of cytokines, macrophages, and mechanical stress on matrix synthesis should be considered along with their effects on degradation. If increases in degradation are matched by increases in synthesis, then the result is simply faster matrix turnover. If synthesis increases more than degradation, then the result may be plaque growth. Such studies may provide insight into atherogenesis, or they may suggest therapies for plaque stabilization, if plaque growth could be carefully controlled.

**Correlation of matrix degradation with plaque weakening and plaque rupture.** Mechanical testing of in vitro models of fibrous cap tissue will help to confirm the effects of biochemically measured matrix loss on physical properties such as moduli and strength of the tissue. Ultimately, of course, the correlation between matrix degradation and plaque rupture will be confirmed if plaque-stabilizing drugs are efficacious in the prevention of acute coronary events.
Appendix A. Characterization of the Collagen-Gel Culture System

A.1 ABSTRACT

The three-dimensional culture system of vascular smooth muscle cells in a collagen-gel matrix is phenotypically and mechanically appropriate for the study of extracellular matrix metabolism in the context of atherosclerotic plaque rupture. This culture system was characterized by measuring proteoglycan and collagen synthesis, size distributions of proteoglycans, and degradation of newly synthesized proteoglycans and collagen.

A.2 INTRODUCTION

The three-dimensional culture system was chosen as a model more similar to the in vivo cell environment than that achieved in a monolayer culture system. Vascular smooth muscle cells (SMCs) in the collagen matrix have an elongated spindle shape similar to those in vivo, while SMCs in monolayer appear more flattened and spread. Electron microscopy of cells in both culture systems reveal abundant rough endoplasmic reticulum and Golgi bodies, and sparse myofilaments in the cytoplasm. This indicates that their phenotypes are more synthetic than contractile. While SMCs in the media of undiseased adult arteries exhibit predominantly a contractile phenotype (sparse synthetic organelles and abundant myofilaments), SMCs from atherosclerotic plaques tend to exhibit a synthetic phenotype. Thus, in terms of morphology and ultrastructure, the collagen-gel culture system appears to be a good model of SMCs in plaques.

The culture substrate also affects cell metabolism. For example, vascular SMCs grown on a layer of collagen gel release a smaller proportion of newly synthesized proteoglycans (PGs) into the culture medium as compared to those grown on tissue-culture plastic, and the ratio of dermatan sulfate to chondroitin sulfate is greater in the cultures on collagen. Vascular SMCs cultured within a collagen gel have lower rates of protein and collagen synthesis compared to those on plastic. In addition, the culture substrate can affect responses to cytokines. For example,
transforming growth factor beta (TGF-β) increases the proportion of collagen in total proteins synthesized by SMCs in both collagen-gel cultures and monolayer cultures on plastic, but the effect is significantly lower in gel cultures.\(^{46}\)

The gel culture system has the added advantage of being amenable to mechanical testing. Compressive loads are easily applied, while compressive moduli and other mechanical properties can be measured with minor modifications to standard test equipment. Tensile characterization is technically more challenging, but also possible. Such measurements will be important in future studies to correlate biochemical degradation of matrix molecules with deterioration of mechanical properties of the matrix, a process which may play a role in atherosclerotic plaque rupture.

A.3 MATERIALS AND METHODS

Cell culture. The culture substrate is a 3-dimensional collagen gel made from Vitrogen 100 (Celtrix). Vitrogen 100 is bovine dermal collagen containing 95-98% type I and the remainder type III collagen. Type I and type III collagens constitute approximately 70% and 20%, respectively, of the total collagen in arterial intima; furthermore, in atherosclerosis, the absolute amount of type I collagen increases approximately 10% while type III decreases approximately 10%.\(^{4}\) Collagen type is significant since SMCs respond differently with respect to morphology and proliferation when cultured in contact with type I versus type III collagen gels.\(^{98}\)

Medial-layer explants were cultured from unused portions of human saphenous veins from coronary bypass surgery at the Brigham and Women's Hospital, Boston, MA. The culture medium, DMEM+ (Dulbecco's Modified Eagle Medium [DMEM] [BioWhittaker] with 10% fetal calf serum [Hyclone], 25 mmol/L Hepes, 100 U/mL penicillin, 100 µg/mL streptomycin, 1.25 µg/mL amphotericin B, and 2 mmol/L L-glutamine) is selective for SMCs over endothelial cells.\(^{59}\) SMCs from the explants were grown in monolayers in tissue-culture flasks. When confluent, they were split 1:3 using 0.25% bovine pancreatic trypsin (Worthington Biochemical Corp.) and 0.5 mmol/L EDTA in Hanks' balanced salt solution without calcium or magnesium (Gibco
BRL). The cells were cultured in monolayers for 3 to 6 passages before final trypsinization for use in experiments.

SMC-collagen gels were formed four at a time by combining 4.5 mL of Vitrogen 100 (2.9 to 3.1 mg/mL collagen in 0.012 N HCl), 450 µL of 10x modified Earle's balanced salt solution, 90 µL of 1 N NaOH, and 1 mL of SMCs in DMEM++ (DMEM+ supplemented with 0.07 mmol/L ascorbate-2-phosphate [Wako Pure Chemical Industries], 0.1 mmol/L non-essential amino acids [Sigma], and 0.75 mmol/L sodium sulfate). This mixture was placed in 1.25-mL aliquots into four wells of 24-well (16 mm diam.) culture plates (Costar). Each well contained 250,000 to 300,000 cells. After 2 hrs of gelation in a 37°C incubator, 1 mL of DMEM++ was added to each well. To prevent the gel from becoming spheroid under the influence of cell contraction over the course of a 2-week culture, 8 mm-diameter co-culture membranes (Costar) were placed over the gels in early experiments. In later experiments, we found that the gels would retain a disk-like shape in the absence of co-culture membranes if they were detached from the plastic with a thin spatula after gelation. The medium was changed three times a week. Over the course of a 2-week culture in a humid, 37°C, 5% CO₂ incubator, the gels with co-culture membranes contracted to 1 mm thick and approximately 8 mm in diameter, while the gels without co-culture membranes became 1 to 2 mm thick and 4 to 6 mm in diameter. Control gels without cells were formed at the same time as the other gels; they did not contract.

The concentration in DMEM++ of ascorbate-2-phosphate, which is a stable vitamin C analog, reflects the level of ascorbate found in vivo. Ascorbate is required for crosslinking and hydroxylating collagen and also affects glycosaminoglycan (GAG) metabolism.

Non-essential amino acids and sodium sulfate were included in the culture medium to provide sufficient levels of proline and sulfate such that the addition of radiolabeled proline and sulfate for metabolic studies would not significantly change the total amount of proline in the medium.

Radiolabeling and pulse-chase experiments. Radiolabeling was performed on the fourteenth day after the start of gel cultures. For pulse-chase experiments, serum was first removed from the gels on the twelfth day with six 10-minute washes with 1
mL of IT medium (equal volumes of DMEM and Ham's F-12 medium [BioWhittaker], with 12.5 mmol/L Hepes, 100 U/mL penicillin, 100 μg/mL streptomycin, 1.25 μg/mL amphotericin B, 1.5 mmol/L L-glutamine, 0.07 mmol/L ascorbate-2-phosphate, 0.1 mmol/L non-essential amino acids, 0.75 mmol/L sodium sulfate, 1 μmol/L insulin [Collaborative Research], and 5 μg/mL transferrin [Collaborative Research]). IT medium eliminates the hormones and cytokines found in varying amounts in fetal calf serum, such as platelet-derived growth factor and TGF-β, while providing known amounts of insulin, transferrin, and other molecules required for supporting biosynthesis.

Collagen and other proteins were labeled with tritiated L-proline (Amersham). Proteoglycans were labeled with sodium 35S-sulfate (DuPont NEN). The incorporation experiment used 25 μCi/mL 35S-sulfate and 100 μCi/mL 3H-proline. The pulse-chase experiment used 75 μCi/mL 35S-sulfate and 200 μCi/mL 3H-proline.

After the specified labeling period in the incorporation experiment, unincorporated radiolabeled molecules were washed out exhaustively with phosphate-buffered saline (PBS). Washes were pooled with the spent radiolabeled media.

In pulse-chase experiments, IT medium was used for washing after 24 hrs of radiolabeling, and 1 mL of IT medium with or without 100 μg/mL of trypsin was added to gels following the washes. Gels and media were collected at various timepoints. Medium was exchanged for fresh medium with or without trypsin after 24 and 48 hours.

**Gel-filtration chromatography of proteoglycans.** Incorporated and free 35S-sulfate were separated through pre-packed Sephadex G-25M (PD-10) columns (Pharmacia). Prior to chromatography, gels were digested overnight at 60°C in 1 mL of 125 μg/mL of papain (Sigma). Aliquots of gel digests or media were placed in a solution containing 0.17 mg/mL chondroitin-6-sulfate (Sigma) as a carrier and PD-10 elution buffer (4 mol/L guanidine HCl, 0.1 mol/L sodium sulfate decahydrate, 0.1 mol/L Tris, and 0.025 mol/L EDTA, pH 7.0). The loading volume was 0.5 mL, and twenty-five 0.5-mL fractions were collected. Each fraction was mixed with 3 mL of Ecolume scintillation fluid (ICN) and measured for 3H cpm and 35S cpm (Pharmacia LKB 1211 Rackbeta Liquid Scintillation Counter).
The size distribution of newly synthesized PGs was determined by chromatography after radiolabeling for 24 hours with 61 μCi/mL $^{35}$S-sulfate and washing with PBS. PGs were extracted from gels overnight at 4°C with extraction buffer (4 mol/L GuHCl, 1 mmol/L sodium sulfate decahydrate, 50 mmol/L sodium acetate, pH 6.8) and protease inhibitors (10 mmol/L disodium EDTA, 0.3 mol/L aminohexanoic acid, 1 mmol/L phenylmethanesulfonyl fluoride, 15 mmol/L benzamidine-HCl, 10 mmol/L N-ethylmaleimide, 1 μg/mL pepstatin). Total glycosaminoglycan (GAG) contents of the gel extracts and of the pooled media and PBS wash were spectrophotometrically assayed (see below). Free sulfate was removed from both the gel extracts and the media by Centricon-10 filtration (Amicon) followed by PD-10 chromatography. The macromolecular PD-10 fractions were then concentrated by Centricon-10 filtration. Tritiated water (0.1 μCi/mL) and DNA (8 μg/mL) were added as markers for the total and void volumes, respectively.

Chromatography on 90 cm x 6 mm diameter Sepharose CL-2B gel filtration columns (Pharmacia) was performed under dissociative conditions (4 mol/L GuHCl buffer). The loading volume was 0.5 mL, and sixty 0.63-mL fractions were collected. The media were also chromatographed under associative conditions favorable to PG aggregation (0.5 mol/L sodium acetate, 5 μg sodium hyaluronate [Pharmacia] per 100 μg PG, and 100 μg calf articular PG [gift of John Sandy, Shriner's Hospital, Tampa, FL]), according to the method of Sah. The corresponding associative chromatography of the gel extracts was not done.

**Ion-exchange chromatography of proline and hydroxyproline.** Radiolabeled collagen was measured as tritiated hydroxyproline. Portions of media samples and papain-digested gels were hydrolyzed overnight at 110°C in 6 N HCl. Hydrolysates were diluted with deionized water to 0.9 N HCl and combined with carriers (60 μg/mL of proline, 60 μg/mL hydroxyproline). Samples were then chromatographed on 8 cm x 7 mm diameter columns packed with Dowex 50W-X8 ion-exchange resin (Sigma). Loading volume was 0.5 mL, and forty 1-mL fractions were eluted with 0.9 N HCl at a rate of 0.33 mL/min. Each fraction was mixed with 3 mL of Ecollume scintillation fluid and measured for $^3$H cpm and $^{35}$S cpm. Hydroxyproline eluted before proline.
Elastin assay. Since both elastin and collagen contain hydroxyproline, an assay of radiolabeled elastin was performed to determine what proportion of tritiated hydroxyproline represented elastin. SMC-collagen gels were radiolabeled with tritiated proline as described above. The gels were then lyophilized and sequentially digested according to the method of Jones.\textsuperscript{108} In the first digestion, collagen was removed with 50 μg/mL bovine pancreas trypsin (Sigma) in the presence of 5 mg/mL elastase added to adsorb contaminating elastase. After centrifugation and 3 washes with deionized water, the remaining pellet was lyophilized. Elastin was then solubilized from the pellet with 50 μg/mL porcine pancreas elastase (Sigma). After centrifugation and 3 washes, the pellet was lyophilized, and the remaining collagen was digested with 10 μg/mL bacterial collagenase (Worthington). The remaining solids underwent final centrifugation, washes, and lyophilization. Each digestion proceeded for 16 hrs at 37°C in a buffer of 0.1 mol/L Tris and 10 mmol/L CaCl\textsubscript{2}, pH 7.6. Supernatants and washes were pooled at each step and lyophilized. Identical radiolabeled control gels were processed in parallel, with buffer replacing each enzyme solution. The pellets and supernatants were hydrolyzed and chromatographed on Dowex ion-exchange columns as described above.

Semi-quantitative immunoblotting of bovine collagen. Western analysis of SMC-collagen gels was performed to examine the loss of Vitrogen over 15 days of culture. The gels were treated in the same manner as experimental gels, including changes to IT medium. A fresh acellular control gel and SMC-collagen gels of various ages were lyophilized, digested with pepsin (Worthington) in 1 mol/L acetic acid, and neutralized with sodium hydroxide. Serial dilutions of the digested control gel were used as standards. Aliquots of the standards and the digested SMC-collagen gels were electrophoresed on 8% polyacrylamide minigels (Bio-Rad) with 0.1% sodium dodecyl sulfate (Sigma). The maximum amount of Vitrogen loaded in a lane was approximately 0.5 μg. The proteins were transferred to a nitrocellulose membrane (Bio-Rad) and an enhanced chemiluminescence detection system (Amersham) was used to detect Vitrogen. A 5% solution of defatted dried milk in phosphate-buffered saline containing 0.1% Tween-20 was used to block non-specific binding and to dilute the primary and secondary antibodies. The primary antibody was rabbit anti-human
bovine collagen type I (Biodesign International). The secondary antibody was goat anti-rabbit IgG conjugated with horseradish peroxidase (Bio-Rad).

**DNA assay.** As an approximate measure of cell number, the digested gels were fluorometrically assayed for DNA content using Hoechst 33258 dye. Standards were made by adding known quantities of calf thymus DNA (Sigma) to acellular Vitrogen gels, and then lyophilizing and digesting the gels with papain. Triplicate aliquots of digests (75 μL to 150 μL) were mixed with 2 mL of dye in acrylic cuvettes (Sarstedt). Fluorescence at 458 nm was measured using an excitation wavelength of 365 nm with an SLM-AMINCO SPF-500C Spectrofluorometer.

**GAG assay.** Total glycosaminoglycan (GAG) contents of the digested gels were spectrophotometrically assayed using dimethylmethylene blue dye (Polysciences). Standards were made by adding known quantities of chondroitin-6-sulfate (Sigma) to acellular Vitrogen gels, and then lyophilizing and digesting the gels with papain. Triplicate 20-μL aliquots of digests were mixed with 200 μL of dye in flat-bottom 96-well plates (Nunc). Absorbance at 520 nm was measured with a Molecular Devices Vmax Kinetic Microplate Reader.

**A.4 RESULTS**

**Incorporation of sulfate and proline.** Figure A.1 shows the incorporation of sulfate and of proline as hydroxyproline, calculated from measurements of radiolabeled macromolecular sulfate and radiolabeled hydroxyproline in digests of SMC gel cultures. The incorporation rates (0.17 nmol sulfate/μg DNA/day and 0.045 nmol hydroxyproline/μg DNA/day) are linear over the 24-hr labeling period (r²=0.91 and 0.60, respectively) and are comparable to rates published by other workers for SMCs in culture.

**Estimation of elastin synthesis.** The proportion of total hydroxyproline released into solution by sequential trypsin, elastase, and collagenase digestion of SMC-gel cultures was calculated and compared to amounts released by three enzyme-free incubations performed in parallel on control gels. Further calculations compared hydroxyproline release at each step as a fraction of hydroxyproline remaining in the gels. The results are shown in Table A.1. The excess hydroxyproline released by
Figure A.1. *Estimation of proteoglycan and collagen synthesis rates.* Incorporation of (A) sulfate and (B) proline as hydroxyproline into the vascular smooth muscle cell-collagen gel matrix, calculated from measurements of radiolabeled macromolecular sulfate and tritiated hydroxyproline in papain-digested gel cultures (n = 2).
Table A.1. *Tritiated hydroxyproline released by sequential enzymatic digestion of SMC-collagen gels.* Control gels were incubated in parallel in enzyme-free buffer. Values are mean ± SD (n = 3).

<table>
<thead>
<tr>
<th></th>
<th>Trypsin</th>
<th>Elastase</th>
<th>Collagenase</th>
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</thead>
<tbody>
<tr>
<td><strong>Percent of Total Hydroxyproline</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control gels</td>
<td>30.5 ± 0.3</td>
<td>11.2 ± 1.8</td>
<td>7.7 ± 1.5</td>
</tr>
<tr>
<td>Digested gels</td>
<td>80.4 ± 0.8</td>
<td>11.6 ± 1.3</td>
<td>8.0 ± 2.0</td>
</tr>
<tr>
<td><strong>Percent of Remaining Hydroxyproline</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control gels</td>
<td>30.5 ± 0.3</td>
<td>16.1 ± 2.6</td>
<td>13.3 ± 3.0</td>
</tr>
<tr>
<td>Digested gels</td>
<td>80.4 ± 0.8</td>
<td>59.5 ± 8.5</td>
<td>100.0 ± 0.0</td>
</tr>
<tr>
<td>Difference</td>
<td>50.0</td>
<td>43.4</td>
<td>86.7</td>
</tr>
<tr>
<td><strong>Net Percent of Total Hydroxyproline, Based on Difference in Percent of Remaining Hydroxyproline</strong></td>
<td>50.0</td>
<td>8.5</td>
<td>6.9</td>
</tr>
</tbody>
</table>
elastase over controls is 43.4% of remaining hydroxyproline, or 8.5% of total hydroxyproline.

**Loss of bovine collagen during a 2-week culture.** The Western blot of Vitrogen standards and bovine collagen in SMC-collagen gels of increasing age is shown in Figure A.2. All of the SMC-collagen gels appeared to contain more bovine collagen than the one-gel standard. This suggests that the presence of SMCs inhibits the washing out of some of the Vitrogen, probably because SMCs adhere to collagen. The intensity of bands at 120 kD and 130 kD, representing $\alpha_1$ and $\alpha_2$ helices of collagen, remains approximately constant in gels from Day 3 to Day 15 of culture. The intensity of the band at 220 kD, representing two $\alpha_1$ helices ($2\alpha_1$), decreases with progressing age of culture; the $2\alpha_1$ band of Day 15 has roughly one-quarter the intensity of the $2\alpha_1$ band of Day 3 (visual estimate). The total immunoreactive collagen on Day 15 appears to be no less than one-half the amount on Day 3.

A spectrophotometric assay of total hydroxyproline in SMC-collagen gels\textsuperscript{13,14} indicated that the total hydroxyproline content decreases approximately 35% over 14 days of culture (data not shown). This figure is consistent with the data from Western analysis, assuming that most of the hydroxyproline is from Vitrogen. This is a reasonable assumption, based on estimates of collagen synthesis by smooth muscle cells. Using the above result of 0.045 nmol hydroxyproline/µg DNA/day in newly synthesized collagen, one can estimate that 6 µg of collagen would be synthesized in a typical gel over 14 days. If this figure is multiplied tenfold to account for faster rates of synthesis early in the culture (the synthesis rate was measured in 14-day-old cultures), then 60 µg of human collagen would be an upper estimate in a gel after 14 days of culture, since turnover rates are not being considered. SMC-collagen gels start with 2.8 mg of bovine collagen. Loss of 35% after 14 days would leave 1.8 mg. Therefore, bovine collagen accounts for the great majority of the collagen in SMC-collagen gels, even after 2 weeks in culture.

**Size distribution of proteoglycans.** Figure A.3.A compares results from dissociative Sepharose CL-2B chromatography of PGs in gel extracts and media. The area under each curve is scaled to represent the proportions of radiolabeled PGs found in gel extracts and in media. In this particular experiment, 16% of macromolecular $^{35}$S
Figure A.2. Western blot of bovine collagen type I in pepsinized smooth muscle cell (SMC)-collagen gel cultures. Lanes 1 to 5 represent the indicated dilutions of a fresh acellular Vitrogen gel. Lanes 6 to 10 represent undiluted SMC-collagen gel cultures of the indicated ages.
Figure A.3. *Size distributions of newly synthesized proteoglycans.* (A) Sepharose CL-2B chromatography of macromolecular $^{35}$S-sulfate in extracts of smooth muscle cell-collagen gel cultures and in culture media after 24 hrs of radiolabeling. Chromatography was performed under dissociative conditions (4 mol/L guanidine-HCl). (B) Sepharose CL-2B chromatography of macromolecular $^{35}$S-sulfate in culture media after 24 hrs of radiolabeling. Chromatography was performed under dissociative conditions (4 mol/L guanidine-HCl) and under associative conditions favorable to proteoglycan aggregation (0.5 mol/L sodium acetate, 5 μg hyaluronic acid per 100 μg proteoglycan, and 100 μg calf articular proteoglycan).
was estimated to be in the gel extracts. This fraction, however, may vary greatly; the proportion of macromolecular $^{35}$S found in the gels in the sulfate incorporation experiment described above was 56%. The variability may be a result of metabolic differences among SMCs from different sources; this observation serves to emphasize the need for repeating experiments multiple times using different cell sources.

The CL-2B distributions are similar to those of Lark and Wight for PGs synthesized by vascular SMCs. Larger PGs ($K_v \sim 0.3$) tend to enter the media while smaller PGs ($K_v \sim 0.65$) are the dominant species in the gel. The peak around $K_v \sim 0.65$ may represent biglycan and decorin, small PGs that have been identified in the extracellular matrix of atherosclerotic plaques. Decorin is a likely candidate, as it is known to bind to type I collagen fibrils.

Figure A.3.B compares results from dissociative and associative CL-2B chromatography of PGs in media. The data show that associative conditions decrease the large-PG peak at $K_v = 0.3$ and increase the PG-aggregate peak at $K_v = 0$; that is, primarily larger PGs aggregate. The aggregating PGs may be versican, a large PG that binds hyaluronate and has been identified in human aorta. The associative chromatography described here does not indicate the maximum possible aggregation by PGs in the sample, since conditions for aggregation were not optimized.

Pulse-chase of collagen and PGs. Figure A.4 shows the effect of 100 μg/mL of trypsin on loss of newly synthesized PGs, measured as macromolecular $^{35}$S-sulfate, and newly synthesized collagen, measured as tritiated hydroxyproline, from SMC gel cultures. Untreated control gels lost 29% of newly synthesized PGs and 28% of newly synthesized collagen after three days. Treatment with trypsin increased losses to 94% and 79%, respectively, with most of the additional losses occurring during the first 24 hours.

To rule out the possibility that the effects of trypsin on matrix loss might be the result of enzymatic activity released by trypsin-induced cell death, the DNA contents of gel cultures were measured. Figure A.5.A shows that trypsin had no significant effect on DNA content over the 3-day chase period.

GAG contents of gel cultures were assayed to examine the correlation between loss of newly synthesized (radiolabeled) PGs and loss of total PGs. Figure A.5.B
Figure A.4. Effect of trypsin on loss of (A) macromolecular $^{35}$S-sulfate and (B) tritiated hydroxyproline from smooth muscle cell-collagen gel cultures. Cultures were radiolabeled for 24 hrs prior to Hour 0. At Hour 0, unincorporated radiolabel was washed from the cultures, and trypsin was added. Media were exchanged for fresh media with 100 µg/mL trypsin at Hours 24 and 48. For controls (●), n = 3 at each timepoint. For gels treated with trypsin (▲), n = 2 at each timepoint.
Figure A.5. Effect of trypsin on (A) DNA and (B) GAG contents of smooth muscle cell-collagen gel cultures. Media were exchanged for fresh media with 100 μg/mL trypsin at Hours 0, 24, and 48. For controls (●), n = 3 at each timepoint. For gels treated with trypsin (▲), n = 2 at each timepoint.
shows that the GAG content of gels treated with trypsin decreased 60% over 3 days, while that of control gels remained constant.

A.5 DISCUSSION

These results demonstrate that the SMC-collagen gel culture system can be used to study metabolism of collagen and PGs. Collagen synthesized by SMCs are labeled with tritiated proline and measured as hydroxyproline, with the caveat that up to 8.5% of radiolabeled hydroxyproline represents elastin. PGs are labeled with $^{35}$S-sulfate; both matrix-associated and soluble PGs can be characterized by gel-filtration chromatography. Size and ability to aggregate are measurable characteristics of PGs that are particularly relevant to mechanical properties of the extracellular matrix since PGs and their aggregates contribute to compressive resistance.

The pulse-chase experiments with trypsin demonstrate how the culture system is useful for monitoring the degradation of collagen and PGs in response to experimental stimuli. The radiolabeled molecules, however, represent newly synthesized matrix. Freshly secreted matrix molecules may respond differently from more mature matrix, which may have undergone further crosslinking or other modifications that affect their stability in the presence of enzymes. The spectrophotometric GAG assay is an important tool for confirming that degradation of newly synthesized PGs is representative of overall PG degradation.

A similarly convenient assay is not currently available in this laboratory for the measurement of total collagen. The case of collagen is further complicated by the fact that over 95% of the collagen in a two-week-old SMC-gel culture is bovine collagen rather than collagen synthesized by the SMCs. It is not known how SMCs in such an environment might act differently from SMCs in a completely self-synthesized matrix.

Overall, the SMC-collagen gel culture system is a reasonable model for studying matrix metabolism in the context of atherosclerotic plaque rupture, while also providing a means for mechanical testing of the matrix.
Appendix B. Experimental Protocols

B.1 WESTERN ANALYSIS

B.1.1 SOLUTIONS FOR POLYACRYLAMIDE GEL ELECTROPHORESIS

Reagents are from BioRad.

**Acrylamide Solution, 100 mL (make in hood)**
- 22.2 g acrylamide
- 0.6 g bis-acrylamide
- Store at 4°C.

**1.0 mol/L Tris-HCl pH 8.8, 100 mL**
- 12.1 g Tris base (FW=121.1)
- pH to 8.8 with HCl
- Store at room temp.

**5X Electrode Buffer, 1 L**
- 15 g Tris
- 72 g glycine
- 50 mL 10% SDS
- (pH should be about 8.3)
- Store at 4°C.

**100 mg/mL APS**
- 0.1 g ammonium persulfate
- 1 mL dH₂O
- Make fresh each day.

**1.0 mol/L Tris-HCl pH 6.8, 25 mL**
- 3.0 g Tris base (FW=121.1)
- pH to 6.8 with HCl
- Store at room temp.

**6X Sample Buffer, 10 mL**
- 720 µL 1.0 mol/L Tris-HCl pH 6.8
- 5 mL 60% glycerol
- 2.4 mL 10% SDS
- 0.6 mL 2-mercaptoethanol
- 240 µL 5% bromophenol blue
- 1.04 mL dH₂O
- Store at -20°C in 1.5-mL aliquots.

B.1.2 CASTING MINIGELS

See Section B.1.1 for recipes for solutions.

Resolving gel, 12 mL (one 1.5 mm minigel)

<table>
<thead>
<tr>
<th>Polyacrylamide</th>
<th>6.5%</th>
<th>8%</th>
<th>10%</th>
<th>12%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acrylamide solution (room temp)</td>
<td>3.4 mL</td>
<td>4.2 mL</td>
<td>5.3 mL</td>
<td>6.3 mL</td>
</tr>
<tr>
<td>1 mol/L Tris-HCl pH 8.8</td>
<td>4.5 mL</td>
<td>4.5 mL</td>
<td>4.5 mL</td>
<td>4.5 mL</td>
</tr>
<tr>
<td>10% SDS</td>
<td>120 µL</td>
<td>120 µL</td>
<td>120 µL</td>
<td>120 µL</td>
</tr>
<tr>
<td>dH₂O</td>
<td>3.9 mL</td>
<td>3.1 mL</td>
<td>2.0 mL</td>
<td>1.0 mL</td>
</tr>
</tbody>
</table>

Add just before pouring:
- 60 µL | 100 mg/mL APS (0.5 mg/mL) |
- 6 µL | TEMED |

Allow 2 hrs for polymerization.
Stacking gel, 5 mL (one 1.5 mm minigel)

3% 4.5% Polyacrylamide

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume 1</th>
<th>Volume 2</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acrylamide solution</td>
<td>660 µL</td>
<td>1.0 mL</td>
<td>(room temp)</td>
</tr>
<tr>
<td>1.0 mol/L Tris-HCl</td>
<td>625 µL</td>
<td>625 µL</td>
<td>pH 6.8</td>
</tr>
<tr>
<td>0.125 mol/L</td>
<td>50 µL</td>
<td>50 µL</td>
<td>10% SDS</td>
</tr>
<tr>
<td>0.1%</td>
<td>3.7 mL</td>
<td>3.3 mL</td>
<td>dH₂O</td>
</tr>
<tr>
<td>100 mg/mL APS</td>
<td>25 µL</td>
<td>25 µL</td>
<td>(0.5 mg/mL)</td>
</tr>
<tr>
<td>TEMED</td>
<td>5 µL</td>
<td>5 µL</td>
<td>(boil mixture for 1 min just before adding TEMED)</td>
</tr>
</tbody>
</table>

Add just before pouring:

Allow 30 min for polymerization.

B.1.3 SAMPLE PREPARATION AND ELECTROPHORESIS

Sample Preparation
Mix samples with 6x Sample Buffer, 5:1 by volume (typically 25 µL sample and 5 µL 6x Sample Buffer per lane). Cap tightly and place in boiling water for 6 minutes to denature the proteins. Load up to 25 µL per lane in a 1.5-mm, 15-lane minigel. For molecular weight standards, load 4 µL per lane.

Electrophoresis
Stacking: Run at 175 V constant voltage.
Resolving: Run at 200 V constant voltage.

B.1.4 TRANSFER OF PROTEINS TO NITROCELLULOSE

1. Prepare the transfer apparatus just before you are ready to start the transfer so that the buffer stays as cold as possible:
   a. Place (don't drop) a stir bar in the bottom of the chamber.
   b. Pour some transfer buffer into a plastic tray.
   c. Lay down a gray half-holder.
   d. Lay down a sponge.
   e. Lay down two thin blotting sheets or one thick sheet.
   f. Cut the appropriate corners off a nitrocellulose membrane (see Step 6) and lay it down on the blotting sheet. Always handle the nitrocellulose only by the edges, and preferably only on the edge where the top of the gel will be. The top edge should be closer to the hinges of the holder, and the Lane 1 edge should be on the left.
   g. Make sure the nitrocellulose is completely wetted.
2. When the electrophoresis is done, turn off and disconnect the power supply and remove the cover.
3. Take out the upper chamber and pour out the buffer.
4. Lay the upper chamber on top of the lower chamber and pop out the gel holder. Loosen the screws and slide the glass plates out.
5. Slide out one spacer and use it to pry the glass from the gel.
6. Cut the stacking gel away. Cut off the two corners near Lane 1 of the gel. If the gel is going to be probed for collagenase, also cut off the other lower corner.
7. Handling the gel only at the top edge, place it on the nitrocellulose in an orientation to match the cut corners. Wet the surface of the gel and center the gel on the nitrocellulose by sliding a wet gloved finger along the surface of the gel. Be careful not to tear the gel, and make sure the gel is not stretched when you are done. Eliminate any air bubbles under the gel.
8. Lay blotting sheet(s) on the gel, and then lay a sponge on the blotting sheet.
9. Attach the black half-holder by the hinges and close the holder.
10. Place the holder in the transfer chamber with the hinges up and the black side toward the black water ports.
11. Fill the chamber with cold transfer buffer up to the hinges of the holders. Transfer buffer (1.5 L):

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris</td>
<td>4.54 g</td>
</tr>
<tr>
<td>glycine</td>
<td>21.6 g</td>
</tr>
<tr>
<td>methanol</td>
<td>300 mL</td>
</tr>
</tbody>
</table>

Add deionized water for a final volume of 1.5 L. At room temperature, the pH should be between 8.1 and 8.4, but do not adjust the pH!

12. Lift up and slam down each holder a few times to dislodge large bubbles.

13. Set up the chamber on a stir-plate and hook it up to cold water. Turn on the stirrer and the water.

14. Put on the cover, with the black electrode nearer the black water ports.

15. Hook up and turn on the power supply with the voltage turned all the way up and the current set at 200 mA for 45 min (longer for proteins >80 kD). The voltage should be at least 70 V. If it is 71 - 75 V, discard the buffer after this use. Record this voltage.

16. When the 45 min are up, record the voltage. Turn off and disconnect the power supply. Take one holder out and carefully check the completeness of the transfer by lifting one corner of the gel from the nitrocellulose. If the molecular weight markers are not dark on the nitrocellulose and faint or invisible on the gel, carefully re-assemble the apparatus and run longer.

17. If the nitrocellulose is not going to be probed immediately, seal it in a bag, moist and with minimal air bubbles. Store inside a protective cardboard folder at 4 C.

### B.1.5 PROBING AND DEVELOPING BLOTS

1. Make 75 mL Blotto per membrane. To make 100 mL:

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>nonfat dry milk</td>
<td>5 g</td>
</tr>
<tr>
<td>PBS-T</td>
<td>100 mL</td>
</tr>
</tbody>
</table>

2. Incubate membrane, darker side up, in small tray with 35 mL Blotto on shaker for 1 hr.

3. Meanwhile, make 2 L PBS-T per membrane. To make 4 L:

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaH₂PO₄·H₂O</td>
<td>1.56 g</td>
</tr>
<tr>
<td>Na₂HPO₄</td>
<td>4.08 g</td>
</tr>
<tr>
<td>NaCl</td>
<td>34 g</td>
</tr>
<tr>
<td>Tween 20</td>
<td>4 mL</td>
</tr>
<tr>
<td>dH₂O</td>
<td>4 L</td>
</tr>
</tbody>
</table>

Adjust to pH 7.2 with NaOH.

4. Wash membrane in PBS-T at room temperature using large tray (~100 mL each step):

   - 2 quick rinses
   - 1 x 15 minutes on rocker
   - 2 x 5 minutes on rocker

5. Incubate membrane with desired dilution of primary antibody in 15 mL Blotto for 1 hr on shaker at 37C.

6. Wash membrane in PBS-T as above.

7. Incubate membrane with 15 mL Blotto and 25 μL secondary antibody (goat anti-rabbit IgG conjugated with horseradish peroxidase; stored at 4C) for 30 min on shaker at 37C.

8. Wash membrane in PBS-T as above, plus 2 more 5-min washes.

Membranes can be left in PBS-T longer or stored in Saran Wrap™ at 4C.

9. For each membrane, mix 3.5 mL of each ECL (Amersham) reagent in 15-mL tube. Use separate pipets for each reagent.

10. Unwrap membrane carefully; place darker side up in tray; add reagents.

11. Keep tray in motion until bands are visible in the dark or for up to ~3 min.

12. Place a sheet of Saran on counter; put membrane face down on Saran; pull Saran taut under membrane; fold over edges to wrap membrane.

13. Place membrane face up in film cassette.

14. Turn off lights and load film; expose for 1 min as a first attempt.

15. Develop film. Try different exposures as needed.
B.2 GELATIN ZYMOGRAPHY

Quantities are for two full-size gels, 0.75 mm thick, 10% polyacrylamide. Reagents are from BioRad.

1. Pour two resolving gels. See Section B.1 for further details. Recipe for two gels:

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
<th>Conc.</th>
</tr>
</thead>
<tbody>
<tr>
<td>15.9 mL 22.2% acrylamide / 0.6% bis</td>
<td>10%</td>
<td></td>
</tr>
<tr>
<td>3.0 mL deionized water</td>
<td>(10%)</td>
<td></td>
</tr>
<tr>
<td>13.5 mL 1 mol/L Tris, pH 8.8</td>
<td>(0.375 mol/L)</td>
<td></td>
</tr>
<tr>
<td>360 μL 10% SDS</td>
<td>(0.1%)</td>
<td></td>
</tr>
<tr>
<td>3.0 mL 36 mg gelatin dissolved in deionized</td>
<td>(1 mg/mL)</td>
<td></td>
</tr>
<tr>
<td>water (heat to 60C to dissolve)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>180 μL 100 mg/mL ammonium persulfate</td>
<td>(0.5 mg/mL)</td>
<td></td>
</tr>
<tr>
<td>18 μL TEMED</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

2. Pour 4.5% stacking gels. See Section B.1 for further details. Recipe for each gel:

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
<th>Conc.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0 mL 22.2% acrylamide / 0.6% bis</td>
<td>(4.5%)</td>
<td></td>
</tr>
<tr>
<td>625 μL 1 mol/L Tris, pH 6.8</td>
<td>(0.125 mol/L)</td>
<td></td>
</tr>
<tr>
<td>50 μL 10% SDS</td>
<td>(0.1%)</td>
<td></td>
</tr>
<tr>
<td>3.3 mL deionized water</td>
<td></td>
<td></td>
</tr>
<tr>
<td>25 μL 100 mg/mL ammonium persulfate</td>
<td>(0.5 mg/mL)</td>
<td></td>
</tr>
<tr>
<td>5 μL TEMED</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

3. Prepare samples. Typical quantities per lane in a 15-well gel: 50 μL of culture medium + 10 μL of 6x sample buffer. Boil 6 min.

4. Do electrophoresis:
   Set up electrophoresis apparatus and make 2 L of electrode buffer (see Section B.1.1). Pour 1.4 L into the bottom chamber and 0.6 L into the upper chamber.
   Load samples (50 μL/ lane) and molecular weight markers (8 μL/ lane). Run at 15 mA for 12 hrs for good visualization of collagenase and gelatinases.

5. Wash gel twice for 15 min on a rocker at room temp. in 250 mL of a 2.5% aqueous solution of Triton X-100 to remove SDS and renature the enzymes.

6. Incubate gel overnight at 37C with shaking in 250 mL incubation buffer (for gelatin digestion):

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
<th>Conc.</th>
</tr>
</thead>
<tbody>
<tr>
<td>25 mL 1 mol/L Tris, pH 7.3</td>
<td>(0.05 mol/L)</td>
<td></td>
</tr>
<tr>
<td>735 mg calcium chloride dihydrate</td>
<td>(0.01 mol/L)</td>
<td></td>
</tr>
<tr>
<td>833 μL 30% Brij 35</td>
<td>(0.05%)</td>
<td></td>
</tr>
<tr>
<td>474 mL deionized water</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

7. Stain gel for 10 min on a rocker at room temp. in 25 mL of:

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1% w/v Coomassie Brilliant Blue R-250</td>
<td></td>
</tr>
<tr>
<td>10% v/v glacial acetic acid</td>
<td></td>
</tr>
<tr>
<td>30% v/v methanol</td>
<td></td>
</tr>
</tbody>
</table>

8. De-stain gel with 30-min washes of de-staining solution (same as staining solution, but without Coomassie) as needed to visualize bands (approximately 3 washes).

9. Dry between two sheets of porous cellophane on the gel dryer at 60C for 2 hrs.
B.3 ISOLATION OF MONOCYTES

1. Thaw Sigma Histopaque-1077 at RT. 
   Thaw RPMI 1640 + 10% FCS + L-Glu + Pen/Strep (RPMI+) at 37°C.
2. Keep leukopack warm during transport from hospital.
   Save the identification sticker with the blood bank number, if any, in lab notebook.
3. Aliquot 15 mL of Histopaque to 6 sterile 50-mL centrifuge tubes.
4. Wipe the bag with ethanol, then wipe the top with an alcohol swab.
   Wipe scissors or blade with alcohol swab and cut open bag; swab the opening.
5. Use 25-mL sterile pipets to remove cells; do not touch pipet tip to bag.
   Very slowly drip 15 mL of blood against side of tilted tube, over Histopaque.
   Use a fresh pipet each time; put used pipets back in plastic before discarding.
6. Slowly add 10 mL of RPMI+ to blood (this lowers the viscosity).
7. Centrifuge at 2500 rpm for 20 min at room temp.
8. Prepare a 50-mL tube with 40 mL RPMI+.
9. RBCs & granulocytes are at the bottom; monocytes & lymphocytes are at the interface.
   Use a 5-mL pipet to remove the cells at the interface without picking up Histopaque;
   better to miss some cells than to pick up Histopaque.
   Pool and disperse the cells in the 40 mL of RPMI+.
10. Centrifuge at 1500 rpm for 5 min at RT.
11. Remove media and resuspend cells in 12-18 mL of RPMI+ (depending on size of pellet).
12. Prepare 12-18 T-150 flasks with 19 mL of RPMI+ each.
13. Add 1 mL of cells to each flask; put pipet tip in the medium when dispensing cells.
14. Incubate 2 hrs at 37°C. (Meanwhile, thaw PBS, trypsin, Hanks, IT medium [see Appendix A] 
    at 37°C.) The monocytes will adhere and the lymphocytes and contaminating RBCs will not.
15. Remove the media with the undesired cells.
    Wash adherent cells 3 times with 15 mL of warm PBS (+/- Ca&Mg; doesn't matter).
16. Trypsinize (4 mL) 15 min at 37°C, scrape, wash with 4 mL RPMI+, pool, spin, decant.
17. Wash, spin, and decant twice with 10 mL IT medium; resuspend in IT medium; count cells. Note 
    that monocytes are difficult to disperse and will clump together over time.
18. Add 1000 U/mL macrophage colony-stimulating factor (M-CSF) to monocyte cultures.
References


34. Lendon CL, Davies MJ, Born GVR, Richardson PD. Atherosclerotic plaque caps are locally weakened when macrophages density is increased. *Atherosclerosis.* 1991;87:87-90.


42. Raines EW, Dower SK, Ross R. Interleukin-1 mitogenic activity for fibroblasts and smooth muscle cells is due to PDGF-AA. Science. 1989;243:393-396.


