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Increased Expression of Matrix Metalloproteinases *in Vivo* in Scleritis Tissue and *in Vitro* in Cultured Human Scleral Fibroblasts

Nick Di Girolamo,* Andrew Lloyd,* Peter McCluskey,* Marijan Filipic,† and Denis Wakefield*

From the Inflammation Research Unit, School of Pathology, The University of New South Wales, and the Department of Eye Pathology,† Sydney Eye Hospital, Sydney, Australia*

Scleritis is a sight-threatening inflammatory disorder of the eye characterized by the degradation of scleral matrix. Matrix metalloproteinases (MMPs) are ubiquitous proteolytic enzymes important in physiological and pathological processes, the activity of which is stringently controlled by the action of a family of natural antagonists, the tissue inhibitors of matrix metalloproteinases (TIMPs). We hypothesized that enhanced expression of MMPs, without the negative regulatory influence of TIMPs, may be a key feature of tissue destruction in inflammatory eye diseases, such as scleritis. The aim of this study was to localize and characterize cells expressing MMPs and TIMPs in sclera affected by necrotizing scleritis and, in a parallel study, to establish whether cytokines modulate MMP expression in cultured human scleral fibroblasts. **In situ** ***hybridization and immunohistochemical analyses indicated that resident scleral fibroblasts as well as inflammatory cells such as macrophages and T lymphocytes express stromelysin, gelatinase B, and TIMP-1 in necrotizing scleritis tissue. In addition, cytoplasmic immunoreactivity for tumor necrosis factor-α, an inducer of MMPs, was detected in infiltrating inflammatory cells. Cultured scleral fibroblasts stimulated with the combination of interleukin-1α plus tumor necrosis factor-α increased TIMP-1 mRNA twofold above constitutive levels. By contrast, these cytokines induced a sevenfold increase in the steady-state levels of stromelysin mRNA. Using Western blotting, stromelysin and TIMP-1 protein production paralleled mRNA induction in cytokine-stimulated human scleral fibroblasts. Culture supernatants harvested from cytokine-stimulated human scleral fibroblasts were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis gelatin substrate zymography. Our results revealed a prominent 92-kd gelatinolytic band corresponding to gelatinase B, which was inducible with interleukin-1α. These data provide evidence for our hypothesis, that an imbalance between enzyme/inhibitor ratios may be the underlying mechanism of the tissue destruction characteristic of scleritis. Our results demonstrate the potential involvement of MMPs and their modulation by cytokines produced by infiltrating inflammatory cells in destructive ocular inflammation. (Am J Pathol 1997, 150:653–666)***

Scleritis is a destructive inflammatory eye disease characterized by scleral edema and inflammatory cell accumulation in the sclera. Untreated, the inflammatory process may extend to adjacent tissues, causing uveitis, glaucoma, retinal detachment, and perforation of the globe. Scleritis is commonly classified according to its location and severity to include anterior or posterior forms. Anterior scleritis can be further subdivided into diffuse anterior scleritis, which is the most common and can be the least severe form; nodular anterior scleritis, characterized by localized elevations of the sclera; and necrotizing anterior scleritis, the least common but often the most severe and destructive form.[1] Approximately 50% of cases are idiopathic, with the majority of the other cases having an associated systemic connective tissue disorder.[2] A current hypothesis based on immunohistochemical data[3,4] suggests that scleritis represents a localized primary vasculitis, possibly caused by circulating immune complexes. The in-

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Address reprint requests to Professor Denis Wakefield, Inflammation Research Unit, School of Pathology, The University of NSW, Sydney 2052, Australia.

flammatory cell infiltrate seen in scleritis may directly induce tissue destruction in this disease *via* their secretion of proinflammatory cytokines, such as interleukin (IL)-1α and tumor necrosis factor (TNF)-α, which are known to be important modulators of inflammatory processes. These functionally related cytokines are also known to to be inducers of matrix metalloproteinases (MMPs).[5,6]

MMPs are important in normal connective tissue turnover, wound healing, and angiogenesis.[7,8] They have also been implicated in tissue-destructive diseases such as osteoarthritis[9] and rheumatoid arthritis[10] and with the metastatic activity of some tumors.[11–13] MMPs are a family of zinc-containing enzymes with distinct specificities for certain components of the extracellular matrix. They are divided into three main groups according to their substrate specificities. The collagenases are the most specific of the MMPs, cleaving the triple helix of types I, II, and III collagens at a single site. This causes the collagen molecule to unwind and become susceptible to further proteolytic degradation by another group of enzymes called gelatinases, known to degrade types IV, V, VII, IX, and XI collagens.[14] The three collagenases cloned and characterized thus far include a 55-kd enzyme, interstitial collagenase (MMP-1), secreted by many connective tissue cells; a 75-kd highly glycosylated enzyme, neutrophil collagenase (MMP-8), stored in secondary granules of polymorphonuclear leukocytes; and collagenase-3 (MMP-13), expressed in breast carcinomas and by chondrocytes in human osteoarthritic cartilage.[15] The two gelatinases identified thus far include a 72-kd enzyme, gelatinase A (MMP-2), and a 92-kd enzyme, gelatinase B (MMP-9), secreted by macrophages, connective tissue cells, and tumor cells. The third group of proteases include the stromelysins (MMP-3, MMP-10, and MMP-11) with broader substrate specificity and the ability to degrade a range of extracellular matrix components including collagen III, IV, IX, and X, laminin, proteoglycans, and fibronectin.[14] More recently, a novel group of membrane-associated MMPs have been cloned and termed membrane-type MMPs (MT-MMPs).[16]

The expression of MMP genes can be regulated at several levels, including transcriptional modulation, post-translational processing, and *via* the activity of natural inhibitors. Transcription can be regulated by various cytokines[17] and growth factors[18] as well as tumor promoters such as 12-O-tetradecanoyl-phorbol-13 acetate (TPA).[19,20] The similarities in the pattern of transcriptional regulation of individual members of the MMP gene family are likely to be due to the presence of conserved regulatory elements in their promoter regions.[21] MMPs are secreted as inactive proenzymes that require proteolytic cleavage to generate the mature, active enzyme. A third mechanism regulating the extracellular activity of these enzymes is provided by inhibitors of MMPs. Tissue inhibitor of metalloproteinase-1 (TIMP-1), a 28-kd glycoprotein, is considered the major regulator of MMP activity in tissues. TIMP-1 is secreted by many connective tissue cells including fibroblasts,[22] endothelial cells,[23] and epithelial cells.[24] TIMP acts to inhibit MMPs by binding to form an irreversible, 1:1 stoichiometric complex with the active enzyme.

In physiological circumstances, the balance between MMPs and their natural inhibitors is a crucial factor in maintaining homeostasis of extracellular matrix proteins. It has been postulated that in pathological tissue-destructive conditions, there is an imbalance between MMPs and their inhibitors.[25–28] The purpose of this study was to demonstrate whether metalloproteinases are expressed in scleritis tissue and to characterize their cellular sources. Second, sensitive protein assays were used to establish whether cytokine-stimulated cultured scleral fibroblasts secrete inducible amounts of matrix-degrading metalloproteinases.

Materials and Methods

Patients

Formalin-fixed, paraffin-embedded tissue blocks of necrotizing scleritis were obtained from the Department of Eye Pathology, Sydney Eye Hospital, and the Department of Pathology, St. Vincent's Hospital, Sydney, Australia. Nine specimens were collected and included tissue from six females and three males with a mean age of 55 years (range, 34 to 71 years). Four of the nine patients had underlying systemic disorders; the other five had idiopathic scleritis. Table 1 summarizes some of these features.

Preparation of RNA Probes

The plasmid pFas containing the 1.6-kb human stromelysin-1 was a generous gift from Dr. R. Nicholson, Centre for Immunology, St. Vincent's Hospital, Sydney, Australia. A 587-bp *Xba*I/*Hin*dIII fragment of human stromelysin-1 cDNA was subcloned into the pBluescript-II (SK) transcription vector (Stratagene, La Jolla, CA) and sequenced. The new plasmid construct was linearized within the multiple cloning site using the appropriate restriction enzymes and transcribed using both T3 and T7 RNA polymerase to generate sense and antisense cRNA transcripts. *In*

Table 1. *Clinical Features of Patients with Necrotizing Scleritis*

Patient	Age/sex	Source of tissue	Systemic disease
1	63/F	Enucleation	None
2	57/M	Surgical specimen	None
3	48/F	Surgical specimen	None
4	39/F	Enucleation	Rheumatoid arthritis
5	57/M	Enucleation	None
6	71/F	Surgical specimen	Wegener's granulomatosis
7	66/M	Surgical specimen	Rheumatoid arthritis
8	62/F	Enucleation	None
9	34/F	Surgical specimen	Relapsing polychondritis

F, female; M, male.

vitro transcribed RNA was labeled using a nonradioactive technique (digoxigenin, Boehringer Mannheim, Sydney, Australia). The labeling efficiency and concentration of the cRNA probes was compared with prelabeled controls provided by the manufacturer by dot blot and subsequently electrophoresed on 0.8% agarose/formaldehyde gels as a further check of riboprobe integrity and molecular weight (data not shown). A plasmid containing human TIMP-1 (633 bp) in pBluescript-II (SK) and the plasmid p92MOI, containing the cDNA for human gelatinase B (subsequently subcloned in pBSK-II), were generously provided by Dr W. G. Stetler-Stevenson (National Institutes of Health, Bethesda, MD) and were similarly transcribed to generate digoxigenin-labeled sense and antisense riboprobes.

In Situ *Hybridization*

In situ hybridization was performed as previously described.[29] To minimize any accumulated RNAse contamination, six sections were cut and discarded from all tissue blocks. For hybridization, 4-μm sections were cut and placed on 3-aminopropyltriethoxysilane-treated slides (Aldrich Chemicals, Sydney, Australia). Sections were deparaffinized in Histoclear (Medos, Sydney, Australia), rehydrated through decreasing graded ethanol, and left in phosphate-buffered saline (PBS) treated with diethylpyrocarbonate (BDH Chemicals, Kilsyth, Australia). Sections were deproteinized for 20 minutes at room temperature (RT) in 0.2 N HCl and then placed in 0.3% Triton X-100/PBS for 15 minutes at RT. Tissue was then treated with 5 μg/mL nuclease-free proteinase K (Boehringer Mannheim, Sydney, Australia) in 50 mmol/L Tris/HCl, pH 8.0, 1 mmol/L $CaCl_2$ for 30 minutes at 37°C. Digestion was stopped by rinsing in 0.2% glycine. Sections were then post-fixed in freshly prepared 4% paraformaldehyde for 5 minutes and washed in diethylpyrocarbonate-treated PBS three times. Tissue was acetylated in 0.25% acetic anhydride (BDH Chemicals) containing 0.1 mol/L triethanolamine (BDH Chemicals) for 10 minutes to reduce potential nonspecific binding sites, followed by two rinses in 2X standard saline citrate buffer (1X SSC is 150 mmol/L NaCl, 15 mmol/L sodium citrate, pH 7.0). Sections were covered with approximately 30 μL of prehybridization buffer containing 50% deionized formamide, 2X SSC, 1X Denhardt's solution (0.02% Ficoll, 0.02% bovine serum albumin (BSA), 0.02% polyvinylpyrrolidone), 10% dextran sulfate, 250 μg/ml yeast tRNA, and 250 μg/ml heat-denatured salmon sperm DNA in 0.01 mol/L Tris/HCl, pH 7.5, for 2 hours in a humidified chamber at 42°C. Sections were rinsed briefly in 2X SSC at RT, and 30 μl of hybridization buffer (same as prehybridization buffer) containing 50 ng of digoxigenin-labeled probe was added to each slide. Sections were covered with a coverslip and incubated in a humid chamber overnight at 42°C. After hybridization, the coverslips were removed and the sections washed twice (20 minutes each) in 2X SSC at RT. Background was reduced by incubating the slides in 2X SSC containing 100 μg/ml RNAse A (Boehringer Mannheim) for 30 minutes at 37°C. Sections were rinsed in 1X SSC for 15 minutes at 42°C and then for 20 minutes at 42°C in 0.5X SSC. Hybridized probes were detected using a DIG nucleic acid detection kit (Boehringer Mannheim). Briefly, the detection required an anti-digoxigenin antibody and a color-substrate solution consisting of nitroblue tetrazolium salt (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP; Boehringer Mannheim). After detection, the sections were lightly counterstained with neutral red.

Immunohistochemical Analysis

Formalin-fixed scleritis tissue was cut (4 μm), mounted, dried at 37°C on glass slides, and processed for immunohistochemistry. Tissue was deparaffinized in Histoclear (Medos) and dehydrated through a graded series of ethanol. Sections

were incubated in 0.8% pepsin (Dako Corp., Carpinteria, CA) in 0.01 N HCl at 37°C for 20 minutes or in 10 μg/ml proteinase K (Boehringer Mannheim) at 37°C for 20 minutes, followed by two 5-minute washes in 0.05 mol/L Tris-buffered saline (10X stock TBS contains 0.25 mol/L Tris base, 0.25 mol/L Tris/HCl, 8.5% NaCl, pH 7.6). Endogenous peroxidase was quenched with 3% hydrogen peroxide/methanol for 5 minutes and then washed in TBS and the sections incubated with a 1:5 dilution of the appropriate blocking serum for 20 minutes. Human anti-CD68, human anti-CD3 antibody (Dako) and human anti-TNF-α antibody (Genzyme Diagnostics, Cambridge, MA) were used as primary antibodies at dilutions of 1:50, 1:50, and 1:100, respectively, and incubated for 25 minutes, after which a 1:200 dilution of biotinylated secondary antibody, horse anti-mouse (Vector Laboratories, Burlingame, CA) was applied for 20 minutes. Avidin-biotin complex was then added (Vector) for 30 minutes, followed by 0.03% diaminobenzidine tetrahyrochloride. Sections were lightly counterstained with hematoxylin. Specificity of the reaction was verified by omitting the primary antibody and also by using lymph node tissue as a positive control (micrographs not shown). Sections were viewed under a light microscope and photographs taken using Kodak Ektachrome EPP-100 film.

Human Scleral Fibroblast Cultures

Human sclera was obtained from postmortem eyes from subjects with no past history of eye disease as previously described.[30] Explants were grown in 25-cm^2 tissue culture flasks (Nunc, Roskilde, Denmark) containing Eagle's minimal essential medium (EMEM; Sigma, Sydney, Australia) with 10% fetal calf serum (CSL, Melbourne, Australia) and 100 U/ml penicillin and 100 μg/ml streptomycin. Passaged cells were split 1:3 and used at passages 5 to 10 for all experiments. All cell culture media and solutions used were filtered through Zeta-pore filters (Cuno Filter Systems, Blacktown, Australia) to remove any contaminating endotoxin. Endotoxin levels were monitored in the cultures using a limulus amebocyte lysate assay (Associates of Cape Cod, Falmouth, MA). For Northern, Western, and zymography analyses, 1×10^6 cells were seeded into 75-cm^2 culture flasks and grown for 48 hours in EMEM/10% fetal calf serum (at which stage the cells were 80 to 90% confluent). The culture medium was removed, each flask was washed three times with PBS and three times with EMEM, and the cells were left in serum-free medium (EMEM/0.2% BSA) for 24 hours, after which the serum-free medium was removed and fresh serum-free medium added with known concentrations of recombinant human interferon (IFN)-γ (100 U/ml), IL-1α (20 ng/ml), or TNF-α (50 ng/ml; R&D Systems, Minneapolis, MN). Phorbol myristate acetate (PMA; Sigma), a known inducer of MMPs, was used as a positive control stimulus at 1 ng/ml final concentration. The experiments described in Figures 4 to 6 were performed with human scleral fibroblasts (HSFs) from a single subject. Results similar to those described have been obtained with cells derived from other subjects.

Total RNA Extraction from HSFs

For Northern analyses, total RNA was extracted as previously described.[31] Briefly, conditioned medium (CM) was collected and stored in aliquots at −70°C, and the monolayers were washed twice with ice-cold PBS. Denaturing solution (4 mol/L guanidine thiocyanate in citrate/sarcosine/β-mercaptoethanol was added to each flask. Monolayers were scraped with a rubber policeman and homogenized using a 21-gauge needle attached to a 1-ml syringe. The RNA was extracted with equal volumes of phenol/chloroform/isoamyl alcohol (50:48:2) and precipitated with isopropanol. The resulting pellet was dissolved in diethylpyrocarbonate-treated H_2O and the absorbance measured at 260 and 280 nm to determine the purity and concentration of the RNA. Samples were stored frozen at −70°C in small aliquots until used in Northern blot experiments.

Northern Blot Analysis

Stromelysin, gelatinase B, and TIMP-1 steady-state mRNA levels were determined by Northern blotting. Equivalent amounts of total RNA were loaded into each lane (4 μg) of a 0.8% agarose, 2.2 mol/L formaldehyde gel.[32] For each experiment, the gels were stained with ethidium bromide before transfer to check RNA loading and integrity and then viewed again after transfer to check that equal transfer had taken place. The RNA was vacuum transferred onto Hybond N+ nylon membrane (Amersham, Sydney, Australia) in 10X SSC for 2 hours. Blots were hybridized with digoxigenin-labeled antisense RNA probes. Riboprobes were generated as previously described so that approximately equal labeling and probe length was achieved.[29] Membranes were incubated in prehybridization solution consisting of 5X SSC, 50% formamide, 0.02% sodium dodecyl sulfate (SDS), 0.1% lauroylsarcosine, and 2% blocking solution (Boehringer Mannheim) for 2 hours at 45°C and then hybridized overnight under the same con-

ditions with the appropriate riboprobe. Stringency washes included two rinses in 2X SSC/0.1% SDS at RT and twice in 0.1X SSC/0.1% SDS at 65°C. Hybridized probes were detected using an anti-digoxigenin antibody and Lumigen CSPD (Boehringer Mannheim) as the substrate, essentially as described in Dig Systems User's Guide for Filter Hybridization (Boehringer Mannheim). The membranes were then exposed to Kodak X OMAT AR scientific imaging film for 10 minutes at RT. The Northern blots were scanned using a densitometer (Hoefer Scientific, San Francisco, CA) to obtain semiquantitative data.

Protein Estimation

The total protein concentration in each CM sample was determined using the bicinchonic acid protein assay kit (Pierce, Rockford, IL). Briefly, CM samples from cytokine-stimulated HSFs were diluted 1:50 in PBS and 10 μl of this dilution was placed into the wells of a microtiter plate with 200 μl of working reagent (Pierce kit). The plates were incubated at 37°C for 30 minutes and the optical density measured at 540 nm with a Titertek Multiskan Plus (ICN-Flow, Sydney, Australia) microplate reader. The protein content of each sample was standardized against BSA. There was no significant difference ($F = 0.49$, $P < 0.74$) in the amount of total protein in the CM from any cytokine treatment, as determined by analysis of variance.

Western Blot Analysis

Fibroblast CM was electrophoretically separated on a 4% stacking and 10% resolving acrylamide gel under nonreducing conditions. Proteins were transferred to Immobilon-P membranes (Millipore, Sydney, Australia) using a Trans-Blot semi-dry electrophoretic transfer cell (Bio-Rad, Sydney, Australia) for Western blot analysis as previously described.[33] After transfer, the protein membranes were blocked in TBS containing 2% BSA for 1 hour at RT and then incubated for 1 hour, gently shaking with antibodies to either human TIMP-1 (Fuji Chemicals, Togama, Japan) or human stromelysin (Biogenesis, Poole, UK) in TBS/1% BSA containing 0.1% Tween-20. Membranes were then washed three times in TBS/0.1% Tween-20 for 10 minutes each. Goat anti-rabbit alkaline-phosphatase-conjugated secondary antibody (Dako) was added for 1 hour to detect stromelysin and goat anti-mouse horseradish-peroxidase-conjugated secondary antibody (Dako) added to detect TIMP-1. Membranes were washed once for 10 minutes in each of the following: TBS/0.1% Tween-20, TBS, and finally in buffer consisting of: 100 mmol/L Tris/HCl, pH 9.5, 100 mmol/L NaCl, 50 mmol/L $MgCl_2$. Membranes were then incubated with substrate containing NBT/BCIP in the same buffer. To detect TIMP-1, the membranes were washed three times for 10 minutes each in TBS/0.1% Tween-20 after the secondary antibody, and then a chemiluminescent reagent for nonradioactive detection of proteins was added (Dupont, Sydney, Australia) for 1 minute of shaking. Membranes were exposed to x-ray film and developed as for Northern blotting. This method of detection was necessary as we could not detect TIMP-1 protein using the NBT/BCIP substrate.

SDS-Polyacrylamide Gel Electrophoresis (PAGE) Gelatin Zymography

Gelatin substrate zymography was modified from the technique originally described by Heussen and Dowdle.[34] Bovine type B gelatin (Sigma) was added to a standard 10% acrylamide resolving gel mixture at a final concentration of 1 mg/ml. Gelatin was used as the substrate because it is easily incorporated in the gel mixture and is an appropriate substrate for the gelatinases. CM samples were thawed and diluted 3:1 with nonreducing sample buffer (10% SDS, 4% sucrose, 0.25 mol/L Tris/HCl, pH 6.8, with 0.1% bromophenol blue), and 33 μl was loaded immediately without boiling under nonreducing conditions into the wells of a 4% acrylamide stacking gel. Gels were run using a Mini-Slab gel apparatus (Hoefer Scientific) at 90 V through the stacking gel and then at 120 V through the resolving gel at 4°C. After electrophoresis, the gels were soaked in 2.5% Triton X-100 (Sigma), shaking for 30 minutes at RT with one change of detergent solution to remove the SDS and to allow the proteins to renature. Gels were rinsed and incubated overnight at 37°C in substrate buffer (50 mmol/L Tris/HCl, pH 8.0, 5 mmol/L $CaCl_2$, and 0.02% NaN_3) to allow proteinase digestion of the gelatin substrate. After this incubation, the gels were stained for 45 minutes in 0.1% Coomassie blue R-250 (Bio-Rad) in methanol, acetic acid, and water (4:1:5) and then destained in staining solution without stain, photographed, soaked in gel drying solution (30% methanol, 2.5% glycerol), and dried for a permanent record. Because the active site of the latent MMP becomes available after SDS treatment, this method detects gelatinases even in the proenzyme form. Enzymatic activities were identified as clear zones (lytic bands) in a blue-stained background. Broad-range molecular weight standards

(Bio-Rad) were run in adjacent lanes. Metalloproteinase activity was verified by adding various proteinase inhibitors, both to the 2.5% Triton X-100 solution and to the substrate buffer.[35] MMP inhibitors included ethylenediaminetraacetic acid (EDTA) to a final concentration of 10 mmol/L and 1,10-phenanthroline (1 mmol/L) (Sigma). Serine proteinase inhibitors included 2 mmol/L phenylmethylsulfonyl fluoride and 2 mmol/L 4(-2-aminoethyl) benzene-sulfonyl fluoride (Sigma). Finally, leupeptin, an inhibitor of serine and cysteine proteases, was used at 1 mmol/L final concentration. In addition, *p*-aminophenyl mercuric acetate (APMA) was incubated with CM samples at a final concentration of 1 mmol/L for 1 hour at 22°C. APMA is an organomercurial that relaxes the structure of the proenzymes and permits autocatalytic activation with an accompanying reduction in molecular mass of approximately 10 kd.

Results

Localization and Characterization of Stromelysin-, Gelatinase-B-, and TIMP-1-Expressing Cells in Necrotizing Scleritis Tissue

Tissue samples were obtained from nine patients with necrotizing scleritis. Adjacent sections were used for *in situ* hybridization and immunohistochemistry. Stromelysin, gelatinase B, and TIMP-1 mRNA transcripts were localized to several cell types, including large inflammatory cells with extended cytoplasms, inflammatory cells with large nuclei and relatively little cytoplasm, and spindle-shaped fibroblastic cells (Figures 1 and 2). Generally, histology demonstrated granulomatous regions that were surrounded by large cell types. These cells were highly positive for stromelysin (Figure 1A) and gelatinase B (micrograph not shown) mRNA. Using adjacent tissue sections, these cells were characterized as CD68-positive macrophages (Figure 1C). Stromelysin (Figure 1D) and gelatinase B (micrograph not shown) mRNA transcripts were also detected amongst resident fibroblastoid cells. Several diseased scleral tissue samples studied were predominantly infiltrated with T lymphocytes as detected by immunostaining with anti-CD3 (Figure 1E). Using adjacent tissue sections, some of these cells demonstrated gelatinase B mRNA expression (Figure 1F). Essentially little or no expression of these gene transcripts was found in normal uninvolved scleral tissue (away from the inflammation) and among the neutrophilic infiltrate (Figure 1A). Hybridization using sense cRNA probes for stromelysin (Figure 1B) and gelatinase B (micrographs not shown) showed no hybridization signal. Although TIMP-1 mRNA was localized to the similar cell types (Figure 2, A and C), the signal intensity and the number of cell expressing this transcript was often significantly less than the corresponding MMPs. In addition, when we probed normal scleral tissue for TIMP-1, essentially no expression of this gene transcript was observed (micrographs not shown). Normal sclera is relatively avascular and acellular; hence, these results suggest that inflammatory mediators may be responsible for the leukocyte recruitment and the induction of stromelysin, gelatinase B, and TIMP-1 in necrotizing scleritis.

In Vivo *Detection of TNF-α in Diseased Scleral Tissue*

To determine whether proinflammatory cytokines such as TNF-α were expressed in diseased scleral tissue we used a monoclonal anti-human TNF-α antibody. TNF-α immunoreactivity was specifically localized to the cytoplasm of infiltrating inflammatory cells (Figure 3B). When the primary antibody was omitted (Figure 3A) or when the primary antibody was preabsorbed overnight with purified recombinant human TNF-α and incubated with scleritis tissue (Figure 3C), no immunoreactivity could be demonstrated. No immunoreactivity for IL-1α or IL-1β was observed in scleritis tissue using commercial antibodies, suggesting that either no IL-1 was expressed in this tissue or, more probable, the antigen could not be retrieved.

Regulation and Quantitation of Stromelysin, Gelatinase B, and TIMP-1 mRNA in HSFs

HSF cells were cultured under serum-free conditions and stimulated with various proinflammatory cytokines over a 72-hour period. Our results indicate that the addition of IFN-γ, IL-1α, or TNF-α alone did not significantly induce stromelysin or TIMP-1 mRNAs (results not shown). However, potent induction of stromelysin and to a lesser extent TIMP-1 mRNA resulted when these cells were stimulated with a combination of TNF-α and IL-1α (Figure 4A). To quantitate the effect of each cytokine on the relative expression of stromelysin and TIMP-1 mRNA, autoradiographs shown in Figure 4A were subjected to scanning densitometry. The relative inductions were determined by subtracting the constitutive mRNA levels from control unstimu-

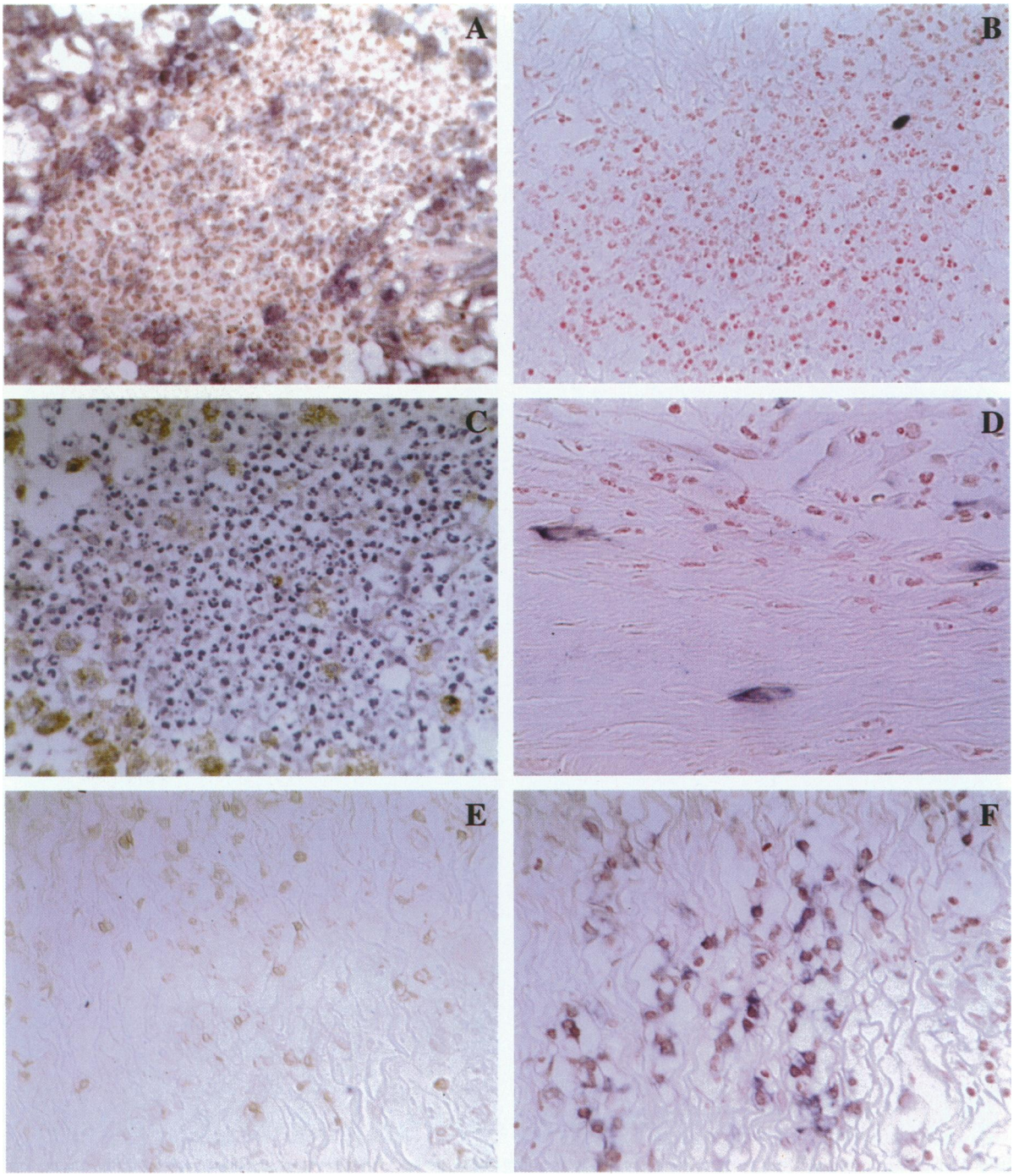

Figure 1. *Localization and characterization of stromelysin and gelatinase B in necrotizing scleritis tissue.* In situ *hybridization was performed on diseased scleral tissue* (**A, B, D,** *and* **F**) *using stromelysin antisense* (**A** *and* **D**), *stromelysin sense* (**B**), *and gelatinase B antisense* (**F**) *digoxigenin-labeled probes. The dark blue cytoplasmic staining indicates zones of hybridization, with neutral red counterstaining establishing the background. To characterize MMP-expressing cells, immunohistochemistry using mouse anti-human CD68* (**C**) *and mouse anti-human CD3* (**E**) *antibodies were used. The brown staining identifies positive immunoreactive cells with hematoxylin-counterstained nuclei* (**C**) *and no counterstaining* (**E**). *Tissue sections in* **A** *to* **D** *were derived from patient 8, and tissue used in* **E** *and* **F**) *was derived from patient 9. Similar results were obtained with tissue sections from other scleritis patients. Magnification, ×250.*

lated cultures, from each time point, for each cytokine used. Constitutive mRNA levels for both genes remained unmodified throughout the time course (data not shown); hence, we chose the 48-hour time point as the reference or baseline level.

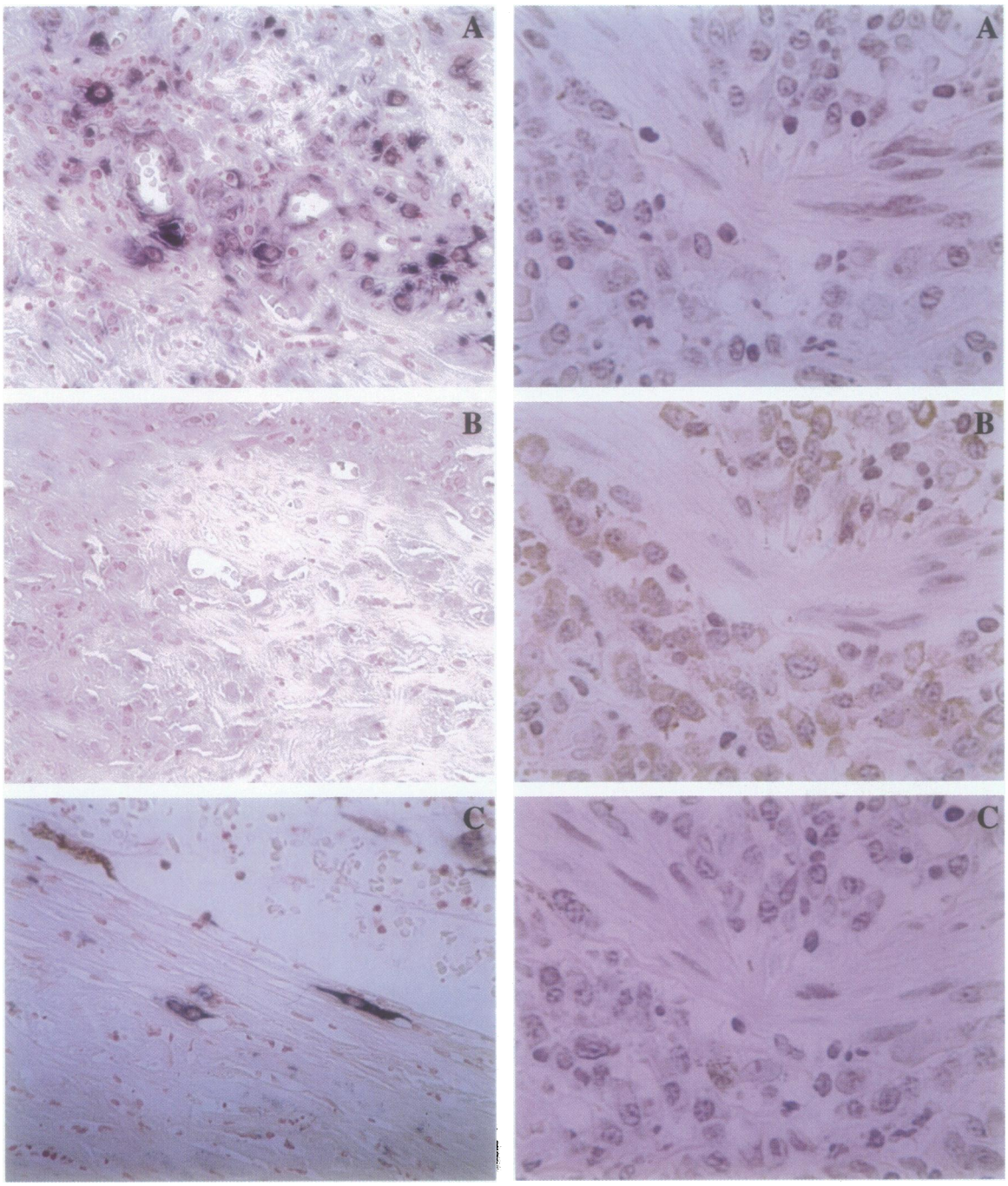

Figure 2 (left). *Localization of TIMP-1 mRNA transcripts in necrotizing scleritis tissue.* In situ *hybridization was performed on diseased scleral tissue as in Figure 1. TIMP-1 transcripts were localized to large rounded macrophage-type inflammatory cells* (**A**) *and to resident spindle-shaped fibroblastoid cells within the sclera* (**C**). *No hybridization signal was revealed when adjacent sections were hybridized with the TIMP-1 sense probe* (**B**). *These tissue sections were derived from patient 3. Similar results were obtained with tissue sections from other scleritis patients. Magnification,* ×*250.*

Figure 3 (right). In vivo *expression of TNF-α in scleritis. Serial sections were prepared for immunohistochemistry using an anti-TNF-α antibody. Brown cytoplasmic immunoreactivity was present in infiltrating inflammatory cells* (**B**). *No reactivity was demonstrated when the primary antibody was omitted* (**A**) *or when the anti-TNF-α antibody was preabsorbed with recombinant human TNF-α* (**C**). *Magnification,* ×*400. Tissue used for this experiment was derived from patient 8. Similar patterns of immunoreactivity were demonstrated with other scleritis tissue samples.*

Densitometric analyses indicate that the addition of TNF-α plus IL-1α induced stromelysin message over sevenfold, with the highest expression measured at 72 hours after cytokine stimulation. In contrast, the combination of both TNF-α and IL-1α induced TIMP-1 message approximately twofold.

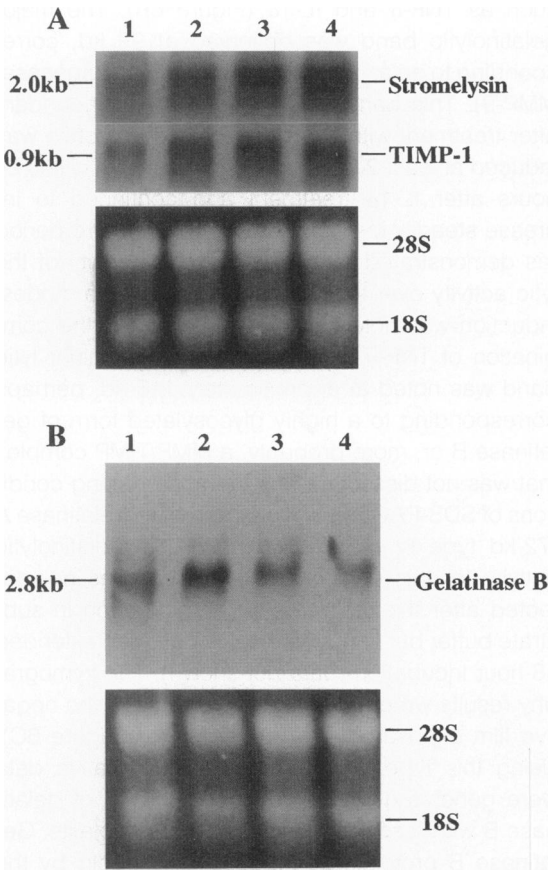

Figure 4. *Induction of stromelysin, gelatinase B, and TIMP-1 mRNA expression in cytokine-stimulated HSFs. Northern blot analyses were performed on total RNA (4 μg) isolated from HSFs cultured under SF conditions over a 72-hour time course. Blots were hybridized with digoxigenin-labeled riboprobes to human stromelysin* (**A, upper blot**), *human TIMP-1* (**A, lower blot**), *and human gelatinase B* (**B**). *In all experiments, the gels were ethidium bromide stained before transfer to check for equal loading and RNA integrity and after transfer (gels not shown) to check for equal transfer. These results are representative of three separate blots.* **A:** *RNA derived from 48-hour control cultures* (**lane 1**) *and from TNF-α- plus IL-1α-stimulated fibroblasts for 24, 48, and 72 hours* (**lanes 2, 3,** *and* **4**). **B:** *RNA derived from 48-hour control cultures* (**lane 1**) *and from IL-1α-stimulated fibroblasts for 24, 48, and 72 hours* (**lanes 2, 3,** *and* **4,** *respectively*).

Interestingly, stromelysin message steadily increased over the 72-hour time course studied, whereas TIMP-1 message peaked at 24 hours after cytokine treatment and then remained constant for the next 48 hours, suggesting that these two genes were discoordinately expressed. Moreover, these results suggest that the induction of metalloproteinase when compared with metallo-inhibitor may be disproportionate.

Although stromelysin and TIMP-1 mRNA was induced by the combination of TNF-α plus IL-1α, gelatinase B message was induced only by IL-1α. Northern blotting performed on three separate occasions revealed a specific transcript at 2.8 kb corresponding to gelatinase B mRNA (Figure 4B). Densitometry demonstrated a twofold induction of this transcript 24 hours after IL-1α treatment, after which the expression returned to basal levels for the remainder of the time course. Essentially no increase in gelatinase B steady-state mRNA expression was demonstrated by the addition of TNF-α or IFN-γ. The combination of TNF-α plus IL-1α induced gelatinase B mRNA to similar levels as IL-1α alone (data not shown).

Detection of Stromelysin and TIMP-1 Protein by Western Blotting

To correlate protein synthesis with mRNA expression, we used CM from HSFs stimulated with the combination of TNF-α plus IL-1α over a 72-hour time course. Frozen aliquots of CM were thawed, and 25 μl of sample was loaded nonreduced into the wells of a discontinuous SDS-PAGE gel. After electrophoresis, the proteins were transferred onto Immobilon-P protein membranes. Immunoblots using mouse anti-human TIMP-1 monoclonal antibody revealed a 28-kd inducible immunoreactive band corresponding to TIMP-1 protein. As indicated in Figure 5 (upper panel), TIMP-1 protein was only faintly detected at 48 hours, (lane 3) but was more abundant 72 hours after cytokine stimulation (lane 4). Unstimulated fibroblasts (Figure 5, upper panel, lane 1) and those stimulated for 24 hours demonstrated no detectable TIMP-1 secretion. Immunoblotting using rabbit anti-human stromelysin antibody resulted in the detection of a specific immunoreactive band that migrated to approximately 56 kd, corresponding to stromelysin protein. Although constitutive levels of stromelysin protein were not detected by this method, stromelysin secretion was induced over the time course and was most abundant 72 hours after cytokine stimulation (Figure 5, lower panel, lane 4). Although quantitation of protein secretion was not possible with these blots, the protein data presented are comparable to the mRNA data from Figure 4A, indicating that stromelysin and TIMP-1 proteins are disproportionately expressed.

Gelatinase B Protein Secretion by Cytokine-Stimulated HSFs

Preliminary zymography assays revealed gelatinolytic activity in CM of cytokine-stimulated HSFs. To confirm that these proteolytic clearance bands were produced by MMPs, zymograms were incubated with low molecular weight protease inhibitors. Incubation of gelatin-containing gels with EDTA or 1,10-

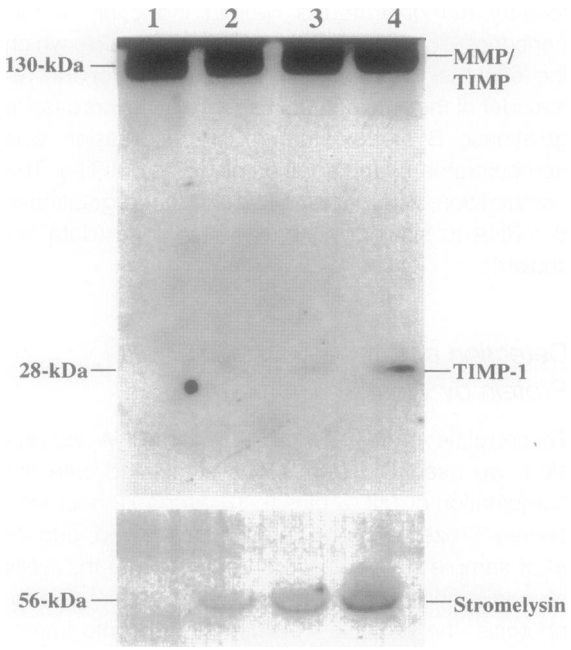

Figure 5. *Western blot analysis for TIMP-1 and stromelysin in CM of cytokine stimulated HSFs. HSFs were stimulated with the combination of TNF-α plus IL-1α. The CM was harvested over the 72-hour time course and processed for immunoblotting for the detection of secreted TIMP-1 protein* (**upper panel**) *and stromelysin protein* (**lower panel**). *CM from 48-hour control cultures* (**lane 1**), *CM from TNF-α- plus IL-1α-stimulated cultures for 24, 48, and 72 hours* (**lanes 2, 3, and 4,** *respectively). The immunoreactive bands around 130 kd are probably MMP/TIMP complexes that were recognized by the TIMP-1 monoclonal antibody. The immunoreactive stromelysin band was detected by NBT/BCIP* (**lower panel**), *whereas a more sensitive chemiluminescent method of detection was necessary to reveal TIMP-1* (**upper panel** *see Materials and Methods*).

phenanthroline (chelators of metal ions) completely inhibited the proteolytic activity in CM derived from IL-1α-stimulated HSFs (Figure 6A, lanes 2 and 6). Inhibitors of serine proteases (AEBSF and phenylmethylsulfonyl fluoride) and leupeptin (inhibitor of serine and cysteine proteases) had little or no effect on the gelatinolytic activity (Figure 6A, lanes 3, 4, and 5, respectively). In addition, the gelatinolytic activity observed in this CM was activated *in vitro* with 1 mmol/L APMA. Our results illustrate the generation of an extra lytic band at approximately 87 kd, corresponding to active gelatinase B (due to the cleavage of the propeptide domain of gelatinase B; Figure 6A, lane 7). In summary, these findings provide strong evidence to suggest that the proteolytic bands represent the activity of members of the MMP gene family.

CM from cytokine-stimulated HSFs was standardized for total protein and cell number and 25 μl of each sample was loaded onto a 4% stacking and 10% resolving gelatin substrate SDS-PAGE for zymographic analysis. HSFs expressed gelatinolytic activity that was induced by the addition of cytokines such as TNF-α and IL-1α (Figure 6B). The major gelatinolytic band was observed at 92 kd, corresponding to gelatinase B (92-kd type IV collagenase, MMP-9). This band of activity was most abundant after treatment with IL-1α. Gelatinase B protein was induced at least 2.5-fold above constitutive levels 24 hours after IL-1α treatment and continued to increase steadily over the next 48-hour culture period (as demonstrated by the increased intensity of the lytic activity over this period). In addition, a modest induction was noted after stimulation with the combination of TNF-α and IL-1α. A second minor lytic band was noted at approximately 116 kd, perhaps corresponding to a highly glycosylated form of gelatinase B or, more probably, a MMP/TIMP complex that was not dissociated by the nonreducing conditions of SDS-PAGE. HSFs also secreted gelatinase A (72-kd type IV collagenase, MMP-2). Gelatinolytic activity for this distinct gene product was not detected after the usual overnight incubation in substrate buffer but could be resolved after an extended 48-hour incubation (data not shown). The zymography results were quantitated by scanning the negative film exposures on a densitometer (Figure 6C). Using this type of analysis, semiquantitative data were generated on the relative induction of gelatinase B with respect to unstimulated fibroblasts. Gelatinase B protein was induced over 4-fold by the addition of IL-1α alone. The combination of TNF-α plus IL-1α produced a comparable induction (approximately 3.5-fold increase) and followed similar kinetics to IL-1α alone. These results suggest that gelatinase B is largely induced by IL-1α alone and not by the combination of TNF-α and IL-1α. TNF-α and IFN-γ treatment alone produced minor alterations (1.5- and 1.3-fold increase, respectively).

Discussion

Necrotizing scleritis is a painful and sight-threatening inflammatory ocular disease characterized by destruction of the sclera. Since it was first documented a century and a half ago,[36] the trigger and the mechanism(s) involved in scleritis remain unknown. Although immune complex deposition may be involved in the pathology of scleritis, the putative antigen(s) are as yet unknown.[3] In addition, Watson et al[37] suggest that activated scleral fibroblasts and inflammatory cells such as macrophages could be responsible for releasing collagenolytic proteases capable of digesting scleral proteins (collagens). Using electron microscopy, Young and Watson[38] were able to demonstrate two possible mechanisms

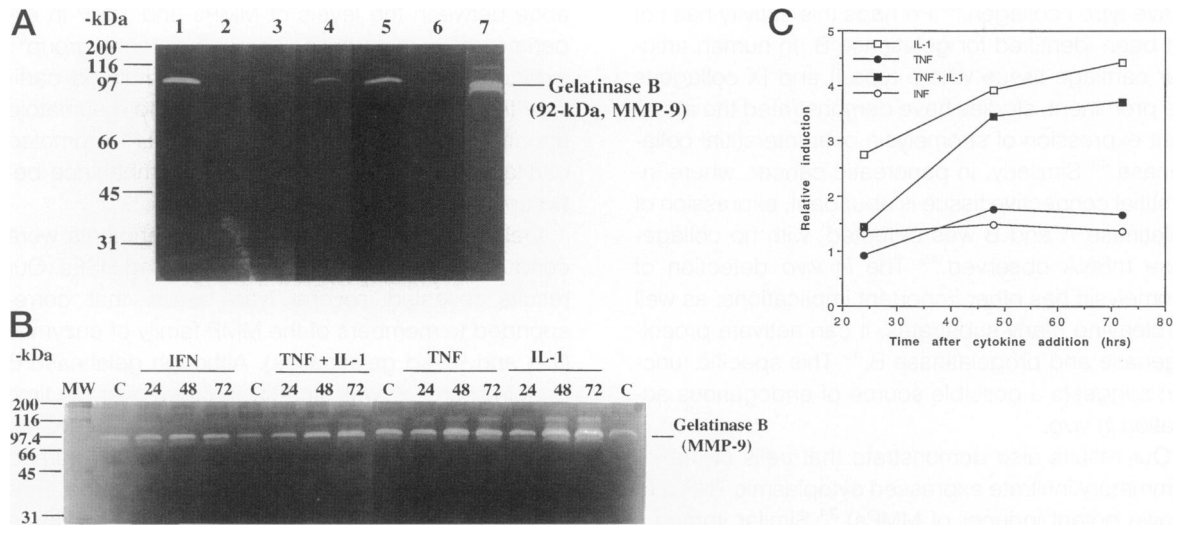

Figure 6. *Gelatinolytic activity in CM from cytokine-stimulated HSFs.* A: *CM from IL-1α-induced HSFs* (lanes 1 *to* 7) *was subjected to SDS-PAGE gelatin zymography. Control CM* (lane 1) *was not treated with any protease inhibitor but incubated in substrate buffer (as described in Materials and Methods). EDTA and 1,10-phenanthroline (potent MMP inhibitors) completely abolished gelatinolytic activity in the CM sample* (lanes 2 *and* 6, *respectively). The addition of 4 (-2-aminoethyl) benzene-sulfonyl fluoride and phenylmethylsulfonyl fluoride* (lanes 3 *and* 4) *and leupeptin* (lane 5), *serine and cysteine protease inhibitors, respectively, had little or no effect on gelatinase activity. APMA* (lane 9) *activated progelatinase B as demonstrated by the extra band of proteolytic activity.* B: *CM from cytokine-stimulated HSFs were loaded onto SDS-PAGE gelatin substrate gels and processed for zymography. CM derived from unstimulated control cultures (C) or from cultures stimulated with 100 U of IFN-γ, TNF-α plus IL-1α, TNF-α, or IL-1α (all for 24, 48, and 72 hours) were reacted with gelatin substrate gels to determine the effect of these cytokines on gelatinase B secretion.* C: *Negative film exposures were scanned using a densitometer to quantitate gelatinase B protein secretion by cytokine-stimulated fibroblasts.*

of collagen degradation in the scleral stroma in several cases of advanced necrotizing scleritis. First, they observed phagocytosis of collagen fibrils by activated stromal fibroblasts and macrophages. Second, they observed the solubilization and unraveling of collagen fibrils in the scleral stroma in the absence of infiltrating leukocytes. The authors concluded that resident stromal fibroblasts, which demonstrated a variety of morphological changes in necrotizing scleritis, were probably the major source of proteolytic enzymes (of unknown class) that caused collagen degradation. We are the first group to have identified and localized this class of collagen-degrading enzymes in scleritis.[29]

In the present study we performed *in situ* hybridization on tissue derived from nine patients with necrotizing scleritis and localized abundant expression of matrix-degrading enzymes such as stromelysin, gelatinase B, and the MMP inhibitor (TIMP-1) to resident scleral fibroblasts and inflammatory cells such as macrophages and T lymphocytes. Recently, several investigators have demonstrated the synthesis and regulation of gelatinase B from human T lymphocytes in response to various cytokines[39] and have postulated that the expression of these proteases may be responsible for tissue-destructive processes or perhaps may aid in their extravasation through basement membranes and migration into deeper connective tissue.[40] We have previously shown that HSFs secrete 10 to 50 times the amount of gelatinase B as would the same number of leukocytes (unpublished observation). The concept of autodestruction may be a valid one, in which the production of various proinflammatory cytokines by infiltrating leukocytes may account for the increased expression of MMPs and the abundant scleral matrix degradation evident in this ocular disorder.

Scleral matrix consists of approximately 80% collagen by weight, of which type I collagen is predominant, with smaller amounts of collagen types II and III. The collagen is strengthened by elastic fibers with proteoglycan matrix completing the microstructure of the sclera. It was recently demonstrated that type II collagen exists in a co-polymeric assembly with type IX and XI collagens.[41] Although the MMPs localized in this study have little or no activity against the interstitial collagens, these proteinases may influence scleral collagen fibril composition through interactions with type IX and XI collagens as well as other extracellular components such as fibronectin and elastin. Other studies have shown that protein extracts from chicken sclera contain predominantly gelatinase A activity, with no other MMP activity detected.[42] Although *in situ* hybridization for gelatinase A was not performed in this study, its activity was detected in HSF CM by zymography assays. Recently, gelatinase A has been characterized as an interstitial collagenase due to its ability to cleave

native type I collagen.[43] Perhaps this activity has not yet been identified for gelatinase B. In human articular cartilage tissue where type II and IX collagens are prominent, studies have demonstrated the abundant expression of stromelysin over interstitial collagenase.[44] Similarly, in pancreatic cancer, where interstitial connective tissue is abundant, expression of gelatinase A and B was detected, with no collagenase mRNA observed.[45] The *in vivo* detection of stromelysin has other important implications; as well as cleaving many substrates, it can activate procollagenase and progelatinase B.[46] This specific function suggests a possible source of endogenous activation *in vivo*.

Our results also demonstrate that cells of the inflammatory infiltrate expressed cytoplasmic TNF-α (a known potent inducer of MMPs).[24] Similar immunostaining patterns have been observed in inflammatory cells of chronic granulomatous skin lesions[47] and granulomatous lymphadenitis.[48] Using this evidence, an *in vitro* culture model of scleritis was established to demonstrate the regulation of stromelysin, gelatinase B, and TIMP-1 expression by proinflammatory cytokines such as IL-1α and TNF-α at both the protein and mRNA levels.

To determine the relative expression of MMPs and TIMP-1 mRNAs in our *in vitro* culture model, total RNA was isolated from cytokine-stimulated fibroblasts. Our results indicate that stimulation of HSFs by inflammatory cytokines such as IFN-γ, TNF-α, and IL-1α have minor modulatory effects on the induction either of stromelysin or TIMP-1 gene expression. However, the addition of the combination of TNF-α plus IL-1α had a potent effect and induced stromelysin approximately sevenfold. Interestingly, TIMP-1 was only maximally induced twofold over the 72-hour time course (Figure 4A). These results imply that TNF-α and IL-1α in combination are potent inducers of stromelysin mRNA expression. Of note was the fact that the induction of TIMP-1 mRNA by these two cytokines was fivefold less than the metalloenzyme, suggesting that these genes may be discoordinately expressed by HSFs. Both TNF-α and IL-1α may be involved in the induction of a disproportionate enzyme/inhibitor ratios, favoring tissue-destructive processes *in vivo*.

The findings of this study are in accordance with recent reports by MacNaul et al,[25] who reported a dissociated expression of interstitial collagenase (MMP-1), stromelysin, and TIMP-1 by human rheumatoid synovial fibroblasts. In addition, they demonstrated the synergistic effects of TNF-α plus IL-1α on the expression of MMPs in cultured synovial fibroblasts. Pelletier et al[27] reported a possible imbalance between the levels of MMPs and TIMP in experimental osteoarthritis. Later, the same group[28] extended their findings using IL-1-stimulated cartilage from patients with osteoarthritis and rheumatoid arthritis. Their results suggested that IL-1 promoted cartilage degradation by causing an imbalance between metalloenzyme and its inhibitor.

Gelatin substrate zymography experiments were conducted on CM from cytokine-induced HSFs. Our results revealed several lytic bands that corresponded to members of the MMP family of enzymes (72- and 92-kd gelatinases). Although gelatinase B protein secretion was steadily induced over the time course, mRNA expression was increased twofold after 24 hours of IL-1α stimulation and then returned to basal levels over the next 48-hour period. The apparent induction of gelatinase B protein (Figure 6B) may, however, reflect the accumulation of relatively stable progelatinase B in CM samples rather than induction of protein synthesis, as the increased expression of gelatinase B mRNA occurred only 24 hours after cytokine treatment.

In summary, this study demonstrates the localization of stromelysin, gelatinase B, and TIMP-1 mRNA to resident scleral fibroblasts and inflammatory cells such as macrophages and T lymphocytes present in necrotizing scleritis tissue. Using an *in vitro* culture model and sensitive zymography assays, several members of the MMP family of proteinases were identified and characterized. Regulation and induction of secreted stromelysin, gelatinase B, and TIMP-1 was demonstrated by stimulating cultured HSFs with proinflammatory cytokines. Although both stromelysin and TIMP-1 were induced by TNF-α and IL-1α treatment, stromelysin expression was observed in greater abundance as compared with its inhibitor as detected *in vitro* by mRNA studies and *in vivo* by *in situ* hybridization. These results also suggest that the inducing effect of TNF-α and IL-1α on MMP and TIMP mRNA steady-state levels takes place at the pretranslational level. We hypothesize that this altered balance between metalloenzymes and their inhibitors may be a potential mechanism of tissue destruction in necrotizing scleritis. In addition, TNF-α present in the inflammatory lesion may be a key mediator of the inflammatory response and potential modulator of MMP expression.

Currently, therapy for scleritis is unsatisfactory and consists of the systemic administration of anti-inflammatory or immunosuppressive agents. The demonstration of MMP expression in diseased scleral tissue suggests that therapies aimed at inhibiting MMP activity may be beneficial in controlling this collagen-degrading disorder. Evans

and Eustace[49] addressed this possibility with a study in which they successfully treated a patient with severe necrotizing scleritis with a topical solution of 0.5% sodium versenate (EDTA, a potent inhibitor of MMP activity). Together this data may help to better understand the pathogenesis and treatment of this potentially blinding inflammatory ocular disease.

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