Metalloproteinases and Tissue Inhibitor of Metalloproteinases in Mesothelial Cells

Cellular Differentiation Influences Expression

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Abstract

Mesothelial cells play a critical role in the remodeling process that follows serosal injury. Although mesothelial cells are known to synthesize a variety of extracellular matrix components including types I, III, and IV collagens, their potential to participate in matrix degradation has not been explored. We now report that human pleural and peritoneal mesothelial cells express interstitial collagenase, 72- and 92-kD gelatinases (type IV collagenases), and the counterregulatory tissue inhibitor of metalloproteinases (TIMP). Our initial characterization of the mesothelial cell metalloenzymes and TIMP has revealed: (*a*) they are likely identical to corresponding molecules secreted by other human cells; (*b*) they are secreted rather than stored in an intracellular pool; (*c*) a primary site of regulation occurs at a pretranslational level; (*d*) phorbol myristate acetate, via activation of protein kinase C, upregulates expression of collagenase, 92-kD gelatinase, and TIMP, but has no effect on expression of 72-kD gelatinase; and (*e*) lipopolysaccharide fails to upregulate the biosynthesis of either metalloproteinases or TIMP. Of particular interest is the observation that the state of cellular differentiation has a striking influence on the expression of metalloenzymes and TIMP, such that epithelioid cells display a more matrix-degradative phenotype (increased 92-kD gelatinase and decreased TIMP) than their fibroblastoid counterparts. We speculate that mesothelial cells directly participate in the extracellular matrix turnover that follows serosal injury via elaboration of metalloproteinases and TIMP. Additionally, the reactive cuboidal mesothelium which is characteristic of the early response to serosal injury may manifest a matrix-degradative phenotype favoring normal repair rather than fibrosis. (*J. Clin. Invest.* 1993. 91:1792–1799.) Key words: collagenase • differentiation • gelatinase • mesothelial cells • metalloproteinases

Introduction

Mesothelial cells line the major body cavities (pleural, pericardial, and peritoneal) and the organs enclosed within, forming a simple squamous epithelial lining layer. They exhibit several distinctive features that may relate to their mesodermal embryologic origin, rather than the endodermal or ectodermal origin of most epithelial tissues. For example, these cells display a mitogenic response to transforming growth factor-β and platelet-derived growth factor (1), which is unusual for epithelial cells. Mesothelial cells also have a striking degree of plasticity in their state of differentiation. They may adopt either an epithelioid or fibroblastoid morphology, and express cytoskeletal elements characteristic of both cell types, in vitro and in vivo (2–5).

Serosal injury may occur at the pleural, peritoneal, or pericardial surfaces and the strategically located mesothelial cells play a central role in the remodeling process that ensues (6, 7). Mesothelial cells undergo significant morphologic changes during the repair process, adopting a "reactive" cuboidal morphology shortly after serosal injury (8). With normal repair, they revert to a squamous morphology and no significant functional impairment results. When severe mesothelial cell injury occurs, the likelihood of fibrosis and its pathophysiologic consequences increases (8). It has been demonstrated that mesothelial cells have the capacity to directly participate in fibrin deposition and fibrinolysis (9–11). They can also synthesize a variety of extracellular matrix components including types I, III, and IV collagens (12–14), but their potential to participate in matrix degradation during the repair process has not been investigated.

A significant body of evidence suggests that a family of metalloproteinases and the counterregulatory tissue inhibitor of metalloproteinases (TIMP)[1] play an important role in tissue remodeling (15). The human metalloproteinases include interstitial collagenase, neutrophil collagenase, stromelysin, 72- and 92-kD gelatinases (type IV/type V collagenases), and the recently described PUMP, or putative metalloproteinase (matrilysin), each with its own preferred matrix substrates (16). We have found that human pleural and peritoneal mesothelial cells express interstitial collagenase, 72- and 92-kD gelatinases, and TIMP. Furthermore, metalloproteinase and TIMP expression is modified by the state of differentiation of mesothelial cells, such that epithelioid cells manifest a more matrix-degradative phenotype than their fibroblastoid counterparts.

Methods

Materials. Tissue culture plasticware was obtained from Costar (Cambridge, MA); fetal bovine serum from Hyclone Laboratories, Inc. (Logan, UT); medium 199, formaldehyde, cycloheximide, dextran sulfate, PMA, staphylococcal protein-A Sepharose CL-4B, gelatin, HBSS, *Escherichia coli* LPS, Triton X-100, murine anti-vimentin monoclonal an-

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Received for publication 19 May 1992 and in revised form 16 November 1992.

J. Clin. Invest.

0021-9738/93/04/1792/08 $2.00
Volume 91, April 1993, 1792–1799

1. *Abbreviation used in this paper:* TIMP, tissue inhibitor of metalloproteinases.

tibody, rhodamine-conjugated rabbit anti-mouse IgG antibody, MOPS, EDTA, TCA from Sigma Chemical Co. (St. Louis, MO); acrylamide, bisacrylamide, Coomassie Brilliant Blue R-250, glycine, agarose, Tris, SDS, protein molecular weight standards, mercaptoethanol, protein assay dye reagent, ammonium persulfate from Bio-Rad Laboratories (Richmond, CA); IFN-γ, IL-1β and TNF-α from Genzyme Corp. (Cambridge, MA); [^{35}S]methionine (sp act > 1,100 Ci/mmol) from ICN (Irvine, CA); [α-^{32}P] dCTP (sp act ~ 3,000 Ci/mmol) from Amersham Corp. (Arlington Heights, IL); Nytran 0.2-μm nylon membrane from Schleicher & Schuell, Inc. (Keene, NH); guanidine thiocyanate and transblot apparatus from Bethesda Research Laboratories (Gaithersburg, MD); hydrocortisone from Calbiochem-Behring Corp. (San Diego, CA); phenol, proteinase K, G-25 Sephadex columns, and random primer labeling kit from Boehringer-Mannheim Biochemicals (Indianapolis, IN); EGF from Collaborative Research (Beverly, MA); murine PKK-1 monoclonal anti-cytokeratin antibody from Labsystems (Helsinki, Finland); OPTI-FLUOR from Packard (Meriden, CT); Enlightening from New England Nuclear (Boston, MA); Auto Probe III Immuno-histochemical Detection System and aqueous hematoxylin from Biomeda Corp. (Foster City, CA); LP-9 human peritoneal mesothelial cells from the Aging Cell Respiratory of the National Institute on Aging (Camden, NJ); polyclonal antisera to interstitial collagenase (17), TIMP (18), and 92-kD gelatinase (19) raised as described previously; polyclonal anti-human fibronectin antibody from Telios (La Jolla, CA); plasmids containing a 2.0-kb human collagenase cDNA fragment (20) and a 3.9-kb human TIMP cDNA fragment (21) from American Type Tissue Collection (Rockville, MD); the 0.74-kb CHO-B cDNA fragment (22) was kindly provided by Dr. W. T. Garvey (VA Medical Center, Indianapolis, IN).

Tissue culture. Primary pleural mesothelial cell cultures were established from pleural fluid obtained from four adult males, with congestive heart failure (n = 3) and sterile parapneumonic effusion (n = 1). The fluid was centrifuged at 300 g and the cell pellet resuspended in medium 199 supplemented with 10% heat-inactivated fetal bovine serum, EGF (20 ng/ml), hydrocortisone (0.5 μg/ml), penicillin (100 U/ml) and streptomycin (100 μg/ml). All experiments with pleural mesothelial cells were performed on cells of passages 3–5. LP-9 human peritoneal mesothelial cells (23) were cultured in the same medium. Experiments were performed on LP-9 cells of passages 7–10. Tissue culture plates were coated with culture grade gelatin to improve cell adherence. For most experiments, cells were grown to confluence in complete media. The monolayers were then washed with HBSS and changed to serum-free medium, without EGF or hydrocortisone. After a 6-h period, fresh serum-free medium was added and the experimental period begun. Conditioned medium and cell lysates were collected 24 h later unless otherwise noted. Experiments testing the effects of cytokines were harvested at the 48-h timepoint. This longer incubation period necessitated inclusion of 1% serum throughout the experimental period to prevent detachment of the cells from the culture plates.

For the experiments focusing on state of differentiation, LP-9 cells were grown to confluence in serum-containing medium without EGF or hydrocortisone. At near confluence, cells were maintained in serum-containing medium to favor epithelioid morphology or changed to medium supplemented with delipidized serum (24) to facilitate transition to fibroblastoid morphology. Experiments were begun after differences in cellular morphology were distinct, typically 48–72 h later.

Immunologic assays. Collagenase and TIMP were quantified by ELISA using polyclonal antibodies to collagenase and TIMP purified from human skin fibroblasts as previously described (18, 25). These assays have nanogram sensitivity and recognize the respective proteins whether they are present in a free state, bound to one another, or bound to matrix substrates. Results were normalized to the amount of protein in the cell lysate (26).

Biosynthetic labeling and immunoprecipitation. Confluent mesothelial cells were washed with HBSS and transferred to serum-free medium for 6 h. The medium was then changed to methionine-free medium supplemented with [^{35}S]methionine (75 μCi/ml) and the cells incubated for an additional 24 h. Supernatants were collected and clarified by centrifugation. Cell layers were washed with HBSS and lysates prepared by scraping the cells into 1 ml of lysis buffer (1% Triton X-100, 0.5% deoxycholic acid, 0.02% sodium azide, 0.15 M NaCl, 20 mM Tris, pH 7.8). The samples were preabsorbed with 50 μl of a 10% suspension of staphylococcal protein A-Sepharose CL-4B, and divided into several equal portions.

Immunoprecipitation was then performed by adding protein A-Sepharose beads that had been preincubated with either a 75-fold dilution of rabbit anti-human collagenase, rabbit anti-human TIMP, rabbit anti-human 92-kD gelatinase, rabbit anti-human fibronectin, or nonimmune sera. After overnight incubation at 4°C the precipitates were washed, solubilized by boiling briefly in SDS sample buffer, and subjected to PAGE (10% gel) under reducing conditions. The gel was processed for autofluorography with Enlightening as directed by the manufacturer.

To assess total incorporation of [^{35}S]methionine, proteins were precipitated from an aliquot of each lysate by the addition of 100 μg of carrier bovine serum albumin and TCA to a final concentration of 10%. The precipitates were collected on fiberglass filters by vacuum and washed free of unincorporated [^{35}S]methionine with ethanol. The filters and 10 ml of OPTI-FLUOR were then placed in scintillation vials and radioactivity measured in a model LS-4000 scintillation counter (Beckman Instruments, Inc. (Palo Alto, CA).

Northern blot analyses. Total RNA was prepared by acid guanidine thiocyanate-phenol-chloroform extraction (27) and quantitated by measuring absorbance at 260 nm. RNA was fractionated on 1% agarose/2.2 M formaldehyde gels, transferred to nylon membranes by capillary action, and cross-linked to the membrane by exposure to UV light. The nylon membranes were then pre-hybridized at 37°C for 4–6 h in 5× SSC, 50 mM Na_2HPO_4, 10× Denhardt's, 2.5% dextran sulfate, 50% formamide, 0.75% SDS, 10 μg/ml herring sperm DNA. The following cDNA probes were labeled with [α-^{32}P]dCTP by the random primer method: (*a*) 510 basepair collagenase cDNA fragment derived by the polymerase chain reaction (template, linearized plasmid containing the collagenase cDNA fragment; primers, (+) strand, 961–982 bp, AAGACAGATTCTACATGCGCACA and (−) strand 1464–1439 bp, AGTTGAACCAGCTATTAGCTTTCTGG); (*b*) linearized plasmid containing the full-length TIMP cDNA; and (*c*) CHO-B cDNA fragment (0.74 kb) separated from vector DNA by restriction enzyme digestion and preparative agarose gel electrophoresis and then purified with the Prep-a-Gene kit used according to the manufacturer's instructions. Hybridization conditions were as follows: 5× SSC, 20 mM Na_2HPO_4, 1× Denhardt's, 10% dextran sulfate, 50% formamide, 0.5% SDS, 10 μg/ml herring sperm DNA at 42°C overnight. After hybridization, the membranes were washed at high stringency and exposed to XAR film (Eastman Kodak Co., Rochester, NY) at −70°C with an intensifier screen. Autoradiograms were scanned with an Ultrascan XL enhanced laser densitometer (LKB Instruments, Inc., Gaithersburg, MD). Densitometric measurements for collagenase and TIMP bands were normalized to the corresponding CHO-B band. We have found that the CHO-B mRNA level consistently correlates with the amount of RNA per lane, as judged by the intensity of the ribosomal RNA bands in the ethidium-bromide stained gel.

Substrate gel electrophoresis (28). Aliquots of conditioned medium, prepared for analysis without boiling or reduction, were applied to 10% polyacrylamide gels impregnated with 1 mg/ml gelatin. After electrophoresis, the gel was incubated in 2.5% (vol/vol) Triton X-100 for 20 min and then overnight at 37°C in 50 mM Tris, pH 8.0, containing 5 mM $CaCl_2$ and 1 μM $ZnCl_2$. Clear zones of lysis due to gelatinase activity were identified by staining the gel with 0.125% Coomassie Blue.

Immunohistochemistry. Cells grown in T-75 flasks were washed with PBS and fixed with absolute methanol for 10 min at room temperature. The flasks were then broken apart and the bottoms cut into several pieces. Staining for fibronectin was performed using minor modifications of the ABC technique (29) as described in the Auto Probe III immunohistochemical detection system. The primary rabbit anti-fibronectin antibody was used at a 1:250 dilution, with substitu-

tion of an identical dilution of nonimmune rabbit serum serving as control. Aqueous hematoxylin was used as a counterstain.

Photomicroscopy. Living cells were photographed with an inverted transmitted light microscope (E. Leitz Inc., Rockleigh, NJ) equipped with a model MPS12 Microcamera (Wild Heerbrugg Instruments Inc., Farmingdale, NY) and a model MPS05 Mikrophot exposure meter (Nikon Inc., Garden City, NY) using Pan X-400 black and white film (Eastman Kodak Co.). Stained cells were photographed with a Zeiss Photomicroscope II equipped with an MC63 camera using Ektachrome 64T color reversal film (Eastman Kodak Co.).

Results

Collagenase and TIMP secretion by mesothelial cells. Our studies initially focused on mesothelial cell expression of interstitial collagenase and TIMP. We found no detectable spontaneous release of immunoreactive collagenase from unstimulated pleural mesothelial cells by ELISA assays. In contrast, unstimulated cells secreted substantial quantities of TIMP into the culture medium. Exposure of pleural mesothelial cells to PMA induced marked secretion of interstitial collagenase in a dose-dependent manner, while only modestly upregulating TIMP production (Fig. 1). These actions of PMA were apparently mediated by activation of protein kinase C, since the inactive structural analogue, 4α-PMA, had no effect. LPS had no significant effect on collagenase or TIMP production.

Peritoneal mesothelial cells constitutively produced significantly greater amounts of TIMP than pleural cells (43.5±4.5 vs. 4.4±0.49 μg TIMP/mg protein in the cell lysates, mean±SE, $n = 4$). However, they responded to PMA (and not LPS) in a fashion very similar to the pleural mesothelial cells (data not shown).

Biosynthesis of collagenase and TIMP. Immunoprecipitation of labeled proteins from pleural (Fig. 2) and peritoneal (not shown) mesothelial cell supernatants showed constitutive synthesis and secretion of 29-kD TIMP, but not collagenase.

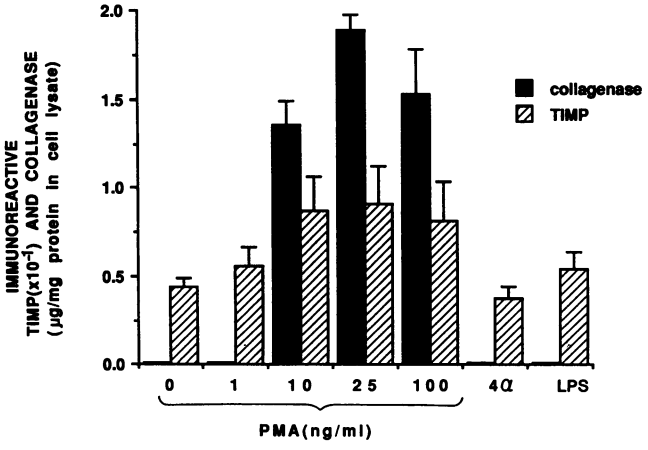

Figure 1. Production of interstitial collagenase and TIMP by pleural mesothelial cells. Confluent pleural mesothelial cells were changed to serum-free medium for 6 h and then PMA (0, 1, 10, 25, or 100 ng/ml), 4α-PMA (25 ng/ml), or LPS (1 μg/ml) was added. After 24 h, supernatants and lysates were collected. TIMP and collagenase were quantified by ELISA and normalized per milligram of protein in the cell lysate ($n = 4$ for each). Note the potent effect of PMA, particularly on collagenase expression. Neither 4α-PMA nor LPS had significant effect on collagenase or TIMP expression.

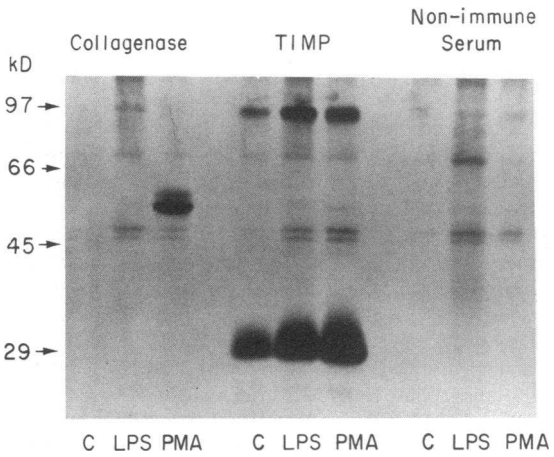

Figure 2. Immunoprecipitation of interstitial collagenase and TIMP from biosynthetically labeled pleural mesothelial cells. Cell cultures, unstimulated and exposed to LPS (1 μg/ml) or PMA (25 ng/ml), were incubated in medium supplemented with [^{35}S]methionine for 24 h. Labeled secreted proteins were immunoprecipitated from equal aliquots of the conditioned medium. There was roughly equivalent incorporation of [^{35}S]methionine into TCA-precipitable protein in the cell lysates (control, 0.84×10^4 cpm/μg protein; LPS, 1.54×10^4 cpm/μg protein; PMA, 1.1×10^4 cpm/μg protein). Note the constitutive synthesis of TIMP and the marked accumulation of newly synthesized collagenase in response to PMA.

Exposure to PMA resulted in marked accumulation of newly synthesized 56- and 52-kD proenzyme doublet of interstitial collagenase as well as a moderate increase in the amount of TIMP. The labeled 92-kD protein that coprecipitated with the anti-TIMP antibody likely represents the 92-kD gelatinase which was complexed to TIMP (see below) (19, 28). LPS had no reproducible effect on collagenase or TIMP biosynthesis. Although Fig. 2 suggests that LPS upregulates TIMP biosynthesis, several other experiments (both pleural and peritoneal mesothelial cells with TIMP quantified by immunoprecipitation and ELISA) failed to demonstrate an effect of LPS on TIMP expression.

Analysis of steady-state collagenase and TIMP mRNA levels. Similar to the studies on protein expression, Northern blot analyses showed constitutive expression of 0.9-kb TIMP mRNA by pleural and peritoneal mesothelial cells, but no constitutive expression of collagenase mRNA (Fig. 3). PMA induced a striking accumulation of 2.5-kb collagenase mRNA and a modest increase in steady-state levels of TIMP mRNA (Fig. 3). Concomitant incubation of peritoneal mesothelial cells with cycloheximide (5 μg/ml), an inhibitor of protein synthesis, blocked the PMA-induced increase in collagenase mRNA ~ 95% (determined by densitometric analysis) suggesting that synthesis of a secondary regulatory protein was necessary for this PMA effect on gene expression.

Expression of 72- and 92-kD gelatinases. We next investigated mesothelial cell expression of other members of the metalloproteinase family. Gelatin substrate gels showed that pleural (Fig. 4) and peritoneal (not shown) mesothelial cells have the capacity to produce 72- and 92-kD gelatinases. Both cells constitutively secreted a 72-kD gelatinase. PMA had no effect on 72-kD gelatinase expression, but did induce production of the 92-kD gelatinase. Biosynthetic labeling and immu-

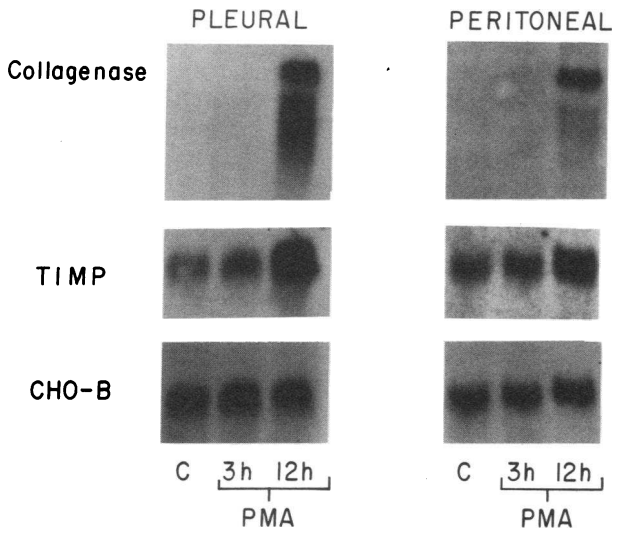

Figure 3. Northern analyses of mesothelial cell collagenase and TIMP mRNA levels. Total RNA was prepared from pleural and peritoneal mesothelial cells exposed to PMA (25 ng/ml) for 3 or 12 h. Note the basal expression of TIMP mRNA and the striking accumulation of collagenase mRNA in response to PMA. The constitutive CHO-B signal demonstrates roughly equivalent amounts of RNA per lane.

Table I. Effect of Cytokines on Production of Collagenase and TIMP by Pleural Mesothelial Cells

Cytokine	Immunoreactive material in cell lysate	
	Collagenase	TIMP ($\times 10^{-1}$)
	μg/mg protein	
None	1.07±0.39	2.13±0.14
TNF-α (200 U/ml)	2.40±0.28	5.22±0.24
TNF-α (2,000 U/ml)	3.59±0.47	8.51±0.69
IL-1β (10 U/ml)	0.82±0.22	5.49±1.63
IL-Iβ (100 U/ml)	1.08±0.25	7.31±1.01
TNF-α + IL-Iβ (200 U/ml and 10 U/ml, respectively)	6.36±1.30	10.18±0.47
IFN-γ (10 U/ml)	0.86±0.05	3.22±0.17

Confluent pleural mesothelial cells were changed to medium containing 1% serum and EGF (10 ng/ml) for 6 h and then TNF-α, IL-Iβ, or IFN-γ was added to achieve the final concentrations noted above. After 48 h, supernatants and lysates were collected. Collagenase and TIMP were quantified by ELISA and normalized per mg protein in the cell lysate. Data shown are the mean±standard error, $n = 3$ for each manipulation.

noprecipitation showed that this increase in 92-kD gelatinase activity was due to biosynthesis of the metalloproteinase (data not shown). Again, the inactive structural analogue, 4α-PMA, had no effect, suggesting that the PMA action was mediated by activation of protein kinase C. Inclusion of 10 mM EDTA in the incubation buffer abolished the 72- and 92-kD gelatinase activities, as would be expected for metalloproteinases. No activity was detected in the cell lysates suggesting that the gelatinases were not stored in an intracellular pool. LPS failed to modify expression of the 72- or 92-kD gelatinase.

Effect of TNF-α, IL-1β, and IFN-γ on metalloproteinase and TIMP expression. Because macrophages and their secretory products appear to play an important role in serosal injury and repair (7, 30), we next examined the effects of TNF-α, IL-1β and IFN-γ on metalloproteinase and TIMP expression. We found that TNF-α increased production of both collagenase and TIMP, whereas IL-1β increased only the expression of TIMP (Table I). The addition of TNF-α and IL-1β together resulted in increases in collagenase and TIMP beyond that observed in response to either cytokine alone. While these effects were considerable, they were of smaller magnitude than that induced by PMA. Gelatin substrate gels demonstrated that TNF-α and IL-1β each increased expression of the 92-kD gelatinase (Fig. 5). Again the combination of the two cytokines resulted in synergistic stimulation of 92-kD gelatinase production.

Effect of cellular differentiation on metalloproteinase and TIMP expression. With manipulation of in vitro culture conditions, mesothelial cells can adopt either a cuboidal epithelioid appearance or a flattened fibroblastoid morphology (4). In the next series of experiments focused on peritoneal mesothelial cells, we investigated the effect of cellular differentiation on metalloproteinase and TIMP expression. Fig. 6 shows the striking difference in morphology of LP-9 peritoneal mesothelial cells under different culture conditions. Mesothelial cells grown in delipidized serum adopted a fibroblastoid morphology and expressed predominantly vimentin whereas cells grown in normal serum displayed an epithelioid morphology and expressed cytokeratins as well as vimentin (not shown), consistent with previous reports (2, 4). With respect to metal-

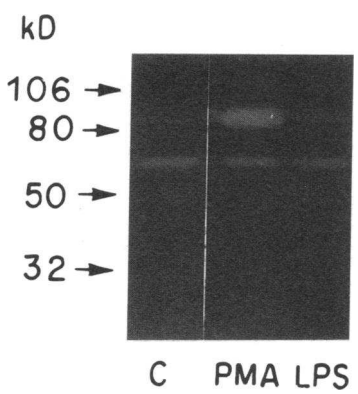

Figure 4. Substrate gel electrophoresis of secreted mesothelial cell metalloenzymes. Analysis was performed on an aliquot (20 μl) of conditioned medium from pleural mesothelial cell cultures unstimulated or exposed to PMA (25 ng/ml) or LPS (1 μg/ml) for 24 h. Note the constitutive expression of 72-kD gelatinase, which is unaffected by PMA or LPS, and the marked increase in 92-kD gelatinase in response to PMA.

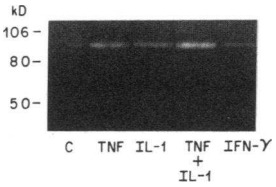

Figure 5. Substrate gel electrophoresis of secreted mesothelial cell metalloenzymes. Analysis was performed on an aliquot (20 μl) of conditioned medium from pleural mesothelial cell cultures, unstimulated or exposed to TNF-α (200 U/ml), IL-1β (10 U/ml), TNF-α plus IL-1β (200 U/ml and 10 μ/ml, respectively), or IFN-γ (10 U/ml) for 48 h. Note the upregulation of 92-kD gelatinase in response to TNF-α and IL-1β and the apparent synergistic effect when the two cytokines are combined. The 72-kD gelatinase, though not clearly visualized in this picture, was readily detectable in concentrated samples and its expression did not appear to be significantly altered by the cytokines (not shown).

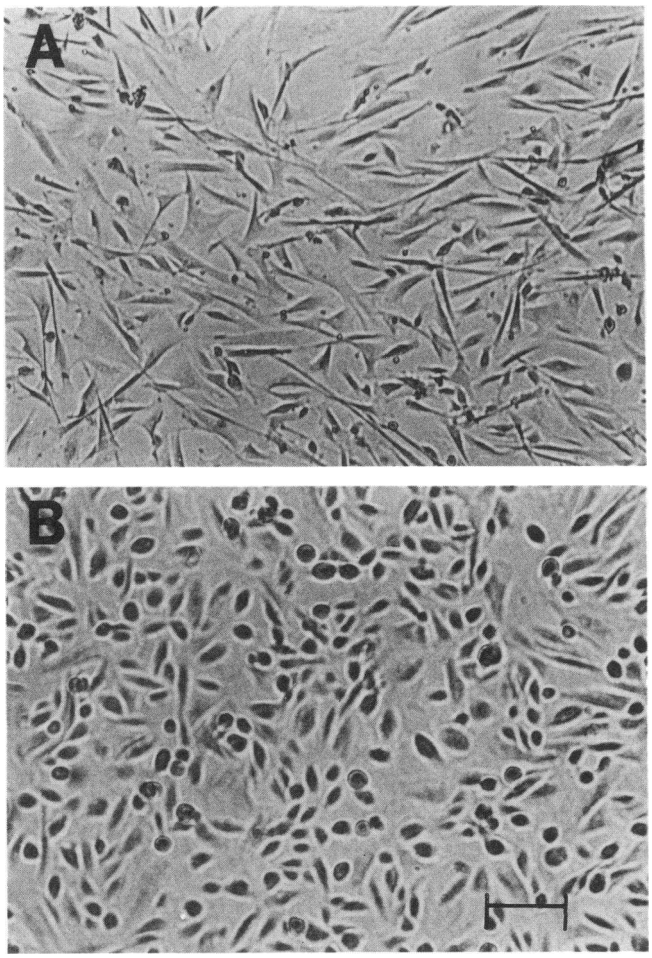

Figure 6. Phase-contrast photomicroscopy. Peritoneal mesothelial cells grown in medium containing delipidized serum (*A*) displayed a spindle-shaped fibroblastoid morphology as compared with cells grown in normal serum (*B*) which displayed a cuboidal epithelioid morphology. Bar, 100 μm.

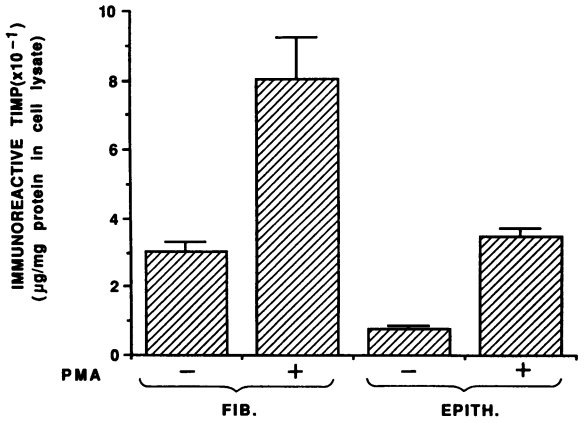

Figure 7. TIMP expression by peritoneal mesothelial cells: fibroblastoid vs. epithelioid cells. The amount of TIMP in conditioned medium from fibroblastoid and epithelioid cell cultures was quantified by ELISA and normalized per milligram of protein in the cell lysate ($n = 5$ for each). Note that epithelioid cells secreted less TIMP (basal and in response to PMA) than fibroblastoid cells.

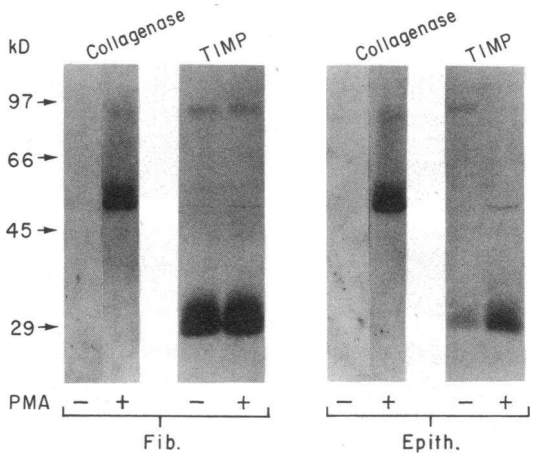

Figure 8. Immunoprecipitation of interstitial collagenase and TIMP from biosynthetically labeled peritoneal mesothelial cells: fibroblastoid vs. epithelioid cells. Fibroblastoid peritoneal mesothelial cells and epithelioid cultures were labeled with or without the addition of PMA (25 ng/ml). There was roughly equivalent incorporation of [^{35}S]-methionine into TCA-precipitable protein in the cell lysates (fibroblastoid: control, 1.7×10^4 cpm/μg protein; PMA, 1.1×10^4 cpm/mg protein; and epithelioid: control, 2.2×10^4 cpm/μg protein; PMA, 1.7×10^4 cpm/μg protein). Note the diminished production of newly synthesized TIMP in the epithelioid cultures, unstimulated as well as PMA-treated, as compared to the fibroblastoid cells. Both fibroblastoid and epithelioid cells synthesized approximately equal amounts of interstitial collagenase in response to PMA.

loproteinase and TIMP production, we found that epithelioid cells produced significantly less TIMP, both constitutively and in response to PMA (Figs. 7 and 8). In contrast, however, PMA induced significantly more 92-kD gelatinase from the epithelioid cultures (Fig. 9). Amounts of collagenase elaborated in response to PMA were similar in both fibroblastoid and epithelioid cultures (Fig. 8). In sum, when induced to differentiate from the same progenitor mesothelial cells, epithelioid cells displayed a more matrix-degradative phenotype (increased 92 kD gelatinase, decreased TIMP) than fibroblastoid cells. In contrast, fibroblastoid cells exhibited a more matrix-producing phenotype than epithelioid cells. By using fibronectin expression as a general marker of extracellular matrix production, we found significantly greater accumulation of newly synthesized fibronectin in fibroblastoid cultures than epithelioid cultures (Fig. 10). Immunocytochemistry also showed greater cell-associated and matrix-associated fibronectin in fibroblastoid cultures as compared to epithelioid cultures (Fig. 11).

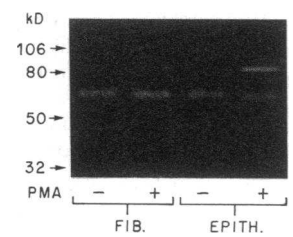

Figure 9. Substrate gel electrophoresis of secreted metalloproteinases by peritoneal mesothelial cells: fibroblastoid vs. epithelioid cells. An aliquot (20 μl) of conditioned medium from control and PMA (25 ng/ml)-stimulated fibroblastoid and epithelioid cell cultures was analyzed by gelatin substrate gel electrophoresis. Note the PMA-induced upregulation of 92-kD gelatinase expression in the epithelioid cells but not the fibroblastoid cells.

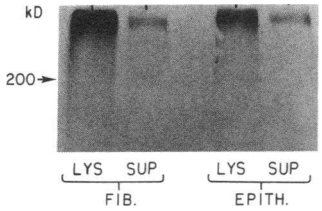

Figure 10. Immunoprecipitation of fibronectin from biosynthetically labeled peritoneal mesothelial cells: fibroblastoid vs. epithelioid cells. Fibroblastoid peritoneal mesothelial cell and epithelioid cultures were incubated in medium supplemented with [^{35}S]methionine for 24 h. Fibronectin was then immunoprecipitated from an aliquot of the culture supernatants and cell lysates. There was roughly equivalent incorporation of [^{35}S]methionine into TCA-precipitable protein in the cell lysates (fibroblastoid, 3.85×10^4 cpm/μg protein; epithelioid, 2.33×10^4 cpm/μg protein). Note the marked accumulation of newly synthesized fibronectin in the fibroblastoid cell lysates.

Discussion

Timely matrix deposition and resorption are critical for effective wound repair. The key role of the lining mesothelial cell in repair of serosal injury has been clearly delineated (6–8). Mesothelial cells have the capacity to synthesize a variety of extracellular matrix components including types I, III, and IV collagen (12–14). However, the present report provides the first evidence of their potential to participate in degradation of collagen and other matrix components. We have found that human mesothelial cells (pleural and peritoneal) express interstitial collagenase, 72- and 92-kD gelatinases (type IV/type V collagenases) and the counterregulatory inhibitor TIMP. Thus, these cells are likely to directly participate in matrix degradation during wound repair. Furthermore, expression of these products is modified by the state of differentiation of the cells, such that epithelioid cells manifest a more matrix-degradative phenotype than their fibroblastoid counterparts.

Our initial identification and characterization reported herein suggests that the mesothelial cell metalloproteinases and TIMP are identical to the corresponding proteins elaborated by other human cell types. In support of this possibility, we have found that the mesothelial cell products have the characteristic molecular weights by gel analysis and are recognized by specific antibodies developed against antigens derived from other cellular sources. This report adds to the body of evidence that a variety of cells have the capacity to express metalloenzymes and TIMP (16).

Although the regulation of metalloproteinase and TIMP expression is cell-type specific, several common features have emerged which are also shared by mesothelial cells. First, mesothelial cell metalloproteinases were secreted rather than stored in an intracellular pool, typical of all cell types other than the neutrophil (31, 32). Second, a primary site of mesothelial cell metalloenzyme regulation appeared to reside at a pretranslational level. Third, PMA (presumably via activation of protein kinase C) upregulated expression of interstitial collagenase, 92-kD gelatinase and TIMP, but had no effect on production of the 72-kD gelatinase (33). The action of PMA on collagenase gene expression required intermediate protein synthesis. Although not specifically investigated herein, it is likely that this regulatory process involves the transcriptional activating factors *c-fos* and *c-jun,* as has been reported in other cell types (34–36).

While PMA has proven to be a useful tool to demonstrate the capacity of mesothelial cells to express metalloproteinases and TIMP, ongoing work in our lab is aimed at elucidating the key physiologic modulators and their mechanisms of action. Since macrophages and their secretory products appear to play an important role in serosal injury and repair (7, 30), we focused our initial attention on TNF-α, IL-1β, and IFN-γ. We found that TNF-α increased production of interstitial collagenase and that both TNF-α and IL-1β upregulated expression of 92-kD gelatinase. Both cytokines also increased mesothelial cell production of TIMP. This concomitant induction of metalloproteinases and TIMP has been observed in some other cell types (37, 38), whereas in other cells the cytokines induce metalloproteinases without affecting TIMP production (39, 40). The addition of TNF-α and IL-1β together resulted in increased expression of interstitial collagenase, 92-kD gelatinase, and TIMP beyond that observed with either cytokine alone. TNF-α and IL-1β have previously been reported to have a synergistic effect on a number of cellular responses (41–43), most notably on the synovial cell expression of the metalloproteinase stromelysin (40).

LPS, a potent stimulator of metalloproteinase biosynthesis in mononuclear phagocytes (44), had no significant effect on

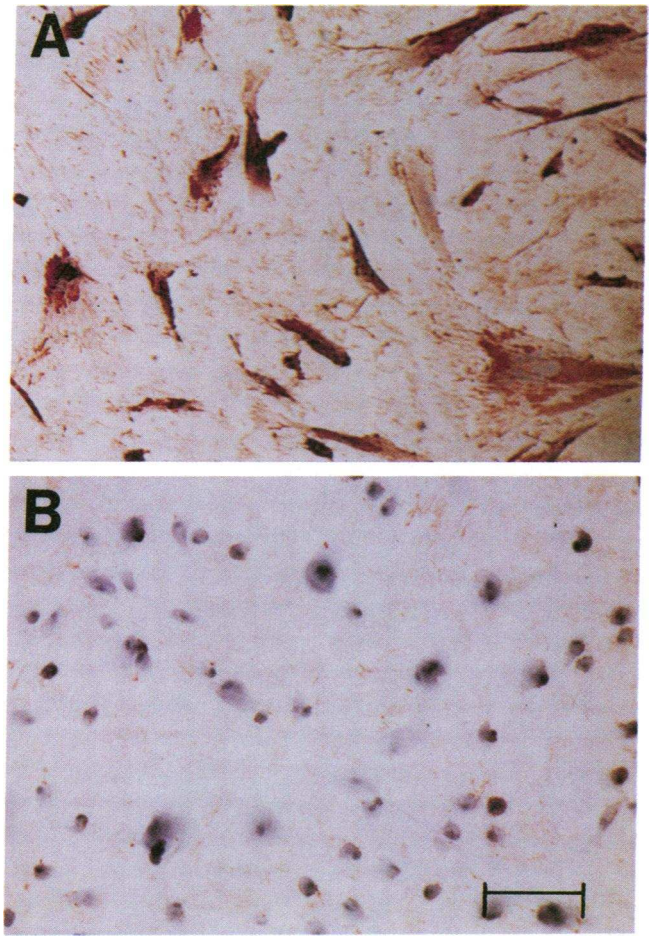

Figure 11. Immunocytochemical staining of peritoneal mesothelial cell cultures for fibronectin: fibroblastoid vs. epithelioid cells. Note the prominent cell-associated and matrix-associated reddish-brown staining for fibronectin in the fibroblastoid cultures (*A*), as compared with the epithelioid cultures (*B*). Bar, 100 μm. Controls in which an identifical dilution of non-immune sera was substituted for the primary antibody showed virtually no staining (not shown), confirming the specificity of the staining pattern.

mesothelial cell metalloenzyme production. The presence of serum in the incubating medium did not confer LPS responsiveness, suggesting that a deficiency of LPS binding protein was not the explanation. Since LPS has been demonstrated to have other biological effects on mesothelial cells (11, 45), cell surface receptors are presumably present. Analogous to our findings, TNF-α but not LPS, induces pleural mesothelial cell production of IL-8 (46). Further investigation of the differing response to LPS between mononuclear phagocytes and mesothelial cells may provide insight into postreceptor LPS signal transduction mechanisms.

Of particular interest is the observation that the state of cellular differentiation has a striking influence on mesothelial cell expression of metalloproteinases and TIMP. Epithelioid mesothelial cells secreted significantly more 92-kD gelatinase in response to PMA than fibroblastoid cells. There was also markedly decreased TIMP expression by epithelioid cells. In contrast, the constitutive production of 72-kD gelatinase and PMA-induced collagenase biosynthesis were not altered by state of differentiation. Thus, the 92-kD gelatinase and interstitial collagenase were not coordinately regulated in this setting. When assessed for extracellular matrix production, fibroblastoid cells synthesized and accumulated much more fibronectin than epithelioid cells. Taken as a whole, the epithelioid cells displayed a more matrix-degradative phenotype (increased 92-kD gelatinase, decreased TIMP) as compared to their matrix-producing fibroblastoid counterparts. We speculate that the cuboidal mesothelium characteristic of the early response to serosal injury (8) may manifest matrix-degradative properties which favor normal repair as opposed to the initiation of fibrosis.

In summary, we have found that human mesothelial cells have the capacity to express metalloproteinases and TIMP and are therefore likely to directly participate in the matrix turnover that follows serosal injury. Furthermore, the state of differentiation of the cells has a marked influence on metalloproteinase and TIMP expression, such that cells with epithelioid morphology adopt a more matrix-degradative phenotype. Further investigation of the modulation of mesothelial cell metalloproteinases and TIMP may provide new insights into the mechanisms of serosal injury and repair as well as rational strategies to promote a favorable outcome.

Acknowledgments

The authors gratefully acknowledge Ms. Ina Kim for typing the manuscript and Dr. Brent Brown for assistance in obtaining human pleural mesothelial cells.

This work was supported by National Institutes of Health (NIH) Clinical Investigator Awards HL-02370 (Dr. Marshall) and AR-01787 (Dr. Petersen); NIH grant HL-37615 (Dr. Hoidal); NIH grant HL-29594 (Dr. Welgus); NIH grant HL-46440, Council for Tobacco Research, U.S.A., Inc., and DVA research funds (Dr. Campbell). Dr. Campbell is a Clinical Investigator of the Department of Veterans Affairs.

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