Identification of TIMP-2 in Human Alveolar Macrophages

REGULATION OF BIOSYNTHESIS IS OPPOSITE TO THAT OF METALLOPROTEINASES AND TIMP-1*

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We have identified the metalloproteinase inhibitor TIMP-2 as a secreted product of human alveolar macrophages. In contrast to human fibroblasts, TIMP-2 was released from macrophages free of any apparent complexed metalloproteinases. Also in marked distinction to fibroblasts, TIMP-2 secretion from mononuclear phagocytes was subject to modulation by a variety of agents. TIMP-2 was synthesized by macrophages placed in culture under basal conditions in amounts approximately 30% of those secreted by fibroblasts on a per cell basis. The additions of lipopolysaccharide, denatured type I collagen, and zymosan to culture medium each resulted in a dose-dependent and profound decrease in macrophage TIMP-2 protein production and steady-state mRNA levels. In contrast, all of these agents markedly enhanced the biosynthesis of macrophage interstitial collagenase and TIMP-1 as assessed by analysis of identical cell and conditioned media samples. In human fibroblasts, TIMP-2 biosynthesis was unaffected by interleukin-1, tumor necrosis factor-α, platelet-derived growth factor, and phorbol ester despite the massive collagenase stimulation induced by each of these agents. We conclude that TIMP-2 is a potentially important mononuclear phagocyte product whose biosynthesis is regulated in a distinct and completely opposite manner to that of collagenase and TIMP-1.

The secretion of its own battery of metalloproteinases and inhibitors.

The capacity of human mononuclear phagocytes to produce metalloproteinases and TIMP-1 has been clarified by several recent studies (5–8). As these cells undergo differentiation from monocytes to resident tissue macrophages, their proteinase and matrixmetamorphosis shifts from an intracellularly stored group of neutrophil-like serine proteinases to a highly regulatable and secreted battery of metalloenzymes (7, 9–11). A prototypic resident macrophage, the alveolar macrophage, has been shown to produce an interstitial collagenase identical to that secreted by human fibroblasts and in quantities 20–30% of the latter cell type (8, 12). Stromelysin-1 is produced at similar levels, but 72-kDa type IV collagenase is only a very minor, at times even undetectable macrophage product, especially compared with its prominent release by fibroblasts (5). Instead, macrophages elaborate a highly related 92-kDa type IV collagenase whose secretion from fibroblasts is only rarely observed (13, 14). Interestingly, the 92- and 72-kDa type IV collagenases display very similar catalytic activities and are indistinguishable in their range of susceptible matrix substrates. Macrophages and fibroblasts also produce large quantities of the counterregulatory inhibitor TIMP-1, whose function appears to involve control of the enzymatic activity of interstitial collagenase, stromelysin, and 92-kDa type IV collagenase (5, 8). The potential physiologic importance of macrophage metalloproteinase production is underscored by recent in situ hybridization studies demonstrating high levels of stromelysin mRNA in lipid laden macrophages of atherosclerotic plaques (15) and of interstitial collagenase mRNA in macrophage-like cells of the rheumatoid synovial pannus (16). In this report, we have examined the production of TIMP-2 by alveolar macrophages in an effort to further define the spectrum of metalloproteinases and inhibitors produced by human mononuclear phagocytes, to gain insight into mechanisms regulating their expression, and ultimately to delineate the role of these cells during inflammation. TIMP-2 is a newly characterized metalloproteinase inhibitor of M, 21,000 that is primarily responsible for controlling the activity of 72-kDa type IV collagenase (17–21). Heretofore, production of TIMP-2 has been documented only in human fibroblasts in which it is found complexed with the large amounts of 72-kDa type IV collagenase simultaneously secreted by such cells (17–21). Furthermore, its biosynthesis in this cell type has been reported to be largely constitutive and unmodified by a variety of cytokines and growth factors, except for TGF-β (19). We now report that TIMP-2 is also produced constitutively by human alveolar macrophages; however, it is secreted from macrophages uncomplexed to higher M, metalloproteinases. Remarkably, protein and steady-state mRNA levels of TIMP-2 are markedly diminished by exposure to lipopolysaccharide, denatured type I collagen, and zymosan (typical macrophage-
activating agents, factors that simultaneously cause marked stimulation in the cell's production of interstitial collagenase and TIMP-1.

**EXPERIMENTAL PROCEDURES**

**Materials**—Bovine serum albumin, phorbol 12-myristate 13-acetate (PMA), Trition X-100, and protein A-Sepharose were obtained from Sigma. Lipopolysaccharide (LPS), derived from *Escherichia coli* cell walls and purified by trichloroacetic acid extraction, was also purchased from Sigma (catalogue no. L 4255). Recombinant human IL-1 was kindly provided by Peter Lomedico (Hoffmann-La Roche). Recombinant human TNF-α and human PDGF were purchased from R & D Systems, Inc. (Minneapolis, MN). Zymosan was obtained from Sigma. Type I collagen was isolated from sheep-skin digested rat tail tendon and purified as described previously (22). Collagen was denatured by heating to 60 °C for 15 min. The amount of endotoxin present in the collagen and zymosan preparations was quantified by the limulus amebocyte lysate assay (QCL-1000, Whittaker M. A. Bioproducts, Walkersville, MD). The highest concentrations of collagen and zymosan used (10 μg/ml and 1.0 mg/ml, respectively) contained less endotoxin (collagen = 0.028 μg/ml, zymosan = 0.014 ng/ml) than our 10% fetal calf serum-containing medium (0.048 ng/ml).

**Purification of TIMP-2**—Specific Antiserum to TIMP-2—TIMP-2 was purified in our laboratory with 72-kDa type IV collagenase from the serum-free conditioned medium of human skin fibroblasts grown to confluence in monolayer culture (23, 24). This material was used to mark the electrophoretic migration of TIMP-2 in reverse zymography and to test monospecificity of the TIMP-2 antibody (below).

Rabbit polyclonal antiserum to human TIMP-2 was kindly provided by Keith Langley of Amano Biologicals (Thousand Oaks, CA). This antiserum exhibited complete monospecificity for TIMP-2, as assessed by Western blot of TIMP-1, TIMP-2, interstitial collagenase, stromelysin, and 92- and 72-kDa type IV collagenases. Only TIMP-2 was recognized. Furthermore, metabolic labeling of human fibroblasts and exposure of the labeled proteins to antisera precipitated the desired protein complexes.

**Cells and Cell Culture Conditions**—Human alveolar macrophages were harvested from healthy adult volunteer cigarette smokers (who smoked more than one pack/day) by using saline bronchoalveolar lavage. More than 95% of the cells obtained were macrophages as determined by differential counting of Wright-stained cytocentrifuge preparations. The remaining cells were primarily lymphocytes; fibroblasts were not observed. The lavaged cells were washed three times in Hanks' balanced salt solution and plated in Linbro six-well cluster plates (Flow Laboratories, Inc., McLean, VA) at a concentration of 2 × 10^6 cells/well. The cells were incubated for 1 h at 37 °C to allow attachment. The Hanks' balanced salt solution was then exchanged for culture medium (1:1 [v/v] Ham's F-12/Dulbecco's modified Eagle's medium containing 10% fetal calf serum), and the cell cultures were maintained in humidified 95% air, 5% CO₂ (12).

Human skin fibroblasts were obtained from American Type Culture Collection (Rockville, MD, CRL-1467). The cells were grown in 10% fetal calf serum-containing medium until visual confluence.

**Generation of Conditioned Medium**—All media samples analyzed were conditioned in the presence of [³⁵S]methionine for 24 h. To determine the effects of LPS (2.5 μg/ml), zymosan (100–500 μg/ml), and gelatin (10 μg/ml), the cells were exposed to these agents for 24 h before labeling and then again during the study period. To begin the study period, culture medium was replaced with otherwise identical methionine-free medium containing 50 μCi/ml [³⁵S]methionine (Amersham Corp.), and cells were labeled for the next 24 h. After collection, the samples of conditioned medium containing the labeled proteins were stored at −70 °C until analysis.

**Generation of Cell Lysates**—Cells were radiolabeled as described above, and cell lysates were obtained to determine if TIMP-2 was present within the cell, on the cell surface, or in the extracellular matrix. The conditioned medium was removed, cells were washed three times with phosphate-buffered saline, and then cells were lysed with Triton X-100 (0.1% [ω/ω] Triton X-100 and 1.5 mM phenylmethylsulfonyl fluoride, 20 mM EDTA, 5 mM benzamidine, and 2 mM peptatin). The lysates were incubated for 18 h at 25 °C, and the supernatants were then analyzed for the presence of radio-labeled TIMP-2. Lysates prepared in this manner contained cytoplasmic, membrane, and extracellular matrix constituents.

To assess TIMP-2 susceptibility to any membrane-bound proteases, labeled conditioned medium (containing newly synthesized TIMP-2) was removed from basal macrophages and incubated for 24 h with cells that had been maintained in culture for 48 h in the presence or absence of LPS. Cell lysates and supernatants were then obtained to determine the fate of the added TIMP-2.

**Single-Well Gel Electrophoresis**—Aminopeptidase gel electrophoresis (25) was performed to assess the effects of LPS and zymosan on the production of 72-kDa type IV collagenase using this enzyme's capacity to efficiently degrade denatured type I collagen or gelatin (23). Aliquots of serum-free conditioned medium were first subjected to immunoprecipitation with specific antiserum to 72-kDa type IV collagenase (13) to remove this major source of macrophage gelatinolytic activity. The supernatant was then applied without reduction to a 10% polyacrylamide slab gel impregnated with 1 mg/ml gelatin. Polyacrylamide gel electrophoresis was performed at 4 °C. After electrophoresis, the gel was incubated in 2.5% (v/v) Triton for 30 min and overnight in 50 mM Tris, pH 8.0, containing 5 mM CaCl₂ and 1 μM ZnCl₂ at 37 °C. The gel was then stained with a solution of 0.125% Coomasie Blue.

To demonstrate the functional activity of TIMP-2 in macrophage-conditioned medium, reverse zymography was performed (26). Samples concentrated by dialysis and lyophilization were applied to the polyacrylamide gel and then processed in an identical manner to that described above, except for the addition of rat uterine explant-conditioned medium (kindly provided by J. J. Jeffrey, Albany, NY) to the Tris-Ca-Zn buffer during the 37 °C overnight incubation. This medium has spontaneous gelatinase activity, so proteinase inhibitors like protease of blocking gelatin degradation appear as dark blue stained bands.

**Immunologic Assays**—Enzyme-linked immunosorbent assays for human interstitial collagenase and TIMP-1 were performed by our laboratory as described previously (27, 28). These assays have nanogram sensitivity and employ polyclonal antibodies which recognize free enzyme or inhibitor with equal avidity to that of each complexes with one another.

**Immunoprecipitation of Labeled Proteins**—Polyclonal antisera to human interstitial collagenase (27) and TIMP-1 (28) were generated as described previously. A rabbit polyclonal antibody to human TIMP-2 was kindly provided by Amano Biologicals. The procedure for immunoprecipitation of metalloproteinases and TIMP-1 has been reported previously (5). Processed samples were applied to 10 or 12% polyacrylamide slab gels, and electrophoresis was performed as described by King and Laemmli (29). The gels were equilibrated with EN'HANCE (Du Pont-New England Nuclear) for 1.5 h, rinsed in cold distilled water for 1 h, and then dried under vacuum. The dried gels were exposed to Kodak XAR-5-X-Omat film (Eastman Kodak Co., Rochester, NY) at −70 °C.

**RNA Purification and Analysis**—Total cellular RNA was isolated by the guanidinium phenol extraction method (30). Northern hybridization was performed essentially as described previously (10). The DNA probe used (also obtained from Keith Langley, Amano Biologicals, (17) and TIMP-1 cDNA (obtained from David Carmichael, Synergen, Boulder, CO (31) were used as probes.

**RESULTS**

To determine whether human alveolar macrophages produce TIMP-2, cells were metabolically labeled, and newly synthesized proteins were subjected to immunoprecipitation with TIMP-2-specific antiserum. As shown in Fig. 1, macrophages constitutively synthesized a single immunoreactive species which migrated at 21 kDa, the molecular mass of TIMP-2. Furthermore, this band corresponded in Mₐ to a zone of metalloproteinase inhibitory activity demonstrated by reverse zymography of macrophage-conditioned medium (data not shown). The electrophoretic migration of macrophage TIMP-2 was compared with the corresponding protein produced by metabolically labeled human fibroblasts (Fig. 1). Identical electrophoretic mobility was observed. However, in the case of fibroblast-conditioned medium, as reported previously (18, 24), large amounts of 72-kDa type IV collagenase were co-precipitated (Fig. 1, lane 4) because most TIMP-2 is secreted already bound to this metalloenzyme. Interestingly, and in contrast, there was no evidence of any species co-precipitating with TIMP-2 from the macrophage-conditioned medium (Fig. 1, lane 2). These results suggest that most, if
not all, TIMP-2 secreted by human alveolar macrophages is in a "free" or unbound form, rather than in a complex with 72-kDa type IV collagenase or any other metalloenzyme. This finding is perhaps explained by the very low production of the 72-kDa metalloproteinase by human macrophages (5). Based upon simultaneous immunoprecipitation of labeled macrophage versus fibroblast-conditioned media, mononuclear phagocytes appeared to possess approximately 30% of the fibroblast's TIMP-2 synthetic capacity on a per cell basis (data not shown) despite their considerably smaller size.

To gain insight into factors regulating the production of TIMP-2 by human alveolar macrophages, cells were exposed to LPS (2.5 μg/ml), denatured type I collagen (gelatin, 10 μg/ml), or zymosan (500 μg/ml in A; 100 μg/ml in B). All cultures were performed in serum-containing media. Labeled proteins were immunoprecipitated with specific antiserum to TIMP-2, TIMP-1, and interstitial collagenase as indicated. Panels A and B represent identical experiments (except for zymosan concentration) performed on two separate individuals.

Likewise, its synthesis also increased dramatically upon exposure of the cells to LPS, gelatin, and zymosan. In contrast, TIMP-2 was secreted by basal macrophages in readily detectable amounts. However, exposure of the same cells to LPS, gelatin, and zymosan followed by immunoprecipitation of the same labeled conditioned media samples with TIMP-2-specific antiserum revealed that each agent largely abolished TIMP-2 biosynthesis. This identical pattern of regulation was observed in several experiments, indicating that control of TIMP-2 production in the human alveolar macrophage is essentially opposite to that of interstitial collagenase and TIMP-1.

To eliminate the possibility that TIMP-2 was sequestered within the cell, on the cell surface, or in the extracellular matrix, cell lysates were obtained by detergent treatment (see "Experimental Procedures") from macrophages exposed to [35S]methionine. Lysates from cells cultured in the absence of LPS contained no detectable TIMP-2 by immunoprecipitation (data not shown).

Experiments were also performed to determine if secreted or cell-associated proteinases induced by LPS degraded newly synthesized TIMP-2. Labeled control medium (which contained spontaneously secreted 35S-labeled TIMP-2) was incubated with: 1) LPS-conditioned versus control media for 24 h at 37 °C; and 2) LPS-treated versus control macrophages for 24 h at 37 °C. There was no appreciable difference between the amounts of TIMP-2 recovered from the LPS-conditioned versus the control media nor were there differences in TIMP-2 levels following exposure to LPS-treated versus untreated cells. In addition, cell lysates (from experiment 2) contained no detectable TIMP-2 (data not shown). Finally, our culture conditions always included 10% fetal calf serum, even during metabolic labeling when dialyzed serum was employed. Therefore, our results indicating suppression of TIMP-2 production by macrophage-activating agents reflect real changes in actual TIMP-2 biosynthesis, a conclusion proven by the steady-state mRNA data presented later.

Since 72-kDa type IV collagenase is co-secreted with TIMP-2 in human fibroblasts, we examined the effects of LPS, gelatin, and zymosan on the production of this enzyme in macrophages. Serum-free conditioned medium was collected, immunoprecipitated with antiserum to 92-kDa type IV collagenase (to remove this major source of macrophage gelatinolytic activity), and the supernatants analyzed by gelatin substrate zymography. None of these agents affected production of 72-kDa type IV collagenase by human macrophages (data not shown).

To more completely characterize the effects of LPS on TIMP-2 production, time course and dose-response determinations were performed. As shown in Fig. 3, macrophages exposed to [35S]methionine for 6, 24, and 48 h constitutively synthesized only a very small amount of interstitial collagenase. Upon the addition of LPS, a prominent induction of this enzyme was observed by 48 h. This considerable temporal delay in response to endotoxin has been noted previously by Welgus and co-workers (8, 12) and by others (32). When the identical conditioned media samples were immunoprecipitated with TIMP-2-specific antiserum, basal synthesis of TIMP-2 was apparent by 24 h and increased considerably over the next 24 h. In contrast, the addition of LPS prevented TIMP-2 biosynthesis at any time point examined.

A dose titration was performed to compare concentrations of endotoxin that inhibit the production of TIMP-2 with those capable of stimulating collagenase biosynthesis. As shown in Fig. 4, LPS concentrations >0.001 μg/ml caused a dose-dependent increase in collagenase production while si-
Thesis. Macrophages were incubated in serum-containing culture medium including [35S]methionine and with (LPS) or without (-LPS) added LPS (2.5 μg/ml). Newly labeled proteins were then immunoprecipitated with specific antisera to TIMP-2 (top panel) and interstitial collagenase (bottom panel).

FIG. 3. Time course of LPS inhibition of TIMP-2 biosynthesis. Macrophages were incubated in serum-containing culture medium including [35S]methionine and with (+LPS) or without (-LPS) added LPS (2.5 μg/ml). Newly labeled proteins were then immunoprecipitated with specific antisera to TIMP-2 (top panel) and interstitial collagenase (bottom panel).

Multaneously inhibiting TIMP-2 biosynthesis. These inverse effects became increasingly prominent with progressively higher concentrations of endotoxin in serum-containing medium.

To determine whether the inhibition of TIMP-2 biosynthesis observed concomitantly with metalloproteinase induction in human alveolar macrophages is a general biological phenomenon, we examined the effects of phorbol ester and IL-1 on human skin fibroblasts. These two agents have a well-characterized capacity to stimulate interstitial collagenase production in this cell type. As shown in Fig. 5, human fibroblasts secreted only small amounts of collagenase basally, but levels were increased dramatically upon exposure of the cells to PMA or IL-1. When the same labeled conditioned media were subjected to immunoprecipitation with TIMP-2 antisemum, production of the inhibitor remained unchanged. Similar results were also observed using TNF-α and PDGF (data not shown); both cytokines caused marked up-regulation in collagenase production, consistent with previous reports (3, 4, 33), whereas TIMP-2 biosynthesis was unaltered.

Finally, Northern hybridizations were performed to assess macrophage TIMP-2 gene regulation. Total cellular RNA was purified from 2.5 × 10^6 macrophages exposed to LPS, gelatin, or no agent for 48 h. Equal amounts of RNA (verified by ethidium bromide staining (data not shown)) were subjected to Northern analysis. As shown in Fig. 6, control macrophages contained readily detectable steady-state mRNA levels for TIMP-2. However, following exposure to LPS (Fig. 6A) or gelatin (Fig. 6B), TIMP-2 mRNA was markedly diminished corresponding to the observed decrease in this protein’s production. In contrast, when identical samples of total RNA were hybridized to TIMP-1 cDNA, this mRNA was undetectable in basal macrophages, but its levels increased markedly following exposure to LPS (Fig. 6A) and gelatin (Fig. 6B). Therefore, TIMP-2 and TIMP-1 are regulated in opposite directions by macrophage activating agents at the gene level.
DISCUSSION

The data in this report provide further insight into the capacity of human macrophages to control extracellular matrix turnover by expanding their metalloproteinase/metalloprotease inhibitor repertoire to include TIMP-2. Interestingly, in human fibroblasts a large proportion of TIMP-2 is secreted in complex with 72-kDa type IV procollagenase, although the precise site of complex formation remains undefined. Similarly, the 72-kDa enzyme, a major product of fibroblasts, is released almost entirely in complex with TIMP-2. In contrast to fibroblasts, human mononuclear phagocytes are capable of producing only very limited amounts of the 72-kDa enzyme but elaborate large quantities of a highly related 92-kDa type IV collagenase. We (5) and others (13) have previously shown that most of this latter macrophage metalloproteinase is released in zymogen form already complexed with TIMP-1. As demonstrated in the metabolic labeling and immunoprecipitation experiments shown in Fig. 1, TIMP-2 is secreted by human alveolar macrophages free of apparent association with any higher Mr species. In particular, neither interstitial collagenase, stromelysin, nor 92-kDa type IV collagenase (all major secreted products of the human alveolar macrophage) are co-precipitated. These data suggest that unlike the case for human fibroblasts, TIMP-2 is released by human macrophages predominantly in a free or unbound state. If this situation is also found in vivo, it would suggest that secreted macrophage antiproteases may be capable of modifying the activity of 72-kDa type IV collagenase produced by neighboring tissue fibroblasts. In this regard, it has recently been demonstrated that the activated fibroblast 72-kDa type IV collagenase requires a second associated TIMP-2 molecule (in addition to the one that is secreted in complex with) for complete inhibition of catalytic activity (20, 21, 24).

Very little is known about the regulation of TIMP-2 production. In human fibroblasts, like its closely associated 72-kDa type IV collagenase, TIMP-2 biosynthesis has been documented to occur constitutively in vitro, but such basal synthetic rates have largely resisted modification by phorbol esters, cytokines, and growth factors (19, 24). Indeed, our results shown in Fig. 5 confirm these observations using IL-1, PMA, TNF-α, and PDGF on human fibroblasts, all of which dramatically up-regulate interstitial collagenase biosynthesis without affecting TIMP-2 production. The only agent capable of modifying TIMP-2 release by human fibroblasts reported thus far is TFG-β, which specifically decreases TIMP-2 steady-state mRNA levels. In their study (19), Stetler-Stevenson et al. noted a divergent regulation of TIMP-1 and TIMP-2 by TGF-β, with production of TIMP-1 being stimulated following exposure of fibroblasts to the matrix-inducing growth factor.

Our results studying the regulation of TIMP-2 production in human alveolar macrophages are striking. These mononuclear phagocytes express TIMP-2 constitutively. However, exposure of the cells to LPS, denatured type I collagen, and zymosan (all macrophage-activating agents that potentially stimulate metalloproteinase production from such cells) abolishes the synthesis of TIMP-2. Furthermore, for LPS and gelatin, we have shown parallel diminution in TIMP-2 mRNA levels with secreted protein (Fig. 6). Interestingly, all three macrophage-activating agents also simultaneously induce increased levels of TIMP-1. These data demonstrate that in the macrophage, in clear contrast to the fibroblast, production of TIMP-2 is readily subject to control by a variety of regulatory signals. The data also add to the already compelling case supporting the existence of cell type specificity with regard to both the spectrum of secreted metalloproteinases and inhibitors and the regulatory agents capable of modifying their release. Finally, and perhaps most importantly, TIMP-2 biosynthesis in the macrophage appears to be regulated in a completely opposite manner to the production of TIMP-1 and interstitial collagenase.

We believe these observations will serve as a foundation for the further study of the biological role of TIMP-2 secreted by the human alveolar macrophage in the control of extracellular matrix turnover. The potential contribution of the state of cellular differentiation, so critical for determining mononuclear phagocyte capacity for metalloproteinase expression, must now be carefully examined for TIMP-2. Most importantly, the intracellular mechanisms responsible for the totally divergent regulation of this newly described metalloproteinase inhibitor, in comparison with TIMP-1 and the metalloproteinases, must be elucidated. Our future studies will address these issues.

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