Divergent regulation of 92-kDa gelatinase and TIMP-1 by HBECs in response to IL-1β and TNF-α

P. M. Yao, B. Maitre, C. Delacourt, J. M. Buehler, A. Harf, and C. Lafuma

Institut National de la Santé et de la Recherche Médicale Unité U296 and Département de Physiologie, Faculté de Médecine, 94010 Créteil; and Département de Biologie Cellulaire et Moléculaire, Commissariat à l’Energie Atomique, 91191 Gif-sur-Yvette, France

Yao, P. M., B. Maitre, C. Delacourt, J. M. Buehler, A. Harf, and C. Lafuma. Divergent regulation of 92-kDa gelatinase and TIMP-1 by HBECs in response to IL-1β and TNF-α. Am. J. Physiol. 273 (Lung Cell. Mol. Physiol. 17): L866–L874, 1997.—In this study, we investigated the question of whether human bronchial epithelial cells (HBECs) contribute to the regulation of 92-kDa gelatinase activity by secreting tissue inhibitor of metalloproteinase (TIMP)-1. We investigated expression of 92-kDa gelatinase and TIMP-1 in response to lipopolysaccharide (LPS) and to the proinflammatory cytokines interleukin (IL)-1β and tumor necrosis factor (TNF)-α. Confluent HBECs from explants were cultured in plastic dishes coated with type I and III collagen. We demonstrated that TIMP-1 was expressed at both the protein and mRNA levels by primary cultures of HBECs. Gelatin zymography of HBEC-conditioned media showed that exposure of HBECs to LPS, IL-1β, or TNF-α induced a twofold increase in the latent form of 92-kDa gelatinase production, as well as its activation. Also, quantitative reverse transcriptase (RT)-polymerase chain reaction (PCR) demonstrated a twofold increase in the 92-kDa mRNA level in response to both cytokines. In contrast, TIMP-1 production evaluated by immunoblotting was unchanged in the presence of LPS and IL-1β and was clearly decreased in the presence of TNF-α. Quantitative RT-PCR demonstrated that TIMP-1 mRNA levels remained unchanged in response to LPS or IL-1β but decreased by 70% in the presence of TNF-α. All of these results strongly suggest that the control mechanisms regulating the expression of 92-kDa gelatinase and TIMP-1 by HBECs in response to inflammatory stimuli are divergent and result in an imbalance between 92-kDa gelatinase and TIMP-1 in favor of the metalloproteinase. Such an imbalance may contribute significantly to acute airway inflammation.

Matrix metalloproteinases; inflammatory cytokines; lung; tissue inhibitor of metalloproteinase-1; human bronchial epithelial cells; interleukin-1β; tumor necrosis factor-α

Matrix metalloproteinases (MMPs) play an important role in the proteolytic degradation of the extracellular matrix (ECM), both in physiological processes, such as tissue remodeling, angiogenesis, wound healing, etc., and during pathological events, such as tumor invasion and metastatic progression (18). Regulation of the role of MMPs in ECM degradation occurs mainly at three levels. At the transcriptional level, MMP expression is precisely controlled by various cytokines acting through positive or negative regulatory elements of its genes. After secretion from the cell, MMP activity is controlled both by proteolytic activation of latent proenzymes and by interactions with their specific inhibitors.

Naturally occurring inhibitors, tissue inhibitors of metalloproteinases (TIMPs), are important controlling factors in the actions of MMPs and tissue destruction in disease processes. The balance between the levels of activated MMPs and of free TIMPs plays an important role in the regulation of MMP activity, effectively controlling the amount of connective tissue breakdown. Alterations in this equilibrium affect angiogenesis (20), cell growth (13), cell differentiation (9), and embryonic development (21). Four distinct TIMP molecules (TIMP-1, TIMP-2, TIMP-3, and TIMP-4) have been isolated, cloned, and characterized in several species (2, 6, 12, 17). The major inhibitor, a glycoprotein with an apparent molecular size of 30 kDa called TIMP-1, is encoded by a single 0.9-kb mRNA. It is produced by a variety of human tissues and by many tumor cell lines (5, 19, 32). TIMP-1 inhibits MMPs by forming a 1:1 complex (stoichiometry) with activated interstitial collagenase (MMP-1), stromelysin (MMP-3), 72-kDa gelatinase (MMP-2), 92-kDa gelatinase (MMP-9), and pro-MMP-9 in a noncovalent fashion.

Expression of the genes for MMPs and TIMPs in different normal and tumor cells is regulated by a variety of physiological and pharmacological agents in a manner that may be cell-type and tissue specific. The effects of interleukin (IL)-1β, tumor necrosis factor (TNF)-α, and lipopolysaccharide (LPS) on the biosynthesis and secretion of MMPs and TIMPs have been studied in a variety of cell lines. These factors induce the production of high levels of some MMPs. The activity of MMPs is also controlled by TIMPs, which also depend on the presence of the same cytokines in the microenvironment. It has been shown that synthesis and secretion of TIMPs occur in several types of MMP-producing cells, such as human alveolar macrophages, monocytes, skin fibroblasts, and some tumoral cells (4, 19, 28, 32). In a previous study (34), we demonstrated that primary cultures of human bronchial epithelial cells (HBECs) constitutively express major 92-kDa matrix gelatinase and minor 72-kDa gelatinase, both of which may be involved in basement membrane degradation and remodeling. We therefore addressed the question of whether HBECs also contribute to the regulation of the extracellular activity of these enzymes via secretion of TIMP-1.

In this study, we investigated the basal expression of TIMPs by primary cultures of HBECs, as well as the effects on 92-kDa gelatinase and TIMP-1 expression of Escherichia coli LPS endotoxin and of the proinflammatory cytokines IL-1β and TNF-α. We found that HBECs secreted not only 92-kDa gelatinase but also TIMP-1 under basal conditions. In the presence of IL-1β, TNF-α, or LPS, 92-kDa gelatinase activity increased sharply, whereas TIMP-1 was unchanged or diminished in response to LPS and to both cytokines. The imbalance...
between 92-kDa gelatinase and local TIMP-1 activities from HBECs in inflammatory pathological conditions suggests that metalloproteinases from HBECs may play a prominent role in the pathological remodeling of the bronchial epithelium in response to inflammatory events.

MATERIALS AND METHODS

Cell Cultures

Human bronchial epithelial biopsies were obtained by fibroscopy in 18 patients investigated for bronchopulmonary carcinoma. Biopsies were taken at a distance from the tumor. For each specimen, pathological examination confirmed that the bronchial mucosa was normal. All procedures were reviewed and approved by the Hospital Henri Mondor Committee for the Protection of Rights of Human Subjects, and written informed consent was obtained to authorize the study.

HBECs were cultured as previously described (34). Briefly, two or three explants (~0.5 × 0.5 mm in size) were placed on sterile plastic dishes coated with collagen G matrix (type I and III collagen; Polylabo). The explants were covered with 600 µl of Dulbecco’s modified Eagle’s medium (DMEM)-F-12 (1:1) medium (Life Technologies) and were incubated for 24 h. Two milliliters of culture medium were then added to each dish. Culture medium consisted of serum-free DMEM-F-12 (1:1) supplemented with 2% Ultroser G (Sepracor), 1% antibiotics (10,000 U/ml penicillin G sodium, 10,000 µg/ml streptomycin sulfate, and 25 µg/ml amphotericin B), and 2 mM glutamine (Life Technologies). Explants were placed in a humidified incubator at 37°C under 5% CO2 in air. The culture medium was changed every 3–4 days. Explants were cultured for 2 wk until confluence of HBECs. Also, the same explants were transferred successively to new, sterile, coated plastic dishes, at 5- to 8-day intervals, to initiate new primary HBEC cultures. As described in our previous publication (34), the confluent HBECs exhibited a flat polygonal shape and were closely opposed, as is typical of cultured epithelial cells. The beating of cilia was easily identified under a light microscope as localized movement of medium over the cells. Moreover, the epithelial nature of all cultured bronchial cells was previously confirmed by staining with antibody to cytokeratin, the characteristic component of epithelial cell intermediate filaments. Also, HBEC cultures did not stain with anti-fibroblast or KP1 antibodies, indicating that contamination by nonepithelial cell types did not occur.

Normal human mammary fibroblasts cultured at confluence on plastic dishes were used as reference cells for the human TIMP-1 assay by reverse transcriptase (RT)-polymerase chain reaction (PCR). Human alveolar macrophages were recovered from bronchoalveolar lavage specimens from four patients with adult respiratory distress syndrome (ARDS) and were used as reference cells for the human 92-kDa gelatinase assay by RT-PCR.

For measurement of the gelatinases and TIMP-1 activities as well as isolation of total cellular RNA (RNA T), HBEC cultures were incubated at confluence with Ultraser G-free culture medium in the presence of 0.2% lactalbumin for 24 h. These cultures were or were not subsequently exposed to LPS (1 µg/ml), IL-1β (100 U/ml), or TNF-α (100 U/ml; Sigma) for 24 additional hours. To establish biological relevance, a few cell cultures were carried out for investigating the time course response (at 6, 12, and 24 h) or dose response (10, 50, 100, and 500 U/ml) in the case of TNF-α, which has been shown to be more reactive.

Zymography and Reverse Zymography

The HBEC culture medium was harvested and stored at −20°C until use. Collected medium was resolved by 8% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) in the presence of 1 mg/ml porcine skin gelatin. The Laemmli method was followed without reducing agents or procedures involving boiling. After electrophoresis, the gel was washed for 30 min in 2.5% Triton X-100 at room temperature to remove SDS. The gel was then incubated overnight at 37°C in reaction buffer [100 mM tris(hydroxymethyl)amino methane (Tris)·HCl and 10 mM CaCl2, pH 7.4]. After staining with Coomassie brilliant blue R 250, gelatin-degrading enzymes were identified as clear zones of lysis against a blue background. Molecular weights of gelatinolytic bands were estimated using prestained molecular weight markers.

Activities in the gel slabs were quantified using semiautomated image analysis (National Institutes of Health Image 1.52), which quantifies both the surface and the intensity of lysis bands after scanning the gels. Results were expressed as arbitrary units per 24 hours per 10² cells. To check that this method for measuring enzymatic activity on zymograms was linear over the range of activities in unknown samples, we evaluated activities for increasing volumes of culture medium and found that arbitrary units obtained with the image analysis system increased linearly with the volume of the samples (r = 1.00; see Ref. 10).

TIMP-1 secreted into the culture medium was detected using reverse zymography (11). Briefly, 25 µl of 20-fold concentrated conditioned media were resolved by 11.5% SDS-PAGE in the presence of 1 mg/ml porcine skin gelatin. The standard zymographic method was modified as follows: SDS from the gel by incubation of the gel for 45 min at 37°C in conditioned medium from p-aminophenylmercuric acetate-activated rabbit skin fibroblasts, which provided a source of activated metalloproteinases capable of degrading the gelatin in the gel. The gel was then incubated and stained in the same way as standard zymography. Protection of the gelatin in the gel by the presence of TIMPs led to the production of relatively dark bands against a lighter background. Recombinant TIMP-1 was used as the reference (Valbotech).

Immunoblotting

Aliquots of 20-fold concentrated HBEC-conditioned media were separated by 11.5% SDS-PAGE in the presence of 10% β-mercaptoethanol and were transferred to an Immobilon-P filter (0.45 µm polyvinyldene difluoride; Millipore). Nonspecific staining was blocked by incubating the transfers for 90 min in Tris-buffered saline (TBS) containing 5% nonfat dried milk. The transfers were then incubated overnight with polyclonal rabbit antibody against human TIMP-1 (Valbotech) diluted 1:500 in TBS. Polyclonal rabbit antibody against human TIMP-3 (CliniSciences) diluted 1:500 in TBS was also used to investigate the presence of TIMP-3 because its molecular mass (24–25 kDa) is near that of TIMP-1. The blots were washed three times in TBS-0.05% Tween 20 and were incubated for 90 min with biotinylated goat anti-rabbit immunoglobulin G diluted 1:1,000 as the second antibody (Dako). The blots were visualized using alkaline phosphatase and fast red TR/Naphthol AS-MX (Sigma).

RNA Extraction

RNA T was extracted from HBECs, human macrophages, and fibroblasts using Trizol reagent (LifeTechnologies) according to an improvement to the single-step RNA isolation method developed by Chomczynski and Sacchi (7a). RNA T
was quantified at 260/280 nm, and the integrity of the samples was checked by 1.5% agarose gel electrophoresis. Reproducible amounts of 8–15 µg RNA were obtained from 10⁶ cells, and aliquots were stored in sterile microfuge tubes at -80 °C until use.

Quantitative RT-PCR of TIMP-1 mRNA

Primer design and synthesis. For RT-PCR experiments, sense and antisense primers were designed using previously published cDNA sequences for human TIMP-1 (6). Specific sense and antisense primers with closely similar fusion temperature values >55 °C were selected as follows: sense primer 5’-GGGGACACCAGAAGTCAACCAGA-3’, antisense primer 5’-CTTTTCAGCAGCTTGGAGAGCTAGAGTTTTCGC-3’, which begin at base +140 and +517. The positions of the 5’-ends of the primers are numbered from the adenine, thymine, guanine initiation codon of the TIMP-1 gene. The TIMP-1 primer corresponded to a cDNA fragment of 400 bases. These primers (synthesized and purified by Eurogentec) were also checked for minimal self-priming and upper/lower dimer formation.

Sense and antisense primers for human 92-kDa gelatinase were selected as previously described in our recent work (34). RT step. To minimize sample handling and contamination, RT and PCR steps were performed sequentially in the same reaction tube. To a final volume of 25 µl, the following compounds were added: 3 µl of 10X PCR buffer (200 mM Tris·HCl, pH 8.3, 500 mM KCl, 15 mM MgCl₂, and 1 mg/ml gelatin), 10 µl dilution buffer for RNA (10 µl of 1 M Tris, pH 8.3, 20 µl of 0.1 M dithiothreitol (DTT), 1 µl RNasin, 100 µl bovine serum albumin, and 80 µl H₂O), 10 µl RNA obtained from cultured HBECs (5, 10, and 20 ng), and 2 µl corresponding downstream primer (10 pmol). After heating for 2 min at 80 °C in the thermocycler to break up secondary structures, the tubes were equilibrated at 42 °C. Each sample was supplemented with 25 µl of RT mix containing 2.5 µl of 10X PCR buffer, 1.25 mM each of dATP, dCTP, dTTP, and dGTP (16 µl), 100 mM MgCl₂ (1.5 µl), and 100 mM DTT (4 µl) with or without 200 units of Moloney murine leukemia virus (Life Technologies). The final volume was 50 µl. The RT reaction lasted 45 min and was carried out at 42°C to prevent excessive mispriming and possible RNA refolding. After completion of RT, the temperature was raised to 96 °C for 30 s to inactivate the enzyme and to denature the RNA-DNA hybrid. The temperature was then equilibrated at 80 °C.

PCR. The amplification reaction was initiated by adding 50 µl of a mix containing 5 µl of 10X PCR buffer, 2 µl of upper primer (10 pmol), 0.3 µl of Taq polymerase (1.5 units; Life Technologies), 0.3 µl of [α-32P]dCTP (3 µCi/μmol; Amersham), and 42.4 µl of H₂O. The final volume was 100 µl. Samples were overlaid with mineral oil and were subjected to the following sequential steps: denaturation at 96 °C for 30 s, annealing at 60 °C for 30 s, and extension at 72 °C for 45 s. Thirty cycles were performed for TIMP-1 assay, and the last amplification was followed by a final 10-min elongation step at 72 °C.

To ensure that the amplification products were generated from the RNA and were not contaminating cellular DNA, we performed PCR directly on RNA that had not been subjected to the RT step. Other negative controls included PCR amplification of all the RT reagents except RNA. A positive control for TIMP-1 mRNA expression was also included in the assay and consisted of RNA harvested from human fibroblasts (2.5 ng). The positive control for 92-kDa gelatinase mRNA expression was RNA harvested from human aortic macrophages (10 ng). PCR products (3 µl) were resolved by 5% PAGE with 0.5X TBE (100 mM Tris, 90 mM boric acid, and 1 mM EDTA) and were analyzed by autoradiography. Band sizes were previously verified by 2% agarose gel electrophoresis with 0.5X TBE in the presence of molecular mass markers.

Characterization of RT-PCR products. We characterized RT-PCR products issued from HBECs and fibroblasts, after digestion with specific restriction enzymes, i.e., Nci I and Nco I (Life Technologies). The expected fragments (133 and 267 bp after digestion by Nci I, 122 and 278 bp by Nco I) were analyzed by 2% agarose gel electrophoresis.

Generation of the internal PCR standard. The quantitative RT-PCR assay that we developed required availability of a specific internal DNA standard corresponding to the TIMP-1 RNA target. This internal DNA standard was obtained by amplification of a foreign DNA fragment from the ampicillin resistance gene in the plbluescript II SK plasmid (Stratagene), using two composite primers. Each composite primer was composed of the corresponding target gene primer sequence attached to a short segment of nucleotides that hybridized to the opposite strand of the foreign DNA fragment as follows:

Sense composite primer 5’-GGGGACACCAGAAGTCAACCAGA-3’

Antisense composite primer 5’-CTTTTCAGCAGCTTGGAGGAGCTAGAGTTTTCGC-3’

Because the internal standard contained the primer target RNA (in the primer mixture), the inner end was the internal standard primer, whereas the outer end was the target RNA primer. As expected, a 585-bp fragment was obtained as the internal DNA standard for TIMP-1. Finally, both the internal standard and the target were amplified using the same target primer.

To perform quantitative PCR, serial dilutions of known quantities of internal DNA standard (10⁴–10⁵ molecules for which a linear dose response was obtained) were added to PCR amplification tubes containing constant amounts of target RNA. After resolution by 2% agarose gel to verify the size of the bands, PCR products were resolved by 5% PAGE. The quantities of amplified internal standard or amplified target RNA in each tube were compared by autoradiography and were evaluated using the same semiautomated image analysis procedure that was used for zymograms. Finally, the amount of target mRNA was evaluated by interpolation between the limits of the linear standard curve.

Quantitative RT-PCR for 92-kDa gelatinase was carried out using RNA as previously described (34). Briefly, 10 ng RNA obtained either from HBECs with or without LPS, IL-1β, or TNF-α exposure or from macrophages were sub-
Constitutive and LPS or Cytokine-Modulated Production of TIMP-1 by HBECs

Reverse zymography. Under basal conditions, reverse zymography of 20-fold concentrated media from HBECs demonstrated constitutive secretion of an ~30-kDa molecular mass band related to TIMP-1 (Fig. 1), with no lower molecular mass band (TIMP-2). TIMP-1 activity appeared to be unchanged in response to LPS and IL-1β or slightly diminished in response to TNF-α. As expected, the molecular mass of recombinant human TIMP-1 (21 kDa) was lower than that of the protein secreted by HBECs. This apparent discrepancy is due to posttranslational modification of the enzyme involving addition of several oligosaccharide side chains. This modification is absent in cell-free translation.

Western blot analysis. Both recombinant unglycosylated human TIMP-1 (recombinant TIMP-1) used as a positive control and 20-fold concentrated HBEC-conditioned media were recognized by polyclonal rabbit antibody against human TIMP-1 (Fig. 2). This result confirmed that cultured HBECs at confluence can constitutively secrete a 30-kDa protein corresponding to TIMP-1, which was detected above by reverse zymography. The immunoreactive band corresponded to the level of total TIMP-1 protein because Western data were obtained in the presence of β-mercaptoethanol, which disrupts any pro-92-kDa gelatinase/TIMP-1 complexes. TIMP-1 content was unchanged in response to LPS and IL-1β, whereas it was clearly diminished in the presence of TNF-α. No response was observed with the same membrane using preimmune serum (negative control). Moreover, all assays using polyclonal anti-TIMP-3 failed to demonstrate any presence of TIMP-3 (24–25 kDa), even in the 20-fold concentrated culture medium and whatever the experimental conditions (basal conditions or in the presence of LPS, IL-1β, or TNF-α).

Evidence of TIMP-1 mRNA expression by HBECs. RT-PCR for TIMP-1 from HBECs was successful and provided a single band of the expected size (400 bp). Also, our results clearly showed that RT-PCR was RNA dose dependent (Fig. 3A). PCR performed directly on RNA not subjected to the RT step and run in parallel with the test samples was negative. RT-PCR controls consisting of RNA harvested from human mammary fibroblasts (reference cells for TIMP-1) were positive.

Characterization of RT-PCR products from HBECs by a set of restrictive enzymes yielded two bands of the expected sizes (133 and 267 bp with Nco I and 122 and 278 bp with Nco I), thus definitively identifying the amplification products as TIMP-1 (Fig. 3B).

Development of a semiquantitative RT-PCR. We determined the optimal experimental conditions for quantifying TIMP-1 mRNA levels. For this, incremental amounts (100–105 molecules) of specific internal DNA standard (585 bp for TIMP-1) were amplified together with a constant amount (2.5 ng) of the target RNA (Fig. 4). Two serial bands of the expected size were observed. Under our experimental conditions, and with no more than 105 molecules of internal DNA standard, the amount of amplified target remained constant, whereas the amount of amplified internal DNA standard increased linearly as a function of the initial concentration of internal DNA. For all used internal DNA standard amounts between 103 and 105 molecules, the calculated amounts of amplified target were constant. With >105 molecules of internal DNA standard, the reaction became competitive and difficult to quantify. We consequently chose to evaluate the amount of
amplified target by direct interpolation to the coamplified internal DNA standard within the limits of the linear standard curve. Finally, to evaluate the TIMP-1 mRNA level, quantitative RT-PCR carried out on HBECs was performed via coamplification with 5 × 10^4 molecules of the corresponding specific internal DNA standard.

Evaluation of TIMP-1 mRNA levels in HBECs using quantitative RT-PCR: Modulation by LPS and inflammatory IL-1β and TNF-α cytokines. Coamplification with specific internal DNA standard and scanning analysis of autoradiograms (Fig. 5) clearly showed that HBECs produced about five times less TIMP-1 mRNA than human fibroblasts (4.8 × 10^4 mRNA molecules/10 ng RNA_T for HBECs vs. 26 × 10^4 mRNA molecules/10 ng RNA_T for human fibroblasts). LPS- or IL-1β-exposed HBECs exhibited similar TIMP-1 mRNA levels than nonexposed cells (4.5 and 4.7 × 10^4, respectively, vs. 4.8 × 10^4 mRNA molecules/10 ng RNA_T). In contrast, the level of TIMP-1 mRNA decreased in response to TNF-α, with a 70% decrease in the TIMP-1 mRNA steady-state level compared with the constitutive level (1.4 × 10^4 vs. 4.8 × 10^4 mRNA molecules/10 ng RNA_T).

LPS- and Cytokine-Stimulated Production of 92-kDa Gelatinase by HBECs

Zymography. As expected under basal conditions (Fig. 6), the gelatinase activities investigated were detected in four forms as follows: a major band of 92 kDa produced by the proform of gelatinase B (MMP-9), a barely visible 88-kDa band corresponding to the active form of gelatinase B, and two minor bands at 72 and 68 kDa corresponding to the pro and active forms of gelatinase A (MMP-2), respectively. Production of 92-kDa gelatinase production as a latent form increased sharply (2-fold) and similarly in the presence of LPS, IL-1β, or TNF-α. Also, some activation of 92-kDa gelatinase (as activated form, 88 kDa) was observed in the presence of LPS, IL-1β, or TNF-α. Semiautomated image analysis quantification of the gelatinolytic bands showed that levels of pro-92-kDa gelatinase increased about twofold compared with basal levels (Table 1). Neither LPS nor inflammatory cytokines modified 72- or 68-kDa gelatinase activities.

A time course study (Fig. 7A) showed that the activity of 92-kDa gelatinase was increased in a linear manner as a function of time (6, 12, and 24 h) after the addition of TNF-α (100 U/ml) in the culture medium. A parallel experiment demonstrated that the 92-kDa gelatinase activity was systematically increased about
Evaluation of 92-kDa mRNA levels in HBECs using quantitative RT-PCR: Modulation by LPS and inflammatory IL-1β and TNF-α cytokines. As in our earlier study (37), coamplification with specific internal DNA standard and scanning autoradiogram analysis (Fig. 8) showed that 1) the level of 92-kDa mRNA from HBECs was comparable to the level of 92-kDa gelatinase from human alveolar macrophages (3.2 × 10^4 mRNA molecules/10 ng RNA_T from HBECs vs. 3.7 × 10^4 mRNA molecules/10 ng RNA_T from human alveolar macrophages) and 2) the level of 92-kDa mRNA from LPS-stimulated HBECs was identical to the level from nonstimulated HBECs (3.2 × 10^4 mRNA molecules/10 ng RNA_T). In contrast, compared with nonstimulated cells, the levels of 92-kDa mRNA produced by IL-1β- and TNF-α-stimulated HBECs were increased about twofold (5.6 × 10^4 vs. 3.2 × 10^4 mRNA molecules/10 ng RNA_T).
TIMP-2 may occur more specifically with the 72-kDa gelatinase pro form, the absence of TIMP-2 may be relevant to the very low level of constitutive expression of 72-kDa gelatinase, as well as to the almost nonexistent positive regulation of 72-kDa gelatinase in response to LPS and to the proinflammatory cytokines IL-1β and TNF-α.

Modulation of TIMP-1 Expression by LPS, IL-1β, and TNF-α in HBECs

Because there is firm evidence that LPS, IL-1β, and TNF-α acutely influence inflammatory processes in the airways, we investigated the effects of LPS, IL-1β, and TNF-α exposure on the modulation of constitutive TIMP-1 expression by HBECs. Our reverse zymography data showed that exposure to LPS, IL-1β, or TNF-α either diminished or failed to modify TIMP-1 production. Also, quantitative RT-PCR failed to demonstrate any changes in TIMP-1 mRNA levels from HBECs exposed to LPS or IL-1β. Similar unresponsiveness of the TIMP-1 mRNA level to LPS and IL-1β has been reported in normal aortic smooth muscle cells and human monocytes (4, 15). As with undifferentiated monocytes (5), it can be speculated that HBECs lack an intracellular mediator required for LPS to induce upregulation of TIMP-1 synthesis, such as one or more transcriptional activating factors that subserve this function in other cell types such as fibroblasts (27).

In contrast, quantitative RT-PCR showed a 70% decrease in the TIMP-1 mRNA level from TNF-α-exposed HBECs. This result is in accordance with the diminution of TIMP-1 protein level in response to TNF-α, as observed by immunoblotting. In contrast to immunoblotting, reverse zymography only showed a slight decrease in TIMP-1 protein activity; this may reflect some difficulty in estimating the exact value of data from reverse zymography. Other authors found, on the contrary, that the expression of TIMP-1 was markedly upregulated by TNF-α in human myeloblastic HL-60 leukemia cells (16). These divergent results suggest cell-type specific control mechanisms for the expression of TIMP-1.

Upregulation of 92-kDa Gelatinase for HBECs by IL-1β and TNF-α

Several recent studies found that inflammatory cytokines (IL-1β and TNF-α) stimulated 92-kDa gene expression (24, 25). More specifically, IL-1β and TNF-α induced production of 92-kDa gelatinase by normal human fibroblasts (30) and by osteosarcoma and fibrosarcoma cell lines (22). Also, exposure of U-937 monoblastoid cells to TNF-α was responsible for production of 92-kDa gelatinase, which increased the ability of the cells to break through reconstituted basement membrane (31). Also, HL-60 leukemia cells exposed to TNF-α released 92-kDa gelatinase, and this promoted bone marrow egress and evasion of leukemia cells into peripheral tissues (26). It is well known that IL-1β and TNF-α play a central and often synergistic role in the

### Table 1. Quantitative evaluation of gelatinase B activities on the gelatin zymograms

<table>
<thead>
<tr>
<th>Gelatinase, kDa</th>
<th>Basal Secretion</th>
<th>LPS</th>
<th>IL-1β</th>
<th>TNF-α</th>
</tr>
</thead>
<tbody>
<tr>
<td>92</td>
<td>16</td>
<td>17</td>
<td>19</td>
<td>18</td>
</tr>
<tr>
<td>88</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>3</td>
</tr>
</tbody>
</table>

Semiquantitative image analysis of the surface and intensity of lysis bands (Fig. 6) was carried out. Results are expressed as arbitrary units ·24 h. Human bronchial epithelial cells (HBECs) were cultured in control medium (1a–6a). HBECs from the same subjects were cultured in the presence of lipopolysaccharide (LPS; 1b and 2b), interleukin (IL)-1β (3b and 4b), or tumor necrosis factor (TNF)-α (5b and 6b).
Fig. 8. Divergent expression of 92-kDa gelatinase in HBECs exposed to LPS, IL-1β, and TNF-α. RNA**T** (10 ng) from HBECs cultured with or without 1 µg/ml LPS, 100 U/ml IL-1β, or 100 U/ml TNF-α and 10 ng RNA**T** from alveolar macrophages (MAC) were reverse transcribed and coamplified with a constant amount (5 x 10⁵ molecules) of the specific internal DNA standard. Two serial bands of the expected sizes (491 bp for internal DNA standard and 303 bp for HBECs and MAC) were observed. Level of 92-kDa mRNA from nonstimulated HBECs was comparable to the level of 92 kDa from LPS-stimulated HBECs and from macrophages, whereas the expression of 92-kDa mRNA was increased about twofold after exposure to IL-1β or TNF-α.

orchestration of acute inflammatory responses in the lung; they also contribute to the pathogenesis of a large number of acute and chronic infectious and noninfectious inflammatory diseases of the human lung (1, 29). It was therefore of interest to determine whether the inflammatory cytokines IL-1β and TNF-α contributed to the regulation of MMP expression in HBECs.

Our results clearly show that IL-1β and TNF-α enhance 92-kDa gelatinase expression at both the protein and the mRNA levels. Also, they confirm our recent works (34) that demonstrated marked upregulation of 92-kDa gelatinase production and activation in response to LPS, with only minimal changes in mRNA levels due to an increase in the half-life of the specific mRNA. Moreover, time course and dose-response studies carried out in the presence of TNF-α clearly demonstrate that 1) the 92-kDa gelatinase is linearly neosynthesized by HBECs as a function of time and is actively secreted as early as during the first 6 h of cell culture and 2) HBECs have the capacity to amplify the production of 92-kDa gelatinase in response to a combination of proinflammatory cytokine TNF-α as weak as 10⁻¹² M, thus supporting some physiological relevance. Indeed, the inducible upregulation of 92-kDa gelatinase from HBECs in response to the inflammatory cytokines IL-1β and TNF-α as well as to E. coli LPS strongly supports the hypothesis that 92-kDa gelatinase may be involved not only in the repair of damaged human respiratory epithelium (3) but also in inflammatory pulmonary processes such as acute lung injury. Increased production of 92-kDa gelatinase by HBECs may contribute to focal degradation of the subepithelial basement membrane, as well as to cell-matrix disruption and to detachment of epithelial cells.

**Imbalance Between 92-kDa Gelatinase and TIMP-1 Expression by HBECs**

Exposure to LPS endotoxin or to the proinflammatory cytokines IL-1β and TNF-α induced upregulation of 92-kDa gelatinase expression by HBECs, with no change or a slight decrease in TIMP-1 expression by the same cells. These divergent regulations suggest that the presence of endotoxin and inflammatory cytokines shifts HBECs toward a matrix degradation phenotype.

In addition to elevated 92-kDa gelatinase expression by HBECs in response to LPS, IL-1β, or TNF-α, our study demonstrated that a proportion of 92-kDa gelatinase underwent extracellular activation, as demonstrated by increased production of the 88-kDa active form. Gelatinase and other MMPs are secreted as latent proforms. The conversion of latent metalloproteinases to active enzymes is an important step for the regulation of metalloproteinase proteolytic activity. Our results suggest that inflammatory cytokines and LPS can induce a high rate of conversion of latent gelatinase to its active form (30%) and that these factors are not present under normal physiological conditions. Activation mechanisms of 92-kDa gelatinase are somewhat controversial and may involve other MMPs such as matrixins (14) or free oxygen radicals (23).

In conclusion, the marked upregulation and activation of 92-kDa gelatinase, the unchanged expression or restrictive modulation of TIMP-1, and the absence of detectable TIMP-2 and TIMP-3 production by HBECs in response to inflammatory conditions may induce an imbalance between gelatinase and TIMPs in favor of the metalloproteinase, thus promoting extensive degradation of specific macromolecular components of the ECM underlying HBECs as well as detachment of these cells. Thus HBECs, which are known to release a number of cytokines, such as IL-1β and IL-8 (8), would also be capable of directly modulating the turnover of basement membrane and ECM, particularly during inflammatory processes such as acute lung injury.

We thank Drs. François Lebargy and Dr. Bruno Housset for providing the biopsies and Sabine Hérigault and Jeanine L’Hour-Menard for technical assistance.

*Address for reprint requests: C. Lafuma, Institut National de la Santé et de la Recherche Médicale Unité 296, F-ACMédicine, 8, rue du Gi Sarrail, 94010 Créteil, France.

Received 26 December 1996; accepted in final form 2 J July 1997.

**REFERENCES**


L874  TIMP-1 SECRETION FROM HUMAN BRONCHIAL EPITHELIAL CELLS


