Expression of Matrix Metalloproteinase Gelatinases A and B by Cultured Epithelial Cells from Human Bronchial Explants*

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To investigate the role of human bronchial epithelial cells (HBECs) in the maintenance and remodeling of the extracellular matrix, we evaluated the expression by HBECs of 72- and 92-kDa gelatinases under basal conditions and after exposure to bacterial lipopolysaccharides (LPS). Confluent HBECs from explants were cultured in plastic dishes coated with type I and III collagens. Gelatin zymography of HBEC-conditioned media showed constitutive major 92-kDa and minor 72-kDa gelatinases recognized by specific human antibodies and totally inhibited by the metalloproteinase inhibitor EDTA. The identification of the two matrix metalloproteinases was confirmed by quantitative reverse transcription-polymerase chain reaction. Identical patterns of gelatinase expression were observed with repetitive primary cultures issued from the same explants. Zymography showed that exposure of HBECs to LPS induced 2- and 20-fold increases in 92-kDa gelatinase production and activation, respectively, as well as a smaller increase in activated 68-kDa gelatinase. With [3H]gelatin substrate, elevated metallogelatinolytic activity (138 μg of hydrolyzed gelatin/48 h/10^6 cells) was also observed, whereas no activity was detected in the absence of LPS. A human epithelial cell line (16HBE 146) exhibited the same basal profile of gelatinase activity, but this profile remained unchanged after exposure to LPS. Quantitative reverse transcription-polymerase chain reaction demonstrated only minimal changes in 92-kDa mRNA levels in response to LPS, but the half-life of 92-kDa gelatinase mRNA was increased with exposure to LPS. In contrast, concomitant slight increases in 72-kDa gelatinase protein and mRNA were found, suggesting that the control mechanisms regulating the expression of 92- and 72-kDa gelatinases by HBECs in response to LPS are divergent. All these data allowed us to propose that HBECs may be actively involved in the physiological and pathophysiological remodeling of the airway basement membrane.

Human bronchial epithelial cells (HBECs)

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§ The abbreviations used are: HBECs, human bronchial epithelial cells; MMP, matrix metalloproteinase; LPS, lipopolysaccharide; APMA, γ-amino-phenyl-mercuric acetate; RNAi, total cellular RNA; RT-PCR, reverse transcription-polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; bp, base pair; TIMP, tissue inhibitor of metalloproteinases.

important role in normal growth and development as well as in normal extracellular matrix turnover, thereby contributing to the maintenance of the structural and functional integrity of the lung (1–3). HBECs may also be involved in responses to bronchial tree insults during inflammatory remodeling or wound healing. Injury to the bronchial epithelial surface can result in sloughing of epithelial cells, leading to partial exposure of the basement membrane. The mechanisms responsible for the repair of airway injuries are not well understood. However, restoration of normal airway function and architecture may require the prompt and orderly repair of any epithelial defects. It has been established that bronchial epithelial cells can repair following injury, including mechanical trauma (4–6), toxin exposure (7, 8), and exposure to inflammatory cell mediators (6). During re-epithelialization after bronchial injury, bronchial epithelial cells may detach from the basement membrane and migrate to cover the exposed connective tissue (4–6). This process is probably affected by matrix metalloproteinases (MMPs), known to degrade most matrix macromolecular components.

MMPs form a group of neutral proteinases that can be divided into three subgroups (for review, see Ref. 9): collagenases (MMP-1, MMP-8, and MMP-13), stromelysins and matrilysins (MMP-3, MMP-10, MMP-11, and MMP-7), and type IV collagenases (MMP-2 and MMP-9). The newly described membrane-bound metalloproteinase, MT-MMP (MMP-14), and metalloelastase (MMP-12) have not as yet been assigned to a particular group since they do not conveniently fall into one of these three categories. Type IV collagenases specifically degrade basement membrane type IV collagen as well as anchoring fibril type VII collagen. Also, type V collagen as well as gelatin, elastin, laminin, or fibronectin can serve as minor substrates for both of the type IV collagenases. Although the substrate specificities of MMP-2 (gelatinase A/72-kDa gelatinase) and MMP-9 (gelatinase B/92-kDa gelatinase) seem similar, the two enzymes are known to be synthesized by different cells in vitro. The 72-kDa form is synthesized principally by dermal and gingival fibroblasts, endothelial cells, and osteoblasts, whereas the 92-kDa form is produced mainly by inflammatory cells including polymorphonuclear leukocytes, macrophages, eosinophils (10), and lymphocytes (11); by various tumor cells such as fibrosarcoma HT1080 or leukemic cell HL–60 (12); and by normal cells such as placental cytotrophoblasts, keratinocytes, osteoclasts (13, 14), and amnion epithelial cells (15). Bronchial epithelial cells overlie the subepithelial basal lamina. The basal lamina is unique in that it is composed predominantly of type IV collagen and laminin. Type V collagen, proteoglycans, and glycoproteins such as entactin are additional constituents. Type IV and V collagens are structurally organized into a nonfibrillar, multilayer network that is resistant to nonspecific proteolytic degradation. Since matrix gelatinases degrade type IV and V col-
Ras oncogene-transformed human bronchial epithelial cells secreted a 72-kDa gelatinase (16), but this form was perhaps a specialized product of the transformed cells. Recently, 92-kDa gelatinase mRNA was detected in normal pulmonary tissue and bronchial epithelium by in situ hybridization (17). Finally, the expression of 72-kDa gelatinase was demonstrated in bovine tracheal gland serous cells, specifically located at the periphery of some tracheal gland acini and involved in gland development (18).

This study was carried out to examine the usefulness of cultured human bronchial epithelium explants as a model for the study of epithelial function under basal conditions and cultured human bronchial epithelium explants as a model for gland development (18). In one study, Ha-ras oncogene-transformed human bronchial epithelial cells secreted a 72-kDa gelatinase (16), but this form was perhaps a specialized product of the transformed cells. Recently, 92-kDa gelatinase mRNA was detected in normal pulmonary tissue and bronchial epithelium by in situ hybridization (17). Finally, the expression of 72-kDa gelatinase was demonstrated in bovine tracheal gland serous cells, specifically located at the periphery of some tracheal gland acini and involved in gland development (18).

This study was carried out to examine the usefulness of cultured human bronchial epithelium explants as a model for the study of epithelial function under basal conditions and after stimulation by Escherichia coli lipopolysaccharide (LPS) endotoxin. To date, LPS is the most potent inducer of metalloproteinase biosynthesis and secretion by a variety of cell lines. A novel finding from our study is that a 92-kDa gelatinase is located at the periphery of some tracheal gland acini and involved in gland development (18).

EXPERIMENTAL PROCEDURES

Materials

APMA, 10× trypsin, Hanks’ balanced salt solution, and Fast™ fast red TR/naphthol AS-MX phosphate tablets were from Sigma. Dulbecco’s modified Eagle’s medium/Ham’s F-12 medium (1:1), antibiotics, gluta-mine, Trizol™ reagent, and Moloney murine leukemia virus reverse transcriptase were from Life Technologies, Inc. Utermöhr G was from Searcro S.A. Collagen G was from Biochrom KG. The monoclonal antibodies of anti-human fibroblast, anti-macrophage (KP1), and anti-nectin antibodies to specify epithelial cell type. Anti-macrophage (CD68 or KP1) and anti-fibroblast antibodies were also used as specific markers for macropores and fibroblasts to investigate culture purity. All these antibodies were murine monoclonal anti-human IgG. After washing in phosphate-buffered saline, pH 7.4, nonspecific antibody binding was blocked by overnight incubation in 20% human AB serum. HBECs were recovered from bronchoalveolar lavage specimens from four patients with adult respiratory distress syndrome. These macrophages were used as reference cells for human 92-kDa gelatinase assay by RT-PCR and as positive control cells for immunocytochemical studies.

Isolation of Human Macrophages—Human alveolar macrophages were recovered from bronchoalveolar lavage specimens from four patients with adult respiratory distress syndrome. These macrophages were used as reference cells for human 92-kDa gelatinase assay by RT-PCR and as positive control cells for immunocytochemistry.

Immunocytochemistry

Primary cultures of HBECs at confluence were stained for anticytokin antibodies to specify epithelial cell type. Anti-macrophage (CD68 or KP1) and anti-fibroblast antibodies were also used as specific markers for macrophages and fibroblasts to investigate culture purity. All these antibodies were murine monoclonal anti-human IgG. After washing in phosphate-buffered saline, pH 7.4, nonspecific antibody binding was blocked by overnight incubation in 20% human AB serum. HBECs were recovered from bronchoalveolar lavage specimens from four patients with adult respiratory distress syndrome. These macrophages were used as reference cells for human 92-kDa gelatinase assay by RT-PCR and as positive control cells for immunocytochemistry.

Partial Purification of Gelatinases

Gelatinases from crude HBEC supernatants were concentrated and purified by substrate affinity chromatography on gelatin-Sepharose. For this purpose, 11 ml of pooled Utermöhr G-free culture medium were loaded onto a gelatin-Sepharose affinity column (10 × 1 cm) equili-brated with 50 mM Tris-HCl, 5 mM CaCl₂, 0.05% Brij-35, and 0.02% NaN₃, pH 7.6 (equilibration buffer), supplemented with 0.5 mM NaCl. The bound fraction containing the gelatinolytic activity was eluted with 5% (v/v) dimethyl sulfoxide in equilibration buffer containing 1 mM NaCl. The flow rate was 35 ml/h, and 2-ml fractions were collected. Protein content was determined by the method of Bradford (20) with bovine serum albumin as the protein standard. Gelatinolytic activity and gelatinase type were then determined by zymography and immunoblotting, respectively.

Zymography

The HBEC culture medium was harvested and stored at −20 °C until use. Collected medium was resolved by 8% SDS-PAGE in the presence of a 10× sample buffer (1× sample buffer + 4× sample buffer). The method of Sambrook (21) was followed, excluding any reducing agents or boiling procedures. 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-HCl, 10 mM CaCl₂, pH 7.4). After staining with Coomassie Brilliant Blue R-250, gelatin-degrading proteolytic zymogens were visualized as clear zones of lysis against a blue background. Molecular masses of gelatinolytic bands were estimated using prestained molecular mass markers.

Activities in the gel slabs were quantified using semiautomated image analysis (NIH Image 1.52), which quantifies both the surface and the intensity of lysis bands after scanning of the gels. Results are expressed as arbitrary units/24 h/10⁶ 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...
Gelatinase Secretion from Human Epithelial Cells

TABLE I

Composition of oligonucleotide primers

| Oligo primers | Sequence | Position | Tm  °C |
|---------------|----------|----------|-------|
| 92-kDa gelatine | Oligo sense | 5'-GTGCTGGGCTGCTTGGTCTG-3' | +37 | 64 |
| Oligo antisense | 5'-GTGCCCCTCCAGAGTGGTGAAT-3' | +339 | 58 |
| 72-kDa gelatine | Oligo sense | 5'-CGGCGTCGCCCATCATACAATG-3' | -140 | 64 |
| Oligo antisense | 5'-TGATTCGAGAAAACCGCAGTGG-3' | +260 | 62 |

obtained with the image analysis system increased linearly with the volume of the samples (r = 1.00) (33).

The pattern of proteinase inhibition was investigated by adding one of the following to the incubation buffer: 2 mM phenylmethylsulfonyl fluoride (final concentration) as a serine proteinase inhibitor, 2 mM N-ethylmaleimide as a cysteine proteinase inhibitor, or 10 mM EDTA as a metalloproteinase inhibitor.

Gelatinase Assays on Radiolabeled Gelatin

Free gelatinase activity was assayed using radiolabeled gelatin as the substrate. Gelatin was radiolabeled with [3H]jactein anhydride according to Caswton and Baret (22). Specific activity was 880 kBq/μg. To measure the free form of gelatinase in the presence of 50 μg of acetylated [3H]gelatin, aliquots of HBEC culture media were tested with or without 1 mM APMA (incubation at 37 °C for 2 h). The proteolytic reaction was allowed to proceed for 48 h at 37 °C and pH 7.4 in the presence of toluene to prevent bacterial contamination, and gelatinase assays were performed as described previously (23).

Immunoblotting

Aliquots of partially purified and concentrated HBEC-conditioned media were separated by SDS-PAGE and transferred to an Immobilon-P filter (polyvinylidene difluoride, 0.45 μm). Nonspecific staining was blocked by incubating the transfers for 90 min in TBS containing 5% nonfat dry milk. The transfers were then incubated overnight with rabbit polyclonal antiserum against human 92- and 72-kDa gelatinases diluted 1:500 in TBS. The blots were washed three times in TBS, 0.05% Tween 20 and incubated for 90 min with biotinylated goat anti-rabbit IgG diluted 1:1000 as the secondary antibody. The blots were visualized using alkaline phosphatase and FastSt 3500 fast red TR/naphthol AS-MX.

RNA Extraction

Total RNA was extracted from HBECs, fibroblasts, or alveolar macrophages using Trizol reagent according to an improvement to the single-step RNA isolation method developed by Chomczynski and Sacchi (24). Total RNA 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 of RNA were obtained from 10 6 cells, and aliquots were stored in sterile microcentrifuge tubes at -80 °C until use.

Quantitative RT-PCR of 92- and 72-kDa Gelatinase mRNAs

Primer Design and Synthesis—For RT-PCR experiments, sense and antisense primers were designed using the previously published cDNA sequences for human 92- and 72-kDa gelatinases (16, 25). Specific primers with Tm of 65 °C were selected. Each pair of upstream and downstream primers had closely similar Tm values (Table I). They were also checked for minimal self-priming and upper/lower dimer formation. The primers were synthesized and purified by Eurogentec.

Reverse Transcription 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 10 × PCR buffer (200 mM Tris·HCl, pH 8.3, 500 mM KCl, 15 mM MgCl2, and 1 mg/ml gelatin), 10 μl of dilution buffer for RNA (10 μl of 1 M Tris, pH 8.3, 20 μl of 0.1 M dithiothreitol, 1 μl of RNasein, 100 μl of bovine serum albumin, and 870 μl of H2O), 10 μl of RNA, obtained from cultured HBECs (10 and 100 ng for 92- and 72-kDa gelatinases, respectively), and 2 μl of the corresponding downstream primer (10 pmol). After heating for 2 min at 90 °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 mixture containing 2.5 μl of 10 × PCR buffer, 16 μl of a 1.25 mM concentration of each dNTP, 1.5 μl of 100 mM MgCl2, and 4 μl of 100 mM dithiothreitol with or without 200 units of Moloney murine leukemia virus reverse transcriptase.

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.

Polymerase Chain Reaction—The amplification reaction was initiated by adding 50 μl of a mixture containing 5 μl of 10 × PCR buffer, 2 μl of upper primer (10 pmol), 0.3 μl of Taq polymerase (1.5 units), 0.3 μl of [α-32P]dCTP (3 μCi/μmol), and 42.4 μl of H2O. The final volume was 100 μl. Samples were overlaid with mineral oil and 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. Twenty-five to thirty-five cycles were performed for 92- or 72-kDa assays. In every case, 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 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, Positive controls for 72- or 92-kDa mRNA expression were also included in the assays and consisted of RNA harvested from human fibroblasts and human macrophages, respectively. PCR products were resolved by 5% PAGE with 0.5 × TBE (100 mM Tris, 90 mM boric acid, and 1 mM EDTA) and analyzed by autoradiography. Band sizes were previously verified by 2% agarose gel electrophoresis with 0.5 × TBE in the presence of molecular mass markers.

Characterization of RT-PCR Products—We characterized the RT-PCR products issued from HBECs, fibroblasts, and macrophages following digestion with specific restriction enzymes, i.e. Alul for 72-kDa gelatinase and PvuII for 92-kDa gelatinase. The expected fragments (103 and 297 bp after digestion by Alul and 93 and 210 bp by PvuII) were analyzed by 2% agarose gel electrophoresis.

Generation of the Internal PCR Standard—The quantitative RT-PCR assay that we developed required the availability of two specific internal DNA standards corresponding to the 92- and 72-kDa gelatinase RNA targets. These internal DNA standards were obtained by amplification of foreign DNA fragments issued from the ampicillin in resistance gene in the Bluescript IIISK plasmid 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 strands of the foreign DNA fragment (Table II). Since each internal standard contained 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, two fragments of 586 and 491 bp were obtained as the internal DNA standards for 72- and 92-kDa gelatinases, respectively. Finally, both the internal standard and the target were amplified using the same target primers, and we verified that the heterologous DNA fragments and their related target genes were amplified with similar efficiency in the presence of [α-32P]dCTP (data not shown). This result is consistent with previous findings by Wang et al. (26) showing that amplification efficiency was mainly determined by the primer sequences.

To perform quantitative RT-PCR, serial dilutions of known quantities of internal DNA standard (10 6 to 100 molecules for a linear PCR response was obtained) were added to PCR amplification tubes containing constant amounts of target RNA. After resolution by 2% agarose gel electrophoresis to verify the size of the bands, PCR products were resolved by 5% polyacrylamide gel electrophoresis. The quantities of amplified internal standard or amplified target RNA, in each tube were compared by autoradiography and evaluated using the same semiautomatic image analysis program that was used for zymograms. Finally, the amount of target mRNA was evaluated by extrapolation between the limits of the linear standard curve.

RESULTS

Cellular Morphology and Immunocytochemistry

Bromochloroformide did not adhere to plastic culture dishes. To increase explant adhesion in primary culture, culture surfaces were coated with collagen G. Phase-contrast microscopic observation showed that HBECs migrated outward in approximately monolayer fashion, forming a halo around the original piece of explant. The outgrowth appeared within 2 days, progressed for up to 2 weeks, and could be maintained with functional ciliated cells for >2 weeks. The number of ciliated cells
was greatest in the areas closest to the explant and on the outward-growing cells. The cells exhibited a flat polygonal shape and were closely opposed, as is typical of cultured epithelial cells (Fig. 1, A–C). The beating of cilia was easily identified under a light microscope as localized movement of medium over the cells. Confluent cultures (90–95% of the dish area) were obtained within 14–16 days when two to three explants were placed in a ring-like manner in the dish; the cultures gradually took on a mosaic-like appearance, with domers similar to those reported with human tracheal gland cells grown on collagen film (27). Each domer comprised 50 cells. The epithelial nature of all cultured bronchial cells was confirmed by staining with antibody to cytokeratin, the characteristic component of epithelial cell intermediate filaments. All cells in 2-week-old cultures were stained with anti-cytokeratin antibodies, whereas suppression of the primary antibody prevented staining (Fig. 2). Also, HBEC cultures did not stain with anti-fibroblast or KP1 antibodies, indicating that contamination by nonepithelial cell types did not occur (data not shown).

### Constitutive and LPS-stimulated Production of Gelatinases by HBECs

Zymography on SDS-gelatin was used to determine whether HBECs secreted gelatin-degrading metalloproteinases. Under basal conditions (Fig. 3A), gelatinase activities investigated in 10 subjects were detected in four main forms: a major band at 92 kDa produced by the pro form of gelatinase B (MMP-9); two minor bands at 72 and 68 kDa corresponding to the pro and active forms of gelatinase A (MMP-2), respectively; and a barely visible 88-kDa band corresponding to active form of gelatinase B. A high molecular mass enzyme (greater than 200 kDa) was also detected and was perhaps due to the presence of gelatinase dimers. All the bands were observed in Ultroser G-free culture medium conditioned for 24 h. Identical gelatinase patterns were obtained with repetitive primary cultures from the same bronchial explants. In contrast, loss of gelatinase production was observed as soon as the second passage following trypsinization (Fig. 3B).

In the presence of LPS, the production of 92-kDa gelatinase was clearly increased as well as that of its active form (88 kDa). Semi-automated image analysis quantification of the gelatinolytic bands showed that latent 92-kDa gelatinase and its active form were increased 2- and 20-fold, respectively, as compared with basal levels (Table III). LPS did not modify minor latent 72-kDa gelatinase activity, but clearly increased the 68-kDa active form (Fig. 3A and Table III). Identical results were obtained in the other six subjects.

EDTA completely inhibited the activity of all the gelatinases, whereas phenylmethylsulfonyl fluoride and N-ethylmaleimide did not (Fig. 4), consistent with previous findings suggesting that the 92- and 72-kDa gelatinases belong to the matrix metalloproteinase family. Moreover, incubation of aliquots of HBEC culture medium in the presence of APMA organomercurial activated major 92-kDa gelatinase into smaller bands of 88 and 67 kDa (data not shown), also confirming that this metalloproteinase belongs to the matrix metalloproteinase family. Interestingly, the cell line 16HBE14o showed the same profile of basal expression as the primary explant cultures, i.e. a major 92-kDa band and a minor 72-kDa band. However, none of the LPS concentrations studied had any stimulating effect on gelatinase production or activation (Fig. 5). This led us to use primary cultures of bronchial explant for our matrix gelatinase studies rather than cell line 16HBE14o, which appears to express a different phenotype.

### Gelatinase Assays on Radiolabeled Gelatin

Using [3H]gelatin, no free gelatinolytic activity was detected in HBEC culture media under basal conditions (Fig. 6). This result demonstrated that, under these conditions, the presence of metalloproteinase inhibitors such as TIMPs was sufficient to prevent free forms of activated gelatinases. When the assays were performed in the presence of 1 μg APMA, low levels of free gelatinolytic activity were detected, indicating that the amount of matrix metalloproteinase inhibitor was only just sufficient to counterbalance activated gelatinase forms. In contrast, when HBECs were cultured in the presence of LPS, free gelatinolytic activity (138 μg of gelatin hydrolyzed per 48 h/106 HBECs) was readily demonstrated, suggesting that the TIMP amount was not sufficient to counterbalance the excess of ac-
activated gelatinase forms produced under the effect of LPS. Moreover, the addition of APMA did not amplify this response, suggesting that neither the LPS-induced 92-kDa pro form increased nor the 72-kDa pro form was converted into an activated form, probably because of the ability of TIMP1 and TIMP2 to form complexes with 92- and 72-kDa gelatinases, respectively, and to prevent their activation by APMA (28–30).

Western Blot Analysis

Both purified human 92-kDa gelatinase used as a positive control and partially purified concentrated HBEC-conditioned media were recognized by antibody against human 92-kDa gelatinase (Fig. 7A). A smaller band (70 kDa) corresponding to autoactivation or a degradation product was also recognized by the 92-kDa gelatinase antibody. This product of activation was similar to that described following activation of gelatinase B in neoplastic cell types (25, 31, 32). Moreover, antibody against human 92-kDa gelatinase did not recognize purified human 72-kDa gelatinase, confirming the absence of cross-reactivity. Also, no response was observed with the same membrane using preimmune serum (negative control).

Fig. 2. Immunocytochemical characterization of cultured HBECs with anti-cytokeratin antibody. A, the primary culture of HBECs at confluence was stained with the anti-cytokeratin antibody to specify epithelial cell type. The anti-cytokeratin antibody was murine monoclonal anti-human IgG1 and was used at a dilution of 1:50. A negative control experiment was performed by suppressing the primary antibody and using 10% AB serum. The secondary antibody (rabbit antibody against mouse IgG) was used at a 1:50 dilution in TBS. Alkaline phosphatase activity was visualized using a Fast™ fast red TR/naphthol AS-MX phosphate tablet. HBECs were counterstained with Harris’ hematoxylin. A, all HBECs in 2-week-old cultures were stained with the anti-cytokeratin antibody (magnification × 500). B, the suppression of the primary antibody prevented staining (magnification × 300).

Both purified human 72-kDa gelatinase used as a positive control and partially purified concentrated HBEC-conditioned medium were recognized by antibody against human 72-kDa gelatinase (Fig. 7B). All these results confirmed that cultured HBECs at confluence can constitutively secrete 92- and 72-kDa proteins corresponding to gelatinases A and B, respectively, detected by zymography.

Reverse Transcription-Polymerase Chain Reaction

Evidence of 92- and 72-kDa Gelatinase mRNA Expression by HBECs—We were successful in performing RT-PCR for 92- and 72-kDa gelatinases from HBECs. In every case, we obtained single bands of the expected sizes, i.e. 303 and 400 bp for 92- and 72-kDa gelatinase, respectively. Also, our results clearly showed that RT-PCR was RNAi, dose-dependent (Fig. 8). PCR performed directly on RNAi not subjected to the reverse transcription step and run in parallel with the test samples was
negative. RT-PCR controls, consisting of RNA<sub>T</sub> harvested from human mammary fibroblasts (reference cells for 72-kDa gelatinase) or human alveolar macrophages (for 92-kDa gelatinase), were positive. Characterization of the RT-PCR products from HBECs by a set of restrictive enzymes yielded the two bands of the expected sizes (103 and 297 bp with AluI and 93 and 210 bp with PvuII), thus definitively identifying the amplification products as 72- and 92-kDa gelatinases (Fig. 9).

Development of Semiquantitative Reverse Transcription-Polymerase Chain Reaction—We determined the optimal experimental conditions for quantifying gelatinase mRNA levels. For this, incremental amounts (10<sup>3</sup> to 10<sup>7</sup> molecules) of specific internal DNA standards (586 and 491 bp for 72- and 92-kDa gelatinases, respectively) were amplified together with a constant amount of target RNA<sub>T</sub> (Fig. 10). Two serial bands of the expected sizes were observed. Under our experimental conditions and under the limit of 10<sup>5</sup> 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 its initial concentration. For all internal DNA standard amounts between 10<sup>3</sup> and 5 × 10<sup>4</sup> molecules, the calculated amounts of amplified target were constant. Beyond 5 × 10<sup>4</sup> 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 extrapolation to the co-amplified internal DNA standard, within the limits of the linear standard curve. Finally, for each evaluation of 92- and 72-kDa gelatinase mRNA levels, quantitative RT-PCR carried out on HBEC RNA<sub>T</sub> was performed via co-amplification with 10<sup>4</sup> molecules of the corresponding specific internal DNA standard.

Evaluation of 92- and 72-kDa Gelatinase mRNA Levels in HBECs Using Semiquantitative RT-PCR and Modulation by LPS—Co-amplification with specific internal DNA standards and scanning analysis of autoradiograms clearly showed that the level of 92-kDa gelatinase mRNA from HBECs was comparable to the level of 92-kDa gelatinase from unstimulated human alveolar macrophages (1.24 × 10<sup>4</sup> mRNA molecules/10 ng of RNA<sub>T</sub> from HBECs versus 1.31 × 10<sup>4</sup> mRNA molecules/10 ng of RNA<sub>T</sub> from human alveolar macrophages) (Fig. 11A). In contrast, when compared with reference normal human fibroblast cell cultures, the level of 72-kDa mRNA produced by HBECs was ~30-fold smaller than that of human fibroblasts (4.3 × 10<sup>3</sup> mRNA molecules/100 ng of RNA<sub>T</sub> for HBECs versus 1.3 × 10<sup>4</sup> mRNA molecules/10 ng of RNA<sub>T</sub> for human fibroblasts) (Fig. 11B).

HBECs cultured in the presence of LPS showed only a slight increase in the level of 92-kDa gelatinase mRNA (1.2 and 1.6 × 10<sup>4</sup> mRNA molecules/10 ng of RNA<sub>T</sub> without and with LPS, respectively) (Fig. 11A). This finding differs strikingly from the large increase in 92/88-kDa gelatinase activity evidenced byzymography. In contrast, LPS-exposed HBECs exhibited slight but significant increases in both protein and mRNA levels of 72-kDa gelatinase as compared with nonexposed cells (6.4 × 10<sup>3</sup> versus 4.3 × 10<sup>3</sup> mRNA molecules/100 ng of RNA<sub>T</sub>; p < 0.001) (Fig. 11B).

The lack of an effect on 92-kDa gelatinase transcription in LPS-stimulated HBECs suggests that up-regulation is con-

**Fig. 3. Basal secretion and LPS stimulation of gelatinase production by cultured HBECs.** Evaluation was carried out by zymography. At confluence, cultured HBECs were incubated for 24 h with Ultrase G-free medium in the presence of 0.2% lactalbumin and were treated with or without 1 μg/ml LPS for an additional 24 h. Then, each culture medium (20-μl aliquots corresponding to 10<sup>6</sup> cells) of confluent HBECs was subjected to SDS-PAGE/gelatin electrophoresis. A: lanes 1–4, HBECs from four different subjects, cultured in control medium; lanes 5–8, HBECs from the same subjects, cultured in the presence of 1 μg/ml LPS. Arrows indicate the positions of 200-, 92-, 88-, 72-, and 68-kDa gelatinase activities. B: lanes 1–4, successive primary cultures of HBECs from one human bronchial explant (the same explant was transferred successively to new dishes, at 5–8-day intervals, to initiate new primary cultures of HBECs); lane 5, first passage following HBEC trypsinization; lanes 6 and 7, second passage following HBEC trypsinization.

**Fig. 4. Constitutive expression of gelatinases by HBECs and identification as metalloproteinases.** After PAGE/gelatin electrophoresis of conditioned media from HBECs, gelatin substrate gels were incubated in 100 mM Tris incubation buffer without inhibitor (Control) or in the presence of 10 mM EDTA, 2 mM phenylmethylsulfonyl fluoride (PMSF), or 2 mM N-ethylmaleimide (NEM). Gels were then studied as described previously. All gelatinases, including the 92- and 72-kDa gelatinases, were EDTA-inhibitable, but resistant to inhibition by other proteinase inhibitors. Prestained molecular mass markers are in the first lane.

| Gelatinase | Basal secretion | LPS stimulation |
|-----------|----------------|----------------|
| 92-kDa    | 88             | 180            |
| 88-kDa    | 4              | 140            |
| 72-kDa    | 15             | 17             |
| 68-kDa    | 2              | 17             |

**Table III**

Quantitative evaluation of gelatinase A and B activities on gelatin zymograms

Semiquantitative image analysis of the surface and intensity of lysis bands (Fig. 3A) was carried out. Results are expressed as arbitrary units/24 h/10<sup>6</sup> cells. HBECs were isolated from four different subjects and cultured without LPS (1a–4a) or with LPS (1b–4b).
controlled by affecting the decay rate of the enzyme transcript. To assess this, LPS-treated and nontreated cells were exposed to actinomycin D to inhibit new transcription, and the decay of 92-kDa mRNA was tracked by semiquantitative RT-PCR. To minimize cytotoxic effects, exposure to actinomycin D was limited to 6 h. The result showed that steady-state 92-kDa mRNA levels dropped in the two groups (LPS-treated and nontreated cells) with coexposure to actinomycin D. The transcript turnover rate was estimated by linear regression analysis. The half-life of 92-kDa gelatinase mRNA in the nontreated cells was 4 h, whereas in the LPS-treated cells, gelatinase mRNA decayed at a slower rate of 5.8 h (Fig. 12).

DISCUSSION

Our results clearly show that HBECs produce two gelatinolytic enzymes. Several findings indicate that these enzymes are members of the matrix metalloproteinase family. (i) They were secreted as major 92-kDa and minor 72-kDa proenzymes that were activated by organomercurials such as APMA. (ii) Their activity was inhibited by chelators such as EDTA, but not by phenylmethylsulfonyl fluoride or N-ethylmaleimide. (iii) They were recognized by specific antibodies. (iv) RT-PCR amplified fragments that had sizes identical to controls (human alveolar macrophage 92-kDa and human mammary fibroblast 72-kDa gelatinases) and that were characterized by a specific set of restriction enzymes.

FIG. 5. Production of gelatinases by 16HBE14o cells. 16HBE14o cells (passage 16) were cultured at confluence for 24 h with 0.1, 0.5, 1.0, 5.0, or 10 µg/ml LPS or without LPS (controls). Aliquots of each culture medium (12 µl corresponding to 6 x 10^4 cells) were investigated by zymography. Arrows indicate the positions of 92- and 72-kDa gelatinase activities.

FIG. 6. Gelatinase assays on [3H]gelatin. Culture media from confluent HBECs cultured without (control) or with 1 µg/ml LPS were investigated for free gelatinolytic activity. Results are expressed as micrograms of gelatin hydrolyzed per 48 h/10^6 HBECs and are presented as the means of triplicate determinations. Bars = S.D. (p < 0.01). Assays were performed with or without prior gelatinase activation by 1 mM APMA.

FIG. 7. Western blot analysis. Aliquots of HBEC-conditioned media were analyzed by immunoblotting to define more precisely the matrix metalloproteinase property of HBEC 92- and 72-kDa gelatinases. A, 200 ng of purified gelatinases (92- and 72-kDa gelatinases as references; lanes 1 and 5, respectively) and 10, 20, and 30 µg of purified conditioned media from HBECs, corresponding to 6.5, 13, and 20 µg of proteins, respectively (lanes 2-4), were immunoblotted under reduced conditions with rabbit polyclonal antiserum against human 92-kDa gelatinase. The absence of cross-reactivity between 72-kDa gelatinase and the rabbit polyclonal 92-kDa gelatinase antibody was demonstrated (lane 5). The specificity of the 92-kDa band recognized by the immune serum was confirmed by the negative result obtained with preimmune serum (lane 6). Arrows indicate the migration of molecular mass standards. B, 200 ng of purified 72-kDa gelatinase as reference (lane 1) and 20 µg of purified conditioned media from HBECs (lane 2) were immunoblotted under reduced conditions with rabbit polyclonal antiserum against human 72-kDa gelatinase.

FIG. 8. Reverse transcription and polymerase chain reaction. RNA extracted from cultured HBECs, human mammary fibroblasts, or human alveolar macrophages was studied by RT-PCR. A, serial dilutions of RNA from HBECs (corresponding to 10, 50, and 100 ng) and 10 ng of RNA from human macrophages were reverse-transcribed (+RT) and subsequently amplified using 25 PCR cycles. Note that a single band of the expected size (303 bp) was obtained from both targets. B, serial dilutions of RNA from HBECs (corresponding to 10, 100, 500, and 1000 ng) and 10 ng of RNA from human fibroblasts were reverse-transcribed (+RT) and subsequently amplified using 35 PCR cycles. Note that a single band of the expected size (400 bp) was obtained from both targets. The control assays were performed either in the absence of reverse transcriptase (−RT) or in the presence of RNA, but in the presence of reverse transcriptase and Taq polymerase. PCR products were resolved by 5% PAGE and analyzed by autoradiography.

Basal Expression of HBEC Gelatinases—In their basal state, primary cultures of confluent HBECs constitutively secreted both predominant 92-kDa progelatinase and minor 72-kDa
enzymes: PvuI and AluI for 92- and 72-kDa gelatinase RT-PCR products, respectively. The fragments were fractionated on 2% agarose gel stained with ethidium bromide. A, the expected band sizes for 92-kDa gelatinase were 303 bp (uncut control) and 93 + 210 bp (PvuI). B, the expected bands for 72-kDa gelatinase were 400 bp (uncut control) and 103 + 297 bp (AluI). The first lanes show the molecular mass markers, MAC, human alveolar macrophages; FB, human mammary fibroblasts.

progelatinase as well as low levels of 88- and 68-kDa active forms. The specific activity of 72-kDa gelatinase against gelatin is 25 times lower than that of 92-kDa gelatinase (48). This may account for the weaker zymography signal of 72-kDa gelatinase as compared with 92-kDa gelatinase. However, quantitative RT-PCR demonstrated high levels of 92-kDa gelatinase mRNA as compared with 72-kDa gelatinase mRNA. Predominant 92-kDa gelatinase expression by HBECs is in accordance with recent in situ hybridization studies (17) demonstrating 92-kDa gelatinase mRNA in normal human bronchial epithelium.

The minor constitutive expression of 72-kDa gelatinase in HBEC cultures was probably due to epithelial cells since cultures were devoid of fibroblasts. This hypothesis is supported by the fact that the human bronchial epithelial cell line (16HBE14o–) used in our study exhibited the same basal gelatinase profile as primary HBEC cultures. These results suggest that the expression of 72-kDa gelatinase alone in immortal Ha-ras-transformed human bronchial epithelial cells (16) may correspond to a switch of 92- to 72-kDa gelatinase.

We observed a low but significant level of free gelatinolytic activity in [3H]gelatin assays on HBEC-conditioned media following APMA activation under basal conditions. This strongly suggests that the amount of gelatinase inhibitors such as TIMPs is too small to fully compensate for the activation of both progelatinases by APMA. Studies are under way to characterize and evaluate TIMP expression by cultured HBECs.

The ability of HBECs to express the two gelatinases was highly conserved in the primary confluent cultures from successive seedings of the initial human bronchial explants, suggesting that the normal HBEC phenotype is maintained under these culture conditions. In contrast, a dramatic decrease in gelatinase expression occurred in secondary cultures, with rapid HBEC mortality or apoptosis as soon as the second passage. Studies of the regulation of these epithelial cells should be limited to primary cultures.

Modulation of HBEC Gelatinase Expression by LPS—Since LPS is well known to acutely influence inflammatory processes in airways (33), we investigated the effect of LPS exposure on the modulation of constitutive gelatinase expression by HBECs. Our zymography results clearly showed that exposure to LPS increased the production of 92-kDa gelatinase. However, quantitative RT-PCR demonstrated only small changes at the mRNA level, suggesting that LPS can modify a post-transcriptional process, e.g. mRNA stabilization. Indeed, with co-exposure to actinomycin D as the RNA synthesis inhibitor, steady-state 92-kDa gelatinase mRNA levels dropped at 6 h post-exposure both in control and LPS-treated cells, but the gelatinase mRNA decayed at a slower rate in treated cells than in control cells, supporting that the half-life of gelatinase mRNA increases with exposure to LPS. A recent study of cultures of U937 cells, a human monocyte-like cell line, in the presence of 2.5 μg/ml LPS yielded similar results and suggested that LPS may stimulate 92/88-kDa gelatinase by increasing the half-life of the specific mRNA (34).

In contrast, the 72-kDa gelatinase mRNA level in LPS-exposed HBECs was slightly but significantly increased as compared with nonexposed cells, and this result was consistent with the protein increase evidenced by zymography. Although 72-kDa gelatinase is very similar to 92-kDa gelatinase in terms of structure, substrate specificity, and properties, there may be striking differences regarding tissue specificity and regulation of expression (25).

Also, LPS exposure seems to stimulate an uncharacterized...

Fig. 9. Specific characterization of RT-PCR products. The products obtained by RT-PCR were digested with specific restriction enzymes: PvuI and AluI for 92- and 72-kDa gelatinase RT-PCR products, respectively. The fragments were fractionated on 2% agarose gel and stained with ethidium bromide. A, the expected band sizes for 92-kDa gelatinase were 303 bp (uncut control) and 93 + 210 bp (PvuI). B, the expected bands for 72-kDa gelatinase were 400 bp (uncut control) and 103 + 297 bp (AluI). The first lanes show the molecular mass markers, MAC, human alveolar macrophages; FB, human mammary fibroblasts.

Fig. 10. Determination of RT-PCR quantitative range. A, 92-kDa mRNA determination. Serial dilutions of specific internal DNA standards (DNAs) (corresponding to 103, 5 × 103, 104, 5 × 104, and 105 molecules) were amplified together with reverse-transcribed products from 10 ng of human macrophage target RNA (MAC). Two serial bands of the expected sizes (491 bp for the internal DNA standard and 303 bp for human macrophage target RNA) were observed. B, 72-kDa mRNA determination. Serial dilutions of specific internal DNA standards (corresponding to 103, 2 × 103, 5 × 103, 104, and 5 × 104 molecules) were amplified together with reverse-transcribed products from 10 ng of human fibroblast target RNA (FB). Control assays were performed in the absence of DNAs and RNAa, or DNAs and reverse transcriptase (-RT), or DNAs alone PCR products (3 μl) were resolved by 5% PAGE and analyzed by autoradiography. Two serial bands of the expected sizes (586 bp for the internal DNA standard and 400 bp for human fibroblast target RNA) were observed. Band sizes were previously verified by 2% agarose gel electrophoresis in the presence of molecular mass markers.
Gelatinase Secretion from Human Epithelial Cells

**Fig. 11.** Evaluation by semiquantitative RT-PCR of 92- and 72-kDa mRNAs from cultured HBECs and LPS modulation. A, evaluation of 92-kDa mRNA. 10 ng of RNA from HBECs (from four different subjects) cultured with or without 1 μg/ml LPS as well as 10 ng of RNA from alveolar macrophages (MAC) were reverse-transcribed and co-amplified with a constant amount (10^6 molecules) of the specific internal DNA standard. Two serial bands of the expected sizes (491 bp for the internal DNA standard and 303 bp for HBECs and alveolar macrophages) were observed. The bands of the internal standard were the same across the range of amplified samples, indicating a constant amplification rate. HBEC bands were slightly enhanced in the presence of LPS as compared with the control, indicating minimal change in the transcriptional level of 92-kDa gelatinase. B, evaluation of 72-kDa mRNA: 100 ng of RNA from HBECs (from the same four subjects as above) cultured with or without 1 μg/ml LPS as well as 10 ng of RNA from human fibroblasts (FB) were reverse-transcribed and co-amplified with a constant amount (10^6 molecules) of the specific internal DNA standard. Two serial bands of the expected sizes (586 bp for the internal DNA standard and 400 bp for HBECs and human fibroblasts) were observed. The 586-bp bands were unchanged, indicating a constant amplification rate across studied samples. Amplification of 400-bp bands was more marked both in human fibroblasts as compared with HBECs and in HBECs cultured in the presence of LPS as compared with control cells.

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200-kDa EDTA-inhibitable gelatinase. A similar large molecular mass band has already been observed in studies of neutrophil and macrophage gelatinases (35, 36), but it is still unclear whether it represents a distinct enzyme species, a dimeric 92-kDa enzyme, or a form of the 92-kDa enzyme that is post-transcriptionally modified, e.g. by extensive glycosylation.

Finally, LPS exposure of HBECs induces activation of both 92- and 72-kDa gelatinases. The agents currently known to activate 92-kDa progelatinase are plasmogen derived from plasminogen activation, stromelysin MMP-3, cathepsin G, leucocyte elastase, mast cell proteases, and some oxidants (37). Activation of secreted 72-kDa progelatinase may involve matrixin MMP-7 (38) or a membranebound metalloproteinase (39). Further investigation is needed to elucidate the pathways of activation of progelatinases expressed by HBECs. Also, the LPS-induced net increase in activated gelatinase forms as evidenced by zymography was accompanied by an elevation of free gelatinolytic activity as measured by assays using [3H]gelatin substrate. This result suggests that an imbalance may develop between gelatinases and their specific inhibitor TIMPs, in the microenvironment of HBECs, as a result of factors related to infections, e.g. bacterial endotoxin. This imbalance may promote in situ action of HBEC gelatinases and raise the issue of the biological role of these enzymes.

Possible Biological Roles for HBEC Gelatinases—The two matrix gelatinases have specific affinity for the subepithelial basal lamina, a specialized nonfibrillar connective tissue structure that anchors epithelial cells to parenchymal surfaces. It has been recently demonstrated that T-lymphocyte gelatinases A and B mediate the invasion of the basement membrane by tumor cells in vitro (40). Also, human keratinocytes secrete type IV collagenase during migration in vitro (41), whereas keratinocytes secrete the two gelatinases during early human wound healing in vivo (42). Even under normal circumstances, alveolar macrophages and polymorphonuclear leukocytes can secrete 92-kDa gelatinase (43). Constitutive production of major gelatinase B and minor gelatinase A by HBECs may contribute to the basal remodeling of the subepithelial basal lamina as well as to the epithelial tubular morphogenesis possibly involved in the remodeling of bronchial mucosa following injury and inflammation (44). Indeed, several in vitro studies have shown that cultures of epithelial cells from different origins including mammary glands (45) and respiratory epithelium may organize into tubular structures (46). Extracellular matrix-degrading proteases such as 72-kDa gelatinase have been shown to regulate mammary epithelial function during involution (47). Also, gelatinase B may be needed during the process of HBEC migration through the extracellular matrix during bronchial epithelial cell physiological remodeling and physiopathological repair. Inducible up-regulation of latent 92-kDa gelatinase and increased activation of this enzyme may be involved in detaching the cells from the basement membrane. Degradation of both type IV and VII collagens may be involved in this process. Type VII collagen is the major structural component of the anchoring fibrils that are critical for epidermal adhesion in the basement membrane zone. In addition, stimulated 92-kDa gelatinase production by HBECs may interfere with the degradation of type XVIII collagen (10), a 180-kDa large extracellular and collagenous portion of transmembrane protein located in the hemidesmosomes of bronchial epithelial cells, thus promoting cell-matrix disruption and detachment of epithelial cells.

In summary, immunological, enzymatic, and RT-PCR data show that primary cultures of human bronchial epithelial cells constitutively express major 92-kDa matrix gelatinase as well as minor 72-kDa matrix gelatinase. These results strongly suggest that these enzymes may be involved in the turnover and degradation of the subepithelial basement membrane as well as in epithelial cell-cell interactions. Moreover, the mechanisms responsible for up-regulation in response to LPS may be different for these two enzymes and may be involved in inflammatory pulmonary processes such as acute lung injury.

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REFERENCES
1. Dufour, S., Duband, J.-L., and Thiery, J.-P. (1986) Biol. Cell 56, 1–18
2. Shah, S., Rickard, K. A., Ertl, R. F., Robbins, R. A., Linder, J., and Reinhard, S. I. (1989) Am. J. Respir. Cell Mol. Biol. 1, 13–20
