Biosynthesis of $\alpha_1$-Proteinase Inhibitor by Human Lung-derived Epithelial Cells*

Joanna Cichy‡, Jan Potempa‡, and James Travis¶

From the 3Department of Microbiology and Immunology, Institute of Molecular Biology, Jagiellonian University, 31-120 Krakow, Poland and 5Department of Biochemistry and Molecular Biology, University of Georgia, Athens, Georgia 30602

Fax: 706-542-1738.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The abbreviations used are: $\alpha_1$-PI, $\alpha_1$-proteinase inhibitor; Achy, $\alpha_1$-antichymotrypsin (Achy); antithrombin III, $\alpha_2$-antiplasmin; C1 inhibitor, plasminogen activator inhibitor; heparin cofactor II, protein C inhibitor; cortisol-binding globulin, thyroxine-binding globulin, and angiotensinogen (1). As a principal inhibitor of neutrophil elastase (HNE), an enzyme which degrades components of the extracellular matrix, $\alpha_1$-PI is involved in the control of turnover of connective tissue. The respiratory tract is particularly vulnerable to damage by proteolytic enzymes, and deficiency has been correlated with disturbances in lung function (2). Although the lung also contains other antiproteases capable of inhibiting HNE, $\alpha_1$-PI contributes >90% of the functional anti-elastase protection of the alveolar walls (3). During tissue injury or inflammation when elevated proteinase activity is present, the role of $\alpha_1$-PI seems to be particularly important and is reflected by an increase in the plasma concentration of this inhibitor under such conditions, as part of the acute phase reaction. Although the liver appears to be the primary source of $\alpha_1$-PI, this inhibitor has also been shown to be synthesized by extracellular lung cells, including cells of epithelial origin (4).

Some data have already been accumulated that indicate that the extrahepatic production of specific serpins is likely to be more significant than had previously been believed. For example, Achy, which increases dramatically in concentration in plasma during the acute phase reaction can be produced by epithelial cells originating from jejunum (4), lung, breast, or skin (5). Indeed, model lung-derived epithelial HTB55 cells synthesize comparable amounts of Achy to those from hepatic-derived HepG2 cells. Furthermore, such synthesis can be up-regulated by a number of factors, including oncostatin M (OSM), interleukin-1 (IL-1), and glucocorticoid analog, dexamethasone (5).

The human airway epithelium, with a surface of 1–2 m², has the potential of exposure to a variety of factors that may trigger an inflammatory response (6). However, the contribution of lung epithelial cells to the local production of $\alpha_1$-PI has never been clarified. In the current study, a variety of inflammatory mediators were tested as potential stimulators of $\alpha_1$-PI in a lung-derived HTB55 cell line, as well as in normal bronchial epithelial cells.

EXPERIMENTAL PROCEDURES

Materials—Human recombinant interleukin-1β (IL-1) (specific activity, 1 × 10⁷ units/mg) was donated by Dr. D. Schenk, Athena Neurosciences, Inc. (San Francisco, CA). Human recombinant interleukin-6 (IL-6) (specific activity, 1 × 10⁶ units/mg), and OSM (specific activity, 4.7 × 10⁶ units/mg) were kindly provided by Immunex (Seattle, WA). Human leukemia inhibitory factor (LIF) from conditioned media of Chinese hamster ovary cells, expressing recombinant LIF at 10⁵ units/ml, was a generous gift of Dr. H. Baumann (Buffalo, NY). Dexamethasone, concanavalin A (ConA; type IV), $\alpha_2$-macroglobulin, and methyl-$\alpha$-D-glucopyranoside were purchased from Sigma. Serum and lung lavage fluid (LLF) (a generous gift of Dr. R. Senior, Washington University, St. Louis, MO) were obtained from a single, 28-year-old Caucasian male smoker in good health.

Cell Culture—HTB55 (Calu-3) human lung adenocarcinoma and HepG2 human hepatoma cell lines were obtained from the American Type Culture Collection (Rockville, MD). Normal human bronchial epithelial cells were purchased from Clonetics (San Diego, CA). HTB55 cells were cultured in Eagle’s MEM supplemented with 0.1 mM non-essential amino acids and 1 mM sodium pyruvate (all from Life Technologies, Inc., whereas HepG2 cells were cultured in Dulbecco’s modified Eagle’s medium. Both media contained, in addition, 100 units/ml penicillin G, 100 μg/ml streptomycin (Life Technologies, Inc.), and 10% fetal bovine serum (Atlanta Biologicals, Norcross, GA). Bronchial cells were cultured in serum-free bronchial epithelial cell basal medium containing 0.5 mM human epidermal growth factor, 5 μg/ml insulin, 0.5 μg/ml each hydrocortisone and epinephrine, 10 μg/ml transferrin, 0.5 mg/ml triiodothyronine, and 0.4% v/v bovine pituitary extract (all from Clonetics, San Diego, CA). Cells were plated, allowed to grow to confluence before assay, and then treated with various stimulating factors.

Northern Blot Analysis—Total RNA was isolated as described previously (7, 8). Northern blot analysis was carried out by electrophoresis of RNA samples in 1% agarose gels containing 2.2% formaldehyde, followed by capillary transfer (9) to Hybond-N membranes (Amersham Corp.). Hybridization with 32P-labeled probes was performed overnight at 65 °C in a mixture containing 1 M NaCl, 1% SDS, and 10% dextran sulfate. The following probes were used; 1-kilobase EcoRI-EcoRI recognized by Dr. R. Senior (Washington University, St. Louis, MO).

Received for publication, December 4, 1996

This paper is available online at http://www-jbc.stanford.edu/jbc/

8250
stimulation of the culture medium was replaced at 24 h by fresh medium containing either at 24 or 48 h after factor(s) addition. In the latter case, however, units/ml IL-1, 10 units/ml LIF, 50 ng/ml OSM, and 10
incubated in serum-free MEM supplemented with 50 ng/ml IL-6, 100
of GAPDH (to rocket immunoelectrophoresis using antiserum to a
scanned by densitometry, and a
rehybridization to the GAPDH probe (A)). Samples of cell medium collected at 24 h were subjected to rocket immunoelectrophoresis using antisera to α-1-PI. Gel was stained with Coomassie Blue to demonstrate precipitin lines (C).

stringtion fragment of human α-1-PI cDNA (10) and 0.5-kilobase PstI-PstI restriction fragment of human GAPDH cDNA (8). These probes were labeled using the Megaprime labeling kit (Amersham). Autoradiographs were then scanned by densitometry, and α-1-PI mRNA was normalized to amounts of GAPDH (B). Samples of cell medium collected at 24 h were subjected to rocket immunoelectrophoresis using antisera to α-1-PI. Gel was stained with Coomassie Blue to demonstrate precipitin lines (C).

| Treatment | α-1-PI secretion μg/ml × 10³ cells |
|-----------|---------------------------------|
| HTB55     |                                 |
| Control   | 0.91 ± 0.06                     |
| IL-6      | 0.97 ± 0.16                     |
| LIF       | 0.86 ± 0.17                     |
| OSM       | 1.41 ± 0.16                     |
| Dexamethasone | 1.02 ± 0.30             |
| Dexamethasone + IL-6 | 1.09 ± 0.02       |
| Dexamethasone + LIF | 1.06 ± 0.06            |
| Dexamethasone + OSM | 1.86 ± 0.15              |

* ND, not detected.

nonreactive fraction expressed as a percentage of total α-1-PI.

Separation of Isoforms of α-1-PI—The separation of glycoforms of α-1-PI was achieved by affinity chromatography using ConA-4B Sepharose column. After application of aliquots of serum, LIF, or HTB55 cell medium, the ConA-nonbinding fraction was eluted with Tris-HCl buffer, pH 7.6, containing 0.15 M NaCl and 1 mM each of MgCl₂, CaCl₂, and ConA-bound material was desorbed with 0.2 M α-methyl-D-mannoside in the same buffer. Fractions were dialyzed against 25 mM ammonium bicarbonate, lyophilized, and dissolved in 1:10 of the original volume. The total amount of recovered α-1-PI in each fraction was measured by rocket immunoelectrophoresis using a specific antisera against α-1-PI, whereas the composition of glycoforms was determined by affinoimmunoelectrophoresis with ConA.

Enzymatic Treatment and Western Blot Analysis—Aliquots of serum, LIF, the ConA-nonbinding fraction of LLF, and HTB55 cell medium, all containing defined amounts of α-1-PI, were incubated with equimolar amounts of HNE (a generous gift of Athens Research and Technology, Athens, GA) for 30 min at room temperature in 20 mM Tris-HCl, pH 7.5, 0.15 M NaCl. Untreated or HNE-treated samples were then subjected to 9% SDS-PAGE (12). α-1-PI was visualized by enhanced chemiluminescence (ECL, Amersham) after electrotransfer to a nitrocellulose membrane and incubation with antibodies. 1:2000 dilution of rabbit antianti-1-PI antibodies (Dako) and 1:2000 dilution of donkey anti-rabbit IgG antibodies conjugated to horseradish peroxidase (Amersham) were used for Western blot analysis.

Biochemical Labeling, Immunoprecipitation, and Fluorography—Confluent monolayers of HTB55 or normal bronchial cells were stimulated for 18 h with 50 ng/ml OSM. The cells were then rinsed and incubated for 4 h in the presence of methionine-free medium containing OSM and 200 μCi/ml [³⁵S]methionine/cysteine (Tran³⁵S-label) (ICN Biomedicals, Inc., Costa Mesa, CA). Aliquots of medium were pre-treated with preimmune serum and Pansorbin (Calbiochem-Novabiochem, La Jolla, CA), as described previously (9). The supernatants were then incubated overnight at 4 °C in 20 mM Tris-HCl, pH 7.5, 140 mM NaCl, 1% Triton X-100, with excess anti-α-1-PI antibody. Immune complexes were precipitated with protein A-agarose (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), washed, released by boiling in Laemmli sample buffer, and examined on SDS-PAGE. Bands were detected by fluorography as described elsewhere (13).

RESULTS

Regulation of α-1-PI Synthesis in HTB55 Cells—Both inflammatory cytokines and the glucocorticoid analog dexamethasone were tested for their ability to regulate the synthesis of α-1-PI in lung adenocarcinoma HTB55 cells. As demonstrated in Fig. 1, A and B, expression of α-1-PI was stimulated with OSM and to a lesser extent by dexamethasone. Although the up-regulation of α-1-PI synthesis by OSM was also noted at the protein level, the effect of dexamethasone on α-1-PI protein synthesis was variable as demonstrated by a high S.D. value (Table I). As might be expected, these two factors acted additively for the
up-regulation of α₁-PI expression, which was also observed at the protein level (Fig. 1; Table I). However, no stimulation of α₁-PI synthesis was obtained when IL-1 was given alone, although this cytokine appeared to enhance the effect of either OSM or dexamethasone. In fact, the most significant up-regulation of α₁-PI synthesis was noted when OSM, dexamethasone, and IL-1 were given in combination. No significant stimulation of α₁-PI expression was observed following either IL-6 or LIF treatment (Fig. 1), although both cytokines were capable of stimulating α₁-PI production in HepG2 cells (Table I).

Stimulation of α₁-PI Expression in Normal Bronchial Epithelial Cells—Northern blot analysis of RNA extracted from normal bronchial epithelial cells indicated that α₁-PI mRNA levels were substantially increased by OSM or a combination of OSM and IL-1, and somewhat by IL-1 alone, whereas IL-6 had no effect (Fig. 2A). The stimulatory effect of OSM and OSM in combination with IL-1 on α₁-PI message expression correlated with corresponding changes in secretion (Fig. 2C). In the case of OSM, the up-regulation of α₁-PI synthesis was observed as early as 2 h after addition of this cytokine, with maximum stimulation occurring after 8 h, and no further significant increase in α₁-PI production after prolonged time of cell exposure to OSM (Fig. 3). The increase in synthesis of α₁-PI by OSM-exposed cells was also concentration-dependent and occurred only at cytokine concentrations above 10 ng/ml, reaching a maximum at about 50 ng/ml and remaining approximately at the same level at higher OSM concentrations (Fig. 3A).

Analysis of α₁-PI Secreted by Hepatic and Epithelial Cells—CAIE with ConA revealed that three microheterogeneous forms of α₁-PI were secreted by liver-derived HepG2 cells, whereas only one was secreted by lung-derived HTB55 cells (Fig. 4). ConA binds with bi- but not with tri- or tetra-antennary carbohydrate structures, and in the case of multiheteroglycan proteins, the degree of reactivity with this lectin depends on the number of bi-antennary structures present on the molecule (14). The three variants of α₁-PI present in HepG2 medium can thus be described as either nonreactive (the electrophoretic migration of this form in a gel containing ConA is not affected), weakly reactive (retarded electrophoretic mobility in ConA-agarose gel), and strongly reactive with ConA (immobilized in ConA-agarose gel electrophoresis), respectively. The α₁-PI secreted by HTB55 cells was only of the ConA-nonbinding type, as confirmed by affinity chromatography using ConA-4B Sepharose, where all of the applied α₁-PI passed directly through the column (0.6 × 7 cm) without being bound or even retarded.

Analysis of α₁-PI in Serum and LLF—α₁-PI appears to enter...
the lung by diffusion from blood plasma (2). However, CAIE analysis of \( \alpha_1 \)-PI from serum and LLF of the same healthy donor demonstrated a higher proportion of the ConA-nonbinding \( \alpha_1 \)-PI glycoform in LLF, which may reflect its local synthesis (Fig. 5). Using planimetry, it was found that the amount of the ConA-nonbinding \( \alpha_1 \)-PI fraction, expressed as percentage of total \( \alpha_1 \)-PI, was about 12% higher in LLF (17.2 ± 1.5 in LLF versus 4.8 ± 0.9 in serum, mean ± S.D. of three separate determinations), and the difference was independent of the concentration of ConA and methyl-\( \alpha \)-D-glucopyranoside, parameters known to affect the separation on CAIE (11). ConA CAIE analysis of serum mixed with HTB55 cell medium resembled that of LLF with respect to \( \alpha_1 \)-PI (Fig. 5). A decrease in ConA reactivity appeared to be specific for \( \alpha_1 \)-PI, because there was no difference in the ConA reactivity of AGP derived from the same serum and LLF (Fig. 5). It is important to note that, in contrast to \( \alpha_1 \)-PI, AGP was not synthesized by lung-derived HTB55 cells (data not shown). To exclude a possibility that differences in ConA reactivity observed between \( \alpha_1 \)-PI from serum and LLF were due to inactivation of this inhibitor, \( \alpha_1 \)-PI from LLF was separated on a ConA-Sepharose 4B column, and the nonbinding fraction of the inhibitor was tested for the ability to form a complex with HNE. As demonstrated in Fig. 6, this fraction of \( \alpha_1 \)-PI did form such a complex.

Inhibition of HNE by \( \alpha_1 \)-PI Derived from Lung Epithelial Cells—To examine if lung epithelial cell-derived \( \alpha_1 \)-PI exhibits inhibitory activity comparable to serum \( \alpha_1 \)-PI, the formation of an SDS-stable complex with HNE was studied in detail. For this experiment, the condition medium from OSM-stimulated HTB55 cells was subjected to rocket immunoelectrophoresis to determine the concentration of \( \alpha_1 \)-PI, and the medium was then incubated with various amounts of HNE. At 0.2 HNE: \( \alpha_1 \)-PI molar ratio, a shifted (\( \alpha_1 \)-PI-HNE complex) band appeared (Fig. 7A). At higher concentrations of HNE, the intensity of the shifted band increased, and native \( \alpha_1 \)-PI decreased, with all detectable \( \alpha_1 \)-PI being in complex with HNE when the concentration of this enzyme was equimolar to \( \alpha_1 \)-PI (Fig. 7A).

This result was similar to that observed when human serum \( \alpha_1 \)-PI was reacted with HNE (Figs. 6 and 7).

To determine if \( \alpha_1 \)-PI secreted by normal bronchial epithelial cells was functionally active, we compared its ability to form a complex with HNE to that exhibited by HTB55 cell-derived \( \alpha_1 \)-PI. As illustrated in Fig. 7B, at a molar excess of HNE over \( \alpha_1 \)-PI, both HTB55- and normal bronchial cell-derived \( \alpha_1 \)-PI was detected in complex with HNE, concomitant with the disappearance of the band corresponding to native inhibitor. The additional band visible in case of bronchial cells most probably represents nonspecific binding because it can be removed by an additional preclearing step using preimmune serum (data not shown).

**Discussion**

At sites of inflammation or tissue injury, proteolytic enzymes, released from phagocytes, may directly contribute to the host inflammatory response as well as to tissue destruction. The implication of these proteinases, including HNE, in the pathogenesis of lung emphysema as well as the finding that extrahepatic cells produce \( \alpha_1 \)-PI suggest that locally synthesized \( \alpha_1 \)-PI is likely to have an effect on the antielastase screen of lung tissue.

In this study, we demonstrated that cells originating from the respiratory tract epithelium are capable of \( \alpha_1 \)-PI synthesis and that several mediators of inflammation increase the expression of this inhibitor in these cells. However, regulation of \( \alpha_1 \)-PI in HTB55 and normal bronchial epithelial cells differs significantly from that in hepatic cells. IL-6-type cytokines (IL-6, OSM, LIF, IL-11, and ciliary neurotrophic factor) upregulate the production of \( \alpha_1 \)-PI in hepatocytes (Ref. 15; Table 1), whereas OSM and to a lesser extent IL-1, but not IL-6 or LIF, exert a significant increase in \( \alpha_1 \)-PI levels in lung-derived epithelial cells (Figs. 1 and 2). This strong effect of OSM and the lack of effect of IL-6 and LIF on production of another inhibitor of serine proteinases, Achy, was also observed in epithelial cells originating from the lung (16). However, IL-1 appears to be a much more potent stimulator of Achy than \( \alpha_1 \)-PI synthesis in these cells. It is important to note that this difference in the effect of OSM and IL-1, on Achy expression, compared with that of IL-6, was also observed in breast-derived epithelial cells (5).

Many of the overlapping biological responses of IL-6-related cytokines, including stimulation of \( \alpha_1 \)-PI production in hepatoma cells, have been explained by the presence of a common subunit, gp130, in cytokine receptors, or in the case of OSM and LIF, by the finding that both cytokines bind with high affinity to the same receptor, the latter consisting of two subunits, the...
Lung Epithelial Cells as a Source of α1-PI

Fig. 6. Western blot analysis of α1-PI. Aliquots of serum, LLF, Vc fraction of LLF obtained by affinity chromatography on ConA-Sepharose 4B column (LLFConA), and HTB55 culture medium collected 24 h after OSM (50 ng/ml) and dexamethasone (10−6 m) addition (HTB55) were subjected directly to 9% SDS-PAGE or were incubated with HNE (as described under "Experimental Procedures") and then separated on SDS-PAGE. The bands were visualized by immunodetection of α1-PI, using enhanced chemiluminescence.

Fig. 7. Analysis of complex formation between epithelial cell-derived α1-PI and HNE. In A, HTB55 cells were incubated for 24 h in MEM supplemented with 50 ng/ml OSM. Aliquots of cell medium or pure serum-derived α1-PI (left panel) were incubated with indicated amounts of HNE (HNE:α1-PI molar ratio is presented) as described under "Experimental Procedures" and then subjected to Western blot analysis. Bands were visualized by enhanced chemiluminescence. In B, HTB55 and normal bronchial epithelial cells were incubated for 18 h in MEM and bronchial epithelial cell basal medium, respectively, both supplemented with 50 ng/ml OSM. Cells were subjected to radiolabeling for 4 h with [35S]methionine/cysteine. Aliquots of cell medium were then incubated with the indicated amounts of HNE as in A (HNE:α1-PI molar ratio is presented) and immunoprecipitated with human anti-α1-PI. Immunoprecipitates were subjected to SDS-PAGE followed by fluorography.

α1-PI in this synthetic mixture resembled that in LLF.

It is possible that the difference in the proportion of ConA-nonreactive α1-PI in LLF when compared with serum is due to selective transportation into, or concentration within, the lung. This is, however, less likely because the ConA reactivity of AGP was unchanged in LLF. We also examined whether the faster migration of LLF-derived α1-PI on ConA CAIE might be due to modifications of serum-derived α1-PI within the lung. This was important in view of the fact that differences in the migration of α1-PI on agarose gel electrophoresis and crossed immunoelectrophoresis in lung-derived fluids, when compared with serum, have been described, and in those studies, α1-PI originating from the respiratory tract appeared to be inactive (22, 23). However, our results of SDS-PAGE analysis demonstrate that the ConA-nonreactive fraction of α1-PI was active as an inhibitor of HNE (Fig. 6), which suggests that although α1-PI is modified within the lung, such a modification does not seem to be manifested by an increase in the amount of ConA-nonreactive α1-PI.

The data we have obtained also show that α1-PI is produced as a fully active inhibitor by lung-derived epithelial cells (Fig. 7), which strongly supports a role for these cells in the local synthesis of α1-PI. It is, however, a matter of further analysis to establish other sources of α1-PI in the lung, as well as to examine the significance of local production of this inhibitor in protecting the lung tissue from irreversible destruction during inflammatory episodes.

Acknowledgments—We thank C. Land for technical assistance and Dr. T. Kordula for review of the manuscript.

REFERENCES

1. Travis, J., and Salvesen, G. (1983) Annu. Rev. Biochem. 52, 655–709
2. Crystal, R. G. (1990) J. Clin. Invest. 85, 1343–135
3. Gadek, J. E., Zimmerman, R. L., Feisi, G. A., Rennard, S. J., and Crystal, R. G. (1981) J. Clin. Invest. 68, 889–898
4. Molmenti, E. P., Ziambaras, T., and Perlmutter, D. H. (1993) J. Biol. Chem. 268, 14116–14124
5. Cichy, J., Potempa, J., Chawla, R. K., and Travis, J. (1995) FEBS Lett. 359, 262–266
6. Weiss, S. J., Lampert, M. B., and Test, S. T. (1983) Science 222, 625–628
