Stimulatory Effect of Inflammatory Cytokines on α_1-Antichymotrypsin Expression in Human Lung-derived Epithelial Cells

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Abstract

Although it is a well known fact that hepatocytes are the primary source of plasma proteinase inhibitors, including α_1-antichymotrypsin, this protein has also been detected in lung epithelial cells, which may suggest its local production. We have demonstrated that lung-derived epithelial cells are capable of synthesizing high levels of α_1-antichymotrypsin. In normal bronchial epithelial cells, as well as in the HTB55 human adenocarcinoma cell line, α_1-antichymotrypsin synthesis was under the control of inflammatory cytokines, of which oncostatin M was the most potent stimulator. This finding is consistent with a role for this inhibitor in protecting the lung epithelium from damage by chymotrypsin-like enzymes released from phagocytes such as neutrophils following pathogen invasion. (*J. Clin. Invest.* 1995. 95:2729–2733.) Key words: serpin • oncostatin M • bronchial epithelial cells • HTB55 cell line • inflammation

Introduction

Tissue injury normally results in both a qualitative and quantitative change in the proteins present in human plasma. In man such alterations include an elevation in the levels of C-reactive protein, serum amyloid A, α_1-antichymotrypsin (Achy),[1] α_1-proteinase inhibitor, fibrinogen, α-1-acid glycoprotein, haptoglobin and C3 complement, as well as a decrease in albumin and transferrin (1). These proteins known as acute phase proteins (APP) are predominantly synthesized by the liver. Their production appears to be modulated primarily by cytokines released at the site of injury, although factors generated in tissues distal to the site, such as glucocorticoids, are also involved (1, 2). Over the past several years, a number of cytokines with hepatocyte stimulating activity have been identified. These include IL-6, IL-1, and TNF-α, of which IL-6 has been recognized as the principal regulator of most APP genes (1, 2). More recently, oncostatin M (OSM), leukemia inhibitory factor (LIF), IL-11, and cilary neurotrophic factor have also been shown to stimulate APP synthesis in a manner similar to IL-6 (2).

The physiologic role of many APP remains unclear, but most of them likely prevent the tissue damage that is associated with inflammation. Such a role has been postulated for Achy, the controlling inhibitor of cathepsin G and other chymotrypsin-like enzymes (3). Studies of human lung secretions have demonstrated that the concentration of Achy is higher than can be predicted by simple diffusion from blood plasma (4). This indicates that this inhibitor may be synthesized locally within the lung, possibly by alveolar macrophages that have been shown as one potential source of this inhibitor (5). The purpose of this study has been to establish whether cells originating from lung epithelium are capable of Achy production under inflammatory conditions. This concept is based on a number of observations, including the fact that (*a*) Achy is detected in bronchial epithelial tissues (6) and (*b*) several epithelial cell lines synthesize high levels of Achy (7–10). If epithelial cells are capable of Achy synthesis, it seems likely that in the case of lung inflammation mediators generated locally might stimulate Achy production in both hepatocytes and epithelial cells. In the current study normal bronchial epithelial cells as well as HTB55 lung adenocarcinoma cells were examined for expression of Achy. Both type of epithelial cells were found to be strongly responsive to OSM, one of the most potent stimulators of Achy synthesis in cells of hepatic origin.

Methods

Materials. All cytokines used during the course of this study were purified molecules with determined specific activity. Human recombinant IL-1β (specific activity 1×10^7 U/mg) was donated by Dr. D. Schenk from Athena Neurosciences Inc. (San Francisco, CA) while IL-6 (specific activity 1×10^7 U/mg) and OSM (specific activity 4.7×10^7 U/mg) were kindly provided by Immunex (Seattle, WA). Human LIF from conditioned media of Chinese hamster ovary cells expressing recombinant LIF at 10^5 U/ml was a generous gift of Dr. H. Baumann (Roswell Park Cancer Institute, Buffalo, NY). Dexamethasone (DEX), PMA, and LPS from *Escherichia coli* 026:B6 were purchased from Sigma Immunochemicals (St. Louis, MO).

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 Corp. (San Diego, CA). HTB55 cells were cultured in Eagle's MEM supplemented with 0.1 mM nonessential amino acids solution and 1 mM sodium pyruvate (all from GIBCO BRL, Gaithersburg, MD). HepG2 cells were cultured in DMEM. Both media contained, in addition, 100 U/ml penicillin G, 100 μg/ml streptomycin (GIBCO) and 10% FBS (Atlanta Biologicals Inc., Norcross, GA). Bronchial cells were cultured in serum-free bronchial epithelial cell basal medium containing 0.5 ng/ml human EGF, 5 μg/

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1. *Abbreviations used in this paper:* APP, acute phase proteins; DEX, dexamethasone; ECL, enhanced chemiluminescence; HepG2, human hepatoma; HTB55, human lung adenocarcinoma; OSM, oncostatin M.

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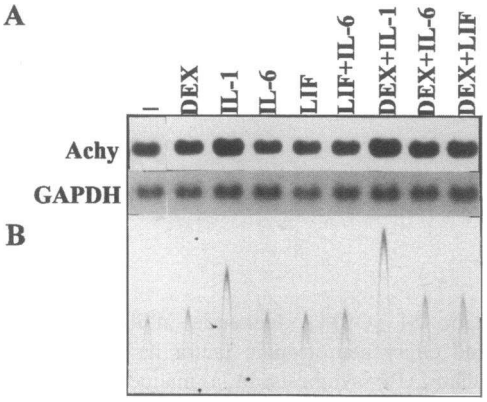

Figure 1. Effect of different factors on the synthesis of Achy in HTB55 cell line. HTB55 cells were grown in MEM containing 10% FBS. Cells were stimulated in serum-free MEM supplemented with 50 ng/ml IL-6, 100 U/ml IL-1, 10 U/ml LIF, and 10^{-6} M DEX. (*A*) Accumulation of Achy mRNA. 5 μg of total cellular RNA extracted at 24 h was separated on gel and transferred to nylon membrane. The blot was hybridized to ^{32}P-labeled Achy probe and then, to demonstrate equal loading, rehybridized to GAPDH probe. (*B*) Achy secretion. Samples of culture media collected at 48 h (from 24–48-h incubation period) were subjected to rocket immunoelectrophoresis using antiserum to Achy. Gel was stained with Coomassie blue to visualize precipitin lines.

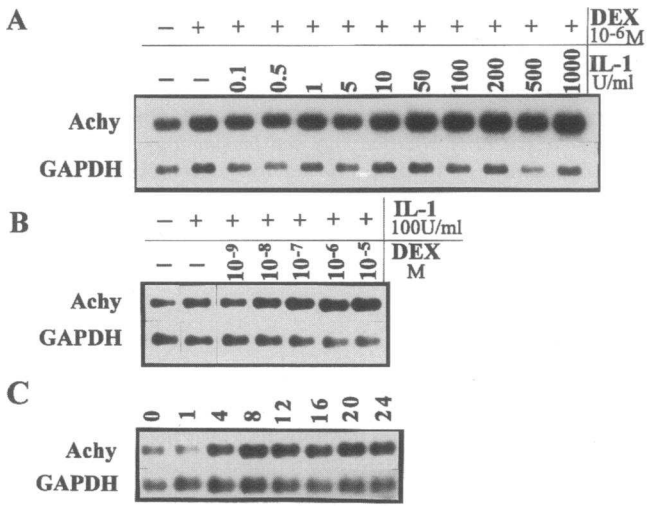

Figure 2. Dose- and time-dependent stimulation of Achy expression in HTB55 cells treated with IL-1 and DEX. Cells were incubated for 24 h in serum-free MEM containing indicated amounts of IL-1 and DEX (*A* and *B*), or were stimulated with 100 U/ml IL-1 and 10^{-6} M DEX for indicated times (*C*). Total cellular RNA was then isolated and subjected to Northern blot analysis. Blots were hybridized to the Achy probe and rehybridized to the GAPDH probe.

ml insulin, 0.5 μg/ml hydrocortisone and epinephrine, 10 μg/ml transferrin, 0.5 ng/ml triiodothyronine, 0.1 ng/ml retinoic acid, and 0.4% vol/vol bovine pituitary extract (all from Clonetics). Cells were plated, allowed to grow to confluency, and incubated for 24 h in MEM (see above description for HTB55 cells) supplemented with 10% FBS and for 24 h in serum-free MEM followed by treatment with indicated stimulating factors.

Northern blot analysis. Total RNA was isolated by the method of Scherrer and Darnell (11) with the modification of Rose-John (12). Northern blot analysis was carried out by electrophoresis of RNA samples in 1% agarose gels containing 2.2 M formaldehyde followed by capillary transfer (13) to Hybond-N membranes (Amersham Corp., Arlington Heights, IL). Hybridization with ^{32}P-labeled probes was performed overnight at 65°C in a mixture containing 1 M NaCl, 1% SDS, and 10% dextran sulphate. The following probes were used; 1.4-kb EcoRI-EcoRI restriction fragment of human Achy cDNA (14), (a generous gift of Dr. H. Rubin, University of Pennsylvania, PA), 0.5-kb PstI-PstI restriction fragment of human GAPDH cDNA (12), and 1.2-kb PstI-XhoI restriction fragment of human IL-6R cDNA (15) (kindly provided by Dr. T. Kishimoto, Osaka University, Osaka, Japan). The probes were labeled with Megaprime Labeling Kit (Amersham).

Determination of protein concentration. The amount of Achy released into the medium was determined by rocket immunoelectrophoresis, as described previously (16).

Biosynthetic labeling and immunoprecipitation. Confluent monolayers of HTB55 cells were stimulated for 24 h with 50 ng/ml OSM and 10^{-6} M DEX. The cells were then rinsed and incubated for 3 h in the presence of methionine-free medium containing stimulating factors and 250 μCi/ml [^{35}S]methionine/cysteine (Tran ^{35}S-label), (ICN Biomedicals, Inc., Costa Mesa, CA). Aliquots of medium were pretreated with preimmune serum and Pansorbin (Calbiochem-Novabiochem, La Jolla, CA), as previously described (13). The supernatants were than incubated overnight at 4°C in 20 mM Tris-HCl, pH 7.5, 140 mM NaCl, 1% Triton X-100, 5 mM EDTA, with excess anti-Achy antibody. Immune complexes were precipitated with protein A–Agarose (Sigma), washed, released by boiling in 0.1% SDS and 1% β-mercaptoethanol or Laemmli sample buffer, and either treated enzymatically (see below) or examined directly on SDS-PAGE.

Enzymatic treatment, fluorography and Western blot analysis. Purified human plasma Achy (kindly provided by MILES Inc., Berkeley, CA) was boiled for 5 min in the presence of 0.1% SDS and 1% β-mercaptoethanol. After addition of Triton X-100 (final concentration 1%) the plasma inhibitor and ^{35}S-labeled Achy from HTB55 cells, prepared as described above, were treated at 37°C for 24 h with either 50 U/ml *N*-glycosidase F (Boehringer Mannheim Corp., Indianapolis, IN) in 0.2 M sodium phosphate, pH 8.0, and 20 mM EDTA, or with 20 U/1 neuraminidase (Sigma) in 50 mM sodium acetate, pH 5.5, and 1 mM calcium chloride. Samples were then subjected to 9% SDS-PAGE according to Laemmli (17). Achy was detected by fluorography as described elsewhere (18) or visualized by enhanced chemiluminescence (ECL) (Amersham) after electrotransfer to a nitrocellulose membrane and incubation with antibodies. 1:2,000 dilution of rabbit anti-Achy antibodies (Dako, Carpinteria, CA) and 1:2,000 dilution of donkey anti–rabbit IgG antibodies conjugated to horseradish peroxidase (Amersham) were used for Western blot analysis.

Results

Stimulation of α_1-antichymotrypsin production in HTB55 cell line. The factors which regulate the production of APP in cells of hepatic origin were studied as potential modulators of Achy synthesis in the HTB55 cell line. Fig. 1 shows that Achy was synthesized in control cells and that its synthesis was increased mainly by IL-1 in combination with DEX. Production of Achy was also affected by IL-1 and DEX individually, as well as by combinations of either IL-6 or LIF with DEX (see also Fig. 5). Achy mRNA levels paralleled the changes in levels of secreted Achy. Significantly, neither IL-6 or LIF up-regulated the production of Achy in the HTB55 cell line when given individually and the medium tested after 2, 8, 18, and 24 h. However, as previously reported (26), both were found to be stimulatory when tested on human hepatoma HepG2 cells (data not shown).

The effect of IL-1 in combination with DEX was studied in detail. IL-1 and DEX mediated an increase in Achy synthesis, visible at mRNA levels, in a dose-dependent fashion at concentrations ranging from 10–1,000 U/ml for IL-1 in the presence of 10^{-6} M DEX (Fig. 2 *A*) and from 10^{-8} to 10^{-5} M DEX in

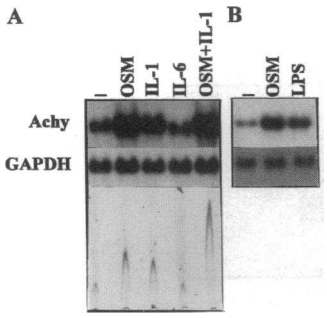

Figure 3. Regulation of Achy synthesis in bronchial epithelial cells. Cells were incubated in serum-free MEM supplemented with 50 ng/ml IL-6, 100 U/ml IL-1, and 50 ng/ml OSM (*A*), or in MEM containing either 10% FBS or 50 ng/ml OSM and 100 ng/ml LPS (*B*). Total cellular RNA isolated at 24 h was subjected to Northern blot analysis. Blots were then hybridized to the Achy probe (upper panels *A* and *B*) followed by rehybridization to the GAPDH probe. Samples of cell medium collected at 24 h were subjected to rocket immunoelectrophoresis using antiserum to Achy (*A, bottom*).

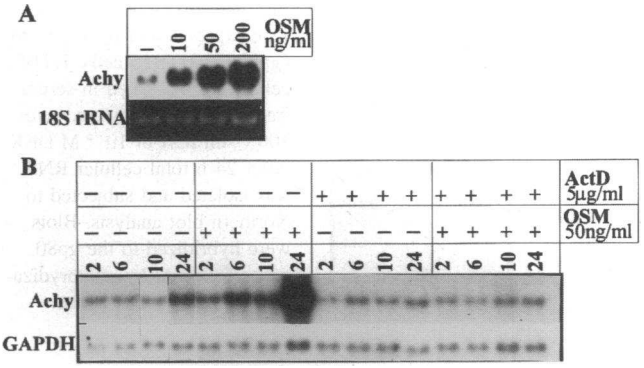

Figure 4. Dose- and time-dependence of Achy mRNA induction by OSM in bronchial epithelial cells. Cells were incubated in serum-free MEM containing indicated amounts of OSM for 24 h (*A*), or were stimulated with 50 ng/ml OSM and/or 5 μg/ml Actinomycin D for indicated times (*B*). Total cellular RNA was then isolated and subjected to Northern blot analysis. Blots were hybridized to the Achy probe (upper panels *A* and *B*). Similar amount of ethidium bromide stained 18S rRNA is visualized in bottom panel of *A*, whereas blot rehybridized to the GAPDH probe is shown in bottom panel of *B*.

the presence of 100 U/ml IL-1 (Fig. 2 *B*). The up-regulating effect of IL-1 and DEX was evident within 4–8 h and did not change significantly up to 24 h (Fig. 2 *C*). This was confirmed by analysis of the amount of secreted Achy where differences between control and stimulated cells were first observed at 8 h, reaching a maximum at 12 h and remaining constant up to 24 h (data not shown).

We also examined the effect of LPS, a factor known to be a mediator of inflammation. LPS increased Achy mRNA levels at a concentration of 5 μg/ml but was not effective at a 10 times lower concentration.

Stimulation of Achy synthesis in bronchial epithelial cells. Northern blot analysis of RNA extracted from bronchial epithelial cells cultured in bronchial epithelial cell basal medium indicated that Achy transcripts were barely detectable in untreated cells but substantially induced by OSM. To compare the responsiveness of HTB55 and normal bronchial epithelial cells, we also tested the latter cells in the same medium. Since these cells do not grow in MEM, they were first cultured in bronchial epithelial cell basal medium and, after reaching confluency, incubated in MEM. Changing of the medium resulted in an elevation of Achy levels which, however, remained relatively stable after reaching a maximum. As demonstrated in Fig. 3 *A*, OSM and to a lesser extent IL-1 upregulated Achy synthesis in bronchial epithelial cells, IL-6, however, having no effect. OSM and IL-1 appeared to act additively which was clearly observed at the protein level. LPS in the presence of serum was also capable of stimulating Achy expression, although its effect was less apparent than that of OSM (Fig. 3 *B*). As illustrated in Fig. 4 *A*, maximal cell response was observed at 200 ng/ml OSM, the highest examined concentration, but was also evident at a concentration of 10 ng/ml. The increase in Achy synthesis was clearly noted at 6 h after OSM addition, with maximum stimulation occurring at 24 h (Fig. 4 *B*). To determine if up-regulation of Achy levels involved transcription or posttranscriptional events, cells were treated with the transcriptional inhibitor actinomycin D and activated by OSM (or not activated in case of control cultures). Fig. 4 *B* demonstrates that in the presence of inhibitor, Achy mRNA levels were equal in control and OSM-stimulated cells, which indicates that OSM treatment did not lead to an increase in stability of the reporter mRNA.

Effect of OSM on stimulation of Achy synthesis in HTB55 cells. Because of the absence of any significant response to IL-6 by HTB55 cells, we wished to determine if they could be stimulated by OSM to increase Achy production, such as was previously demonstrated with normal bronchial epithelial cells. This was deemed important in view of the fact that IL-6 and OSM trigger similar biological responses, including up-regulation of Achy in cells of hepatic origin. Fig. 5 shows that OSM alone, or in combination with DEX, significantly stimulated production of Achy, IL-6 alone, again, having no effect (also demonstrated in Fig. 1). The effect of OSM was observed either at mRNA (Fig. 5 *A*) or protein levels (Fig. 5 *B*). However, treatment of cells with IL-6 and OSM together, did not increase the effect of OSM when examined at 24 h.

Since the lack of responsiveness to IL-6 might result from the absence of the IL-6 receptor (gp80), we also performed Northern blot analysis of gp80 mRNA levels in HTB55 cells treated with DEX and/or IL-1. These factors were chosen because of (*a*) their ability to up-regulate gp80 expression (19, 20) and (*b*) their stimulatory effect on HTB55 cells, as mani-

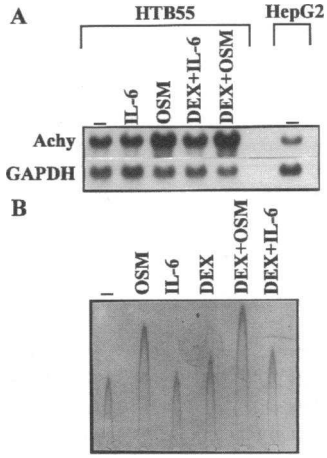

Figure 5. Comparison of OSM and IL-6 effect on Achy synthesis in HTB55 cell line. HTB55 cells were incubated in serum-free MEM supplemented with 50 ng/ml IL-6, 50 ng/ml OSM and/or 10^{-6} M DEX. (*A*) Total cellular RNA isolated at 24 h was subjected to Northern blot analysis and blot was hybridized to the Achy probe followed by rehybrydization to the GAPDH probe. For comparison right panel shows Achy mRNA levels in unstimulated HepG2 cells, cultured as described in Methods. (*B*) Samples of supernatants collected at 48 h (from 24–48-h incubation period) were subjected to rocket electrophoresis using antiserum to human Achy.

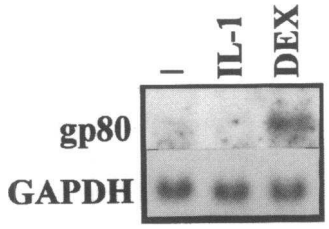

Figure 6. Effect of DEX and IL-1 on expression of IL-6 receptor (gp80) in HTB55 cells. HTB55 cells were incubated in serum-free MEM supplemented with 100 U/ml IL-1 or 10^{-6} M DEX. After 24 h total cellular RNA was isolated and subjected to Northern blot analysis. Blots were hybridized to the gp80 probe followed by rehybridization to the GAPDH probe.

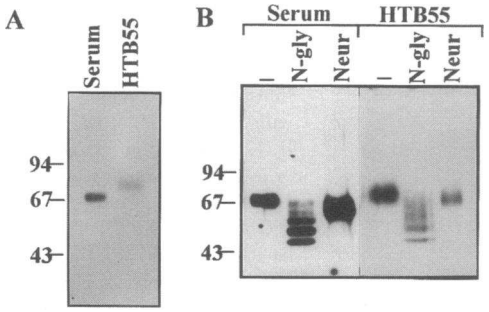

Figure 7. Analysis of Achy produced by HTB55 cells. (*A*) Comparison of molecular mass of serum- and HTB55-derived Achy. HTB55 cells were incubated for 24 h in MEM supplemented with 50 ng/ml OSM and 10^{-6} M DEX. Serum Achy (*Serum*) and aliquots of cell medium (*HTB55*) were subjected to Western blot analysis. Bands were visualized by ECL. (*B*) Enzymatic treatment. HTB55 cells were incubated as in *A*. Cells were then subjected to metabolic labeling as described in Methods. After immunoprecipitation ^{35}S-labeled Achy from HTB55 cells and serum Achy were treated with 50 U/ml *N*-glycosidase-F (*N-gly*) or 20 U/l neuraminidase (*Neur*). Achy was detected by ECL (*Serum*) or fluorography (*HTB55*). The position of molecular mass markers is indicated.

fested by Achy expression. As demonstrated in Fig. 6, gp80 was expressed in HTB55 cells following DEX stimulation. However, IL-1 was neither capable of increasing gp80 gene expression (Fig. 6) nor intensifying the effect of DEX (data not shown).

Characterization of Achy produced by HTB55 cells. As shown in Fig. 5 and Table I, the amount of Achy produced by HTB55 and HepG2 cells was surprisingly comparable, both sources having inhibitory activity towards cathepsin G (J. Cichy, unpublished observation). We used condition medium from HTB55 cells to analyze the form of Achy originating from lung epithelium. The presence of a single band in HTB55 supernatants, detected using antibodies against Achy, is demonstrated in Fig. 7 *A*. This band had a different electrophoretic mobility compared to serum Achy (74 versus 68 kD). While this could be due to a variation in sialic acid content, desialylation of Achy by neuraminidase did not eliminate the differences, although a decrease in the molecular masses of each protein was observed. We then performed *N*-glycosidase F digestion to remove Asn-linked oligosaccharides. As shown in Fig. 7 *B*, a partial deglycosylation was obtained, reflected by several bands. These bands probably correspond to Achy with a number of *N*-glycan chains removed. This is assumed on the basis of previous studies where *N*-glycosidase F deglycosylation of serum Achy was performed (21). In accordance with this study five Achy forms were detected, indicating that serum Achy may carry four Asn-linked oligosaccharides. It appears that at least three N-glycosylation sites are occupied in Achy secreted by HTB55 cells (Fig. 7 *B*). Apparently, totally deglycosylated Achy corresponding to the polypeptide chain had a mass of ~ 44.5 kD in both cases.

Table I. Comparison of OSM Effect on Achy Production in HTB55 and HepG2 Cells

Treatment	Achy secretion μg/ml × 10^6 cells	
	HTB55	HepG2
Control	1.1	1.6
OSM	2.5	5.4
DEX	1.5	1.7
OSM + DEX	3.1	9.5

Cells were cultured in serum-free MEM (*HTB55*) or DME supplemented with 10% FBS (*HepG2*). Cells were then stimulated with 50 ng/ml OSM and/or 10^{-6} M DEX. Media and stimulating factors were daily replaced. Samples of supernatants collected at 48 h (from 24–48-h incubation period) were subjected to rocket immunoelectrophoresis using antiserum to human Achy.

Discussion

Different cell lines originating from the epithelium have been shown to synthesize Achy with several regulatory factors, including hormones and cytokines, appearing to mediate an increase in the expression of Achy in these cells. For example, in the MCF-7 breast cancer line synthesis of Achy is up-regulated primarily by IL-1 and estradiol (8), whereas in the Caco2 intestinal cell line IL-6, and to a lesser extent IL-1, stimulate Achy production (10). In the latter case Achy synthesis has been studied in context with the extra-hepatic regulation of APP expression. Many APP are produced in the Caco2 cell line, and their synthesis has been suggested to be regulated by cytokines in a manner characteristic of the acute phase response. However, in all studies performed so far on epithelial cells, Achy production has been investigated using transformed cell lines. The factors regulating Achy expression could be different in such cases, especially in light of the fact that synthesis of this inhibitor as well as the degree of stimulation of its expression appear to vary widely between tumor-derived epithelial cell lines (7). Moreover, Achy has been shown to be an unfavorable prognostic factor for grade 2 and 3 tumors (22).

In the current study we examined factors capable of modulating Achy expression in normal as well as transformed epithelial cells derived from lung. Both types of cells appeared not to respond to IL-6 in terms of Achy synthesis (Figs. 1, 3, and 5). This insensitivity to IL-6 could be due to the lack of a specific receptor and, since IL-6 is produced by HTB55 cells (data not shown), it is possible that the stimulatory effect of IL-1 on Achy synthesis results from up-regulation of the gp80 receptor for IL-6 in these cells. However, there was no correlation between gp80 expression and stimulation of Achy synthesis by IL-1 (Fig. 6). Moreover gp80 mRNA was present in HTB55 cells following DEX stimulation that suggests that the absence

of responsiveness to IL-6 was most likely not due to the lack of a specific receptor. The decrease in response to IL-6 has been reported previously for other human lung epithelial cancer cell lines, where the inhibitory effect of this cytokine on cell growth was studied (20). Significantly, the cell lines tested had specific receptors for IL-6; however, their affinity and number did not correlate with the sensitivity to IL-6 action. HTB55 also failed to respond to LIF which shares some functional similarity with IL-6 and OSM, including similar regulation of APP synthesis in hepatocyte-derived cells (2). Although this similarity is well explained by the presence of a common signal-transducing subunit, gp130, in their receptors (23, 24), there is much less known about differences in the response to these cytokines. At least two explanations have been suggested. These are (*a*) differential expression of the private ligand-specific receptors (23), and (*b*) the triggering of different signaling pathways (24). Data presented in this paper provide an interesting model for further investigation of this problem.

Regulation of Achy in lung-derived epithelial cells in one respect resembles that found in cells of hepatic origin. In that this inhibitor is under the control of OSM in both cases (Figs. 3 and 5), (26). However, responsiveness to either IL-1 or LPS appears to be specifically directed to epithelial cells. Since both factors are considered to be "alarm molecules" of inflammation, these data suggest that synthesis of Achy in lung epithelial cells may be triggered at early stage of this process.

Achy secreted by HTB55 cells was capable of forming complexes with cathepsin G, which is in agreement with findings by others who also demonstrated the inhibitory activity of epithelial cell-derived Achy (7, 9). However, a significant difference with respect to molecular size was found between serum Achy and Achy derived from HTB55 cells (Fig. 7). This most likely resulted from a modified pattern of glycosylation, since the electrophoretic mobility of the polypeptide chain in SDS-PAGE after *N*-glycanase treatment was similar in both cases. This molecular mass is in accordance with that determined previously for recombinant Achy (14). However, other modifications can not be excluded, especially since heterogeneity at the amino terminus has also been reported for Achy (27). Although the physiological significance of this observed variability is unknown, it could provide a tool to establish sources of Achy in lung fluids. Because the respiratory tract is particularly vulnerable to damage by proteolytic enzymes, high levels of Achy and upregulation of its expression by inflammatory cytokines in lung-derived epithelial cells may suggest a contribution by lung epithelium in protecting itself and surrounding tissues from proteolytic attack.

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