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Secretion of α1-antitrypsin by alveolar epithelial cells

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Abstract

We have investigated the ability of alveolar epithelial cells (human A549 cell line and rat type-II pneumocytes) to produce α1-antitrypsin (AAT). Northern blot analysis demonstrated the presence of an AAT-specific mRNA transcript in A549 cells. Unstimulated A549 cells secreted immunoreactive AAT at a rate of 0.51 ± 0.04 ng/10^6 cells/h, with a modified glycosylation compared to serum AAT. AAT formed a complex with neutrophil elastase. Rat type-II pneumocytes secreted immunoreactive AAT. Our results suggest that alveolar epithelial cells could participate in antiprotease defense within the lung through local AAT production.

Key words: Glycosylation; A549 cell line; Type-II pneumocyte; Human; Rat

1. Introduction

Human α1-antitrypsin (AAT) is a 54 kDa glycoprotein which consists of a single polypeptide chain of 394 amino acids containing about 12% glycans [1].

The hepatocyte is considered as the primary source of serum AAT [2–4]. Other cell types have been shown to synthesize AAT in vitro: human blood monocytes [5], pulmonary alveolar and breast milk macrophages [6], polymorphonuclear neutrophils [7], activated lymphocytes [8] and the human intestinal epithelial cell line Caco2 [9].

Current concepts suggest that AAT constitutes the main inhibitor of serine proteases in human and especially of neutrophil elastase and that it plays a key role in lung homeostasis. Particularly, AAT deficiency has been linked with the development of pulmonary emphysema, a disease caused by an imbalance between proteases and protease inhibitors [10].

Interestingly, investigation into the local sources of AAT synthesis within the human lung have focused exclusively on the contribution of the alveolar macrophage. The potential contribution of other major lung cell populations, such as epithelial cells, to the local production of AAT has not been explored. Because type-II pneumocytes isolated from human lung are not easily available, we have investigated the ability of the A549 cell line to synthesize and secrete AAT. This cell line is derived from a patient with lung carcinoma [11] and has been used as a model of human alveolar type-II pneumocytes [12]. In addition, we have evidence for AAT secretion by rat alveolar type-II pneumocytes in primary culture.

Thus, this study should provide the foundation for future investigations into the contribution of lung resident cells to the regulation of the local antiprotease–host defense.

2. Materials and methods

2.1. Reagents

Ham's F12 medium was purchased from Eurobio (Les Ulis, France). Fetal calf serum (FCS) was from Gibco (Cergy-Pontoise, France). Phenylmethylsulfonylfluoride (PMSF) and leupeptin were from Sigma (La Verpillière, France). Endo-β-acetylglucosaminidase-H (EC 3.2.1.96) (*Streptomyces plicatus*) (Endo-H) was from Genzyme (Boston, MA), peptide-N^4-(*N*-acetyl-β-glucosaminyl), asparagine amidase F (EC 3.2.2.18) (*Flavobacterium meningosepticum*) (*N*-glycanase) was from Boehringer (Meylan, France), endo-α-*N*-acetylgalactosaminidase (EC 3.2.1.97) (*Streptococcus pneumoniae*) (*O*-glycanase) was from Oxford GlycoSystems (Oxford, UK) and neuraminidase (EC 3.2.1.18) (*Vibrio cholerae*) was from Sigma. Nitrocellulose membrane (0.45 μm) was from Bio-Rad (Ivry/Seine, France), rabbit anti-human AAT antiserum was from Boehringer, donkey anti-rabbit IgG antibody conjugated to horseradish peroxidase, Hybond N nylon filter and Enhanced Chemi-Luminescent (ECL) substrate were from Amersham (Les Ulis, France). RNAzol was from Bioprobe (Montreuil/bois, France). Rabbit anti-rat AAT antibodies were a gift from Dr. C. Gauthier (Tours, France).

2.2. Cell culture

A549, the human lung epithelial cell line (CCL 185), was purchased from American Type Culture Collection (Rockville, MD, USA). Cells were cultured in 75 cm^2 flasks in Ham's F12 culture medium containing 100 mM L-glutamine, 100 U/ml penicillin, 50 μg/ml streptomycin (complete medium) supplemented with 15% FCS until the cells reached confluency, and kept at 37°C in a humidified incubator with 5% CO_2 in air. To collect serum-free conditioned medium, A549 confluent cells (9×10^6 cells/flask) were rinsed three times with sterile saline and cultured in 10 ml FCS-free complete medium. Cultures were re-fed every 12 h period for 60 h. The supernatants collected on the first 12 h were discarded, while subsequent harvests were collected, centrifuged ($400 \times g$ for 10 min) and stored at −20°C after addition of protease inhibitors (4 mM PMSF, 40 mM leupeptin). Then cells were either submitted to a trypsin treatment and washed to allow their numeration or frozen in nitrogen before RNA study.

Type-II pneumocytes were isolated from male Sprague–Dawley rat lungs and type-II pneumocytes monolayers were used 24 h after adhesion, as previously described [13]. AAT secretion in type-II pneumocyte supernatants was studied over a 24 h period. Supernatants and cells were collected as described for A549 cells.

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2.3. Protein assays

Total protein was measured by the method of Bradford [14] and AAT supernatants levels by an ELISA as described elsewhere [15].

2.4. Enzymatic procedure

Before the enzymatic procedure (neuraminidase treatment excepted), AAT was denatured by boiling cell culture supernatant for 5 min in the presence of 0.1% SDS and 1% β-mercaptoethanol. SDS was next neutralized by Triton X-100 (10-fold excess). Enzymatic treatments were performed at 37°C for 24 h in a total volume of 100 μl. Endo-H was used in 50 mM sodium citrate, pH 5.0, at a concentration of 40 U/l; *N*-glycanase in 0.2 M sodium phosphate, pH 8.6, 13 mM EDTA at 20 U/ml; *O*-glycanase in 100 mM sodium citrate-phosphate, pH 6.0, 100 μg/ml bovine serum albumin at 3 U/l; neuraminidase in 50 mM sodium acetate, pH 5.5, 1 mM calcium chloride at 10 U/l.

2.5. Western blot analysis

Samples were subjected to 9% SDS-PAGE according to Laemmli et al. [16]. Protein electrotransfer onto a nitrocellulose membrane was performed according to Towbin et al. [17]. Incubation time of the following steps was 1 h at room temperature and dilution buffer was 20 mM Tris-HCl, 0.5 mM NaCl, 0.1% Tween 20, pH 7.5. Nitrocellulose was blocked by immersion in 10% (w/v) non-fat dried milk. Human and rat AAT bands were detected with a 1:1,000 dilution of rabbit specific anti-corresponding AAT antibodies followed by a 1:5,000 dilution of donkey anti-rabbit IgG antibodies conjugated to horseradish peroxidase. Western blots were developed by ECL.

2.6. Assay of elastase-binding activity of human AAT

50 μl of A549 cell line supernatant and serum containing 60 ng AAT were incubated with an equal volume of human neutrophil lysate elastase-containing solution. The elastase activity of the solution was 109 U/l when measured with a synthetic substrate [18]. Incubation was carried out for 2 h at room temperature under continuous stirring. Then, the mixture was analysed by Western blotting.

2.7. Detection of human AAT mRNA

The A549 cell line was evaluated for the presence of AAT mRNA transcripts using Northern blot analysis. Total cellular RNA was extracted from A549 cells and HepG2 cells using the RNAzol procedure [19]. 10 μg of total RNA were subjected to 1% agarose-formaldehyde gel electrophoresis and transferred to nylon filters [20]. Filters were hybridized with a ^{32}P-labelled full-length cDNA probe specific for human AAT [21].

2.8. Data analysis

All data are expressed as the mean ± S.E.M.

3. Results

3.1. Human AAT secretion by A549 cells

3.1.1. Characterization Secretion of AAT by A549 cells was qualitatively demonstrated by visualization of the Western blot of a unique 59 kDa band revealed by specific antibodies against human AAT (Fig. 1). This unique band had a different electrophoretic mobility compared to the human serum AAT (54 kDa).

3.1.2. Glycan structural approach of AAT In order to investigate the difference of apparent molecular mass between human serum AAT and A549-secreted AAT, A549 culture supernatants and diluted serum were submitted to different glycosidase treatments followed by Western blot. After *N*-glycanase treatment, a partial deglycosylation of AAT was obtained (Fig 1). Indeed, four bands were observed corresponding to native AAT and AAT with one, two or three missing *N*-glycan chains, suggesting the presence of three *N*-glycan chains in A549-secreted AAT, similar to serum AAT. Moreover, totally deglycosylated AAT, corresponding to the polypeptide chain, was approximately 1 kDa heavier than serum AAT (Fig. 1). Neuraminidase treatment of A549

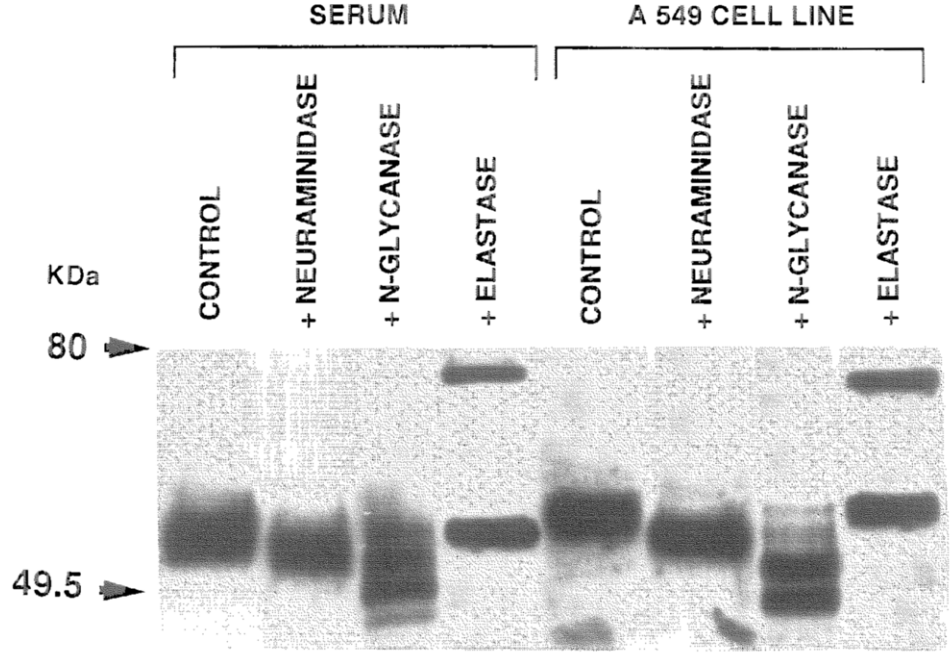

Fig. 1. Western blot analysis of A549 cell supernatant and human serum AAT before and after incubation with glycosidases (*N*-glycanase and neuraminidase) and human neutrophil elastase. AAT bands were visualized by immuno-detection using enhanced chemiluminescence as described in section 2.

cell supernatants and human serum led to a 55 and a 52 kDa AAT band, respectively. The difference of apparent molecular mass between native and desialylated protein was higher for A549 AAT than for serum AAT, indicating an increased sialylation of glycans chains of A549 cell line AAT (Fig. 1). *O*-Glycanase and Endo-H treatment had no effect on A549-secreted AAT, excluding the presence of *O*-glycans and of high-mannose or hybrid type *N*-glycans on the protein (data not shown).

3.1.3. Biological activity of AAT After incubation of A549 cell line supernatant and serum with human neutrophil elastase, Western blot analysis detected an AAT–elastase complex with an apparent molecular mass of 76 and 78 kDa for A549 cell line and serum AAT, respectively (Fig. 1). The presence of uncomplexed AAT is presumably due to SDS-induced dissociation of the complexes [22]. No band was revealed when elastase was incubated with fresh culture medium (data not shown).

3.1.4. Quantification of secreted AAT Secretion of AAT and total proteins was 0.51 ± 0.04 ng/10^6 cells/h and 0.79 ± 0.03 μg/10^6 cells/h ($n = 6$), respectively, and did not significantly vary in the course of the 48 h period. Thus, AAT secretion represented 0.06% of total proteins secreted.

3.1.5. AAT mRNA in A549 cells When total cellular RNA from human A549 was subjected to Northern blot analysis with radiolabeled human AAT cDNA as a probe (Fig. 2), a single 1.60 kb specific AAT mRNA band was present in A549 cells. It was identical in apparent size to that detected in the HepG2 cell line used as a control.

3.2. Rat AAT secretion by type-II pneumocytes

Type-II pneumocytes in primary culture secreted immunoreactive AAT, as observed on Western blot (Fig. 3). A single band with an increased apparent molecular mass (59 kDa) compared to rat serum AAT (54 kDa) was detected.

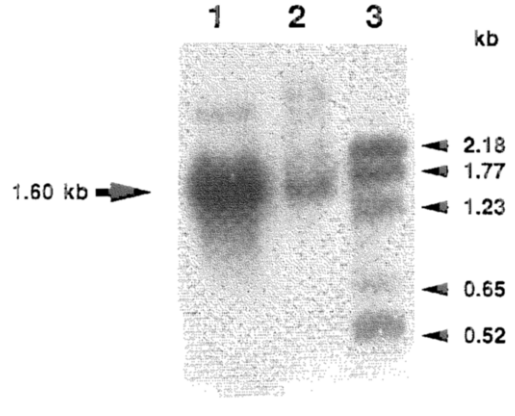

Fig. 2. Detection of AAT mRNA in A549 cells. 10 μg of total cellular RNA isolated from HepG2 cells (lane 1) and from A549 cells (lane 2) were subjected to Northern blot analysis with human AAT cDNA as probe. Molecular mass markers (lane 3) (1.60 kb AAT mRNA transcript indicated).

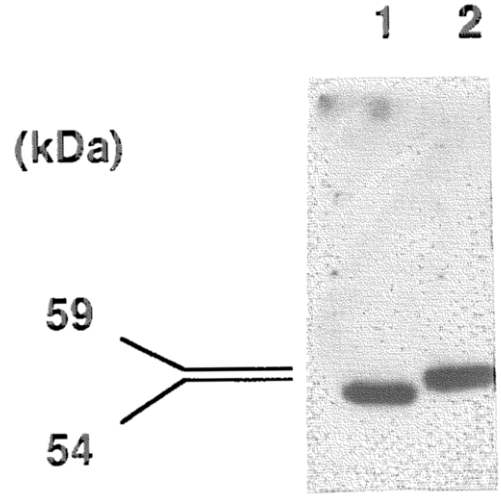

Fig. 3. Western blot analysis of rat serum (lane 1) and rat type-II pneumocytes supernatant (lane 2) AAT after visualization by immunodetection using enhanced chemiluminescence as described in section 2.

4. Discussion

Protease inhibitors are thought to be important in regulating proteases produced by inflammatory cells, such as neutrophils or monocytes, during inflammation. These proteases can be detrimental to the connective tissue and have been implicated in the pathogenesis of lung emphysema. AAT, one of the main protease inhibitors in the lung, is mainly produced by the liver. However, local extrahepatic synthesis has been ascribed to alveolar macrophages [6] and neutrophils [7]. In the present work, we show that AAT is expressed and secreted in an unstimulated human pulmonary epithelial-derived cell line, the A549 cell line. Furthermore, we show that rat type-II pneumocytes in primary culture spontaneously secrete AAT. Taken together, our results indicate that alveolar epithelial cells could participate to the antiprotease defense within the lung through AAT production.

The apparent size of human AAT mRNA is identical to that in the human hepatoma cell line [23]. Western blot analysis of A549-secreted AAT demonstrates a unique band with a greater apparent molecular mass compared to human serum AAT. The observed difference could be due to a modified polypeptide chain. However, the main difference is a result of modified glycosylation of A549 AAT. Taking together the results of the glycosylation study, A549 AAT shares with serum AAT (of hepatic origin, essentially) the presence of three *N*-

glycan chains of complex type and the absence of any *O*-glycan or high-mannose or hybrid *N*-glycan chains. Our data suggest, however, that serum and A549 cell AAT differ by the presence of hypersialylated and highly branched chains in the latter. Instead of producing AAT that contains one tri- and two bi-antennary side chains (as occurs in the liver), A549 processing of AAT may result in tri- and/or tetra-antennary sialylated side chains, which suggests a specific glycosyltransferase action. Such highly branched chains have already been observed in AAT isolated from human hepatoma cell lines [3,4] and from various adenocarcinoma cell lines [24].

We examined the biological activity of AAT synthesized by A549 cells. AAT secreted by A549 cells forms a complex with neutrophil elastase, as does serum AAT, although their glycosylation pattern is different. This result confirms the absence of involvement of glycan chains in the biological anti-elastase activity of AAT. This is in accordance with previous studies in which unglycosylated recombinant human AAT, as well as abnormal AAT secreted by the human hepatoma cell line PLC/PRF/5 [4], were found to be functionally active as protease inhibitors [25].

Measurements concerning levels of A 549 cell secretion were performed over a 48 h period. During this incubation period, AAT and total protein secretion was constant. AAT secretion is low compared to human hepatocyte cell lines [3] and adherent blood monocytes [26]. AAT represented 0.06% of the secreted proteins by the A549 cell line, which is similar to that of mucus protease inhibitor (MPI) (0.1%), and higher than that of elafin (0.004%), two other protease inhibitors secreted by A549 cells [12].

In conclusion, we demonstrate that, in vitro, alveolar epithelial cells secrete AAT. This suggests that, even if hepatocytes remain the primary source of AAT in the body, alveolar epithelial cells could participate in vivo in the local antiprotease screen in the alveolar space.

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