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The elastase-induced expression of secretory leukocyte protease inhibitor is decreased in remodelled airway epithelium

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Abstract

During airway inflammation, proteinases such as human leukocyte elastase are actively secreted. Secretory leukocyte protease inhibitor is a major serine proteinase inhibitor, secreted by bronchial, bronchiolar and lung epithelial cells. We recently identified secretory leukocyte protease inhibitor in human nasal epithelium, exclusively in remodelled areas of the surface epithelium. We now investigated the influence of remodelling and inflammation of the nasal tissue on the in vitro capacity of these cells to respond to human leukocyte elastase. Primary cultures of surface epithelial cells were established from various nasal polyp samples. At confluency, cell cultures were exposed to different human leukocyte elastase concentrations. The secretory leukocyte protease inhibitor immunocytolocalisation, expression and secretion were then investigated. Immunocytochemistry, showed a human leukocyte elastase dose-dependent increase of secretory leukocyte protease inhibitor containing cells and a basal extracellular localization of secretory leukocyte protease inhibitor after incubation with 100 μg/ml human leukocyte elastase. The relative amount of secretory leukocyte protease inhibitor mRNA transcripts increased with respect to the human leukocyte elastase concentration. Nevertheless, the potential stimulation of secretory leukocyte protease inhibitor secretion by human leukocyte elastase was lower in the more remodelled and inflamed tissue. Our results suggest that the contribution of the surface epithelial cells of poorly remodelled tissues to the protection against the deleterious effect of neutrophil proteinases is severely decreased in highly remodelled and inflamed tissues. © 1997 Elsevier Science B.V.

Keywords: Airway epithelium; Inflammation; Airway remodelling; Neutrophil elastase; Secretory leukocyte protease inhibitor

1. Introduction

Airway inflammation is present in most airway diseases such as asthma (Metzger et al., 1987), chronic bronchitis (Thompson et al., 1989), cystic fibrosis (Barton et al., 1976) and is characterized by the presence of activated neutrophils in the airway epithelium. Because neutrophils can potentially release many cytotoxic and proteolytic material, neutrophils have been implicated in the pathogenesis of tissue injury during acute or chronic inflammation (Sibille and Marchandise, 1993). Human leukocyte elastase is actively secreted, especially during inflammatory processes (Weissmann et al., 1980). Human leukocyte elastase is known to be able to injure the airway epithelium (Nahori et al., 1992), to be a powerful inducer of remodelling of the epithelium (Snider et al., 1985) and to be able to stimulate in vitro the expression of secretory leukocyte protease inhibitor by airway and lung cell lines (Abbinante-Nissen et al., 1993; Sallenave et al., 1994). Secretory leukocyte protease inhibitor, a 12 kDa nonglycosylated protein also called antileucoprotease, is the major physiological inhibitor of human leukocyte elastase in the airways (Tegner, 1978). This low-molecular-mass protease inhibitor has been described as a glandular serous cell marker in nasal (Lee et al., 1993), tracheal and bronchial epithelium (Kramps et al., 1981; Tegner and Ohlsson, 1977) and has been identified in some nonciliated cells of the bronchiolar epithelium, where glands are absent (de Water et al., 1986). Willems et al. (1989) showed an increased number of secretory leukocyte protease inhibitor-containing cells in human bronchioles in small airway diseases. Asano et al. (1995) also reported an increased number of secretory leukocyte protease inhibitor-containing cells in human bronchial and bronchio-

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lar structures, together with mucous cell hyperplasia and associated with the existence of acute inflammatory changes in alveolar region. We recently demonstrated the expression of secretory leukocyte protease inhibitor in human adult nasal surface epithelium (Marchand et al., 1995), especially in remodelled areas of the surface epithelium (foldings, basal cell and/or mucous cell hyperplasia), whereas no secretory leukocyte protease inhibitor was ever identified in cells of a normal pseudo-stratified surface epithelium.

In order to find whether the degree of remodelling and inflammation of the nasal tissue could influence the capacity of the surface epithelial cells to respond to human leukocyte elastase, the present study focuses on the relationships between the degree of remodelling and inflammation of the nasal tissue and the capacity of these nasal cells in primary culture to secrete secretory leukocyte protease inhibitor. Furthermore, we have analyzed the dose-dependent effect of human leukocyte elastase on the secretory leukocyte protease inhibitor expression secretion and localization.

2. Materials and methods

2.1. Tissue origin

Human nasal polyps were obtained from 12 patients undergoing nasal polypectomy for nasal obstruction. Immediately after excision, tissue samples were immersed in RPMI medium (Seromed, Biochrom, Berlin, Germany) containing 100 U/ml penicillin, 100 μg/ml streptomycin and 20 mM hydroxyethylpiperazine ethanesulphonic acid (HEPES) (Gibco, Grand Island, NY, USA). The polyp samples were transferred to our laboratory within 2 h in this medium. A sample of each nasal polyp was fixed for 1 h at 4°C in 4% paraformaldehyde, rinsed 3 times for 15 min in phosphate buffer saline (PBS) 0.1 M, then dehydrated through graded concentrations of ethanol and embedded in paraffin. Sections (3 μm thick) were prepared on a microtome and transferred onto gelatin-coated glass slides for standard hematoxylin–phloxin–safran staining.

2.2. Cultures of primary human nasal surface epithelial cells

Primary human nasal surface epithelial cells were isolated from nasal polyps according to the technique described by Chevillard et al. (1993). After rinsing, nasal polyps were incubated overnight at 4°C in a MEM-DVal medium (Gibco) containing 100 U/ml penicillin, 100 μg/ml streptomycin and 0.1% type E pronase (Type XIV, Sigma, St. Louis, MO, USA). The surface epithelium was then separated from the underlying basal lamina by gentle agitation. More complete dissociation of the nasal surface epithelial cells was performed by using a syringe with a needle of 0.5 mm diameter. The cell pellet was obtained by two 10-min centrifugations at $150 \times g$ and resuspended in a minimum volume of the culture medium. Cells were seeded at a density of 10^4–10^5 cells per cm^2 in plastic dishes (22 mm in diameter, Nunc, Denmark) previously coated with a type I collagen gel (Martodam et al., 1979; Chambard et al., 1981; Marchand et al., 1995) at 2.5 mg/ml. Cells were cultured in a moist chamber containing 95% air and 5% CO_2 in a MEM-DVal medium supplemented with 1 μg/ml insulin (Sigma), 1 μg/ml transferrin (Sigma), 10 ng/ml epidermal growth factor (Serva, Heidelberg, Germany), 0.5 μg/ml hydrocortisone (Sigma), 10 ng/ml retinoic acid (Sigma), 100 U/ml penicillin (Gibco) and 100 μg/ml streptomycin (Gibco). The culture medium (1 ml per dish) was changed 24 h after seeding and every 24 h thereafter. The culture supernatants were centrifuged for 10 min at $300 \times g$ and stored at -20°C for further analysis.

2.3. Remodelling and inflammation indexes of the surface epithelium

We observed the entire surface epithelium of each hematoxylin phloxin safran-stained sample and attributed indexes according to the following morphological criteria.

2.3.1. Remodelling index of the surface epithelium

Identification of normal pseudo-stratified epithelium, possibly with areas of hyperplasia, 1; basal cell hyperplasia (more than 3 layers of basal cells) or/and mucous cell hyperplasia (more than 1 mucous cell for 3 ciliated cells), 2; identification of areas of squamous metaplasia, 3.

2.3.2. Inflammation index of the surface epithelium or in the lamina propria

The total number of inflammatory cells was counted. We considered as (1) low density: 30 inflammatory cells/mm^2, (2) moderate density: 30–60 inflammatory cells/mm^2 and (3) high density >60 inflammatory cells/mm^2.

Low density of inflammatory cells, 1; moderate density of inflammatory cells, 2; high density of inflammatory cells, 3.

By addition of the 3 indexes (remodelling index + inflammation index of the surface epithelium + inflammation index in the lamina propria), we calculated a global index which may vary between 3 and 9. Polyps with global index ≤ 6 were considered as poorly remodelled and inflamed (group 1) and polyps with global index >6 were considered as highly remodelled and inflamed tissues (group 2).

2.4. Experimental design

After confluency, the cell cultures were incubated during a 24 h period with either 0, 1, 10 or 100 μg/ml

human leucocyte elastase, prepared according to Martodam et al. (1979). Secretory leukocyte protease inhibitor secretion was evaluated by measuring the secretory leukocyte protease inhibitor concentration (ELISA test) versus the total proteins concentration (Bio-Rad assay) in the culture supernatants. After incubation, supernatants were collected, cultures were rinsed and incubated with the culture medium alone. After a further 24 h period, the cultured cells were processed for either electron microscopy, secretory leukocyte protease inhibitor Northern-blotting, or secretory leukocyte protease inhibitor immunolocalization.

Northern blot analysis of the secretory leukocyte protease inhibitor was carried out when the number of cell cultures was large enough to extract total RNA.

2.5. Northern blot analysis

Total RNA was extracted by using RNAzol treatment (Biogenesis, Bournemouth, UK). 15 μm of total RNA was analyzed by electrophoresis on 1% agarose gels containing 10% formaldehyde and transferred onto nylon membranes (Hybond-N, Amersham, Amersham, UK). The membrane was hybridized with the secretory leukocyte protease inhibitor cDNA probe previously labeled with ^{32}P using random priming synthesis (5×10^8 cpm/μg) (Boehringer-Mannheim, Mannheim, Germany) and the filter was exposed for 1 day. RNA loading was normalized by hybridizing the filter with a ^{32}P-labeled 36B4 probe. 36B4 is a ubiquitous ribosomal RNA. Signal intensities were recorded using an Ultrascan XL laser-scanning densitometer and quantification was made using 36B4 rRNA as internal control. The results were expressed as secretory leukocyte protease inhibitor mRNA transcripts/36B4 rRNA transcripts (secretory leukocyte protease inhibitor). In order to compare the effect of human leukocyte elastase on the secretory leukocyte protease inhibitor gene expression according to the degree of tissue remodelling and inflammation, the results are expressed as the ratio of secretory leukocyte protease inhibitor after adding human leukocyte elastase to secretory leukocyte protease inhibitor in the absence of human leukocyte elastase.

2.6. Enzyme-linked immunosorbent assay (ELISA) for secretory leukocyte protease inhibitor

The concentration of secretory leukocyte protease inhibitor was measured by a non-competitive avidin–biotin immunoenzymatic assay (Francina et al., 1986) using a polyclonal rabbit antibody directed against human secretory leukocyte protease inhibitor as in the immunocytochemistry study (Tournier et al., 1983). Microtitration plates were coated with anti-secretory leukocyte protease inhibitor IgG fractions at a concentration of 10 mg/ml in 0.06 M sodium carbonate-bicarbonate buffer pH 9.6. Secretory leukocyte protease inhibitor purified from human bronchial expectorations or diluted cell culture supernatants in phosphate-buffered saline (PBS) supplemented with 5% normal goat serum) were incubated overnight at 4°C in the microplates. Dilution of all samples in PBS containing normal serum allowed the quantification of secretory leukocyte protease inhibitor in its free form even in the presence of an excess amount of human leukocyte elastase as already earlier observed (Kramps et al., 1984). After washes (3 times 5 min in PBS containing 0.5% Tween 20), biotinylated anti-secretory leukocyte protease inhibitor IgG fraction, diluted 1:500 in the dilution buffer (PBS containing 0.05% Tween 20 and 1% bovine serum albumin), was applied for 3 h at 37°C. Avidin-peroxidase (Sigma) diluted 1:5000 in the dilution buffer was applied for 15 min after 3 washes with the washing buffer. After another cycle of 3 washes, the chromogenic solution (0.037% H_2O_2, 0.1% orthophenylenediamine and 1% imidazol in a citrate buffer pH 5.0) was applied for 2.5 min. Development of the coloration was stopped by adding 100 μl of 3 M HCl in each well. Measurement of the absorbance at 490 nm was performed in a MR5000 microspectrophotometer reader (Dynatech Laboratories, Guernsey, Channel Islands). Each measurement was made in triplicate. The total proteins concentration was measured by using the Bio-Rad protein assay kit. Results were expressed as ng of secretory leukocyte protease inhibitor per μg of total proteins (secretory leukocyte protease inhibitor). In order to compare the effect of human leukocyte elastase on the secretory leukocyte protease inhibitor secretion according to the degree of tissue remodelling and inflammation, the results are expressed as the ratio of secretory leukocyte protease inhibitor after adding human leukocyte elastase to control secretory leukocyte protease inhibitor in absence of human leukocyte elastase.

2.7. Immunocytochemistry

Paraffin sections were dewaxed in xylene and incubated for 5 min with 3% hydrogen peroxide (H_2O_2) in water. After two washes in PBS for 5 min, nonspecific fixation sites were saturated by treating all sections for 10 min with 10% normal goat serum in PBS. This was followed by a 1 h incubation in 3 μg/ml rabbit IgG directed against human secretory leukocyte protease inhibitor (Tournier et al., 1983) in PBS containing 5% normal goat serum (NGS-PBS). The sections were then washed twice for 5 min in PBS and for 10 min in NGS-PBS, and incubated for 1 h with biotinylated donkey anti-rabbit IgG (Amersham) (1:250). The sections were washed twice for 5 min in PBS and for 10 min in NGS-PBS and incubated with streptavidin–biotin–peroxidase complex (Amersham) (1:250). The sections were washed four times with PBS for 5 min and incubated in the dark for 5 min in the chromogenic solution, consisting of 0.1% 3.3′-diaminobenzidine tetrahydrochloride (Sigma), 0.1% H_2O_2 and 0.1% imidazol (Sigma) in distilled water. Enzymatic activity of peroxidase was stopped by immersing the slices in distilled

water. Cells were counterstained with hematoxylin and glass coverslips were mounted on the sections with Aquamount. Sections were visualized with an Axiophot microscope (Zeiss). Negative controls were performed by replacing the secretory leukocyte protease inhibitor antibody by normal rabbit serum IgG at the same concentration (3 μg/ml).

2.8. Electron microscopy

Cell cultures were fixed in 2.5% glutaraldehyde, rinsed in 0.1 M phosphate buffer, rinsed in distilled water, post-fixed 1 h at 4°C in a 1% OsO_4 solution, rinsed in distilled water, embedded in an epon resine and ultra-thin sections were then made. The ultrastructural observations were made by using an Hitachi H300 transmission electron microscope.

2.9. Statistical analysis

A non-parametric Spearman test was used to study the relationship between the remodelling index and the two inflammation indexes of the tissues and between these tissue indexes and secretory leukocyte protease inhibitor production in cultures. Two-way analysis of variance was applied to investigate the effect of the human leukocyte elastase concentration and the effect of the criteria (poorly remodelled/remodelled tissue) on the secretory leukocyte protease inhibitor secretion. In the secretory leukocyte protease inhibitor secretion study, significance was evaluated by using a *t*-test. Differences in the percentages of secretory leukocyte protease inhibitor-containing cells were compared by using a χ^2 test. A $P \leq 0.05$ was considered as significant.

3. Results

3.1. Remodelling and inflammation of the tissue

Indexes for tissue, intra-epithelial and lamina propria inflammation were determined for each of the 12 tissue samples from which the primary cultures were established (Table 1). We observed a positive and significant correlation between the index and the intra-epithelial inflammation index ($r_s = 0.69$; $P = 0.01$), and the index of inflammation in the lamina propria ($r_s = 0.58$; $P = 0.04$), as well as between the intra-epithelial inflammation index and the index of inflammation in the lamina propria ($r_s = 0.83$; $P < 0.01$). We distinguished 2 groups of polyps: group 1, with total indexes ≤ 6, characterized by poorly remodelled and inflamed polyps; group 2, with total indexes > 6, characterized by highly remodelled and inflamed polyps.

3.2. Ultrastructural aspect of the cultured cells after exposure to human leukocyte elastase

Ultrastructural modifications of the cells after exposure to human leukocyte elastase were assessed on the basis of electron microscopic observations. Swelling of the cells and a slight increase in the height of the cultured cell layer were observed after 24 h of incubation with 1 or 10 μg/ml human leukocyte elastase when compared to the control. Partial exfoliation occurred after incubation with 100 μg/ml human leukocyte elastase and intracytoplasmic vacuoles could be detected in the human leukocyte elastase exposed cells (Fig. 1).

3.3. Effect of human leukocyte elastase on the secretory leukocyte protease inhibitor secretion

Under basal conditions, each of the twelve cultures studied secreted secretory leukocyte protease inhibitor. The

Table 1
Remodelling and inflammation indexes of the tissues samples

Culture number	Remodelling index	Intra-epithelial inflammation	Inflammation in the lamina propria	Total index	Group
1	1	1	1	3	1
2	2	1	1	4	1
3	2	2	2	6	1
4	2	2	2	6	1
5	2	2	2	6	1
6	2	2	2	6	1
7	3	3	3	9	2
8	3	3	3	9	2
9	2	2	3	7	2
10	2	2	3	7	2
11	2	3	3	8	2
12	2	3	3	8	2

We attributed an index, an intra-epithelial inflammation index and an inflammation index in the lamina propria for each of the twelve polyp samples used (each index varied from 1 to 3 according to the severity of the phenomenon) (see Section 2). The sum of the index, the intra-epithelial inflammation index, the index of inflammation in the lamina propria, yielded a total index which varied from 3 to 9. We considered the samples with a total index ≤ 6 as poorly remodelled and inflamed polyps (group 1; cultures 1–6) and the samples with a total index > 6 as highly remodelled and inflamed polyps (group 2; cultures 7–12).

secretory leukocyte protease inhibitor concentration was highly variable from one culture to another, either in group 1 (14.6 ± 25.2 ng secretory leukocyte protease inhibitor/μg total proteins) or in group 2 (17.4 ± 31.0 ng secretory leukocyte protease inhibitor/μg total proteins) with no significant difference between the 2 groups.

Two-way analysis of variance showed a significant effect of the human leukocyte elastase concentration ($P = 0.03$) and a significant effect of the remodelling and inflammation degrees of the tissue ($P = 0.02$) on the amount of in vitro secretory leukocyte protease inhibitor secretion. The time-course of the secretory leukocyte protease inhibitor secretion for each group is presented in the Fig. 2. In group 1 (cultures dissociated from poorly remodelled and inflamed polyps), human leukocyte elastase induces a significant increase of secretory leukocyte protease inhibitor secretion at a concentration of 1 μg/ml when compared to the control. The amount of secretory leukocyte protease inhibitor secretion is not significantly different from the control at a concentration of 10 μg/ml of human leukocyte elastase. After incubation with 100 μg/ml of human leukocyte elastase, the mean value of secretory leukocyte protease inhibitor secretion decreases, although no significant difference is observed compared to the control. In group 2 (cultures dissociated from highly remodelled and inflamed polyps), while there was no significant difference after incubation with 1 μg/ml of human leukocyte elastase, there was a significant decrease from the control after incubation with 10 μg/ml of human leukocyte elastase ($P = 0.01$) or with 100 μg/ml of human leukocyte elastase ($P < 0.01$). Differences between group 1 and group 2 were significant after incubation with 1 μg/ml of human leukocyte elastase ($P = 0.05$) and with 10 μg/ml of human leukocyte elastase ($P = 0.03$). There was no significant difference between the two groups after incubation with 100 μg/ml of human leukocyte elastase. In summary, human leukocyte elastase induced an increase of secretory leukocyte protease inhibitor secretion in group 1 (poorly remodelled and inflamed tissues) and a lower increase of secretory leukocyte protease inhibitor secretion

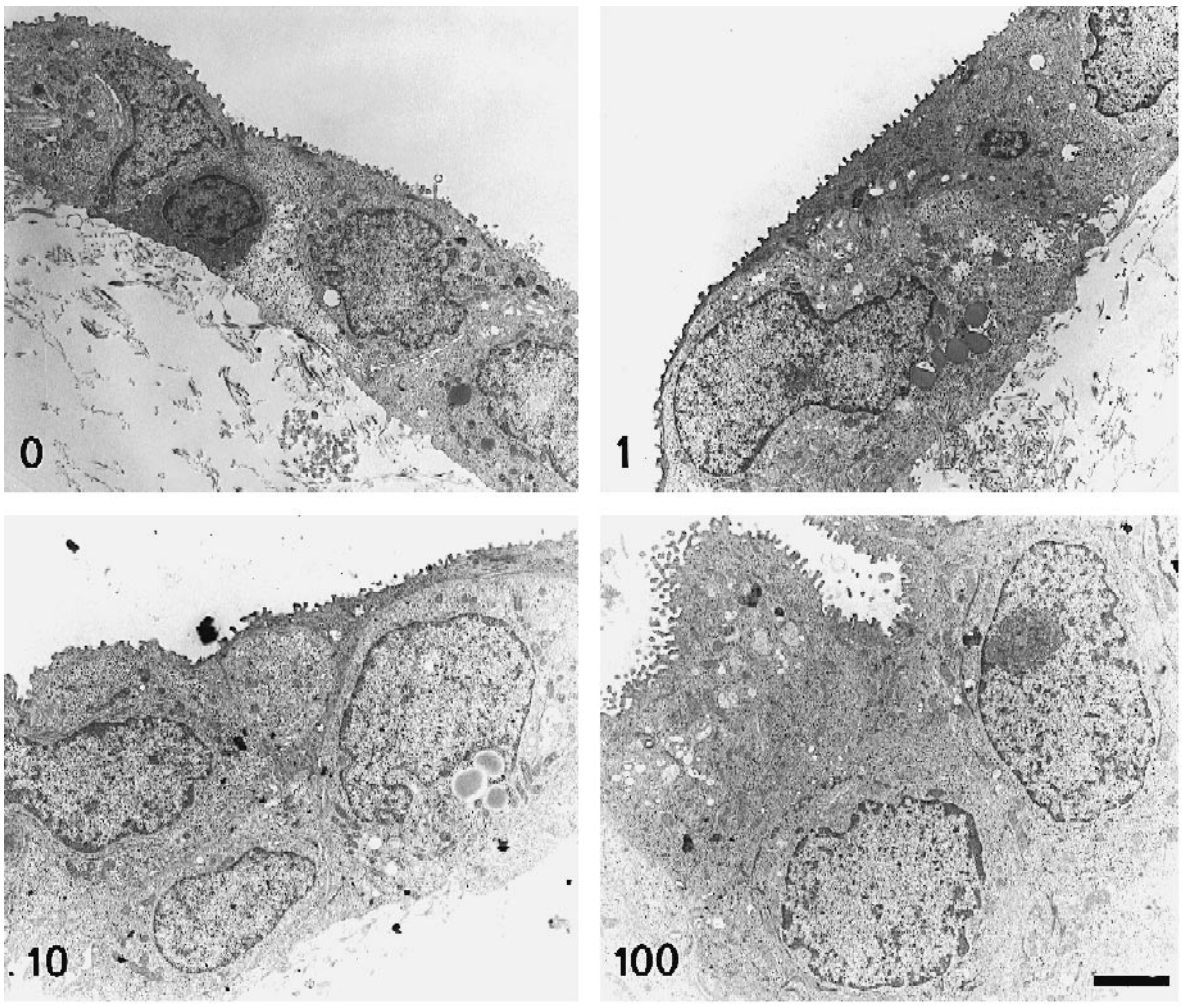

Fig. 1. Ultrastructural aspect of the cultured cells after exposure to human leukocyte elastase. At confluency, surface epithelial cells in primary culture were incubated for 24 h with human leukocyte elastase at 0, 1, 10 or 100 μg/ml. After another 24 h period of incubation with the culture medium alone, the cultured cells were fixed with 2.5% glutaraldehyde and embedded for ultrastructural observations. Intracytoplasmic vacuoles (arrows) are visible after 100 μg/ml human leukocyte elastase exposure (bar = 2 μm).

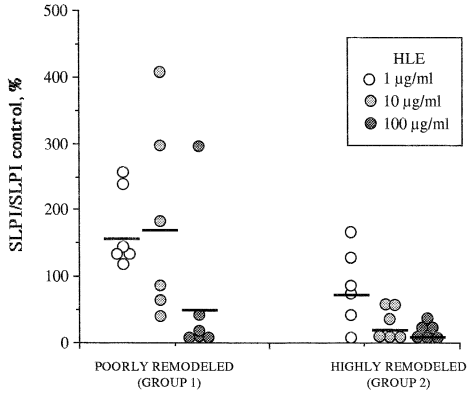

Fig. 2. Effect of human leukocyte elastase on the secretory leukocyte protease inhibitor secretion. Surface epithelial cells, dissociated from 12 different human nasal polyps, were cultured in defined primary culture conditions. At confluency, cells were incubated for 24 h with human leukocyte elastase at various concentrations (0, 1, 10 or 100 μg/ml of culture medium). Supernatants were then collected and secretory leukocyte protease inhibitor concentration and total proteins concentration were determined, respectively, by enzyme-linked immunosorbent assay (ELISA) and BioRad assay. In order to investigate the time-course of secretory leukocyte protease inhibitor secretion, we calculated, for each culture and for each concentration of human leukocyte elastase, the relative amount of secreted secretory leukocyte protease inhibitor as: ng secretory leukocyte protease inhibitor/μg of total proteins divided by the ng secretory leukocyte protease inhibitor/μg of total proteins which was observed in the absence of human leukocyte elastase for the same culture = (secretory leukocyte protease inhibitor/secretory leukocyte protease inhibitor control). The results are presented individually for each group of cultures. The horizontal lines represent the mean value for each group. Group 1: cultures of nasal cells dissociated from poorly remodelled and inflamed polyps ($n = 6$). Group 2: cultures of nasal cells dissociated from remodelled and inflamed polyps ($n = 6$) (see Table 1 for definition of the groups). The two-way ANOVA shows a significant effect of the human leukocyte elastase and of remodelling ($P = 0.04$ and 0.2, respectively).

in group 2 (remodelled and inflamed tissues), compared to group 1.

Interestingly, we observed significant negative correlations between the degree of remodelling of the tissues and secretory leukocyte protease inhibitor secretion after human leukocyte elastase at 10 and 100 μg/ml ($r_s = -0.62$, $P = 0.03$ and $r_s = -0.59$, $P = 0.04$, respectively).

There were significant positive correlations between the secretory leukocyte protease inhibitor secretion in unstimulated cultures and the effect of human leukocyte elastase at 1, 10 and 100 μg/ml on this production ($r_s = 0.86$, $P = 0.001$; $r_s = 0.73$, $P = 0.007$ and $r_s = 0.70$, $P = 0.01$, respectively).

3.4. Effect of human leukocyte elastase on the secretory leukocyte protease inhibitor gene expression

The effect of human leukocyte elastase on transcription of the secretory leukocyte protease inhibitor gene was assessed by Northern-blot analysis of total RNA extracted from 2 cultures of each group. In the absence of human leukocyte elastase, the median value of the amount of secretory leukocyte protease inhibitor mRNA transcripts versus 36B4 rRNA transcripts (secretory leukocyte protease inhibitor control) was 1.4 in the cultures of the group 1 (from poorly remodelled and inflamed polyps) and 1.9 in the cultures of the group 2 (from highly remodelled and inflamed polyps). The effect of human leukocyte elastase on the transcription of the secretory leukocyte protease inhibitor gene was assessed by calculating the relative amount of secretory leukocyte protease inhibitor mRNA transcripts versus 36B4 rRNA transcripts (secretory leukocyte protease inhibitor) after incubation with various concentrations of human leukocyte elastase compared to the amount of secretory leukocyte protease inhibitor mRNA transcripts versus 36B4 rRNA transcripts observed in the same culture in the absence of human leukocyte elastase (secretory leukocyte protease inhibitor control). There was no effect of incubation with 1 μg/ml human leukocyte elastase on the amount of secretory leukocyte protease inhibitor mRNA transcripts in either group. There was, however, a very slight increase of the secretory leukocyte

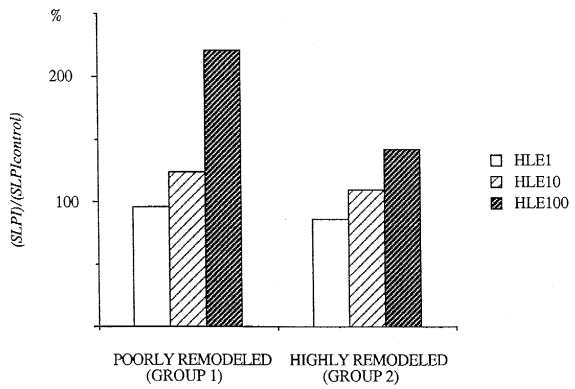

Fig. 3. Effect of human leukocyte elastase on the secretory leukocyte protease inhibitor gene expression. At confluency, surface epithelial cells in primary culture were incubated for 24 h with human leukocyte elastase at 0, 1, 10 or 100 μg/ml. After another 24 h period of incubation with the culture medium alone, total RNA were extracted using RNAzol treatment, separated by electrophoresis on 1% agarose gel and transferred onto a Nylon membrane. Secretory leukocyte protease inhibitor mRNA transcripts were detected by using a ^{32}P-radiolabeled cDNA probe. 36B4 rRNA transcripts were used as control. The relative amount of secretory leukocyte protease inhibitor mRNA transcripts was assessed by calculating the ratio between the intensity of the secretory leukocyte protease inhibitor signal and the intensity of the 36B4 signal, which were previously determined by using a scanning densitometer. We calculated the ratio secretory leukocyte protease inhibitor mRNA transcripts/36B4 rRNA transcripts (secretory leukocyte protease inhibitor) for each concentration of human leukocyte elastase and compared them to the respective ratio secretory leukocyte protease inhibitor mRNA transcripts/36B4 rRNA transcripts which was observed for the same culture in the absence of human leukocyte elastase (secretory leukocyte protease inhibitorcontrol). Results are presented as median values of (secretory leukocyte protease inhibitor)/(secretory leukocyte protease inhibitor control) for group 1 (cultures of cells dissociated from poorly remodelled and inflamed polyps; $n = 2$) and for group 2 (cultures of cells dissociated from highly remodelled and inflamed polyps; $n = 2$).

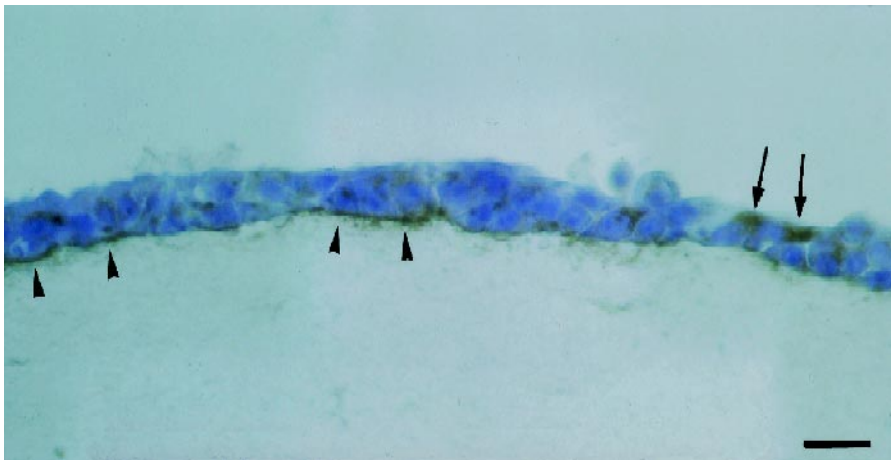

Fig. 4. Immunocytolocalization of secretory leukocyte protease inhibitor. At confluency, surface epithelial cells in primary culture were incubated for 24 h with human leukocyte elastase at 0, 1, 10 or 100 μg/ml. After another 24 h period of incubation with the culture medium alone, the cultured cells were fixed with 4% paraformaldehyde and paraffin-embedded. Secretory leukocyte protease inhibitor was detected on cells sections by indirect immunoperoxidase. After incubation with 100 μg/ml human leukocyte elastase, secretory leukocyte protease inhibitor was identified in the cytoplasm of some cells (arrows), as well as in type I collagen matrix, under the basal side of the surface epithelial cells (arrowheads) in primary culture. (bar = 10 μm).

protease inhibitor gene transcription after incubation with 10 μg/ml human leukocyte elastase and higher stimulation of the transcription of the secretory leukocyte protease inhibitor gene after incubation with 100 μg/ml human leukocyte elastase (Fig. 3). The increase in the amount of secretory leukocyte protease inhibitor mRNA transcripts versus 36B4 rRNA transcripts compared to the control was greater in cultures from group 1 (poorly remodelled and inflamed polyps) than in cultures from group 2 (highly remodelled and inflamed polyps) (increasing up to 2.3 fold in group 1 and 1.5 fold in group 2).

Whereas the relative amounts of secretory leukocyte protease inhibitor mRNA transcripts increased after incubation with 100 μg/ml human leukocyte elastase, the secretory leukocyte protease inhibitor secretion decreased significantly in the cultures from group 2 (highly remodelled and inflamed polyps) and was not significantly different from the control in group 1 (poorly remodelled and inflamed polyps).

3.5. Secretory leukocyte protease inhibitor immunocytolocalization

Localization of the secretory leukocyte protease inhibitor protein was investigated by immunoperoxidase staining. Whatever the human leukocyte elastase concentration used, an intracytoplasmic localization was observed in some cells. After exposure to 100 μg/ml human leukocyte elastase, secretory leukocyte protease inhibitor could also often be identified in the type I collagen matrix, just below the basal side of the surface epithelial cells (Fig. 4). This particular localization of secretory leukocyte protease inhibitor was observed in cultures from poorly remodelled and inflamed polyps (in 3 of 3 cases in group 1) as well as in cultures from highly remodelled and inflamed polyps (in 1 of 2 cases in group 2). Quantification of the percentage of secretory leukocyte protease inhibitor-containing cells in one culture of group 2 showed a significant positive dose-dependent effect of human leukocyte elastase (χ^2 test; $P < 0.05$). This percentage of secretory leukocyte protease inhibitor-containing cells increased from $4 \pm 0.5\%$ without elastase, to $7.5 \pm 3.5\%$ with 1 μg/ml human leukocyte elastase, to $25 \pm 12\%$ with 10 μg/ml human leukocyte elastase and up to $46 \pm 9\%$ with 100 μg/ml human leukocyte elastase. Quantification was made by counting the number of total cells (range values: 70 to 700) and the respective number of secretory leukocyte protease inhibitor-containing cells, on 2 to 3 slices, corresponding to the experiments done with each concentration of human leukocyte elastase.

4. Discussion

This study demonstrated, both that human adult nasal surface epithelial cells in primary culture can express and release the secretory leukocyte protease inhibitor and that human leucocyte elastase may modulate this expression. More interestingly, the capacity of human leukocyte elastase to stimulate the secretory leukocyte protease inhibitor release from the cells appears to depend on the degree of remodelling and inflammation of the tissue from which the cells derive.

Previous in vitro studies have shown the expression of secretory leukocyte protease inhibitor mRNA transcripts (Abbinante-Nissen et al., 1993; Sallenave et al., 1994; Abe et al., 1991) and secretory leukocyte protease inhibitor secretion by human respiratory cell lines (Sallenave et al., 1994; Sallenave et al., 1993) or surface epithelial cells in secondary culture (Emery et al., 1995). Our results are

consistent with the previous findings, since all the primary cultures of nasal surface epithelial cells secreted secretory leukocyte protease inhibitor. We observed huge variations in the amounts of constitutively secreted secretory leukocyte protease inhibitor from one culture to another and no significant difference could be shown under basal conditions according to the degree of remodelling and inflammation of the nasal polyp tissue from which the nasal cells derived. Nevertheless, secretory leukocyte protease inhibitor gene expression appeared to be greater in cultures derived from highly remodelled and inflamed tissues than in cultures derived from poorly remodelled and inflamed ones. On the contrary, there was a significant difference in the responsiveness to human leukocyte elastase according to the initial state of the nasal tissue: human leukocyte elastase increased the amounts of secreted secretory leukocyte protease inhibitor in cultures from poorly remodelled and inflamed tissues, whereas human leukocyte elastase induced a decrease of the amounts of secreted secretory leukocyte protease inhibitor in cultures from remodelled and inflamed tissues.

It is of interest that the basal unstimulated secretory leukocyte protease inhibitor secretion was significantly related to the capacity of the cells to respond to human leukocyte elastase.

The effect of different mediators on the amounts of secretory leukocyte protease inhibitor mRNA transcripts has been investigated in different respiratory cell lines (Sallenave et al., 1994; Abbinante-Nissen et al., 1995; Marayuma et al., 1994). Recently, the anti-inflammatory effect of corticosteroids has been suggested to be related to their ability to stimulate the expression of the secretory leukocyte protease inhibitor gene in a respiratory cell line (Abbinante-Nissen et al., 1995). Sallenave et al. (Sallenave et al., 1994) have shown that interleukin 1β and tumor necrosis factor α, which are cytokines secreted during the onset of inflammation, induce increased expression of secretory leukocyte protease inhibitor by respiratory cell lines derived from Clara cells and type II pneumocytes. Human leukocyte elastase has also been reported to stimulate the secretory leukocyte protease inhibitor gene expression in these cell lines (Abbinante-Nissen et al., 1993; Sallenave et al., 1994). In accordance with these findings, our data showed that human leukocyte elastase at a high concentration (100 μg/ml) stimulates the transcription of the secretory leukocyte protease inhibitor gene by increasing up to 2.3 fold the relative amounts of secretory leukocyte protease inhibitor mRNA transcripts. Stimulation of the secretory leukocyte protease inhibitor gene transcription was less in cultures from highly remodelled and inflamed tissues (only 50% of increase compared to the control), suggesting that transcriptional control of the secretory leukocyte protease inhibitor gene by human leukocyte elastase is less efficient when cells had been exposed to human leukocyte elastase for a long period. The difference that we demonstrate here in secretory leukocyte protease inhibitor responsiveness to human leukocyte elastase according to the degree of inflammation and of the tissue is of pathophysiological relevance. It can be hypothesized that in inflamed and remodelled airway tissue such as nasal polyps, inflammatory cytokines such as interleukin 1β and tumor necrosis factor may be constitutively up-regulated and that the release of these cytokines in response to human leukocyte elastase stimulation may induce the secretion of secretory leukocyte protease inhibitor protein and further down-regulation of the responsiveness of the cells to human leukocyte elastase.

Surprisingly, there was a marked discrepancy in the patterns of secretory leukocyte protease inhibitor mRNA transcripts and secretory leukocyte protease inhibitor secretion after exposure to increasing human leukocyte elastase concentrations, particularly in the group of cultures deriving from highly remodelled and inflamed tissues. Sallenave et al. (1994) have already shown that secretory leukocyte protease inhibitor was decreased in alveolar A549 cell line whereas secretory leukocyte protease inhibitor mRNA transcripts were up-regulated. All these results indicate that, in response to elastase, several types of human respiratory cells increase secretory leukocyte protease inhibitor mRNA transcripts but do not secrete the secretory leukocyte protease inhibitor protein. This observation suggests a possible post-transcriptional or secretory defect or a possible intracellular accumulation of secretory leukocyte protease inhibitor. Another possibility is that in highly remodelled and inflamed tissues, the cells may secrete more secretory leukocyte protease inhibitor toward their basal side than from their apical side.

The expression of secretory leukocyte protease inhibitor by surface epithelial cells dissociated from nasal polyps is consistent with results of our previous in vivo study (Marchand et al., 1995), in which we showed that secretory leukocyte protease inhibitor could be identified in some surface epithelial cells of remodelled epithelia (folding, basal cell and/or mucous cell hyperplasia) of human nasal polyps. Identification of secretory leukocyte protease inhibitor in the surface epithelium of the upper or central airways (Marchand et al., 1995; Willems et al., 1986a) has rarely been described. Van Seuningen et al. (1995) reported that the expression of secretory leukocyte protease inhibitor in normal human bronchial and bronchiolar tissue was restricted to the serous glandular cells. The apparent discrepancy between their results and ours may be explained by the variability in the inflammatory and remodelling characteristics of human nasal polyp tissue (Marchand et al., 1995; Brézillon et al., 1995), which can be also observed in pathological human airway tissue.

Immunocytochemistry showed human leukocyte elastase to increase the percentage of secretory leukocyte protease inhibitor-containing cells in the culture and extracellular basal localization of secretory leukocyte protease inhibitor was often observed after an incubation with 100 μg/ml human leukocyte elastase. This observation sug-

gests that high concentrations of human leukocyte elastase may induce a vectorization of the secretory leukocyte protease inhibitor secretion. In vivo, association of secretory leukocyte protease inhibitor with elastin fibers has been described in human bronchial, bronchiolar and alveolar tissue (Kramps et al., 1989; Willems et al., 1986b). Using a cell culture model in a bi-dimensional chamber, Dupuit et al. (1993) showed that 40% of the secretory leukocyte protease inhibitor was secreted toward the basal side of human tracheobronchial glandular cells. Our results indicate that nasal surface epithelial cells are able to secrete secretory leukocyte protease inhibitor, not only from their apical side into the culture supernatant, but also from their basal side. This observation supports the hypothesis that nasal surface epithelial cells may contribute to the anti-elastolytic burden not only in mucus, but perhaps also in connective tissue. The capacity of defense of the mucosa against human leukocyte elastase therefore seems to proceed in at least two different ways: human leukocyte elastase may induce a phenotypic conversion of the surface epithelial cells, which is expressed by a morphological of the surface epithelium associated with secretory leukocyte protease inhibitor synthesis and secretion at the apical side of the cells, toward the airway lumen. Human leukocyte elastase may also stimulate the secretory leukocyte protease inhibitor secretion by surface epithelial cells on their basal side, toward the connective tissue.

In conclusion, there are several relevant pathophysiological implications of our present results: Human adult nasal surface epithelial cells are able to participate in the biochemical defense of the mucosa by synthesizing secretory leukocyte protease inhibitor. Human leukocyte elastase can induce, in airway surface epithelial cells, a stimulation of secretory leukocyte protease inhibitor gene transcription and protein secretion, but this ability depends highly on the degree of inflammation and remodelling of the nasal epithelial cells. Such phenotypic epithelial changes may limit the capacity of the airway epithelium to participate in the secretory leukocyte protease inhibitor protection of the airway mucosa against the neutrophil burden in chronic inflammatory situations.

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References

AbbinanteNissen, J.M., Simpson, L.G., Leikauf, G.D., 1993. Neutrophil elastase increases secretory leukocyte protease inhibitor transcripts levels in airway epithelial cells. Am. J. Physiol. 265 (Lung Cell. Mol. Physiol. 9), L286–L292.

Abbinante-Nissen, J.M., Simpson, L.G., Leikauf, G.D., 1995. Corticosteroids increase secretory leukocyte protease inhibitor transcript levels in airway epithelial cells. Am. J. Physiol. 268 (Lung Cell. Mol. Physiol. 12), L601–L606.

Abe, T., Kobayashi, N., Yoshimura, K., Trapnell, B.C., Kim, H., Hubbard, R.C., Brewer, M.T., Thompson, R.C., Crystal, R.G., 1991. Expression of the secretory leukoprotease inhibitor gene in epithelial cells. J. Clin. Invest. 87, 2207–2215.

Asano, A., Kida, K., Koyama, T., Wada, H., Izawa, Y., Hosoda, K., Masuda, K., Suzuki, Y., 1995. A morphologic study of lung secretory leukoprotease inhibitor in pneumonia. Am. J. Respir. Crit. Care Med. 151, 1576–1581.

Barton, A.D., Ryder, K., Lourenco, R.V., Dralle, W., Weiss, S.G., 1976. Inflammatory reactions and airway damage in cystic fibrosis. J. Lab. Clin. Med. 88, 423–426.

Brézillon, S., Dupuit, F., Hinnrasky, J., Marchand, V., Kälin, N., Tümmler, B., Puchelle, E., 1995. Decreased expression of the CFTR protein in remodelled human nasal epithelium from non-cystic fibrosis patients. Lab. Invest. 72, 191–200.

Chambard, M., Gabrion, J., Mauchamp, J., 1981. Influence of collagen gel on the orientation of epithelial cells polarity: Follicle formation from isolated thyroid cells and performed monolayers. J. Cell. Biol. 91, 157–166.

Chevillard, M., Hinnrasky, J., Pierrot, D., Zahm, J.M., Klossek, J.M., Puchelle, E., 1993. Differentiation of human nasal surface upper airway epithelial cells in primary culture on a floating collagen gel. Epith. Cell. Biol. 2, 17–25.

de Water, R., Willems, L.N.A., van Muijen, G.N.P., Franken, C., Fransen, J.A.M., Dijkman, J.H., Kramps, J.A., 1986. Ultrastructural localization of bronchial antileukoprotease in central and peripheral airways by a gold-labeling technique using monoclonal antibodies. Am. Rev. Respir. Dis. 133, 882–890.

Dupuit, F., Jacquot, J., Spilmont, C., Tournier, J.M., Hinnrasky, J., Puchelle, E., 1993. Vectorial delivery of newly-synthesized secretory proteins by human tracheal gland cells in culture. Epith. Cell. Biol. 2, 91–99.

Emery, N., Place, G.A., Dodd, S., Lhermitte, M., David, G., Lamblin, G., Perini, J.M., Page, A.M., Hall, R.L., Roussel, P., 1995. Mucous and serous secretions of human bronchial epithelial cells in secondary culture. Am. J. Respir. Cell. Mol. Biol. 12, 130–141.

Francina, A., Cloppet, H., Guinet, R., Rossi, M., Guyotat, D., Gentilhomme, O., Richard, M., 1986. A rapid and sensitive non-competitive avidin–biotin immuno-enzymatic assay for lysozyme. J. Immunol. Methods 87, 267–272.

Kramps, J.A., Franken, C., Meijer, C.J.L.M., Dijkman, J.H., 1981. Localization of low molecular weight protease inhibitor in serous secretory cells of the respiratory tract. J. Histochem. Cytochem. 29, 712–719.

Kramps, J.A., Franken, C., Dijkman, J.H., 1984. Elisa fur quantitative measurement of low-molecular-weight bronchial protease inhibitor in human sputum. Am. Rev. Respir. Dis. 129, 959–963.

Kramps, J.A., Te Boekhorst, A.H.T., Fransen, J.A.M., Ginsel, L.A., Dijkman, J.H., 1989. Antileukoprotease is associated with elastin fibers in the extracellular matrix of the human lung. Am. Rev. Respir. Dis. 140, 471–476.

Lee, C.H., Igarashi, Y., Hohman, R.J., Kaulbach, H., White, M., Kaliner, M.A., 1993. Distribution of secretory leukoprotease inhibitor in the human nasal airway. Am. Rev. Respir. Dis. 147, 710–716.

Marayuma, M., Hay, J.G., Yoshimura, K., Chu, C.S., Crystal, R.G., 1994. Modulation of secretory leucoprotease inhibitor gene expression in human bronchial epithelial cells by phorbol ester. J. Clin. Invest. 94, 368–375.

Marchand, V., Tournier, J.M., Chevillard, M., Polette, M., Beorchia, A., Klossek, J.M., Puchelle, E., 1995. Identification of antileucoprotease in remodelled human adult nasal surface epithelium. Eur. Respir. J. 8, 15–21.

Martodam, R.R., Baugh, R.J., Twumasi, D.Y., Liener, I.E., 1979. A rapid procedure for the large scale purification of elastase and cathepsin G from human sputum. Prep. Biochem. 9, 15–31.

Metzger, W.J., Zavala, D., Richerson, H.B., Moseley, P., Iwamota, P., Monick, M., Sjoerdsma, K., Hunninghake, G.W., 1987. Local allergen challenge and bronchoalveolar lavage of allergic asthmatic lungs: Description of the model and local airway inflammation. Am. Rev. Respir. Dis. 135, 433–440.

Nahori, M.A., Renesto, P., Vargaftig, B.B., Chignard, M., 1992. Activation and damage of cultured airway epithelial cells by human elastase and cathepsin G. Eur. J. Pharmacol. 228, 213–218.

Sallenave, J.M., Silva, A., Marsden, M.E., Ryle, A.P., 1993. Secretion of mucus proteinase inhibitor and elafin by Clara cells and type II pneumocytes cell lines. Am. J. Respir. Cell. Mol. Biol. 8, 126–133.

Sallenave, J.M., Schulmann, J., Crossley, J., Jordana, M., Gauldie, J., 1994. Regulation of secretory leukocyte proteinase inhibitor (secretory leukocyte protease inhibitor) and elastase-specific Inhibitor (ESI/Elafin) in human airway epithelial cells by cytokines and neutrophilic enzymes. Am. J. Respir. Cell. Mol. Biol. 11, 733–741.

Sibille, Y., Marchandise, F.X., 1993. Pulmonary immune cells in health and disease: Polymorphonuclear neutrophils. Eur. Respir. J. 6, 1529–1543.

Snider, G.L., Schur, P.H., Lucey, E.C., 1985. Eglin C a polypeptide derived from medicinal leech prevents human neutrophil elastase-induced emphysema and bronchial gland secretory cell metaplasia in the hamster. Am. Rev. Respir. Dis. 132, 1155–1161.

Tegner, H., Ohlsson, K., 1977. Localization of a low molecular weight protease inhibitor to tracheal and maxillary sinus mucosa. Hoppe-Seyler's Physiol. Chem. 358, 425–429.

Tegner, H., 1978. Quantitation of human granulocyte protease inhibitors in non-purulent bronchial lavage fluids. Acta Otolaryngol. 85, 282–289.

Thompson, A.B., Daughton, D., Robbins, R.A., Ghafouri, M.A., Oehlerking, M., Rennard, S.I., 1989. Intraluminal airway inflammation in chronic bronchitis. Am. Rev. Respir. Dis. 140, 1527–1537.

Tournier, J.M., Jacquot, J., Sadoul, P., Bieth, J.G., 1983. Noncompetitive enzyme immunoassay for the measurement of bronchial inhibitor in biological fluids. Anal. Biochem. 131, 345–350.

Van Seuningen, I., Audie, J.P., Gosselin, B., Lafitte, J.J., Davril, M., 1995. Expression of human mucous proteinase inhibitor in respiratory tract: A study by in situ hybridization. J. Histochem. Cytochem. 43, 645–648.

Weissmann, G., Smolen, J.E., Korchak, H.M., 1980. Release of inflammatory mediators from stimulated neutrophils. New Engl. J. Med. 303, 27–34.

Willems, L.N.A., Kramps, J.A., de Water, R., Stijnen, T., Fleuren, G.J., Franken, C., Dijkman, J.H., 1986a. Evaluation of antileukoprotease in surgical lung specimens. Eur. J. Respir. Dis. 69, 242–247.

Willems, L.N.A., Otto-Verberne, C.J.M., Kramps, J.A., ten Have-Opbroek, A.A.W., Dijkman, J.H., 1986b. Detection of antileukoprotease in connective tissue of the lung. Histochemistry 86, 165–168.

Willems, L.N.A., Kramps, J.A., Stijnen, T., Sterk, P.J., Weening, J.J., Dijkman, J.H., 1989. Antileukoprotease-containing bronchiolar cells. Relationship with morphologic disease of small airways and parenchyma. Am. Rev. Respir. Dis. 139, 1244–1250.