Identification of antileucoprotease in remodelled human adult nasal surface epithelium

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ABSTRACT: Antileucoprotease (ALP) is generally considered as a specific marker for glandular serous cells, and plays a major role in the defence of the respiratory tract against proteolytic damage. Nevertheless, several studies have identified ALP in bronchial and bronchiolar surface epithelial cells, and also an increased number of ALP-containing cells in bronchiolar tissue during the development of pulmonary diseases. In order to define more clearly whether the surface epithelium might be involved in the defence of the respiratory mucosa, we have investigated the expression of ALP by cells of the nasal surface epithelium.

Indirect immunocytochemistry and *in situ* hybridization for ALP were performed on human nasal polyp sections. The height of the surface epithelium, its morphology, and the degree of local inflammation were assessed in parallel.

Surface epithelium morphology was highly heterogeneous. ALP-containing cells were identified, but only in remodelled areas of the surface epithelium (foldings, basal cell and/or mucous cell hyperplasia), with no association to the degree of inflammation.

These results demonstrate that the surface epithelial cells of the human adult nasal mucosa can express ALP in remodelled surface epithelium, and may be actively involved in the biochemical defence of the airways.

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A low molecular weight proteinase inhibitor, found in bronchial secretions and capable of inhibiting leucocyte proteinases [1, 2], is synthesized locally by airway epithelial cells [3]. This proteinase inhibitor, generally referred to as antileucoprotease (ALP) [4], plays a major physiological role in the inactivation of neutrophil elastase bound to elastin [5, 6]. In the respiratory tract, ALP has been localized in the serous cells of the submucosal nasal [3, 7], tracheal and bronchial glands [3, 8], and in some nonciliated cells of the bronchiolar epithelium, where glands are absent [9]. Recent studies have been carried out to estimate the number of ALP-containing cells in relation to pathological situations. WILLEMS *et al.* [10] showed that, in small airway diseases, parenchymal destruction is associated with an increased number of ALP-containing cells in respiratory bronchioles, suggesting a role for ALP in the general defence against inflammatory and destructive processes in the distal airways. The increase in the number of ALP-containing cells was found in areas of mucous cell or squamous cell metaplasia [10]. KIDA *et al.* [11] also reported an increased number of ALP-containing cells in human bronchial and bronchiolar structures, together with

mucous cell hyperplasia, associated with the existence of acute inflammatory changes in the alveolar region.

These data led us to address the question of whether normal and remodelled surface epithelium of the upper airways could express ALP. Using the nasal polyp as a model of inflamed and remodelled respiratory tissue, we investigated the localization of ALP messenger ribonucleic acid (mRNA) transcripts and ALP protein in human nasal tissue, and analysed their distribution in relation to the morphological aspect and the degree of inflammation of the airway epithelium.

Materials and methods

Tissue preparation

Human nasal polyps were obtained from six non-allergic patients undergoing nasal polypectomy for nasal obstruction. These patients did not show any sign of blood eosinophilia, and they presented normal range of total serum immunoglobulin E (IgE), negative radio-allergosorbent tests, and negative skin-prick tests. Most

of the patients (five out of six) had been using anti-inflammatory (corticosteroids) medication just prior to polypectomy. Immediately after excision, tissue samples were immersed in RPMI 1640 medium (Seromed, Biochrom KG, Berlin, Germany), containing 100 U·ml⁻¹ penicillin, 100 µg·ml⁻¹ streptomycin, and 20 mM hydroxyethyl-piperazine ethanesulphonic acid (Hepes)(GIBCO, Grand Island, NY, USA). The polyp samples were transferred to our laboratory within 24 h in this conservation medium.

A sample of each nasal polyp was fixed for 1 h at 4°C in 4% paraformaldehyde, rinsed three times for 15 min in 0.1 M phosphate buffer pH 7.2, then dehydrated through graded concentrations of ethanol and embedded in paraffin. Sections 3 µm thick were then prepared on a microtome and transferred onto gelatin-coated glass slides.

Morphological observations

Paraffin sections from six nasal polyps were analysed along the total observable surface epithelium, using a light microscope equipped with a reticular objective. Four types of surface epithelium (normal or remodelled) were distinguished according to their morphology. The relative occurrence of each morphological type, expressed as a percentage of the whole, was calculated for each sample. The length of the surface epithelium observed varied from 1,160 to 7,900 µm, with approximately 100–800 cells. The height of the surface epithelium in each morphological type of epithelium was calculated in each sample. The mean number of inflammatory cells in the surface epithelium and in the underlying connective tissue was calculated in ALP-positive and ALP-negative areas of five polyp samples. This number of inflammatory cells was assessed from haematoxylin-phloxine-safranin (HPS) stained serial sections.

Immunohistochemistry

Paraffin sections were dewaxed in xylene, and incubated for 5 min with 3% hydrogen peroxide (H₂O₂) in water, to inhibit the endogenous peroxidase activity. After two washes in 0.1 M phosphate buffer (PB) pH 7.4 for 5 min, nonspecific fixation sites were saturated by treating all sections for 10 min with 10% normal goat serum in PB. This was followed by a 1 h incubation in 3 µg·ml⁻¹ rabbit immunoglobulin G (IgG) anti-human antileucoprotease [12] in 0.1 M PB containing 5% normal goat serum (NGS-PB). The sections were then washed twice for 5 min in PB and for 10 min in NGS-PB, followed by a 1 h incubation with biotinylated donkey anti-rabbit IgG (Amersham) (1:250). The sections were washed twice for 5 min in PB and for 10 min in NGS-PB, and incubated with streptavidin-biotin-peroxidase complex (Amersham) (1:250). The sections were washed four times with PB for 5 min and incubated in 0.1% 3,3'-diaminobenzidine tetrahydrochloride (Sigma Chemical), 0.1% H₂O₂, and 0.1% imidazol (Sigma) in distilled water. The sections were then incubated for 5 min in

the dark with the chromogenic solution. The enzymatic activity of peroxidase was stopped by immersing the slices in distilled water. All of the steps were carried out at room temperature. Cells were then counterstained with haematoxylin, and glass coverslips were mounted on the sections with Aquamount. Observations were made using an Axiophot microscope (Zeiss).

Negative controls were performed by either omitting the ALP antibody, or replacing it with normal rabbit serum IgG at the same concentration (3 µg·ml⁻¹).

Hybridization

Nature of the probe. The plasmid pRH 1807, which is a pUC18 derivative that contains the PstI complementary deoxyribonucleic acid (cDNA) insert of pRH 34 was kindly provided by R. HEINZEL [13]; this insert contains the whole coding region for ALP. Northern blot analysis of total ribonucleic acid (RNA) extracted from human respiratory cells revealed a single band of 0.7 Kb [14], which agrees with the results of HEINZEL *et al.* [13] with RNA from human cervix uteri.

In situ hybridization. Paraffin sections of nasal polyps were dewaxed in xylene, transferred in a 2 X standard saline citrate (SSC: 0.3 M NaCl, 0.03 M Tris-Na citrate) solution, and incubated for 10 min in fresh 0.25% acetic anhydride in 0.1 M triethanolamine, pH 8.0. After washings in 2 X SSC, the sections were immersed at 55°C in 50% formamide in 2 X SSC solution for 10 min. The radiolabelled cDNA probe was prepared by the random priming technique (Multiprime DNA, labelling system; Amersham, Little Chalfort, UK) using ³⁵S-labelled dCTP (600 Ci·mM⁻¹, 10 mCi·ml⁻¹, Amersham, UK). Hybridization of this probe was performed in 10 mM Tris pH 7.0 containing 50% formamide, 0.6 M NaCl, 1 mM ethylenediamine tetraacetic acid (EDTA), 1 X Denhardt's solution, 250 µg·ml⁻¹ heat-denatured salmon sperm, 500 µg·ml⁻¹ yeast transfer ribonucleic acid (tRNA), 10% dextran sulphate and 10 mM dithiothreitol. The denatured labelled probe was applied to tissue sections at a concentration of 1 ng·10 µl⁻¹ of the hybridization solution. Denaturation of the probe was carried out by heating at 80°C for 10 min. Each tissue section was covered with 10 µl of the hybridization solution and a siliconized coverslip, and then incubated for 42 h at 42°C in a moist chamber. After hybridization, tissue sections were washed 3 × 5 min at room temperature in 2 X SSC, 3 × 5 min in 1 X SSC, and for 5 min in 0.5 X SSC. Sections were then dehydrated in 50, 70, and 100% ethanol containing 600 mM ammonium acetate, and air dried prior to autoradiography. Sections were dipped into K5 Ilford emulsion, dried and exposed for 28 days at -20°C. They were developed in D19 Kodak, HPS stained and coverslipped. Observations were made by using an Axiophot microscope (Zeiss).

Negative controls for *in situ* hybridization were performed by treating tissue sections with ribonuclease (RNase) (100 µg·ml⁻¹ in 2 X SSC) before prehybridization treatment.

Quantitation of ALP transcripts labelling

The density of labelling obtained by *in situ* hybridization of the ALP cDNA probe over the surface epithelium was quantitatively evaluated. Eleven areas of the surface epithelium were observed. For each area studied, black and white photographs were taken using successive Nomarski differential interference illumination and epifluorescence in dark fields. The quantitative determination of dense markers on the sections was performed on an image analyser (Biocom 500; Les Ulis; France). The program, based on mathematical morphology, automatically detects and counts dense markers [15]. The segmentation of the contours was performed manually with the help of a graphic mouse, in both the surface epithelium and in an area of the connective tissue. The density of markers was expressed as number of markers by μm^2 area.

Relationship between the ALP labelling and the degree of inflammation

This study was performed on 26 microscope fields from five nasal polyp samples previously characterized for ALP immunolocalization on serial sections. Inflammatory cells (lymphocytes and polymorphonuclear neutrophils) were identified from morphological characteristics after HPS staining, in areas where the surface epithelium was known to be ALP-positive or ALP-negative. The results were expressed as number of inflammatory cells- mm^{-2} , in the surface epithelium or in the underlying connective tissue.

Statistical analysis

Nonparametric tests were used. Comparisons of the number of inflammatory cells and of the height of the surface epithelium in normal and remodelled areas were made using a Mann-Whitney test. Comparison of the density of markers after *in situ* hybridization between the surface epithelium and the underlying connective tissue was made using a Wilcoxon test. All the values were expressed as medians with range in brackets and considered to be significantly different at a value of $p < 0.05$.

Results

Morphological aspect of the surface epithelium

In the surface epithelium observed, four morphological types could be distinguished, corresponding to either: 1) normal surface epithelium without any remodelling; 2) invagination or folding of the surface epithelium; 3) basal cell hyperplasia; or 4) mucous cell hyperplasia (fig. 1). Normal surface epithelium was pseudostratified and composed of ciliated cells, mucous cells and basal cells (fig. 1a) (ratio of 1 mucous cell: 5 ciliated cells). In some areas, we occasionally observed foldings (fig. 1b). Basal cell hyperplasia (fig. 1c) was characterized by the presence of at least three layers of basal cells, whilst mucous cell hyperplasia (fig. 1d) was characterized by an increased number of mucous cells in comparison with the normal structure (ratio of >1 mucous cell: 3 ciliated cells). Some areas of remodelled epithelium

Fig. 1. — Classification of the different morphological aspects of the surface respiratory epithelium. a) Normal pseudostratified epithelium, composed of ciliated cells, mucous cells and basal cells. b) Folding of a pseudostratified surface epithelium. c) Area of basal cell hyperplasia, characterized by at least three layers of basal cells. d) Area of mucous cell hyperplasia, characterized by an increased number of mucous cells, in comparison with the pseudostratified surface epithelium.

had both basal and mucous hyperplasia, but were classified in the subtype of basal cell hyperplasia.

Relative percentages of each morphological aspect of the surface epithelium

The relative proportion of each of the four morphological types of surface epithelium defined in figure 1 was calculated in each sample. There were huge differences from one polyp to another. Only three of six polyp samples presented a normal aspect along their entire surface epithelium, this normal aspect representing 10–90% of the whole. Most of the surface epithelium observed exhibited remodelling, and the most frequently identified pattern was basal cell hyperplasia.

Height of the surface epithelium

No significant difference was observed between the height of the surface epithelium, whether it was normal (median 60 μ m; range 40–60 μ m; n=3) or remodelled (median 40 μ m; range 20–80 μ m; n=15).

Immunolocalization of ALP

In the nasal polyp tissue, ALP was generally localized in the glandular epithelium; whereas, the normal surface epithelium contained no ALP (fig. 2a). In the glandular epithelium ALP was exclusively located in the serous cells; whereas, the mucous cells contained no ALP (fig. 2b). Nevertheless, ALP was identified in surface epithelium cells of remodelled areas in some cuboidal cells of folds in the surface epithelium (fig. 2c); in secretory cells in areas of mucous cell hyperplasia (fig. 2d); as well as in some intermediate cells in areas of basal cell hyperplasia (fig. 2e). Negative controls, consisting of incubation without primary antibody or with nonimmune serum replacing the primary antibody, always gave negative results; no staining ever appeared in these sections (data not shown).

In situ hybridization

The presence of ALP transcripts was demonstrated in remodelled areas of the surface epithelium (fig. 3).

Fig. 2. – Immunohistochemical localization of antileucoprotease (ALP) in human nasal polyps. Indirect immunoperoxidase and haematoxylin counterstaining on paraffin sections. The presence of ALP is shown by large arrowheads. A) The surface epithelium (SE) generally contains no ALP. B) In a glandular structure, ALP is localized in the serous cells (S), not in the mucous cells (M). C) Presence of ALP in some surface epithelial cells of a structure appearing as a folding of the surface epithelium. D) Presence of ALP in some secretory cells of a mucous cell hyperplasia area. E) Presence of ALP in some intermediate cells of a basal cell hyperplasia area. (Internal scale bar=20 μ m).

Fig. 3. — Detection of antileucoprotease (ALP) transcripts in the surface epithelium of human nasal polyps. Paraffin-embedded nasal polyp sections stained with haematoxylin-phloxine-safranin (HPS), after *in situ* hybridization of a ^{35}S -labelled complementary deoxyribonucleic acid (cDNA) probe. A) Observations with a Zeiss microscope using Nomarski differential interference illumination. SE: surface epithelium; CT: connective tissue. B) The same area observed in dark field epifluorescence: visualization of the markers. (Internal scale bar=20 μm).

In these areas, the density of markers in the surface epithelium (median 99 μm^{-2} range 41–151 μm^{-2}) was significantly greater than that in the underlying connective tissue (median 21 μm^{-2} range 0–81 μm^{-2}) ($p=0.002$). After incubation with RNase, no hybridization of the probe was observed on the tissue sections (data not shown).

Relationship between the ALP labelling and the morphological aspect of the surface epithelium

No ALP-containing cell appeared in normal areas of pseudostratified surface epithelium, in contrast with the three remodelled structures of the surface epithelium, in which ALP-containing cells were observed. None of these three remodelled aspects of the surface epithelium was systematically associated with the presence of ALP-containing cells: some areas exhibited ALP-containing cells whilst other areas did not.

Relationship between the ALP labelling and the degree of inflammation

No significant difference was observed in the number of intraepithelial inflammatory cells mm^{-2} between ALP-labelled areas (median 64 mm^{-2} ; range 21–115 mm^{-2}) and ALP-unlabelled areas (median 46 mm^{-2} ; range 25–79 mm^{-2}). The same result was observed when comparing the number of inflammatory cells present in the connective tissue underlying ALP-labelled areas (median 206 mm^{-2} ; range 129–941 mm^{-2} ;) and ALP-unlabelled areas (median 264 mm^{-2} ; range 135–503 mm^{-2}).

Discussion

This study clearly shows that human adult nasal surface epithelial cells can express antileucoprotease (ALP), where the epithelium has become remodelled.

Various patterns of surface epithelium were observed in the polyp samples, appearing as normal (pseudostratified) or remodelled (folding of the surface epithelium, mucous cell hyperplasia and/or basal cell hyperplasia) structures. Areas of basal cell hyperplasia and/or mucous cell hyperplasia have already been described *in vivo* and may precede squamous cell metaplasia [16], a more radical alteration of the respiratory mucosa [17, 18], which was not seen in our study. Basal cell and mucous cell hyperplasia are often considered as reversible lesions that occur as adaptive changes of the epithelium, and are thought to play a physiological role in the defence mechanisms of the respiratory mucosa [16]. Foldings of the surface epithelium might be considered as a part of a collecting duct of the glandular epithelium [19, 20]. But it is also possible that some of these structures could be associated with a remodelling process of the surface epithelium, and could represent dynamic invaginations of the surface epithelium in the connective tissue to form new glandular structures [21], as observed during embryogenesis [20, 22].

When investigating the localization of ALP in human nasal polyp sections, we observed a different distribution of this molecule according to the type of mucosa. Whenever the morphological aspect was normal, no ALP-containing cells appeared in the surface epithelium. But ALP-containing cells could be identified in areas with a remodelled morphological aspect. During embryogenesis, some serous cells [23], and ALP-containing cells [24], have been identified in human foetal surface epithelium. Our results show the presence of ALP in some adult surface epithelial cells, suggesting that foetal characteristics might appear in adult respiratory cells. This hypothesis is supported by the findings of JEFFERY *et al.* [20], who have recently described similar characteristics of bronchial secretions during development and also during the pathology of bronchitis.

In order to ascertain whether human surface epithelial cells are able to synthesize ALP, we performed *in situ* hybridization of a cDNA probe on human nasal polyp tissue. ALP mRNA transcripts were identified in the surface epithelium. This observation, associated with

the immunohistochemical localization of the protein, allowed us to conclude that ALP can be expressed not only by glandular serous cells, but also by some surface epithelial cells in specific conditions.

WILLEMS *et al.* [19] have already reported the presence of ALP in some rare isolated human epithelial cells, and also in some cells of structures identified as being collecting ducts. In the nasal turbinate mucosa, LEE *et al.* [7] have recently shown that ALP was only identified in the serous submucosal glands, while no positive immunocytochemical staining was observed in the surface epithelium, including the goblet cells. Our findings are in complete agreement with the previously described localization of ALP in human nasal tissue, since we never observed ALP in normal pseudostratified surface epithelium. The originality of our study is to demonstrate the presence of ALP in areas of human nasal surface epithelial cells which have undergone remodelling.

The ability of human adult nasal surface epithelial cells to form gland-like structures in a collagen gel has been recently demonstrated *in vitro* [25]. *In vivo*, the presence of glandular cell markers in surface epithelial cells of remodelled epithelia have already been described. NIIMI *et al.* [26] demonstrated the appearance of the epithelial membrane antigen and the secretory component of immunoglobulin A (IgA) in human bronchial surface epithelial cells in cases of basal cell hyperplasia and later stages of lesions with stratification and squamous metaplasia of the surface epithelium. These authors suggested that the presence of these two glandular cell markers in remodelled surface epithelia might represent differentiation towards glandular epithelium. Recently, ROGERS *et al.* [27] demonstrated, for the first time, the presence of serous cells in adult human bronchioles, on the basis of morphological criteria. This latter study has been carried out with lung tissues which were macroscopically normal, although derived from smoker and ex-smoker patients with well-localized tumours. It is possible that the identification of serous cells in the surface epithelium of human bronchioles could also be related to the injury of the respiratory mucosa due to smoking, and might not be a normal characteristic of the human bronchiolar epithelium. The expression of an epidermal serine proteinase inhibitor, skin-derived antileucoprotease (SKALP) or elafin, has been demonstrated to be associated with a remodelled phenotype of the skin. A differential expression of this molecule was observed in various epidermal tumours whilst no SKALP has been observed in normal human epidermis [28]. All of these observations support the hypothesis that human surface epithelia might be able to express glandular characteristics, in association with a remodelling process of the surface epithelium.

According to NORLANDER *et al.* [29], the polyp formation appears to be the result of a continuous inflammatory reaction. During inflammatory processes, neutrophil elastase is actively secreted, and this molecule is, to this day, the only molecule thought to be capable of modulating the expression of ALP [30]. The role of neutrophil elastase is to assist in the elimination of foreign

molecules or micro-organisms, but it can also indiscriminately damage the respiratory tissue, and the major physiological role of ALP is the inactivation of neutrophil elastase [4]. ABBINANTE-NISSEN *et al.* [30] demonstrated that neutrophil elastase itself is able to increase by 5–20 fold the levels of ALP mRNA transcripts in airway epithelial cells in culture. Inflammatory stimuli have been demonstrated to stimulate the ALP gene expression by a human bronchial squamous carcinoma cell line [31]. We therefore investigated the possibility that ALP labelling in the surface epithelium could be associated with a local increased degree of inflammation. The stimulation of the expression of ALP by surface epithelial cells does not appear to be associated with an increased number of inflammatory cells in the ALP-positive areas. Nevertheless, it is possible that higher concentrations of elastase might be present in these areas, and we now intend to study *in vitro* the effect of human neutrophil elastase on the expression of ALP by human nasal surface epithelial cells.

Our study, demonstrating the capacity of nasal surface epithelial cells to express ALP, highlights the involvement of the surface epithelium in the preliminary defence mechanisms of the respiratory epithelium. The expression of ALP in remodelled surface respiratory epithelium means that this protein should no longer be considered a specific glandular secretory marker. During epithelial remodelling, it could reinforce the antiprotease screen of the surface epithelium; thereby, protecting both the epithelium and underlying tissues.

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