Inflamm. res. 53 (2004) 230–238
1023-3830/04/060230-09
DOI 10.1007/s00011-003-1240-x

Inflammation Research

Human neutrophil defensins and secretory leukocyte proteinase inhibitor in squamous metaplastic epithelium of bronchial airways

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Received 25 September 2003; returned for revision 3 November 2003, accepted by W. van den Berg 22 December 2003

Abstract. *Objective:* The aim of this study was to analyze a possible contribution of human neutrophil defensins and secretory leukocyte proteinase inhibitor (SLPI) to the induction of airway epithelial changes such as squamous cell metaplasia.
Material and methods: The presence of these molecules and the number of proliferating (Ki-67-positive) epithelial cells was analyzed by immunohistochemistry in bronchial epithelium from subjects with (n = 15) or without (n = 14) chronic obstructive pulmonary disease (COPD).
Results: Our data demonstrate higher numbers of defensin-positive ($p = 0.0001$), elastase-positive ($p = 0.0001$) and Ki-67-positive ($p = 0.0001$) cells in areas with squamous cell metaplasia as compared to areas with intact or damaged epithelium, while the reverse was observed for SLPI expression ($p = 0.002$). No differences were observed between subjects with or without COPD, nor between current smokers and those that had stopped smoking.
Conclusions: These data are in line with a role of defensins in the hyperproliferative phenotype of squamous metaplastic lesions in the airways. This role does not seem to be restricted to patients with COPD.

Key words: Squamous metaplasia – Airway epithelium – Antimicrobial peptides – Proliferation – Airways obstruction

Introduction

Epithelial changes such as squamous metaplasia are frequently observed in smokers, and may be more pronounced in smokers with chronic obstructive pulmonary disease (COPD) [1]. In addition, squamous metaplasia may be an early, potentially reversible, stage in the development of lung cancer [2]. These epithelial changes may be caused directly by cigarette smoke or indirectly by the inflammatory response induced by smoking [3, 4]. This inflammatory process may persist even after smoking cessation for a prolonged time [5]. It is well recognized that chronic airway inflammation plays a major role in the pathogenesis of COPD. Various studies have demonstrated increased numbers of macrophages [6–8], CD8+ T-cells [8, 9] and mast cells [6] in lung tissue of chronic bronchitis and COPD patients. In addition, increased neutrophil numbers were observed in induced sputum and bronchoalveolar lavage (BAL) fluids of these patients [10, 11]. In smokers, increased sputum neutrophil numbers were demonstrated to be associated with a decline in FEV_1 over time [11, 12], suggesting a role for neutrophils in the progression of COPD.

Recent studies have suggested a role for neutrophil defensins and secretory leukocyte proteinase inhibitor (SLPI) in epithelial proliferation and repair processes. The non-enzymatic polypeptides human neutrophil defensins (also designated human neutrophil peptides 1–4 [HNP1-4]) are highly cationic members of the α-defensin subfamily and have an important role in the first line of defense against a broad spectrum of micro-organisms. Neutrophil defensins are stored in the neutrophil's azurophilic granules in high concentrations, and are released from the cell after stimulation. In addition to their antimicrobial activity, defensins have been shown to modulate inflammatory and immunological processes, including complement activation, cytotoxicity, chemotaxis of immature dendritic cells, T cells and monocytes, and induction of epithelial cytokine release (reviewed in [13]). Recent studies demonstrated that defensins increase epithelial proliferation and wound repair [14–16]. Although the functional role of neutrophil defensins has been extensively studied in vitro, little is known about their expression in human airways.

SLPI is a potent inhibitor of serine proteinases such as elastase, and displays antimicrobial properties [17]. In the

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airways SLPI is expressed in goblet and Clara cells of the surface epithelium, and in serous cells of the bronchial glands [18, 19]. Very recent studies indicate a role for SLPI in epithelial cell proliferation and repair. Ashcroft et al. [20] demonstrated that *slpi* knock out mice display a delay in wound healing of the skin, indicating that SLPI may have an essential role in wound repair. In addition, other studies have demonstrated that SLPI may enhance proliferation of lung carcinoma cells [21] and endometrial cells [22]. Zhu et al. [23] have demonstrated that SLPI may promote wound healing by preventing elastase-induced conversion of the growth promoting pro-epithelins into the growth inhibiting epithelins. Since neutrophil defensins increase epithelial expression of SLPI [24], these mediators may act in concert to regulate repair and proliferation.

The aforementioned data indicate that epithelial changes are one of the characteristics of smoking-induced lung disease, not only in COPD but also in i.e. chronic bronchitis without airflow obstruction. Based on in vitro and animal studies, neutrophil defensins and SLPI may play a role in these changes. Therefore the aim of the present study was to quantify the presence of neutrophil defensins and SLPI in intact, damaged and metaplastic areas of airway epithelium and the underlying submucosa of smokers and ex-smokers with or without COPD. In addition, the number of elastase-positive and the number of proliferating epithelial (Ki-67-positive) cells was assessed. Taking into consideration the possible role of neutrophil defensins and SLPI in airway epithelial cell proliferation, we further correlated our findings with the number of proliferating cells in the epithelium.

Material and methods

Subjects

Bronchial tissue used in this study was obtained from patients that underwent a lobectomy or pneumectomy for lung cancer at the Leiden University Medical Center. Anonymized tissue from 15 subjects with COPD and 14 non-COPD subjects were selected using criteria previously described [6]. Briefly, tissue was included in the COPD group when: FEV_1 and FEV_1/FVC ≤ 75% (% predicted), a reversibility in FEV_1 after inhalation of 400 µg salbutamol ≤ 12% of baseline. Non-COPD subjects had an FEV_1 and FEV_1/FVC ≥ 85% (% predicted) and a CO diffusion constant (K_{CO}) ≥ 80% (% predicted). All subjects had a total lung capacity (TLC) ≥ 80% (% predicted), did not suffer from obstruction due to tumors in the central airways and did not have diffuse inflammation. Subjects in the COPD and non-COPD groups were divided in a subgroup of current smokers (COPD n = 7; non-COPD n = 7) and non/ex-smokers (COPD n = 8; non-COPD n = 7). Subjects were considered ex-smokers after cessation of smoking for at least one year before surgery.

Immunohistochemistry

From the selected subjects a formalin-fixed, paraffin-embedded bronchial ring, free of tumor as judged by haematoxylin and eosine (H&E) staining, was selected. For immunohistochemical analysis of HNP1-3, neutrophil elastase, SLPI and the proliferation marker Ki-67, consecutive sections of 4 µm thickness were cut and placed on 3-amino propyltriethoxysilane-coated slides. The sections were then dewaxed in xylene, rehydrated in descending concentrations of ethanol and subsequently endogenous peroxidase activity was blocked by incubation with 0.3% (v/v) H_2O_2 in methanol for 20 min. Slides used for staining of HNP1-3, SLPI or Ki-67 were pretreated for antigen retrieval by boiling in a microwave oven (650 W) for 10 min in a 0.01 M citrate buffer pH 6.0. No antigen retrieval procedure was required for the detection of elastase. The slides were subsequently incubated with mouse monoclonal antibodies against HNP1-3 (clone D1-1, kind gift from Tomas Ganz, UCLA, Los Angeles, CA), SLPI (clone 31, described in [18]), neutrophil elastase (clone NP57, Dako, Glostrup, Denmark) and Ki-67 (clone MIB-1, Immunotech, Marseille, France). Immunoreactivity was detected using standard peroxidase-conjugated strepavidin-biotin complex (sABC) methods and 3-amino-9-ethyl carbazole as a chromogen. Sections from the subjects were stained simultaneously for each marker.

Image analysis

For the analysis of the stainings, digitized images were acquired using a 3-chip color camera. Tissue sections were divided in areas with intact, damaged and metaplastic epithelium. A maximum of 3 randomly selected images from the airway epithelium and the corresponding lamina propria from each of these areas was acquired at a 200× magnification. An epithelial layer was histologically defined intact when the layer consisted of a cell layer of basal and parabasal cells below a superficial cell layer of ciliated cells and damaged when only a layer of basal and/or parabasal cells was present. Squamous cell metaplasia was defined as pseudostratified multilayered epithelium consisting of polygonal cells covered by a flattened layer of squamous cells and an absence of ciliated cells. The lamina propria was defined as the area within 125 µm depth from the epithelial basement membrane (BM). Large vessels, bronchial glands, and muscle tissue were excluded by delineating these structures before analysis. The images were analyzed by interactive counting or by fully automated measurement [25] using the Zeiss Vision KS400 image analysis system (Carl Zeiss, Göttingen, Germany). HNP1-3- and elastase-positive cells were counted in both the epithelial layer and the corresponding lamina propria by interactive measurements or fully automated measurements, respectively, and were expressed as cell counts per 0.1 mm BM length. HNP1-3 stainings were analyzed by interactive measurements, since these showed extracellular matrix staining in a few patients resulting in falls-positive counts when analyzed fully automated. The fully automated measurement module was also used to count the number of Ki-67 cell per 0.1 mm BM length in the epithelial layer. Finally, expression of SLPI in the epithelial layer was determined using a fully automated densitometry measurement module [25], and was expressed as mean density (MD). The (automated) measurement module used in this study has been previously described by us, and was found to be highly reproducible [25]. The mean length of BM analyzed (mean ± SEM) was 1407 ± 28 µm in intact areas, 1296 ± 31 µm in damaged areas, and 1263 ± 45 µm in metaplastic areas. The mean area of the selected epithelium was 78538 ± 1991 μm^2 in intact areas, 19439 ± 755 μm^2 in damaged areas, and 82760 ± 4967 μm^2 in metaplastic areas.

Statistical analysis

Data of cell counts and densitometry obtained from all stainings were log transformed before statistical analysis, since data were not normally distributed. For comparison of HNP1-3, elastase, SLPI, Ki-67 and lung function data in the subgroups, the Student's t-test for unpaired samples was used. Analysis of correlations between the different parameters was assessed using Pearson's correlation coefficient (r). Differences and correlations were considered significant when $p \leq 0.05$.

Results

HNP1-3, SLPI, elastase and Ki-67 in bronchial tissue

HNP1-3 and elastase immunoreactivity was detected in inflammatory cells, mainly neutrophils as judged by morphology (Fig. 1 A and 1 B). Positive cells were present both in

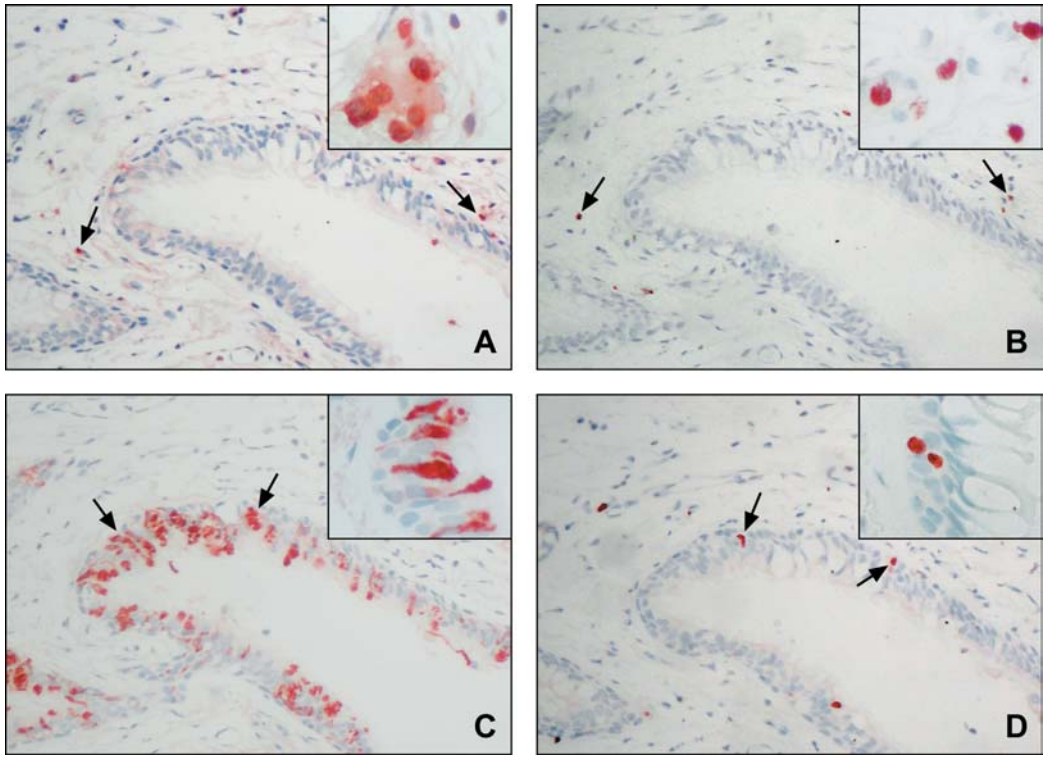

Fig. 1. Microphotographs of immunohistochemical stainings showing representative areas of intact epithelium and underlying submucosa in bronchial airways. Consecutive sections were stained for HNP1-3 (**A**), neutrophil elastase (NE, **B**), SLPI (**C**) and Ki-67 (**D**). Arrows indicate immunoreactive cells; expression of elastase and defensins is shown in inflammatory cells (neutrophils) mainly in the lamina propria. SLPI expression appears to be restricted to goblet cells and Ki-67 expression is shown in the nuclei of basal epithelial cells. Original magnification 200x. Inserted images indicate corresponding positive cells and areas at higher magnification (original magnification: 400×).

the lamina propria, as well as in the epithelial layer. HNP1-3 showed a weak staining of the extracellular matrix in a few subjects. Expression of SLPI was observed in the epithelial layer, particularly in goblet cells and in bronchial gland cells (Fig. 1C). In areas where the epithelial layer was damaged, (para)basal cells were positive in a few subjects. Proliferating cells were detected by staining for the marker Ki-67 (Fig. 1D). In the epithelial layer, Ki-67 positive nuclear staining was predominantly observed in the basal cells.

Expression in areas with intact, damaged, and metaplastic epithelium

The inflammatory response and number of proliferating cells may differ in areas with an epithelial layer that has an intact, damaged or metaplastic phenotype. Therefore, in our analysis a distinction was made between these areas. No difference was observed in the amount of metaplastic and damaged epithelium (% basement membrane length) between COPD and non-COPD subjects, nor between active smokers and ex/non-smokers (data not shown). Epithelial metaplasia was present in 5/15 COPD and 5/14 non-COPD subjects and damaged epithelium in 8/15 COPD and 11/14 non-COPD subjects. In the sections of 5/10 subjects with metaplastic epithelium, also damaged epithelium was present. However, the damaged epithelium of these subjects was in all cases in areas surrounded by intact epithelium at a distance from the metaplastic areas.

Comparison of stainings in intact, damaged and metaplastic epithelium showed a higher number of HNP1-3-positive cells in the epithelial layer (Fig. 2A) of areas with epithelial metaplasia as compared to areas with intact and damaged epithelium, while in the underlying lamina propria a near significant increase was observed (data not shown, $p = 0.077$). Similarly, the number of elastase-positive cells was higher in the metaplastic epithelium (Fig. 2C) and in the underlying lamina propria (data not shown, $p = 0.015$). In contrast, expression of SLPI was lowest in metaplastic epithelium, intermediate in damaged epithelium and highest in intact epithelium (Fig. 2B). The number of proliferating epithelial cells per length basement membrane was highest in metaplastic, intermediate in intact and lowest in damaged epithelium (Fig. 2D). Because squamous metaplasia was only observed in tissue from a subset of patients, we considered the possibility that these parameters were also different in intact epithelium from these subjects when compared to intact epithelium from subjects without apparent metaplastic changes. This possibility was excluded because no differences were observed. However, when intact and metaplastic epithelium within a subject was compared using a paired t-test, higher numbers of HNP1-3- ($p = 0.001$), elastase- ($p = 0.002$) and Ki-67-positive ($p = 0.0001$) cells were observed in metaplastic lesions, while SLPI expression was lower ($p = 0.0004$). These data indicate that the observed differences are related to the phenotype of the epithelium rather than to patient characteristics.

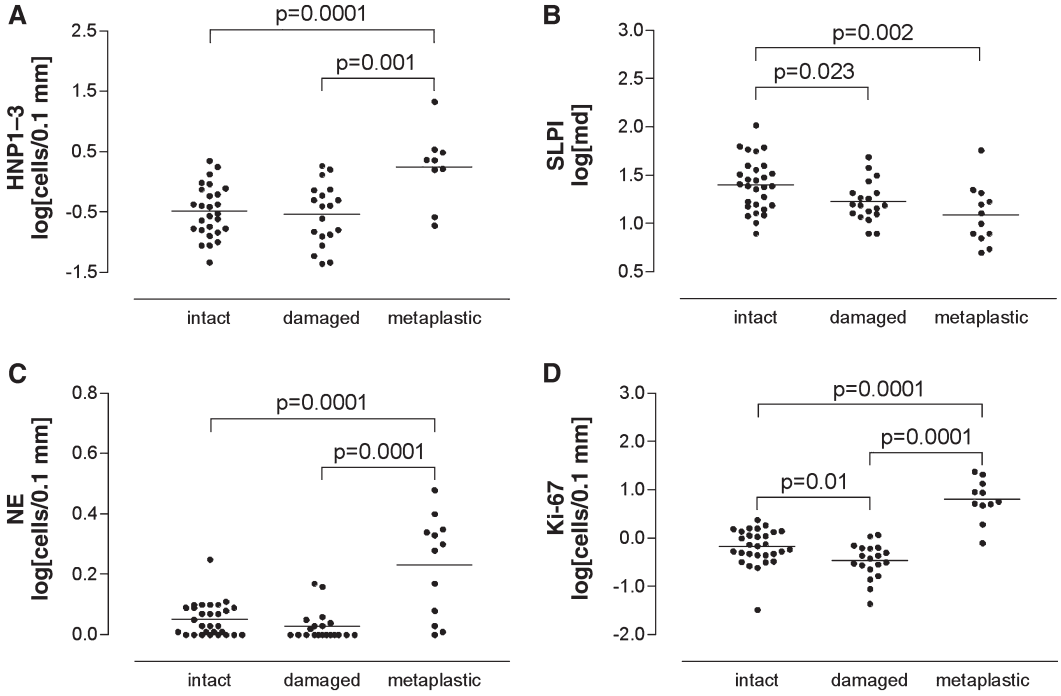

Fig. 2. Quantitative analysis of HNP1-3, SLPI, elastase (NE) and Ki-67 in areas of the large airways with intact, damaged and metaplastic epithelium. Scores of HNP1-3 (**A**), NE (**C**) and Ki-67 (**D**) are expressed as the ^{10}log value of the number of cells in the epithelial layer per 0.1 mm basement membrane length. SLPI is expressed as ^{10}log value of the mean density (md) of the staining in the epithelial layer (**B**). Mean of the scores are indicated by horizontal lines.

HNP1-3, elastase, SLPI and Ki-67 in areas with intact epithelium

Expression of HNP1-3, elastase, SLPI and Ki-67 in areas with intact epithelium was compared in current and non/ex-smokers with or without COPD (Table 2). No difference in any of these parameters was observed when comparing COPD and non-COPD subjects (irrespective of their smoking status), nor between smokers and non/ex-smoker (irrespective of the disease status). Subgroup analysis revealed that the number of HNP1-3-positive cells in the lamina propria of current smoking non-COPD subjects was higher than in current smoking COPD patients.

HNP1-3, elastase, SLPI and Ki-67 in areas with damaged epithelium

Also in areas with damaged epithelium no statistically significance differences in any of the parameters were observed between COPD and non-COPD subjects or between current smokers and non/ex-smokers (Table 3). Expression of SLPI, however, was lower in current smoking COPD patients as compared with current smoking non-COPD subjects, while other parameters did not show any difference in a subgroup analysis.

Subgroup analysis in areas with metaplastic epithelium could not be performed, as the number of subjects in these groups was too small.

Correlation between presence of HNP1-3, elastase, SLPI and Ki-67

Elastase and HNP1-3 are both produced by neutrophils and are released from the azurophilic granules upon stimulation. In addition, HNP1-3 expression has also been described in a subset of T cells [26] and NK cells [27]. Analysis of numbers of NE and HNP1-3 positive cells in the lamina propria adjacent to areas with intact, damaged, and metaplastic epithelium demonstrated a marked significant association between expression of HNP1-3 and elastase, whereas the correlation in areas with intact epithelium was significant but weak (Fig. 3).

Various studies have proposed a role for neutrophil defensins and SLPI in cell proliferation. Therefore we analyzed whether the number of HNP1-3-positive cells and the SLPI expression correlates with the number of Ki-67-positive cells in the epithelial layer. In intact and damaged epithelial layers no correlation was observed between the number of HNP1-3-positive and Ki-67-positive cells. Surprisingly, in areas with metaplastic epithelium, but not in those with intact or damaged epithelium, the number of HNP1-3-positive cells in both the epithelial layer (Fig. 4A) as well as the underlying lamina propria (Fig. 4B) showed a negative correlation with the number of Ki-67-positive cells. SLPI expression, however, did not correlate with the number of Ki-67-positive cells in any of the analyzed areas.

Finally, we also determined whether the analyzed parameters correlated with airflow obstruction. The number of elastase-positive cells and FEV_1/FVC showed a positive correlation in both the epithelial layer ($r = 0.612$, $p = 0.034$) as well as the underlying lamina propria ($r = 0.617$, $r = 0.032$) in areas

Table 1. Subject characteristics.

Sex	Age (years)	FEV_1	FEV_1/FVC	TLC	RV	K_{CO}	py	smoking status
Non-COPD								
M	57	102	99	112	134	83	29	cur
F	74	91	91	107	125	83	50	cur
M	46	109	104	97	87	88	23	cur
M	58	96	92	110	121	90	70	cur
M	58	94	90	95	87	98	35	cur
M	80	98	104	105	122	92	65	cur
M	73	86	96	106	140	81	70	cur
M	64	93	95	110	152	90	20	ex
M	28	99	102	104	143	95	0	non
M	51	97	107	99	92	89	0	non
M	38	100	94	106	100	97	28	ex
M	52	100	100	103	121	105	20	ex
F	59	100	105	90	90	102	28	ex
M	69	110	124	100	102	110	–	ex
Mean	58 ± 3.8	98 ± 1.7	100 ± 2.4	103 ± 1.7	115 ± 6.0	93 ± 2.3	34 ± 6.6	
COPD								
M	69	75	65	110	107	49	–	cur
M	61	75	66	123	155	44	45	cur
M	53	44	70	89	137	57	32	cur
M	65	52	60	112	169	63	55	cur
M	55	56	68	99	131	73	40	cur
M	56	45	60	–	–	81	35	cur
M	46	75	74	97	97	57	–	cur
M	62	49	62	130	223	53	20	ex
M	72	69	64	115	129	40	–	ex
M	65	69	71	116	152	55	20	ex
M	57	47	53	111	170	61	55	ex
F	78	62	61	95	105	42	60	ex
M	73	45	52	114	171	75	–	ex
M	68	64	74	105	102	–	50	ex
Mean	64 ± 2.4	60 ± 3.2	65 ±1.8	109 ± 3.0	140 ± 9.6	59 ± 3.6	40 ± 4.4	
P value	0.177	0.0001	0.0001	0.132	0.040	0.0001	0.470	

FEV_1, TLC, RV and K_{CO} are indicated as the percentage of the predicted value before bronchodilation. FEV1/FVC ratio is indicated as a percentage. py: pack years; F: female, M: male; cur: current smoker.

Table 2. Subgroup analysis of HNP1-3, elastase, SLPI and Ki-67 in areas with intact epithelium in the large airways.

staining	tissue	COPD		non-COPD	
		current-smokers (n = 7)	non/ex-smokers (n = 8)	current-smokers (n = 7)	non/ex-smokers (n = 7)
HNP1-3	EP	–0.72 ± 0.24	–0.34 ± 0.14	–0.39 ± 0.1	–0.57 ± 0.18
	LP	1.19 ± 0.09*	1.41 ± 0.12**	1.52 ± 0.12	1.33 ± 0.18
elastase	EP	0.07 ± 0.03	0.04 ± 0.01	0.06 ± 0.02	0.04 ± 0.01
	LP	0.72 ± 0.16	0.79 ± 0.16	0.96 ± 0.12	0.77 ± 0.09
SLPI	EP	1.27 ± 0.11	1.52 ± 0.11	1.42 ± 0.09	1.36 ± 0.06
Ki-67	EP	–0.22 ± 0.24	–0.09 ± 0.07	–0.18 ± 0.10	–0.23 ± 0.11

Parameters determined in the lamina propria (LP) are indicated as ^{10}log value of the number of cell per 0.1 mm^2 area, and in the epithelium (EP) as ^{10}log value of the number of positive cells per 0.1 mm basement membrane length (for HNP1-3, elastase and Ki-67) or as ^{10}log value of the mean density (for SLPI). *: p = 0.041 as compared with current smokers without COPD. **: p = 0.053 as compared with current smokers with COPD.

Table 3. Subgroup analysis of HNP1-3, elastase, SLPI and Ki-67 in areas with damaged epithelium in the large airways.

staining	tissue	COPD		non-COPD	
		current-smokers (n = 5)	non/ex-smokers (n = 4)	current-smokers (n = 6)	non/ex-smokers (n = 4)
HNP1-3	EP	–0.31 ± 0.37	–0.41 ± 0.27	–0.61 ± 0.28	–0.71 ± 0.09
	LP	1.49 ± 0.15	1.38 ± 0.13	1.66 ± 0.21	1.18 ± 0.12
elastase	EP	0.04 ± 0.04	0.03 ± 0.01	0.05 ± 0.03	0.04 ± 0.01
	LP	0.78 ± 0.12	0.55 ± 0.19	0.89 ± 0.25	0.45 ± 0.09
SLPI	EP	1.05 ± 0.05*	1.30 ± 0.11	1.27 ± 0.07	1.26 ± 0.11
Ki-67	EP	–0.61 ± 0.30	–0.50 ± 0.19	–0.39 ± 0.09	–0.40 ± 0.13

Parameters determined in the lamina propria (LP) are indicated as ^{10}log value of the number of cell per 0.1 mm^2 area, and in the epithelium (EP) as ^{10}log value of the number of positive cells per 0.1 mm basement membrane length (for HNP1-3, elastase and Ki-67) or as ^{10}log value of the mean density (for SLPI). *: p = 0.043 as compared with current smokers without COPD.

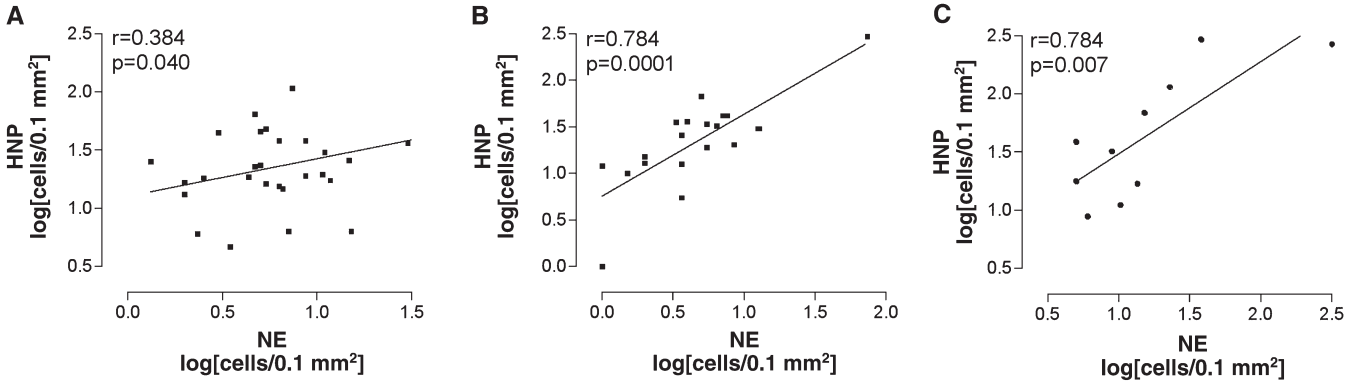

Fig. 3. Correlations between elastase-positive and HNP1-3-positive cells in the lamina propria of areas with intact (**A**), damaged, (**B**) and metaplastic (**C**) epithelium. Scores are expressed as ^{10}log value of the number of cells per 0.1 mm^2 area. Correlations were assessed by linear regression and indicated by the Pearson coefficient (r) and levels of significance (p).

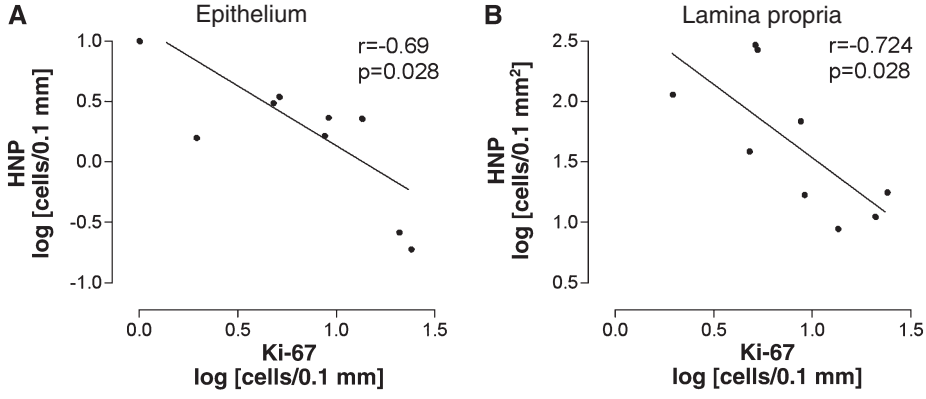

Fig. 4. Correlations between HNP1-3-positive and Ki-67-positive cells in areas with metaplastic epithelium. HNP1-3 scores in the epithelial layer (**A**) and lamina propria (**B**) are expressed as ^{10}log value of the number of cells per 0.1 mm basement membrane length and ^{10}log value of the number of cells per 0.1 mm^2 area, respectively, and Ki-67 scores in the epithelial layer as ^{10}log value of the number of cells per 0.1 mm basement membrane length. Correlations were assessed by linear regression and indicated by the Pearson coefficient (r) and levels of significance (p).

with metaplastic epithelium, while no correlation was found in other areas or with the other parameters analyzed.

Discussion

Our data demonstrate higher numbers of elastase-, HNP1-3- and Ki-67-positive cells in areas with metaplastic epithelium as compared to areas with intact or damaged epithelium, while the reverse is observed for SLPI expression. Subgroup analysis of current and non/ex-smokers with or without COPD demonstrated that the number of neutrophils (elastase-positive cells) measured in areas with intact or damaged epithelium is not significantly different between the groups, while the number of HNP1-3-positive cells in intact areas was higher in smoking non-COPD subjects as compared with smoking COPD patients. The number of HNP1-3-positive cells in intact and damaged areas did not correlate with epithelial cell proliferation, while in metaplastic areas an inverse correlation was observed. In addition, expression of SLPI did not correlate with epithelial cell proliferation or with the number of HNP1-3-positive cells.

Prolonged smoking and the inflammatory process resulting from smoking are important factors that contribute to epithelial injury and subsequent changes. We therefore analyzed intact epithelium, as well as damaged and squamous metaplastic epithelium separately. No differences were observed in HNP1-3- and elastase-positive cells between intact and damaged epithelium, whereas the difference in SLPI- and Ki67-positive cells was small. We cannot exclude that this is in part the result of the selective loss of e. g. infiltrating cells and SLPI producing epithelial cells at sites of epithelial injury that might have obscured potential differences between intact and damaged epithelium. Squamous metaplasia that is frequently observed in smokers, may result from an inadequate repair response following chronic injury. Our data confirm other studies demonstrating that metaplastic epithelium shows higher numbers of proliferating cells than the surrounding non-metaplastic epithelium (reviewed in [28]). A novel finding of the present study is that areas with squamous cell metaplastia are associated with increased numbers of neutrophils. This finding is in line with a mitogenic effect of defensins on epithelial cells [15]. However, when we analyzed the relationship between defensin-positive cells and proliferating cells within the areas of squamous metaplasia, a negative relationship was observed. A possible explanation for this finding is the fact that the mitogenic effect of neutrophil defensins is critically dependent on the local concentration of defensins: defensins increase epithelial proliferation in vitro only at low concentrations, whereas at higher concentrations proliferation is inhibited [15]. This may be relevant in vivo, since various studies have demonstrated levels of neutrophil defensins in lung secretions of patients with inflammatory lung diseases that may be sufficient to potentially cause growth inhibition (reviewed in [13]). We can speculate that, at sites of chronic neutrophilic inflammation (such as that induced by cigarette smoking), proliferation and full repair of the epithelium will be hampered, thus perpetuating the injury cycle.

Whereas there is a negative correlation between defensin-positive cells and Ki-67 positive cells in areas of squamous metaplasia, no such correlation exists between neutrophil elastase-positive and Ki67-positive cells. How do we explain these observations? First, it needs to be noted that the correlation between defensin-positive and elastase-positive cells in the lamina propria is significant but not strong, whereas these parameters did not correlate in the epithelium. Secondly, while both elastase and neutrophil defensins are mainly expressed in neutrophils, these proteins are not exclusively expressed in these cells but also in other cell types. In addition, within the neutrophil, elastase and defensins are stored in different subsets of azurophilic granules [29], and therefore their storage and release may be subject to differential regulation. These considerations may explain the different correlation between Ki-67-positive cells, and elastase- or defensin-positive cells.

Various studies have shown that neutrophil numbers in bronchial tissue are not significantly different when comparing COPD patients with smoking subjects showing no airway obstruction [30], although tissue neutrophilia is associated with exacerbations of COPD [31]. Our results are in line with these findings. It has to be noted that although neutrophils do not appear to predominate in bronchial tissue of stable COPD patients, an increase of these cells in the lumen (bronchoalveolar lavage and induced sputum) of these patients has been described [10]. This intraluminal increase appears to be associated with severity of the disease, as it was shown to be correlated with the degree of airflow limitation [11]. When comparing smokers to never-smokers, Hunninghake et al. [32] demonstrated that neutrophil numbers in BAL fluid and open lung biopsies are significantly higher in smokers. In our study we did not find a difference between actively smoking subjects and those that had stopped smoking for at least a year. These data are in line with other studies indicating that airways inflammation may persist despite smoking cessation [5, 10]. This suggests that cigarette smoking triggers long-term effects on the inflammatory process in the lungs.

Van Wetering et al. [24] demonstrated that neutrophil defensins induce expression of the serine protease inhibitor SLPI in airway epithelial cells. Besides its protective activity against tissue degradation by serine proteinases such as elastase, SLPI may also enhance wound healing [20, 22, 23]. In the present study we observed that SLPI expression is lower in areas with a metaplastic phenotype, where the proliferation index is high, possibly as a result of loss of goblet cells in these areas. It has to be realized that we have analyzed the presence of defensins and SLPI in areas where the metaplastic process is established. Therefore higher numbers of defensin-positive cells and lower SLPI expression may well be a consequence of the metaplastic changes rather than a cause. It has been described that SLPI expression is increased in non-small cell carcinomas (including squamous cell carcinoma) and in plasma from these patients [33]. Our observation that SLPI expression is lowest in squamous metaplastic epithelium, suggests that SLPI is not involved in the early stages of the development of squamous cell carcinomas, and that its expression may be enhanced after transformation of squamous epithelial cells. We also observed that the percentage of area staining positive for SLPI in the squamous metaplastic epithelium is lower (an indirect measure for the number of SLPI-positive cells; data not shown). We therefore cannot formally exclude the possibility that SLPI production

by a limited number of cells plays a role in the development of squamous metaplastic lesions.

What could be the consequence of the low expression of SLPI in squamous metaplastic lesions? Inflammatory processes may increase local expression of SLPI, as demonstrated by the association of an increased number of SLPI expressing epithelial cells in the airways with small airways disease and destruction of alveolar attachments, pathological changes that are observed in patients with COPD [34]. Low expression of SLPI may, however, predispose to the development of inflammatory lung disease. This is based on the observation that low levels of SLPI in the airway secretions are associated with frequent COPD exacerbations [35] and with an increased risk of neonatal chronic lung disease in preterm ventilated infants [36]. The lower expression of SLPI possibly makes epithelial cells more susceptible to injury by proteases such as elastase, thereby perpetuating the cycle of damage and (altered) repair leading to metaplastic changes in COPD patients. Because of the antimicrobial activity of SLPI [17], decreased local expression may also impair the local antimicrobial screen. On the other hand, low levels of SLPI may also have a protective effect against selected infectious micro-organisms. Previous studies have demonstrated that SLPI enhances adherence of *Haemophilus influenzae*, a common respiratory pathogen in chronic bronchitis and COPD patients, by inhibiting autoproteolysis of the *Haemophilus influenzae* adherence protein Hap and thereby facilitating bacterial adherence to the epithelial layer and extracellular matrix [37, 38]. Thus reduced SLPI expression may have a protective effect against selected respiratory pathogens in areas that are susceptible to bacterial colonization.

In conclusion, we have demonstrated that squamous metaplasia, characterized by high proliferative activity, is associated with increased numbers of defensin-positive cells and lower expression of SLPI. This suggests a possible role for defensins in the hyperproliferative phenotype of squamous metaplastic lesions in the airways, a role that appears to be independent of the development of chronic airflow limitation.

Acknowledgements. This study was supported by grants from the Netherlands Asthma Foundation (grants 97.55 and 98.12) and the Netherlands Organization for Scientific Research (NWO; grant 902-11-092). The authors thank Tomas Ganz (UCLA, Los Angeles, CA) for the generous gift of monoclonal antibodies directed against neutrophil defensins.

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