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 FEV1 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
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: FEV1 and FEV1/FVC ≥ 75% (% predicted), a reversibility in FEV1 after inhalation of 400 µg salbutamol ≤ 12% of baseline. Non-COPD subjects had an FEV1 and FEV1/FVC ≥ 85% (% predicted) and a CO diffusion constant (KCO) ≥ 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-aminopropyltriethoxysilane-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) H2O2 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-amin-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 a 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 ± 1911 µm2 in intact areas, 19439 ± 755 µm2 in damaged areas, and 82760 ± 4967 µm2 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 ≤ 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. 1A and 1B). Positive cells were present both in