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ORIGINAL ARTICLE

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# Proliferation and number of Clara cell 10-kDa protein (CC10)-reactive epithelial cells and basal cells in normal, hyperplastic and metaplastic bronchial mucosa

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**Abstract** Clara cell 10-kDa protein (CC10) is an inhibitor of phospholipase $A_2$ and binds to phosphatidylinositol. It may therefore interfere with intracellular signal transduction. Bronchial CC10-reactive cells have been described by several authors. In contrast to the bronchiolar CC10-containing Clara cell, which is a progenitor cell of terminally differentiated airway epithelium, the role of bronchial CC10-reactive cells remains to be elucidated. We assessed the number of bronchial CC10-reactive cells in relation to cytokeratin (CK) expression and proliferative activity in normal, hyperplastic and squamous metaplastic epithelium. Sixty-five human bronchial mucosal specimens were investigated immunohistochemically for CK expression (CK7, CK13 and CK5/6), proliferative activity (MIB-1) and number of CC10-reactive epithelia. The proliferation fraction of CC10-reactive cells was assessed with double staining for MIB-1 and CC10. The proliferation index of the epithelium differed significantly between normal, hyperplastic and metaplastic epithelium. The number of CC10-reactive cells was inversely related to the epithelial proliferation. Bronchial CC10-reactive cells showed no proliferative activity as assessed using immunohistochemical double staining for CC10 and MIB-1. In contrast to normal and hyperplastic epithelium, squamous metaplasia disclosed CK5/6 in all epithelial layers, a loss of CK7 and a gain of CK13. We conclude that CC10-reactive cells have no progenitor role in the bronchial mucosa. However, because the proliferative activity is inversely related to the number of CC10-reactive cells, the CC10 protein may play a role in the regulation of epithelial repair. Squamous metaplasia most likely originates from basal cells.

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## Introduction

In acute and chronic airway disease, epithelial damage and subsequent remodeling induce epithelial hyperplasia and metaplasia [10, 17, 18, 33]. While mucinous hyperplasia alters the quantity and quality of mucous secretion [16, 18, 28], squamous metaplasia reduces mucociliary clearing, causing further aggravation of the disease process due to secrete stasis and bacterial superinfection. Furthermore, squamous metaplasia has been attributed as a precursor lesion of infiltrating squamous cell carcinoma [1, 15]. Thus, epithelial remodeling plays a crucial role in the development and progression of chronic airway disease and neoplasia. The mechanisms leading to normal or alternative defective repair of the airway epithelium are mainly determined by the proliferative capability and ability to differentiate of the progenitor cells of the bronchial mucosa.

The basal cell has been considered to constitute one type of progenitor cell in the human airway epithelium [8, 9]. The Clara cell has been claimed to be the progenitor of the terminally differentiated bronchiolar epithelium [2, 9, 19]. Besides its progenitor role, the Clara cell contains Clara cell 10-kDa protein (CC10), a potent inhibitor of phospholipase $A_2$ [22, 24] and phospholipase C [27], the expression of which originally has been thought to be restricted to the Clara cell. Further investigations have shown that CC10 containing non-ciliated cells can be detected throughout the human tracheobronchial tree [13, 21, 26]. The role of these non-ciliated CC10-reactive bronchial epithelial cells, which are by definition not Clara cells, is so far unknown and requires elucidation.

The present study, therefore, addresses the question as to what extent the two aforementioned cell types – basal cells and bronchial CC10-reactive epithelia – contribute to the development of hyperplasia and metaplasia in the

human bronchial mucosa, i.e., as to whether certain types of hyperplasia or metaplasia can be related to these progenitor cells, because it is still debatable from which cells metaplastic epithelium is derived [19, 23]. Furthermore, we investigated whether the CC10-reactive bronchial cell constitutes a progenitor cell of the bronchial mucosa. In the present study, based on human bronchial mucosal biopsies, we performed an immunohistochemical analysis of differentiation, assessed by means of cytokeratin (CK) expression, and proliferation, recognized by means of a generally accepted proliferation marker, in order to gain more insight into the aforementioned questions.

## Materials and methods

The present study comprises a total of 50 bronchial mucosal biopsies obtained during bronchoscopy from lobar and segmental bronchi of 32 patients who ranged in age from 35 years to 81 years and 21 of whom were females. Biopsies were taken for diagnostic purposes because of hemoptysis, cough lasting for more than 4 weeks or radiographically detected pulmonary infiltrates. We additionally investigated 15 bronchial resection margins obtained from patients who underwent lobar resection due to lung cancer.

Following biopsy or resection, tissues were immediately fixed in 4% formaldehyde. After fixation, the tissues were embedded in paraffin, cut at a thickness of about 5 µm and routinely stained with hematoxylin and eosin (H&E) and periodic acid–Schiff (PAS). The specimens were categorized into four groups. "Normal epithelium" showed no stratification of the columnar epithelial cells, and the basal cells were arranged in a single layer. Increased stratification of the basal cell layer to more than or equal to four layers was classified as "basal cell hyperplasia", and complete replacement of the epithelium by squamous cells was diagnosed as "squamous metaplasia". "Mucinous hyperplasia" was stated when more than 25% of the epithelial cells were PAS-positive goblet cells. Specimens featuring severe dysplasia or carcinoma in situ were excluded from the present study.

### Immunohistochemistry

Immunohistochemistry was performed using a standard avidin–biotin complex (ABC)-peroxidase method using 3,3'-diaminobenzidine (DAB) as a chromogen. Table 1 summarizes the primary antibodies applied together with the dilution of the primary antibody and type of tissue pretreatment used. Microwave pretreatment was performed by heating the deparaffinized and rehydrated sections, immersed in 10 mM sodium citrate buffer (pH 6.0), in a microwave oven at 600 W for three periods of 5 min. For some antibodies, a subsequent mild (0.001%) trypsin pretreatment was performed for 15 min at 37°C [14]. Detection of Clara cells was based on immunohistochemistry by means of a polyclonal antibody directed against human CC10 protein, the generation of which has been described in a previous publication [3].

### Simultaneous MIB-1 and CC10 labeling

Double staining was performed through subsequent immunohistochemical detection of MIB-1 and CC10. First, MIB-1 immunohistochemistry was performed as described above and followed by CC10 immunohistochemistry according to the standard ABC-alkaline phosphatase method using As-bi-Naphthol as a chromogen. This sequence was chosen because preliminary investigations showed this methodology to be superior to the reverse staining sequence [2].

### Quantitative analyses

Quantitative analyses were performed by means of interactive computer-assisted image analysis (Image Pro Plus, Ver 4.0, Media Cybernetics$^{R}$, Silver Spring, Md.) at 800× microscopic magnification. The area of one measuring field was 10,850 µm$^2$, and the number of epithelial cells within one measuring field ranged between 60 and 200. In each case, at least five measuring fields were assessed, and the arithmetic mean (±1×standard deviation) of the quantitative parameters was recorded together with the type of underlying epithelial alteration in a commercially available data base system.

The following quantitative parameters were defined and determined as described below:

1. Relative number of CK5/6$^+$ cells: i.e., the percentage of cells positive for CK5/6 within the total population of epithelial cells
2. Thickness of the CK5/6$^+$ layer: i.e., the thickness in microns of the layer of CK5/6-reactive cells
3. MIB-1 index: i.e., the percentage of epithelial cells with MIB-1-reactive nuclei within the total population of epithelial cells
4. CK5/6 proliferation fraction: i.e., the percentage of CK5/6$^+$ cells within the population of epithelial cells with MIB-1-reactive nuclei
5. Relative number of CC10-reactive cells: i.e., the percentage of CC10-reactive cells within the total population of epithelial cells
6. CC10 proliferation fraction: i.e., the percentage of CC10-reactive bronchial epithelia within the population of MIB-1-reactive epithelial cells

### Measuring process

First, on CK5/6 stained sections, CK5/6-reactive cells were counted together with the total number of epithelial cells, and the rela-

**Table 1** List of antibodies, source, dilution of primary antibody and type of tissue pretreatment; *CC10* Clara cell 10-kDa protein; *CK* cytokeratin

| Antibody (clone) | Source | Dilution | Pretreatment |
|---|---|---|---|
| MIB-1 | Dianova, Hamburg | 1:30 | Microwave |
| CC10 (rabbit antibody) | Prof. G. Suske, IMT[a], Marburg | 1:2500 | – |
| CK5/6 (D5/16B4) | Boehringer, Mannheim | 1:500 | Microwave, 0.001% trypsin |
| CK 7 (Ks 7.18) | Progen, Heidelberg | 1:500 | Microwave, 0.001% trypsin |
| CK13 (DE-K13) | Dako, Hamburg | 1:100 | Microwave |
| CK18 (Ks 18.04) | Progen, Heidelberg | – | Microwave, 0.001% trypsin |
| CK20 (Ks 20.8) | Dako, Hamburg | 1:100 | 0.1% Trypsin 15 min 37°C |

[a] Institute of Molecular Biology and Tumor Research

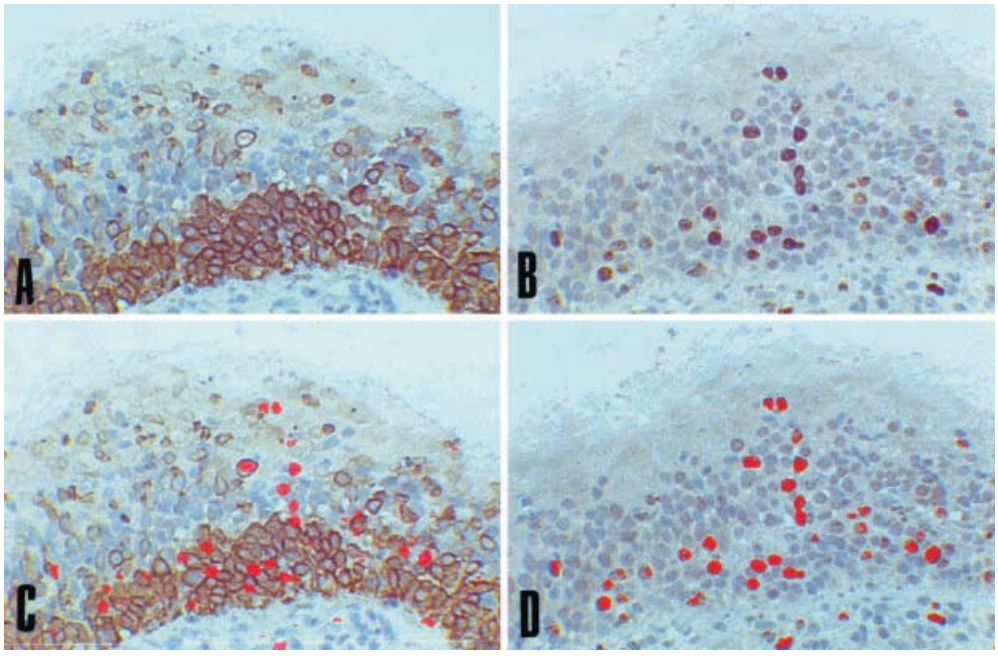


**Fig. 1** Measuring process for the assessment of the cytokeratin (CK)5/6 proliferation fraction. Two sequential step sections are stained for CK5/6 (**A**) and MIB-1 (**B**). MIB-1-reactive nuclei are detected and segmented by means of the image analyzer (*red nuclei*; **D**). The segmented image is superposed to image **A**, resulting in **C**

**Table 2** Expression of the cytokeratins (CKs) investigated in the present study. + positive CK expression; – negative CK expression

| Cytokeratin | Normal epithelium | Squamous metaplasia | Basal cell/ mucinous hyperplasia |
|---|---|---|---|
| CK5/6 | Suprabasal –[a] | Suprabasal + | Suprabasal – |
| Basal + | Basal + | Basal + | |
| CK7 | Suprabasal (columnar) + | Suprabasal – | Suprabasal + |
| Basal – | Basal – | Basal – | |
| CK13 | – | Suprabasal + | – |
| | Basal – | | |
| CK18 | + | – | + |
| CK20 | – | – | – |

[a] Few cells positive

tive number of CK5/6$^+$ cells was computed as described above. In the same microscopic field, the thickness of the layer of CK5/6$^+$ cells and the total thickness of the airway epithelium was measured perpendicularly to the basal membrane. For further analyses, the measuring field was saved as NAMECK5.tif graphic file. Thereafter, the corresponding microscopic measuring field was searched on the step section taken from the same block immunostained for MIB-1, the total number of MIB-1-reactive cells was counted and the MIB-1 index was computed. Finally, the measuring field was saved as a NAMEMIB1.tif graphic file.

In order to assess the CK5/6 proliferation fraction, the two graphic files NAMECK5.tif and NAMEMIB1.tif were superposed and aligned using the graphics overlay mode of the image analyzer system, and the number of MIB-1-reactive cells within the population of CK5/6-reactive cells was counted. The CK5/6 proliferation fraction was then computed as the percentage of cells showing both MIB-1 reactivity and CK5/6 reactivity within the population of MIB-1-reactive epithelial cells (Fig. 1).

On a third corresponding step section, immunohistochemically stained for CC10, the relative number of bronchial CC10-reactive cells was determined as described above for CK5/6$^+$ cells. Finally, sections double stained for MIB-1 and CC10 were used to assess the CC10 proliferation fraction.

### Statistical analyses

Quantitative data are expressed as arithmetic mean ±1×SD. Differences between groups were tested for statistical significance by means of one-way analysis of variance (ANOVA) and, in the case of statistical significance, a Scheffé-test was subsequently performed. A value of $P<0.05$ was defined to be a statistically significant value.

## Results

### Qualitative analyses

The results of the qualitative analysis of the CK expression are summarized in Table 2. Normal airway epithelium displayed a continuous layer of CK 5/6-reactive basal cells covering the basement membrane. The columnar epithelial cells were, except for few tall interspersed cells, negative with CK5/6 but showed a positive reaction for CK7 and CK18. Basal cells were negative with CK7. The columnar epithelium and the basal cells displayed no reactivity with CK13 or CK20.

In basal cell hyperplasia, the multi-layered basal cells showed a strongly positive reaction with CK5/6 like in normal epithelium (Fig. 2). The basal cells additionally appeared to be increased in size. Similar to normal airway epithelium, CK18 was restricted to columnar cells

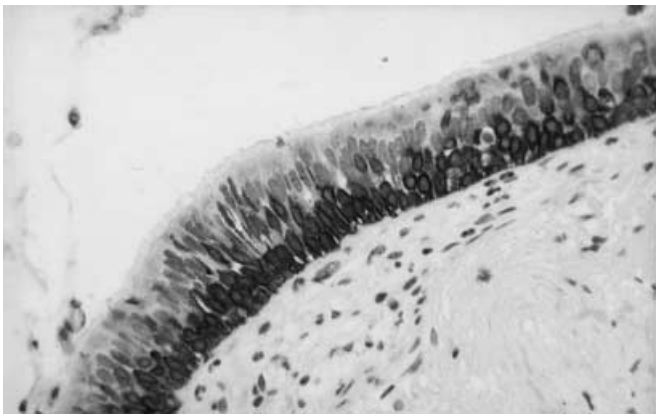

**Fig. 2** Cytokeratin (CK)5/6 immunostaining demonstrates the increased thickness of the basal cell layer (avidin–biotin complex peroxidase; microscopic magnification 400×)

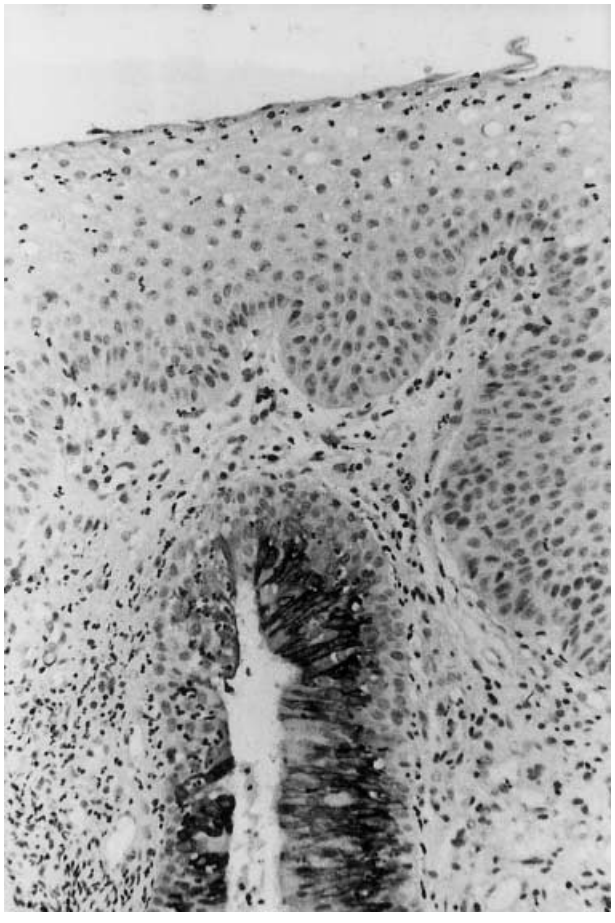

**Fig. 3** In squamous metaplasia, the epithelium is non-reactive for cytokeratin (CK)7, whereas the columnar epithelium of an excretory duct of a bronchial gland reacts positively (avidin–biotin complex peroxidase; microscopic magnification 400×)

in specimens with basal cell hyperplasia. In mucinous hyperplasia, the CK expression corresponded to the pattern observed in normal epithelium in so far as the basal cells were positive for CK5/6 and the goblet cells were

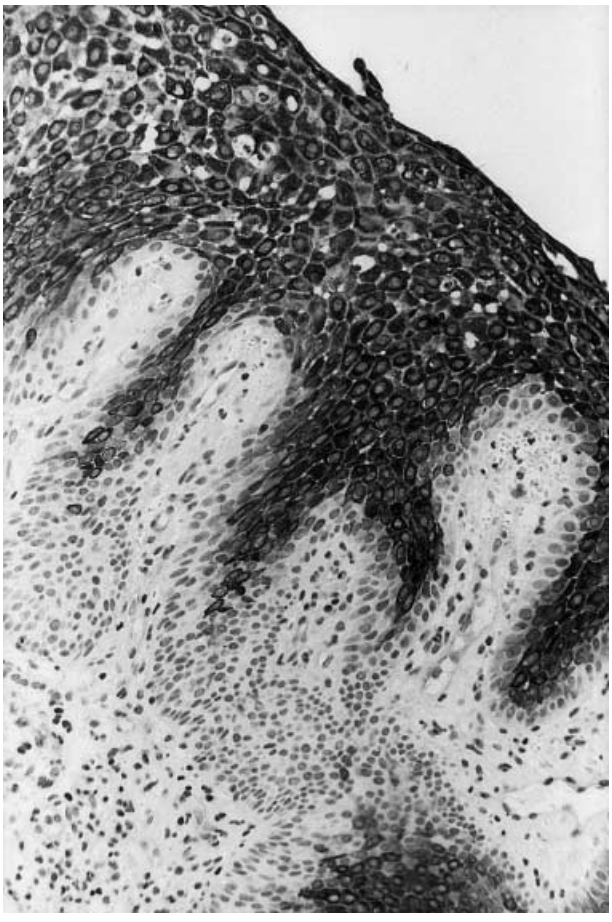

**Fig. 4** Cytokeratin (CK)13 immunohistochemistry demonstrates a strong reactivity of the suprabasal cell layers in squamous metaplasia (avidin–biotin complex peroxidase; microscopic magnification 400×)

positive for CK18. Squamous metaplasia was characterized by a loss of CK 7 (Fig. 3) and CK18 reactivity and a gain of reactivity towards CK13 (Fig. 4), which was restricted to the apical cell layers. The basal cell layer was hyperplastic and showed a positive reaction with CK 5/6. The apical cell layers also disclosed a uniform expression of CK5/6 (Fig. 5).

In the normal bronchial mucosa, CC10-reactive cells were evenly distributed throughout the epithelium. Most of the CC10-reactive cells were non-ciliated, and few of these cells showed small dome-shaped projections. In basal cell hyperplasia and mucinous hyperplasia, the CC10-reactive bronchial cells appeared to be reduced in number. Goblet cells and basal cells were negative for CC10. In squamous metaplasia, no CC10-reactive cells were observed in the epithelium.

MIB-1 immunohistochemistry showed the number of proliferating cells within the epithelium to be increased in basal cell hyperplasia (Fig. 6) and mucinous hyperplasia relative to normal mucosa. The MIB-1-reactive cells were predominantly located in the basal cell layers of the epithelium. Squamous metaplasia showed a huge number of MIB-1-reactive cells show-

**Fig. 5** Cytokeratin (CK)5/6 stains the full thickness of the epithelium in squamous metaplasia (avidin–biotin complex peroxidase; microscopic magnification 400×)

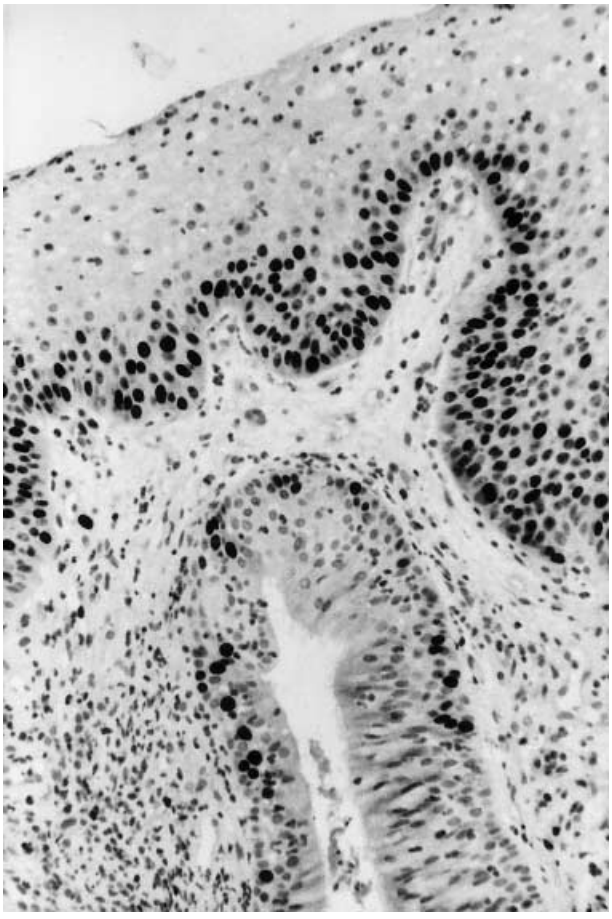

**Fig. 7** In squamous metaplasia, about one-third of predominantly basally located cells are positive for MIB-1 (avidin–biotin complex peroxidase; microscopic magnification 400×)

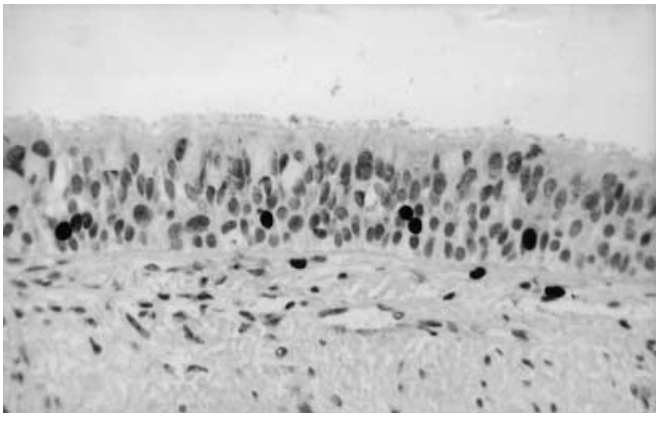

**Fig. 6** In basal cell hyperplasia, MIB-1 decorates few cells within the basal cell layers (avidin–biotin complex peroxidase; microscopic magnification 400×)

ing a similar distribution pattern relative to hyperplasia (Fig. 7).

Double staining for CC10 and MIB-1 showed an inverse relationship between cell proliferation and the number of CC10-reactive bronchial epithelia. Normal epithelium disclosed a low number of MIB-1-reactive bronchial cells and a comparably high number of CC10-reactive cells (Fig. 8), whereas in basal cell hyperplasia, the number of MIB-1-reactive cells was higher, and the number of CC10-reactive cells was reduced. In squamous metaplasia, no CC10-reactive cells were detected, and the number of MIB-1-reactive cells was higher than in the two aforementioned groups. Not a single cell with both a MIB-1-reactive nucleus and CC10-reactive cytoplasm was found in the whole population investigated (Fig. 8).

## Quantitative analyses

In the normal airway mucosa, 16.7% of the total epithelium were CC10-positive bronchial cells, and CK 5/6-reactive basal cells constituted 30.2% of the epithelial cells (Table 3). Of the epithelial cells, 1.4% (85.3% of which were basal cells) were MIB-1 reactive. A significant increase in the number of basal cells (70.7%) was observed in basal cell hyperplasia, whereas the number of CC10-reactive cells significantly decreased to a value of 4.2%. The MIB-1 index increased to about sixfold (8.3%) of

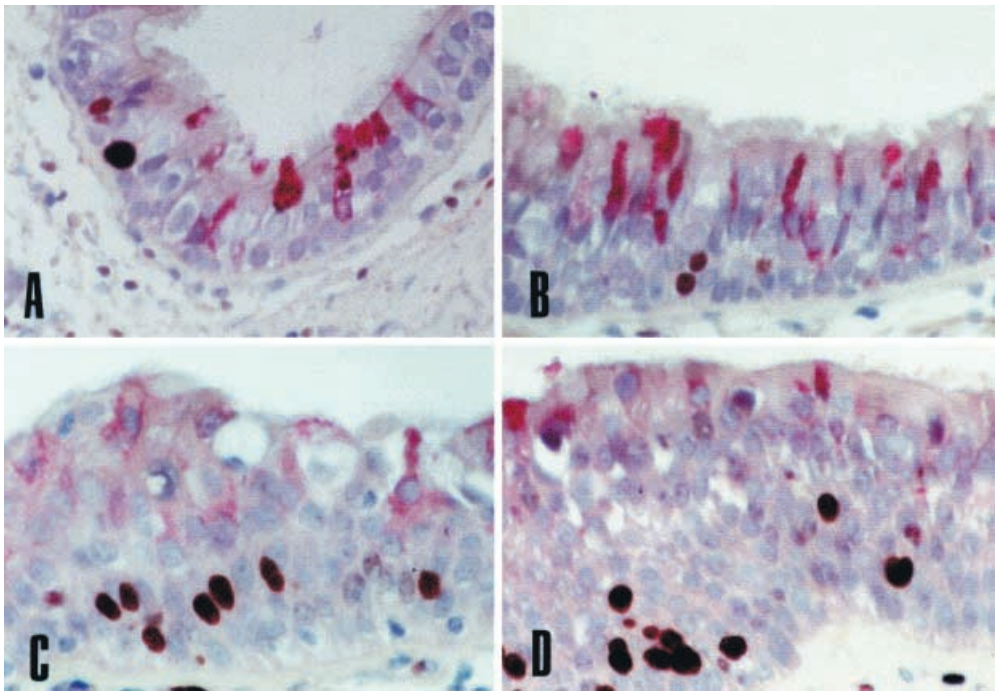


**Fig. 8** In normal bronchial mucosa (**A**), Clara cell 10-kDa protein (CC10)-reactive cells are evenly distributed throughout the epithelium. Not a single cell shows both a MIB-1-reactive nucleus and CC10-reactive cytoplasm. Relative to normal epithelium, basal cell hyperplasia discloses an increased MIB-1 index, whereas the number of CC10-reactive cells is reduced (**B**, **D**). Suprabasal goblet cell are non-reactive with CC10 (**C**)

**Table 3** Relative number of Clara cells and cytokeratin (CK)5/6-reactive cells in the airway epithelium. Data are presented as arithmetic mean ±1×standard deviation. CK5/6 proliferation fraction indicates the percentage of MIB-1-reactive CK5/6-reactive cells among the total population of MIB-1 reactive cells. The CC10 proliferation fraction is defined correspondingly. The MIB-1 index represents the percentage of MIB-1-reactive cells among the total population of epithelial cells

| Histology | *n* | CC10-reactive cells (%) | CC10 proliferation fraction (%) | CK5/6$^+$ cells (%) | CK5/6 proliferation fraction (%) | MIB-1 index (%) |
|---|---|---|---|---|---|---|
| Normal epithelium | 16 | 16.7±6.0 | 0 | 30.2±9.2 | 85.3±7.2 | 1.4±1.0 |
| Mucinous hyperplasia | 8 | 3.0±1.2[a,b] | 0 | 65.1±9.5[a,b] | 96.1±5.1[a] | 11.5±6.1[a,b] |
| Basal cell hyperplasia | 21 | 4.2±3.7[a,b] | 0 | 70.7±11.4[a,b] | 95.9±4.9[a] | 8.3±6.2[a,b] |
| Squamous metaplasia | 20 | 0[a] | 0 | 100[a] | 100[a] | 28.2±15.3[a] |

[a] Statistically significant difference relative to normal epithelium [$P<0.05$; analysis of variance (ANOVA) consecutive Scheffé test]
[b] Statistically significant difference relative to squamous metaplasia ($P<0.05$; ANOVA consecutive Scheffé test)

**Table 4** Thickness of the layer of cytokeratin (CK)5/6-reactive cells and the total epithelium

| Histology | Number | CK5/6 layer (µm) | Total epithelium (µm) |
|---|---|---|---|
| Normal epithelium | 16 | 12.8±3.9 | 47.4±10.4 |
| Mucinous hyperplasia | 8 | 23.9±5.3[a,b] | 55.1±7.4[b] |
| Basal cell hyperplasia | 21 | 25.9±4.1[a,b] | 58.3±4.4[b] |
| Squamous metaplasia | 20 | 88.6±9.7[a] | 88.7±9.7[a] |

[a] Statistically significant difference relative to normal epithelium [$P<0.05$; analysis of variance (ANOVA) consecutive Scheffé test]
[b] Statistically significant difference relative to squamous metaplasia ($P<0.05$; ANOVA consecutive Scheffé test)

the value observed in the normal epithelium, and the CK5/6 proliferation fraction increased significantly to 95.6%. Accordingly, the thickness of the CK5/6$^+$ layer rose significantly from 12.8 µm in normal epithelium to 25.9 µm in basal cell hyperplasia. No statistically significant difference was found in the total thickness of the epithelium in normal mucosa (47.4 µm) and basal cell hyperplasia (58.3 µm; Table 4). Compared with the aforementioned groups, squamous metaplasia disclosed a significantly increased thickness of the total epithelium, making up 88.7 µm. The average MIB-1 index in this group showed a further increase to 28.2% and was sig-

nificantly higher than normal values and those assessed in basal cell hyperplasia. No significant quantitative differences occurred between basal cell hyperplasia and mucinous hyperplasia. No statistically significant differences occurred between resection specimens and specimens obtained using fiber-bronchoscopy. With no cell being double labeled for both markers, MIB-1 and CC10, the CC10 proliferation fraction was zero in all groups investigated.

## Discussion

The present analysis of CKs, a proven system of differentiation markers, confirms and extends previous studies on the CK distribution in normal and pathologically altered respiratory epithelium [1, 7, 12, 15, 23]. Our data have disclosed profound differences between normal bronchial epithelium, basal cell hyperplasia and squamous metaplasia, indicating that these cell populations are differentiating along at least two distinct cell lineages. As to their progenitor cells, it is generally accepted that the Clara cell constitutes a progenitor of the terminally differentiated columnar bronchiolar epithelium [2, 9, 19]. In addition, the basal cell has been claimed to be another progenitor cell in the human airway mucosa, since the proliferative activity of this cell line is significantly higher than that of the Clara cell [8, 9].

Until now, it was unclear to what extent the two putative progenitor cells of the human airway epithelium contribute to normal and defective repair [25]. Moreover, it is debatable whether the bronchial CC10-reactive cell – like the bronchiolar Clara cell – constitutes a progenitor cell of the respiratory epithelium. The present study is the first to correlate the proliferation and numerical proportion of both the CC10-reactive bronchial cells and basal cells to the underlying morphologic alterations of and the CK expression within the airway mucosa.

In accordance with previous investigations [8, 9], the present study shows that the proliferative capacity of the columnar epithelium is low relative to that of the basal cells, even in the normal airway epithelium. With the proliferative capacity of the basal cell being intrinsically higher than that of the columnar epithelium even in normal epithelium [8, 9], basal cell hyperplasia "overgrows" the damaged columnar epithelium and, subsequently, squamous metaplasia develops. In consequence, squamous metaplasia is commonly found in long standing inhalative stress, such as in chronic bronchitis, and after short-term exposure to an extremely high oxidative stress, such as in adult and infantile respiratory distress syndrome, leading to bronchopulmonary dysplasia and complete loss of the Clara cells in the damaged airway regions [3]. Squamous metaplasia and basal cells share the expression of CK5/6, a marker found only sparsely in the columnar respiratory epithelium. We therefore conclude that squamous metaplasia most probably is derived from the basal cells, featuring an additional gain of CK13 expression, indicative of squamous epithelial maturation. This is in agreement with previous studies [15, 23, 31]. The present study suggests that the bronchial CC10-reactive epithelia have no precursor function, because these cells showed no proliferative activity. This fact clearly distinguishes the bronchial CC10-reactive cell from its bronchiolar counterpart, the Clara cell. In the present study, the development of hyperplasia and metaplasia in the bronchial mucosa is paralleled by a gradual loss of the bronchial CC10-reactive epithelia, the number of which therefore is inversely related to an increasing proliferative activity. This suggests that the reduction of the bronchial CC10-reactive cells might play an important role in epithelial remodeling under pathologic conditions. In this context, two different aspects merit further consideration.

First, it has been shown that CC10 is a potent inhibitor of phospholipase $A_2$ [20, 22, 24]. Phospholipase $A_2$ is a key enzyme in the synthesis of arachidonic acid [11, 29] and is able to cause severe damage to biologic membranes leading to the liberation of pro-inflammatory mediators. Loss of CC10-reactive bronchial cells, which are the major source of CC10 in the airway lining fluid, and subsequent reduction of CC10 in the airway lining fluid results in increased inflammation and subsequent epithelial damage. This is likely to result in basal cell hyperplasia and squamous metaplasia since the proliferative activity of basal cells is intrinsically higher than that of the columnar airway epithelium. Reduction and finally complete loss of the bronchial CC10-reactive epithelial cells may therefore be regarded as a sequel of airway damage, which leads to further aggravation of the disease process when repair and regeneration fails. The reduction of CC10-reactive cells occurs in proximal and distal airways and indicates that bronchiolar Clara cells and bronchial CC10-reactive cells share common properties concerning their susceptibility to intrinsic and extrinsic noxious agents [30]. In the bronchioli, the Clara cell constitutes a target of inhaled noxious agents, and the CC10 has been shown to be a sensitive marker of acute pulmonary damage, for example occurring after short-term exposure to fumes in firefighters [5]. In chronic airway disease serum, CC10 levels have been reported to be decreased [4] and, accordingly, histological investigations showed the relative number of Clara cells to be diminished [30].

Second, CC10 is likely to interfere with intracellular signal transduction since it binds phosphatidylinositol [34], inhibits phospholipase C [27] and thus interferes with the IP3 signaling system, which is coupled to either tyrosine kinase or G-protein linked receptors [6]. This assumption has recently been substantiated through transfection studies. Tumor cells transfected with CC10 showed a lower anchorage-independent growth potential compared with that of not transfected cells [32]. The data of the present study are well in line with this concept, since lesions with low counts of CC10-reactive cells had a high proliferative activity and vice versa.

The present study clearly shows that the bronchial CC10-reactive cell has no progenitor function in the bronchial epithelium in a manner comparable to the role

of the Clara cell in the bronchiolar epithelium. However, from the results of the present study, we conclude that the bronchial CC10-reactive epithelial cells play an important role in the development and progression of acute and chronic airway disease.

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