Serum and BAL Clara cell 10 kDa protein (CC10) levels and CC10-positive bronchiolar cells are decreased in smokers


ABSTRACT: Cigarette smoking has diverse effects on the structure and function of the lung. Smoking appears to reduce the levels of Clara cell 10 kDa protein (CC10) in the alveolar lining fluid, but the influence of smoking serum on CC10 levels is still debated, and it has not been clear whether smoking reduces the number of CC10-producing lung cells. The aims of this study were to clarify the influence of smoking on CC10 levels in the alveolar lining fluid and bloodstream, and on the number of CC10-producing lung cells.

CC10 concentrations were measured in sera and bronchoalveolar lavage (BAL) fluids, by means of enzyme-linked immunosorbent assay using monoclonal and polyclonal antibody, and the immunohistochemical expression of CC10 was examined in the lungs of nonsmokers and smokers using the monoclonal antibody, TY-5, against CC10/human urinary protein-1.

CC10 concentrations in sera and in BAL fluids from healthy smokers were significantly lower than in healthy nonsmokers. Immunohistochemical expression of CC10 was found exclusively in nonciliated bronchiolar epithelial cells. As compared to that of nonsmokers, the mean percentage of CC10-positive bronchiolar epithelial cells was significantly decreased in lung tissue specimens obtained from smokers who had normal results in pulmonary function tests.

It was concluded that smoking reduces the proportion of Clara cell 10 kDa protein-producing bronchiolar epithelial cells, resulting in decreased levels of Clara cell 10 kDa protein in the lower respiratory tract and in the bloodstream. The protein is a new blood biochemical and immunohistochemical marker, reflecting structural changes in peripheral airways induced by cigarette smoking.

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Clara cell 10 kDa protein (CC10), which was first described by Singh et al. [1] in 1988, is the predominant product from nonciliated bronchiolar epithelial cells (Clara cells) in respiratory and terminal bronchioles in the lung [2–4]. The protein has also been referred to as Clara cell 16 kDa protein according to the result of electrospray/mass spectrometry [5]. The same group of authors purified human urinary protein-1 (hUP-1), which proved to be identical to CC10 in its amino acid sequences [6]. The protein CC10 in humans is known to be the counterpart of rabbit urotoglobin [7], and to be related to human polychlorinated biphenyl-binding protein [8]. Recently, this protein has been referred to as Clara cell phospholipid-binding protein, since phosphatidylcholine and phosphatidylinositol are bound inside the large internal hydrophobic cavity of the protein [9]. The biological role of CC10 in the lung has remained unclear. However, a highly interesting function of CC10 has been reported; CC10 appears to be a potent inhibitor of phospholipase A₂ (PLA₂) [10, 11] and phospholipase C (PLC) [12], which may regulate the series of inflammatory reaction at affected sites, and is related to anti-inflammatory agents, such as lipocortins and antiflammins [13].

Cigarette smoking has diverse effects on the structure and function of the lung. The peripheral airways of cigarette smokers contain a large number and variety of inflammatory cells [14, 15]. A few studies have investigated the influence of cigarette smoking on human Clara cells [11, 16–18]. Cigarette smoking appears to reduce CC10 levels in the alveolar lining fluid and bloodstream [17, 18]. Recently, Nomori et al. [19] reported contradictory results concerning the influence of cigarette smoking on serum CC10 levels. It has been unclear whether or not cigarette smoking reduces the number of CC10-producing lung cells. We have established monoclonal antibody (MoAb) against CC10/hUP-1 and an enzyme-linked immunosorbent assay (ELISA) using monoclonal and polyclonal antibody against CC10/hUP-1 [20, 21]. The aims of the present study were: 1) to clarify whether or not CC10 levels in sera and in bronchoalveolar lavage (BAL) fluids are influenced by smoking habits; and, 2) if so, to elucidate the mechanism of variation of CC10 in sera and in BAL fluids of smokers and nonsmokers from an immunohistochemical approach.
CLARA CELL PROTEIN IS INFLUENCED BY SMOKING

Materials and methods

Monoclonal antibody

MoAb against CC10/hUP-1 (6D4; immunoglobulin (Ig)G1-κ) was developed as described previously [20], and employed for CC10 quantitation. A MoAb against CC10/hUP-1 (TY-5; IgG1-κ) was newly established, and used for immunohistochemical analysis. Western blot analysis using a urine and BAL fluid sample showed a single band at the molecular mass of 14.4 kDa under nonreducing condition, as well as purified hUP-1 antigen (fig. 1).

Healthy controls

Serum samples were obtained from 107 healthy non-smokers (mean±SD age 43±9 yrs; 51 males and 56 females), and 104 healthy smokers (mean age 44±7 yrs; 58 males and 46 females). There were no significant differences in ages between these groups (male non-smokers 44±10 years; male smokers 43±7 yrs; female nonsmokers 42±8 yrs; female smokers 44±8 yrs). The current cigarette consumption was 24.8±12.5 cigarettes·day⁻¹, the duration of smoking was 22.4±7.4 yrs, and the cumulative cigarette consumption 28.4±16.7 pack-yrs (the number of packets of 20 cigarettes smoked per day multiplied by the years of smoking). The current cigarette consumption was 28.8±13.6 cigarettes·day⁻¹ and 21.9±14.2 pack-yrs, respectively (both p<0.001), whereas the duration of smoking was not (males 23.3±6.7 yrs and females 20.9±7.6 yrs).

BAL fluid samples were obtained from 13 healthy non-smokers (mean age 28±7 yrs; 10 males and 3 females), and 14 healthy smokers (mean age 30±8 yrs; 11 males and 3 females). The current cigarette consumption was 21.3±7.89 cigarettes·day⁻¹, the duration of smoking was 11.2±5.9 yrs, and the cumulative cigarette consumption was 12.1±7.7 pack-yrs.

The healthy subjects had no history of respiratory disease. Their chest radiographic images revealed no abnormalities, and spirometric tests showed normal results (vital capacity (VC) >80% of predicted value, and forced expiratory volume in one second (FEV1) >70% pred). Informed consent was obtained from the healthy subjects.

Lung tissue specimens

Lung specimens were obtained from 20 patients undergoing resection for a peripheral carcinoma. None had a central tumour or obstructive pneumonia. Pulmonary function tests of these patients showed normal spirometric results and normal lung volume. Ten patients were nonsmokers (mean age 55±8 yrs; 6 males and 4 females), and the remaining 10 were smokers (mean age 55±2 yrs; 7 males and 3 females). There was no significant difference in ages between the two groups. The current cigarette consumption was 20.5±11.2 cigarettes·day⁻¹, the duration of smoking was 27.8±6.6 yrs, and the cumulative cigarette consumption was 25.8±11.9 pack-yrs.

BAL procedure

Three 50 mL aliquots of 0.9% sterile saline were instilled into a bronchus in the right middle lobe via a fiberoptic bronchoscope. The BAL fluid was recovered by gentle suction immediately after the infusion of each aliquot. The average recovery ratios of smokers and non-smokers were 64±12 and 66±11%, respectively, showing no significant difference.

BAL samples were centrifuged at 400×g for 10 min. The cells recovered were analysed with total and differential cell counts. The supernatants were collected and then centrifuged again at 550×g for 30 min. The supernatants were collected and cryopreserved at -30°C until use.

BAL fluid albumin concentrations were determined by immunoprecipitation of laser nephelometry. The mean albumin levels in BAL fluids from healthy nonsmokers and smokers were 39.9±12.8 and 48.9±21.0 mg·L⁻¹, respectively, showing no significant difference. The mean concentration of cells in BAL fluid from healthy nonsmokers and smokers were 15.9±8.2 ×10⁴ and 20.3±9.7 ×10⁴ cells·mL⁻¹, respectively, showing no significant difference. The proportions of alveolar macrophages in healthy smokers (94±6%) were significantly increased compared to healthy nonsmokers (86±8%) (p<0.05), and the proportions of lymphocytes in healthy smokers (5±6%) were significantly decreased compared to healthy nonsmokers (13±9%) (p<0.05). There was no significant difference in the proportions of neutrophils in BAL fluids between healthy nonsmokers and smokers.

Fig. 1. Western blot analysis using anti-CC10/hUP-1 monoclonal antibody TY-5 (lanes 1–3) and 6D4 (lanes 4–6). Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed using a 10% separation gel under nonreducing conditions (lanes 1 and 4, concentrated urine; lanes 2 and 5, BAL fluid; and lanes 3 and 6, purified hUP-1 antigen). A single band at the molecular mass of 14 kDa is found in lanes 1–6. CC10: Clara cell 10 kDa protein; hUP-1: human urinary protein; BAL: bronchoalveolar lavage.
Quantitation of CC10

CC10 concentrations were measured with ELISA, as described previously [20, 21]. Briefly, plastic immunoplates (Nunc, Copenhagen, Denmark) were coated overnight at 4°C with MoAb 6D4 at a concentration of 1 mg·L⁻¹ in 50 nM of carbonate buffer (pH 9.6). The unbound antibody was removed with 0.05% Tween 20 in 0.05 M phosphate-buffered saline (PBS) (pH 7.0), and thus coated with 100 µL of 1% bovine serum albumin in PBS (BSA-PBS). To this, 100 µL of standard or diluted samples were added and allowed to react with the coated antibody at 37°C for 90 min. Unbound antigen was removed by washing with 0.05% Tween 20 in 0.05 M PBS. One hundred microlitres of rabbit polyclonal antibody against hUP-1 (Dako, Copenhagen, Denmark) diluted 1 in 100 with BSA-PBS were added, and the mixture was incubated at 37°C for 90 min. The material was washed with the same buffer, 100 µL of horseradish peroxidase-conjugated goat anti-rabbit IgG antibody diluted to 1:2,000 with 1% BSA-PBS added, and the mixture was again incubated at 37°C for another 40 min. After repeated washing with the same buffer, the unbound enzyme conjugate was removed. At this point, 100 µL of enzyme substrate containing 1 mg·mL⁻¹ of o-phenylenediamine in citric buffer (pH 5.0) and including 0.03 % (v/v) hydrogen peroxide was added. Enzyme activity was measured at the absorbance of 492 nm after terminating with 0.05 M phosphate-buffered saline (pH 7.0), and including 0.03% hydrogen peroxide was added. Enzyme activity was measured at the absorbance of 492 nm after terminating with 150 µL of 1.0 M sulphuric acid. Purification of standard antigen was performed as described previously [20, 21]. Average coefficient variations of intra-assay of standard antigen was performed as described previously [20, 21].

Immunohistochemistry

Lung specimens were obtained from patients with lung cancer (less than 3 cm in diameter). Lung specimens without lung cancer were fixed with 10% formalin, and embedded in paraffin. Sections of 5 µm thickness were deparaffinized with xylene. The sections were soaked in absolute methanol containing 0.3% hydrogen peroxide, for 30 min at room temperature, to eliminate endogenous peroxidase activity. The sections were incubated with 1.5% nonimmunized goat serum for 30 min at room temperature, then incubated with MoAb TY-5 (1 µg·slide⁻¹) for 60 min at room temperature, and washed three times with PBS for 30 min. Thereafter, the sections were incubated with biotinylated goat anti-mouse Ig serum for 30 min. The biotinylated goat anti-mouse Ig serum was preapplied to human-Ig-coupled Sepharose-4B to remove nonspecific binding to human tissue. After being washed with PBS, the sections were allowed to react with avidin biotin peroxidase complex (Vector, Burlingame, CA, USA). The enzyme reaction was developed as described previously [22]. Nuclei were lightly counterstained with haematoxylin. Normal mouse Ig was used as a negative control. No significant reaction occurred in this case.

Statistical analysis

Data were expressed as mean±SD. The Mann-Whitney U-test was used to compare paired sets of data. Pearson’s least squares linear regression analysis was used to determine correlations. The level of critical significance was assigned at a p-value less than 0.05.

Results

CC10 concentrations in sera and BAL fluids and their correlation

The average CC10 level in sera from healthy nonsmokers was 11.7±3.9 ng·mL⁻¹ (range 5.2–22.4 ng·mL⁻¹) and that from healthy smokers 7.9±2.8 ng·mL⁻¹ (range 1.6–15.3 ng·mL⁻¹) (fig. 2). The CC10 concentrations in sera from healthy smokers were significantly decreased as compared to healthy nonsmokers (p<0.0001). There were no significant sex-related differences in serum levels between male (11.5±3.6 ng·mL⁻¹) and female nonsmokers (11.9±4.2 ng·mL⁻¹) or between male (7.8±2.9 ng·mL⁻¹) and female smokers (8.0±2.7 ng·mL⁻¹).

The average CC10 level in BAL fluids from healthy nonsmokers was 775±445 ng·mL⁻¹ (range 279–1,890 ng·mL⁻¹) and that from healthy smokers 356±257 ng·mL⁻¹ (range 104–833 ng·mL⁻¹) (fig. 3). The CC10 concentrations in BAL fluids from healthy smokers were significantly decreased as compared to healthy nonsmokers (p<0.01). The average ratio of CC10/albumin in BAL fluids from healthy nonsmokers was 19.4±8.2 ng·µg⁻¹ (range 9.4–32.1 ng·µg⁻¹) and that from healthy smokers was 7.8±5.7 ng·µg⁻¹ (range 2.5–21.2 ng·µg⁻¹). There was a significant difference between the two groups (p<0.001).

The correlation in serum and BAL fluid CC10 values was analysed. There was a significant positive correlation in the values of CC10 in sera and BAL fluids (r= 0.58; p<0.01; n=26) (data not shown).

Fig. 2. – Clara cell 10 kDa protein (CC10) concentrations in sera from healthy nonsmokers and smokers. Horizontal and vertical bars show mean and standard deviation, respectively. A significant difference was found in serum CC10 levels between healthy nonsmokers and smokers (p<0.0001).
CC10 values and smoking habits

There were no significant correlations of serum CC10 values to the current cigarette consumption, the duration of smoking, or the cumulative cigarette consumption in healthy smokers (data not shown). Moreover, BAL fluid CC10 values did not correlate with the current cigarette consumption, the duration of smoking, or the cumulative cigarette consumption in healthy smokers (data not shown).

Examination of immunohistochemical expression of CC10 in the lungs using MoAb against CC10/hUP-1

Assessment of the immunohistochemical expression of CC10 using MoAb TY-5 against human CC10/hUP-1 was performed in lung tissue specimens obtained from nonsmokers and smokers who had normal results of pulmonary function tests. TY-5 reacted with nonciliated bronchiolar epithelial cells but did not react with any other types of cells, including ciliated bronchial and bronchiolar epithelial cells, type I pneumocytes and type II pneumocytes. TY-5 reacted with nonciliated bronchiolar epithelial cells, which are a very minor population of bronchial epithelial cells. Representative photomicrographs are shown in figure 4. CC10 expression was found in 30% of bronchiolar epithelial cells from a nonsmoker (fig. 4a) and in 17% from a smoker (fig. 4b).

The proportion of CC10-positive bronchiolar epithelial cells was evaluated in 30 bronchioles from 10 nonsmokers and in 30 bronchioles from 10 smokers. The proportions of CC10-positive cells amongst the entire bronchiolar epithelial cell population from nonsmokers and smokers were 27±7% (range 15–41%) and 16±5% (range 9–30%), respectively (fig. 5). The difference between the two groups was significant (p<0.001).

CC10-positive bronchiolar epithelial cells and smoking habits

Correlation between the proportion of CC10-positive bronchiolar epithelial cells and the current cigarette consumption, the duration of smoking, and the cumulative cigarette consumption were analysed in the lung specimens from 10 smokers. No significant correlations were observed.
Discussion

CC10 is one of the most abundant proteins produced locally in the lower respiratory tract, as assessed in BAL fluids recovered by BAL [26, 27]. However, different CC10 levels in BAL fluids of healthy subjects have varied considerably between authors, ranging 141–3,620 ng·mL⁻¹ in nonsmokers [11, 17, 24, 25], when determined by means of a latex immunoassay employing polyclonal antibody against CC10/hUP-1. In the present study, immunohistochemical localization of CC10 in healthy smokers was 2.18 in the present study, which was almost the same as the ratio of 2.13 reported by Bernard and co-workers [17].

The variability of mean CC10 values in BAL fluid from healthy subjects may also be influenced by the assays employed and/or the composition of smokers and nonsmokers in each study. It is noteworthy that the present study is the first to demonstrate mean CC10 levels in sera and BAL fluids from healthy subjects, when measured with ELISA employing monoclonal and polyclonal antibody against CC10/hUP-1.

There have been several reports concerning the influence of cigarette smoking on serum CC10 levels. Bernard and co-workers [17, 18] found that, when tested by the Laurell rocket assay using an antiserum specific to CC10, the ratios of CC10/(total protein) in postmortem lung lavage and BAL fluids were 0.14 and 0.17%, respectively. The BAL fluid CC10 level reported by Singh et al. [23] appears to be considerably lower than that in the present study. Mean CC10 levels in BAL fluids from healthy subjects have varied considerably between authors, ranging 141–3,620 ng·mL⁻¹ in nonsmokers [11, 17, 24, 25], when determined by means of a latex immunoassay employing polyclonal antibody against CC10/hUP-1.

The present study confirms that the CC10 concentrations in BAL fluids of healthy smokers are significantly decreased as compared to healthy nonsmokers, when measured with ELISA employing monoclonal and polyclonal antibody against CC10/hUP-1. This result is in keeping with the report by Bernard and co-workers [17]. The ratio of BAL fluid CC10 level in healthy nonsmokers/BAL fluid CC10 level in healthy smokers was 2.18 in the present study, which was almost the same as the ratio of 2.13 reported by Bernard and co-workers [17].

The variability of mean CC10 values in BAL fluid from healthy subjects may also be influenced by uncertainties concerning the actual quantity of epithelial lining fluid recovered by BAL [26, 27]. However, different CC10 levels in sera from healthy controls have been reported in the literature: mean CC10 levels were 7.89 ng·mL⁻¹ (smokers) and 11.7 ng·mL⁻¹ (nonsmokers) in the present study; 122 ng·mL⁻¹ (male nonsmokers) [17]; 63.6 ng·mL⁻¹ (smokers) and 82.0 ng·mL⁻¹ (nonsmokers) [17]; 122 ng·mL⁻¹ [29]; and 118 ng·mL⁻¹ (male nonsmokers) and 167 ng·mL⁻¹ (male smokers) [19]. With the exception of the present study, these values were measured with a latex immunoassay employing polyclonal antibody against CC10/hUP-1. The variability of mean values appears to be due to the specific grade of purification of CC10/hUP-1 [3]. The variability of mean CC10 levels in sera and BAL fluids from healthy subjects may also be influenced by the assays employed and/or the composition of smokers and nonsmokers in each study. It is noteworthy that the present study is the first to demonstrate mean CC10 levels in sera and BAL fluids from healthy subjects, when measured with ELISA employing monoclonal and polyclonal antibody against CC10/hUP-1.

With respect to the influence of sex on serum CC10 levels, contradictory reports have been found [18, 19]. In the present study, serum CC10 concentrations from male and female nonsmokers were compared, showing no significant difference. In contrast with a previous study [18], we were unable to find any influence of age on serum CC10 levels in healthy nonsmokers (data not shown). The conflicting results may be explained by study population, since the present study did not include nonsmokers aged 63 yrs or more. The present results and the report by Bernard and co-workers [17] demonstrate that the serum CC10 concentrations correlate significantly with BAL fluid CC10 concentrations. In this context, in healthy adults, serum CC10 levels appear to reflect CC10 levels in the lower respiratory tract, and serum CC10 levels are not influenced by the release of uteroglobin or hUP-1 from urogenital organs.

Several investigators [11, 23, 30–32] have determined the immunoreactivity of CC10 with polyclonal antibody against CC10/hUP-1. However, to our knowledge, there has been no report concerning the proportion of CC10-positive bronchiolar epithelial cells in relation to the total bronchial epithelial cells in normal lung tissue. In the present study, immunohistochemical localization of CC10 was examined by employing polyclonal antibody against hUP-1 (Dako) and MoAb 6D4 as well as MoAb TY-5. MoAb TY-5 showed good immunoreactivity to nonciliated bronchiolar epithelial cells as compared to MoAb 6D4 and polyclonal antibody against hUP-1 (data not shown). TY-5 and 6D4 recognize the epitope in CC10 molecule, but TY-5 occupies the binding site of CC10 with MoAb 6D4 (data not shown).

The proportion of Clara cells in the adult human lung has not yet been clearly defined. The proportion of Clara cells in the entire bronchiolar epithelial population varies widely between animal species: cat (100%), guinea-pig (74%), mouse (67%), sheep (66%), horse (61%), rabbit

![Fig. 5. Clara cell 10 kDa protein (CC10-positive bronchiolar epithelial cell as a proportion of the total bronchiolar epithelial cells from nonsmokers and smokers. Horizontal and vertical bars show mean and standard deviation, respectively. A significant difference was found in the percentage of CC10-positive bronchiolar epithelial cells between healthy nonsmokers and smokers (p<0.001).](image-url)
(61%), cattle (54%) and rat (25%) [33]. In the present study, we have shown that the proportion of CC10-positive bronchiolar epithelial cells (Clara cells) in relation to total bronchiolar epithelial cells is significantly decreased in smokers (16%) as compared to nonsmokers (28%), although both the smokers and nonsmokers had normal results in pulmonary function tests.

Lumsden et al. [16] reported that when stained with toluidine blue, Clara cell populations in bronchiolar epithelial cells from nonsmokers and smokers were 4.3% (range 0–10.4%) and 8.3% (range 2.0–14.3%), respectively. The results were considerably lower than the present results. The authors reported that counting Clara cells was very difficult, since the impression of Clara cells was of long thin cells, tapering from the apex to the basement membrane. Based on the results in the present study, it can be speculated that cigarette smoking decreases the proportion of CC10-positive bronchiolar epithelial cells and results in decreased CC10 levels in the lower respiratory tract and in the bloodstream.

CC10 has been recently cloned in several species, including humans [1], but its biological role in the lung has remained unclear. However, an important function described in the literature is of great interest: CC10 has remained unclear. However, an important function described in the literature is of great interest. CLARA CELL PROTEIN IS INFLUENCED BY SMOKING

In conclusion, this study indicates that smoking reduces the proportion of Clara cell 10 kDa protein-producing bronchiolar epithelial cells, resulting in decreased levels of Clara cell 10 kDa protein in the lower respiratory tract and bloodstream.

References


