Distribution of Clara cell secretory protein expression in the tracheobronchial airways of rhesus monkeys

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Coppens JT, Van Winkle LS, Pinkerton K, Plopper CG. Distribution of Clara cell secretory protein expression in the tracheobronchial airways of rhesus monkeys. Am J Physiol Lung Cell Mol Physiol 292: L1155–L1162, 2007. First published January 19, 2007; doi:10.1152/ajplung.00454.2006.—Clara cell secretory protein (CCSP) is a protective lung protein that is believed to have antioxidant, immunomodulatory, and anticarcinogenic properties; to be present in all adult mammals; and to be well conserved in rodents, humans, and nonhuman primates. The rationale for this study is to define the distribution and abundance of CCSP in the airway epithelium and lavage fluid of the adult rhesus monkey and to provide information for evaluating CCSP as a marker of Clara cells and as a biomarker of lung health. Lung tissue and lavage fluid from 3-yr-old rhesus monkeys were examined using histopathology and immunohistochemistry. Proximal bronchi, midlevel bronchi, and terminal/respiratory bronchioles were compared for immunohistochemical localization of CCSP in three-dimensional whole mounts as well as in paraffin and Araldite sections. Immunoreactive CCSP was found in nonciliated cells throughout the airway epithelium. Proximal and midlevel airways had the highest labeling. CCSP decreased in distal airways, and respiratory bronchioles had little to no CCSP. CCSP in the most distal airways was in tall cuboidal cells adjacent to the pulmonary artery. Although a large number of cells were present in the terminal bronchioles that would be classified as Clara cells based on morphology (nonciliated cells with apical protrusions), only a small number stained positively for immunoreactive CCSP. Semi-quantitative analysis of Western blots indicated that changes in lavage CCSP are consistent with, and may be predictive of, overall CCSP levels in the airway epithelium in this primate species that is phylogenetically similar to humans.

Macaca mulatta: bronchial epithelium; lung; CC10; CC16; SCGB 1A1

CCSP has been proposed as a useful diagnostic marker of toxicant exposure or of airway epithelial damage. Levels of CCSP in serum have been shown to rise after exposure to ozone (7, 9, 11), smoke (5), or LPS (2). The leading hypothesis is that there is increased leakage of CCSP from the airways into the blood when the epithelial layer is damaged. This has led many to consider serum CCSP as a new noninvasive biomarker of acute epithelial damage (7, 9, 10, 15). To properly assess the potential clinical benefits of CCSP, it is necessary to understand the steady-state distribution of CCSP in the airway and in the various epithelial cell types. The relationship between serum and lavage protein levels, cellular protein, mRNA levels, and airway cellular oxidative damage needs to be defined if CCSP is to be used as a diagnostic tool.

There is a wealth of information on the cellular and spatial distribution of CCSP in rodent models. Data indicate that Clara cells and CCSP are abundant throughout the tracheobronchial tree of rodents. However, information on the cellular and spatial distribution of CCSP in the human is sparse. Transmission electron microscope (TEM) studies indicate that based on morphology, Clara cells are present in only the terminal and respiratory bronchioles of human and monkey lungs (24). But, CCSP is often considered a marker of Clara cells, and in humans, CCSP is found in the lungs from the terminal bronchioles to the main bronchi.

Most of the experimental work that has been used to establish CCSP as a diagnostic tool has been done in rodents. Because there are species differences in lung architecture, lung development, and Clara cell distribution between humans and rodents, it is beneficial when characterizing CCSP as a diagnostic tool to use an animal model that has a lung environment as similar as possible to that of the human. One such animal is the rhesus macaque, which has similar airway branching and cellular distribution/differentiation as humans. The rhesus macaque was used in this study: 1) to define the distribution and abundance of CCSP in the lung tissue and lavage fluid, 2) to correlate CCSP abundance within the lung tissue with CCSP abundance in the airways, and 3) to define the colocalization of CCSP and glycoproteins. This was done using immunohistochemistry with CCSP antibody on whole mounts, Araldite sections, and paraffin sections of the tracheobronchial airways. In this study, CCSP was frequently found colocalized with mucus in what appeared to be goblet cells (8) and was distributed throughout the airway tree.

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MATERIALS AND METHODS

Animals. Four 3-year-old male rhesus monkeys (Macaca mulatta) were used. The animals were raised at the California National Primate Research Center and housed for 2 wk in filtered air chambers before being examined. Animal protocols were reviewed and approved by the University of California, Davis, Institutional Animal Use and Care Committee. Housing and care of monkeys before, during, and after treatment complied with the provisions of the Institute of Laboratory Animal Resources and also conformed to practices established by the American Association for Accreditation of Laboratory Animal Care. Monkeys were sedated with ketamine hydrochloride (5–10 mg/kg im) and then deeply anesthetized with intravenous pentobarbital sodium. The trachea was intubated, and the animal was maintained on positive-pressure ventilation (15.0 ml/kg, 20.0 breaths/min, 10–20 min) until being killed by exsanguination via the systemic aorta. Following exsanguination, lavage was obtained by flushing the right caudal lobe with 120 ml of PBS, the thorax was opened by midline incision, and the entire costal contents were removed en bloc. The trachea and extrapulmonary bronchi were exposed, and the left lobar bronchi were cannulated. Both the left cranial lung lobe, which was used for paraffin embedding, and the costal lobe, which was used for whole lung studies, were fixed by airway infusion with 1% paraformaldehyde at 30 cm hydrostatic pressure. The axial path of the airway tree was exposed by microdissection, and the airway branches were defined. This enabled sampling of the axial airway path at defined airway regions.

Paraffin immunohistochemistry. The 1% paraformaldehyde fixed tissue was embedded in paraffin and sectioned to 5-μm thickness. Sections were deparaffinized in xylene and then rehydrated in a serial dilution of ethanol. Endogenous peroxidase activity was quenched by a 20-min bath in 3% hydrogen peroxide, and antigen retrieval was performed in hot citrate buffer. Sections were blocked with 5% BSA in PBS to prevent nonspecific primary antibody binding. Rabbit anti-human CCSP antibody (Biovendor, Candler, NC) was diluted to 1:1,600 in PBS and incubated with sections for 1 h at room temperature and then overnight at 4°C. To visualize signal, an anti-rabbit ABC immunoperoxidase kit was used along with SG chromagen (Vector Labs, Burlingame, CA) according to the manufacturer's instructions. Stained sections were imaged using an Olympus BX41 microscope with a Q-Color 3 digital camera (Olympus America, Center Valley, PA).

Whole lung immunohistochemistry. Microdissected 1% paraformaldehyde-fixed tissue was washed in five changes of PBS over a period of 2 h. Tissue was then permeabilized in PBS containing 0.3% Triton X-100 for 2 h, rinsed in four changes of PBS over 1 h, placed in 3% hydrogen peroxide for 10 min, and rinsed in four changes of PBS over 1 h. Next, the manufacturer's instructions for the Vector ABC immunoperoxidase kit (Vector Labs) were followed with wash times increased to four changes of PBS over 1 h. Anti-human CCSP (Biovendor) primary antibody was used at 1:400 overnight. The chromagen was immunoperoxidase SG (Vector Labs). The entire process was carried out on an orbital shaker at low speed. Tissues were imaged using a Leica MZFL III microscope (Leica Microsystems, San Jose, CA) and a Q-imaging Retiga-SRV Fast 1394 camera (Q-Imaging, Burnaby, British Columbia, Canada).

Apropos of collection, portions of whole lung tissue were immunohistochemically stained for CCSP, fixed in 1% osmium tetroxide, dehydrated in ethanol, infiltrated with propylene oxide, embedded in Araldite 502 (22), cut to 0.5-μm-thick sections using a diamond knife on a Zeiss micron HN340 (Thornwood, NY), and stained with Azure II stain.

Western blots. Human CCSP (Biovendor) was used as a quantitation standard. Lavage samples were stored at −80°C until used for Western blotting. Samples were combined with 2X loading buffer containing 10% glycerol, 10% 2-mercaptoethanol, 2% SDS, and bromophenol blue in 0.07 M Tris-HCl (pH 6.8, boiled for 5 min). Forty microliters of standard or sample per lane was applied to 4–20% Tris-HCl minigels (Bio-Rad). For 70 min, 140 V was applied to a Bio-Rad Mini Protein II apparatus at 4°C. Gels were placed in transfer buffer not containing SDS for 5 min before being transferred to PVDF membrane at 50 V for 50 min in the same buffer at 4°C. After transfer, membranes were rinsed in double-distilled water for 1 min and placed in 100% methanol for 1 min before being allowed to dry for at least 30 min.

CCSP standard was 90% purified CCSP from human urine (Biovendor). The concentration of the standard was verified using a human CCSP ELISA (Biovendor).

Since lavage total protein concentrations can vary widely, CCSP was reported as weight per volume of lavage fluid instead of as a fraction of the total protein. To determine that changes in CCSP levels were not due to changes in total protein, Bradford assays were run on each of the four lavage samples, and total protein concentrations were compared with CCSP concentrations.

Immunoblotting. Immunoblotting was performed on a tilting shaker. All dilutions and washes were in PBS with 0.05% Tween 20 (PBST) unless otherwise noted. Chemiluminescent detection was performed with an ECL Advance kit (GE Healthcare, Piscataway, NJ, formerly Amersham Bioscience). The blocking agent from the ECL Advance kit was used throughout immunoblotting. Primary rabbit anti-human CCSP (Biovendor) was diluted 1:500 in PBST with 0.5% blocking agent. Horseradish peroxidase-conjugated goat anti-rabbit (Biomedia) secondary antibody was diluted 1:4,000 in PBST with 1% blocking agent. Membranes were rehydrated in methanol, placed in PBST for 5 min, placed in 2% blocking agent for 30 min, rinsed 2X in PBST for 1 min, and incubated with primary antibody solution for ~18 h at 4°C. Membranes were rinsed in six changes of PBST over 50 min, incubated with secondary antibody for 30 min, rinsed in six changes of PBST over 50 min, and developed with the ECL Advance kit according to the manufacturer’s directions. Membranes were scanned with a Typhoon scanner (Molecular Dynamics/GE Health Care, Piscataway, NJ) at 999 V, high sensitivity, and 100-nm resolution. CCSP bands were quantified using ImageJ (Rasband W. S., ImageJ, National Institutes of Health, Bethesda, MD; http://rsb.info.nih.gov/ij/, 1997–2006). Samples found to be outside of the accurate dynamic range for this assay were diluted and rerun, and the dilution was factored into the result.

Sampling. One stained slide was examined at each of the following four airway locations in each of the four monkeys: 1) proximal (4th generation axial airway), 2) midlevel (8th generation axial pathway), 3) distal (immediately before the respiratory bronchiole), and 4) the respiratory bronchiole (50% conducting airway epithelium and 50% respiratory epithelium). Imaged samples were representative of the selected airway level. The samples were subjectively graded as to the fraction of epithelium between the lumen and the basal lamina staining positive for CCSP.

Semi-quantitative immunohistopathology. The abundance of CCSP was estimated and given a grade from 0 to 10, approximating the fraction of epithelial cross section that stained positive for immunoreactive protein, where 0 was considered negative and 10 was epithelium in which every cell was positive and completely covered with immunoreactive stain. Staining intensity was not considered in the grade, and a lightly stained cell was given equal value to a darkly stained cell. In cells that were partially stained, only the stained cell volume was considered.

Alcian blue/CCSP, periodic acid-Schiff/CCSP colocalization. After deparaffinization, selected paraffin sections were stained red for neutral glycoproteins with periodic acid-Schiff (PAS) base reaction or stained blue with Alcian blue (AB) dye for acidic glycoproteins before immunohistochemical staining for CCSP as above. For PAS, slides were placed in 3% acetic acid for 3 min, running deionized water for 3 min, 0.5% periodic acid for 10 min, running deionized water for 5 min, Schiff’s reagent for 10 min, two changes of sulfuric acid for 2 min each, and running deionized water for 5 min before being
coverslipped with Vectashield mounting media with 4’-6-diamidino-2-phenylindole nuclear stain (Vector Labs). In combination with Vectashield mounting media, the PAS reaction product fluoresces red when viewed through a standard green fluorescent filter set. For AB, slides were placed in 3% acetic acid for 3 min and 0.05% AB solution in 3% acetic acid for 2 h, rinsed in deionized water 5 min before immunochemistry, and coverslipped with Cytoseal mounting media (Richard-Allan Scientific, Kalamazoo, MI). Images were taken using an Olympus BH2 microscope and a Q-color 3 digital camera (Olympus America, Center Valley, PA).

Imaging. Images were each universally adjusted using Photoshop 7.0 (Adobe Systems, San Jose, CA) to reflect what was seen through the microscope eyepiece. Extended depth of field images are composite images taken at varying focal depths, which were assembled using Image-Pro Plus 6.0 (Media Cybernetics, Silver Spring, MD). Images were first aligned using the “align” function and then assembled using the “extended depth of field” function with focus regions selected for maximum local contrast.

RESULTS

Immunohistochemistry. The airways in the following four regions were compared: 1) proximal (main axial pathway near the 4th branch point), 2) midlevel (main axial pathway near the 8th branch point), 3) distal (main axial pathway near the point

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Fig. 1. Images of microdissected whole lung tissue from the costal lobe of a 3-yr-old monkey immunohistochemically stained for Clara cell secretory protein (CCSP) and labeled with SG chromogen. The line drawing is a schematic map of the airway indicating where each imaged area was obtained within the axial airway path. A: proximal bronchi near first branch point on the axial pathway. B: midlevel airway at branch point 8. C: distal airway between branch points 12 and 14. D: terminal and respiratory bronchioles after branch point 14. Arrows indicate areas of increased staining near bifurcations.

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Fig. 2. High-resolution light micrographs of CCSP immunostaining in whole lung from the costal lobe embedded in Araldite (A), sectioned at 0.5 μm, and lightly stained with Azure II to determine the morphology of cells positive for CCSP (black precipitate). A–D on the schematic map (left) indicates the location of each image within the sample. Blue numbers indicate airway branch number along the axial pathway. Images at right represent airway levels. In proximal (A), midlevel (B), and distal (C) epithelial locations, a dark reaction product can be seen in the apical portion of some nonciliated cells that protrudes into the lumen (arrowheads). In D, the transition from columnar to cuboidal epithelium, some staining can be seen (arrowhead at left), but no staining is seen in morphologically defined (cuboidal nonciliated) Clara cells. Clara cells (arrows) in D and in E transition from cuboidal airway to squamous respiratory epithelium.
of the 1st alveolar outpockets that fell between the 10th and 15th branch point on the main axial pathway), and 4) respiratory (peripheral airway adjacent to the distal level that is ~50% respiratory outpockets).

Whole mounts. Immunostaining for CCSP in whole lung tissues of 3-yr-old monkeys showed a pattern of CCSP expression within distinct airway cells (Fig. 1). The greatest abundance of CCSP expression was found in proximal (Fig. 1A) and midlevel airways (Fig. 1B). CCSP staining/expression was focal in the distal bronchioles, where the highest concentration of positive staining for immunodetectable CCSP was at bifurcations (Fig. 1C, arrows). Very little positive staining for CCSP was found in or near the respiratory bronchioles.

Araldite sections. Portions of whole lung tissue were immunohistochemically stained for CCSP, embedded in Araldite, and cut to 0.5-μm-thick sections. In Fig. 2, many of the CCSP-positive cells of the proximal, midlevel, and distal airways were nonciliated epithelial cells with mucus cell morphology (columnar cells filled with large, secretory vesicles with an attenuated basal area), and some of these cells appeared to be secreting CCSP-containing substances into the lumen (Fig. 2, A–C). CCSP immunostaining was virtually absent from the transitional zone between the columnar and cuboidal epithelium to the most proximal airways (Fig. 2, D and E). Most of the apical protrusions of nonciliated epithelial cells contained an even distribution of CCSP, but staining was able to penetrate only a few micrometers below the luminal surface. Subsequent embedding and sectioning of the immunohistochemically stained tissue whole mounts revealed that, in spite of a lengthy permeabilization step, immunohistochemical staining of the whole tissue was not able to penetrate far below the luminal surface of intact cells in the complex, pseudostratified epithelium of the rhesus monkey. In Fig. 3, a mucus cell has lifted from the basement membrane. The lifting of the epithelium is a processing artifact that occurred before immunohistochemistry, but it allowed the stain to penetrate the basal-lateral portions of the mucus cell, indicating the presence of CCSP at the basal portion of the cell.

Paraffin sections. On 5-μm-thick paraffin sections, immunoreactive CCSP antigen was detected in nonciliated cells throughout the airway epithelium from the lobar bronchus to the respiratory bronchioles. Figure 4 contains images of the most representative sections for each airway level. Strong staining for CCSP was present in the proximal (Fig. 4A) and the midlevel bronchi (Fig. 4B) with less in the distal bronchi (Fig. 4C). This gradually diminished in a proximal to distal manner; the terminal bronchioles had few positively stained cells. Occasionally, CCSP was detected in the cuboidal cells adjacent to a blood vessel (Fig. 5A). No CCSP was detected in the parenchyma or endothelium, but, on occasion, some staining was detected in the interstitium between a larger blood vessel and a large airway (Fig. 5B) or between two large airways. CCSP immunostaining was also commonly seen in the glands of the proximal and midlevel airways (Fig. 5C). Most CCSP-positive cells expressed CCSP in one of two patterns: evenly throughout the cell or with more intense localization on the luminal side in or near the apex. The overall

Fig. 3. After en bloc immunohistochemistry, portions of the airways were embedded in Araldite, sectioned at 0.5 μm, and lightly stained with Azure II stain. A mucus cell is shown lifted from the basal lamina, exposing the basal portion of the cell that is stained positive for CCSP (arrow).

Fig. 4. Images of paraffin sections of 4 airway levels immunohistochemically stained en bloc for CCSP similar to those used to determine volume density of epithelial CCSP: proximal (axial pathway at the 4th branch point) (A), midlevel (at the 8th branch point) (B), the terminal bronchiole (1–2 generations before the respiratory bronchioles) (C), and the respiratory bronchioles (D).
pattern of expression in paraffin sections was similar to that found in the whole lung. CCSP immunostaining was heaviest in the proximal and midlevel airways lessening in a proximal-to-distal direction. We found CCSP to be present in the trachea of one sample. It was detectable at very low levels in one of two animals examined and is not likely to be a significant source of CCSP in the lung. Therefore, the trachea was not included in this study.

Quantitative analysis of CCSP. The data in Fig. 6A define the approximate fraction of the epithelial cross section that expressed immunoreactive CCSP at each airway level. Each data point is the added airway scores for one of the four animals. The data were separated by animal, and the scores for the four airway levels were added together to show the variance in total epithelial CCSP for each animal. In Fig. 6B, these data were overlaid with the concentration of the protein in lavage fluid for each animal. The correlation (regression analysis indicated an $R^2$ value of 81.5) may indicate a relationship between immunoreactive CCSP in airway epithelial cells and levels of CCSP found in the lavage fluid of the rhesus monkey.

Since lavage total protein concentrations can vary widely, CCSP was reported as weight per volume of lavage fluid instead of as a fraction of the total protein. Each lavage sample was obtained at the time of necropsy when 120 ml of PBS was used to flush the right caudal lobe. To determine that changes in CCSP levels were not due to changes in total protein, Bradford assays were run on each of the four lavage samples. When total protein levels were compared with CCSP concentrations, lavage total protein concentrations varied by more than 100-fold between samples, but changes in total protein did not correlate with changes in CCSP concentration (data not shown).

Colocalization of CCSP and glycoproteins. Slides immunohistochemically stained for CCSP were counterstained with AB for acidic glycoproteins or PAS for neutral glycoproteins. CCSP colocalized with both AB and PAS staining to some extent at all airway levels. Figure 7, A–D, indicates that colocalization of PAS and CCSP staining does occur within...
individual nonciliated cells, but not in all cells. In the proximal to midlevel bronchi, most nonciliated cells stained positive for both AB and CCSP. Figure 7, E–H, shows the proximal epithelium and an adjacent gland immunohistochemically stained for CCSP. Figure 7 shows staining for AB (Fig. 7E), immunohistochemistry (IHC) CCSP (Fig. 7F), and IHC CCSP and AB (Fig. 7G). Figure 7H is a magnified portion of Fig. 7G to show detail of CCSP-stained cells. Arrows in Fig. 7, G and H, indicate dual labeling of AB and CCSP. In the respiratory bronchioles, AB staining was less intense in the nonciliated airway cells than staining for CCSP. Although some of the submucosal glands stained positive for CCSP, many did not. All glands observed were positive for AB stain. The general pattern of expression of AB staining in the nonciliated airway epithelial cells was heavy in the proximal airways and gradually decreasing in a proximal-to-distal direction. PAS staining was less prominent than AB but followed a similar pattern with far less staining in the distal airways than AB.

DISCUSSION

Our long-term goal is to establish whether CCSP is a good diagnostic tool for airway disease. Here we evaluate the rhesus monkey as an appropriate animal model for humans in the study of CCSP. To our knowledge, this is the first study of the rhesus monkey lung to 1) define the distribution of CCSP in cells in a semiquantitative manner, 2) determine if lavage CCSP levels correlate with immunohistochemical detection of CCSP distribution and abundance in the airway epithelium, and 3) to determine the phenotype of CCSP-expressing cells in the airway. The present study demonstrates that, as in human lungs, CCSP is not exclusively present in Clara cells, at least not by the morphological definition of Clara cells used by investigators who have done previous work looking at human and primate Clara cells (16, 21, 24, 31). We found that the epithelial CCSP for each animal paralleled CCSP levels measured in lavage fluid from the same animal. This adds to existing evidence that changes detected in CCSP levels in lavage fluids may reflect changes in lung tissue expression of CCSP. Other investigators have reported colocalization of CCSP and mucins (8, 30) in the human lung similar to what we report here in the monkey. These findings suggest CCSP expression in rhesus monkey airway epithelium is outside the classic lineage defined by TEM in distal airways by Plopper et al. (23, 24).
When comparison is made of airways throughout the tracheobronchial tree that are identified by their branching history, immunoreactive CCSP was detected throughout the intrapulmonary airway tree from the lobar bronchus to the respiratory bronchioles. Immunopositive cells were most abundant in the proximal and midlevel bronchi where they were uniformly dispersed. However, these cells were especially abundant at bifurcations. The distribution of CCSP we found in the monkey lung is similar to that of the human as reported by Singh in 1992 (29) and Engelhardt in 1994 (12), increasing proximally with the highest levels in the cartilaginous bronchioles. Unlike in the rat, where submucosal glands are rare and contain no CCSP, several investigators have previously reported CCSP in the epithelial cells of the submucosal glands (19) and gland ducts (3, 30, 32) of humans. In looking at newborn human infant lungs, Asabe et al. (3) found CCSP in the bronchioles and the bronchi, as we did in monkeys. However, Asabe et al. found the highest concentrations of CCSP in the bronchioles, although they did not define the precise airway level they examined. In 1999, Boers et al. (8) looked at CCSP in adult human lungs (median subject age, 61 years). They reported that CCSP was present throughout the airway but with a higher density of positive cells in the respiratory bronchioles, lower levels in the bronchi, and fewer numbers of CCSP-positive cells overall compared with what is seen here in the monkey. However, Boers et al. defined airways solely by their diameter and not by airway level or proximity to the alveoli. Given the variations we found in CCSP levels between the bronchi and trachea, and the abrupt reduction in levels in the respiratory bronchioles, we believe that defining location by airway level is the most accurate way to compare humans and nonhuman primates. Differences in protein expression patterns among the Asabe et al. and Boers et al. studies and our study may also be due to subject ages. The human subjects Boers et al. examined were near the end of life expectancy, whereas the monkeys used here were just reaching sexual maturity. Expression patterns may also have been affected by the fact that these monkeys were housed in a controlled environment in filtered air for the last 2 wk of their lives, which could effect the steady state. Future experiments will address differentiating CCSP expression of the maturing airways of the rhesus monkey.

CCSP was present in lavage of all four monkeys, and quantitation of CCSP in lavage fluids reflected the overall levels detected immunochemically in sections from the airway epithelium and may be indicative of airway injury. Arsalane et al. reported decreased CCSP in lung homogenate and bronchoalveolar lavage fluid of rats acutely exposed to ozone (1) and for rats exposed to LPS (2). Given the many differences between rats and humans, it is difficult to say how applicable these data are to humans or monkeys. The data from our study in the rhesus monkey provide evidence that, in the steady state, bronchoalveolar lavage fluid levels of secreted CCSP could be indicative of overall levels of the protein within the lung epithelium. It is important to know that noninvasive techniques can be used to determine the status of CCSP in the airways, particularly since we understand little about how CCSP is regulated. Our results quantifying lavage CCSP gave us a mean value of 0.23 ng/μL (SD = 0.12), which is similar to what Petrek et al. (20) found (0.39 ng/μL) in the bronchoalveolar lavage fluid of normal nonsmoking humans using a latex immunoassay. Other investigators have found CCSP concentrations of 3.6 ng/μL (6) and 3.18 ng/μL (6) in human bronchoalveolar lavage fluid using latex immunoassays.

The morphology of CCSP-positive cells as seen in Araldite sections ranged from mucus cells (also indicated using AB/PAS and CCSP containing on paraffin sections) in the columnar epithelium of the proximal and midlevel airways to tall, cuboidal Clara cells in the distal airways and respiratory bronchioles. In the respiratory bronchioles where morphologically defined Clara cells reside, immunoreactive CCSP was detected less frequently and tended to be more abundant adjacent to pulmonary veins and bifurcations where the epithelium is also taller than in surrounding areas. It could be that CCSP is not a Clara cell-specific marker in the monkey lung or this could be evidence of the plastic nature of lung Clara cells as demonstrated in mice by Evans et al. (13). As Evans et al. also mention, in addition to mucin glycoconjugates, the Schiff’s reaction stains other nonmucous cellular materials that are commonly found in mucus cells when not filled with mucin. This makes precise localization of mucin within the cell difficult when using PAS.

The localization of CCSP to the bifurcations resembles the pattern of neuroendocrine cell distribution other investigators have found in the rhesus monkey (17, 25) and in rats (4). Although we did not stain with a nerve-specific marker to confirm colocalization of neuroendocrine and Clara cells, such future studies may be beneficial in determining whether there is a relationship between these two potential progenitor cells.

There are little data available on the arrangement of CCSP-producing cells in the human lung from gestation to adolescence. Since it is difficult to control experimental variables such as genetics, past exposure, diet, health history, and even age when working with human subjects, it becomes essential to have a controllable model that can closely represent the human lung. Given the close phylogenetic relationship and other similarities between the rhesus monkey and humans, detailed surveys such as this one can do much for our understanding the role of CCSP in the human airway. We believe the rhesus monkey is a good model, and studies using the rhesus monkey bridge the gap between studies in rodents, which are phylogenetically distant from humans, and human studies where it is difficult to obtain the same depth of information.

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