American Journal of Pathology, Vol. 156, No. 1, January 2000

Neuroepithelial Bodies of Pulmonary Airways Serve as a Reservoir of Progenitor Cells Capable of Epithelial Regeneration

Susan D. Reynolds,* Adam Giangreco,* John H. T. Power,† and Barry R. Stripp*

From the Department of Environmental Medicine, University of Rochester, Rochester, New York; and the Department of Human Physiology,† The Flinders University of South Australia, Adelaide, South Australia*

Remodeling of the conducting airway epithelium is a common finding in the chronically injured lung and has been associated with increased risk for developing lung cancer. Pulmonary neuroendocrine cells and clusters of these cells termed neuroepithelial bodies (NEBs) play a central role in each of these processes. We previously developed an adult mouse model of airway injury and repair in which epithelial regeneration after naphthalene-induced Clara cell ablation occurred preferentially at airway branch points and gave rise to nascent Clara cells. Continued repair was accompanied by NEB hyperplasia. We now provide the following evidence that the NEB microenvironment serves as a source of airway progenitor cells that contribute to focal regeneration of the airway epithelium: 1) nascent Clara cells and NEBs localize to the same spatial domain; 2) within NEB, both Clara cell secretory protein- and calcitonin gene-related peptide-immunopositive cells are proliferative; 3) the NEB microenvironment of both the steady-state and repairing lung includes cells that are dually immunopositive for Clara cell secretory protein and calcitonin gene-related peptide, which were previously identified only within the embryonic lung; and 4) NEBs harbor variant Clara cells deficient in cytochrome P450 2F2-immunoreactive protein. These data suggest that the NEB microenvironment is a reservoir of pollutant-resistant progenitor cells responsive to depletion of an abundant airway progenitor such as the Clara cell. *(Am J Pathol 2000, 156:269–278)*

Conducting airways of the lung are lined by a variety of specialized epithelial cells that fulfill critical functions necessary for the viability of air-breathing organisms. These functions include clearance and, in some cases, metabolism of inhaled environmental agents, regulation of lung fluid balance, and renewal of the epithelium after injury.[1] Altered airway epithelial cell composition that is associated with acute or chronic lung injury has the potential to significantly impact these functions.[2,3] Among the many agents that cause lung injury, pneumotoxic environmental pollutants contribute significantly to morbidity and mortality in a significant fraction of the human population.[4,5] Mechanisms by which these pollutants exert their effects involve cellular injury leading to dysregulation of growth and/or differentiation of epithelial cells. Associated changes in airway epithelial cell composition often involve transient or persistent hyperplasias such as squamous cell, neuroendocrine cell, basal cell, and secretory cell hyperplasia. These changes to the conducting airway epithelium increase the risk of developing either small-cell or non-small-cell lung cancers in smokers[6–11] and in nonsmokers.[12,13]

Both pulmonary neuroendocrine (PNE) cells and Clara cells have been proposed as progenitors for the genesis of small-cell and non-small-cell lung cancers. Clara cells represent the most abundant secretory cell type of distal airways in the human lung and of both proximal and distal airways in the lungs of rodents and rabbits.[14] In addition to their secretory function, Clara cells are a major site for cytochrome P450-mediated oxidation of both endogenous and xenobiotic lipophilic compounds in the lung,[15,16] and they serve as the principal source of progenitor cells for repopulation of airways after injury by inhaled oxidant pollutants.[17] A morphological derivative of the Clara cell, referred to as a type-A cell, enters the cell cycle after oxidant-induced airway injury.[18] Type-A cells lack the differentiated ultrastructural features, such as smooth endoplasmic reticulum and secretory granules, that are characteristic of mature Clara cells.[17,18] Cells with Clara-like characteristics but reduced secretory protein expression have been identified in association with preneoplastic lesions and represent a subpopulation of non-small-cell lung cancers.[19–21]

Supported by a pilot project grant from the University of Rochester Environmental Health Sciences Center (ES01247). A. G. was supported by a toxicology training grant (ES07026) and by a summer research fellowship provided by Dr. Mark Utell, Pulmonary and Critical Care Mecicine, University of Rochester.

Accepted for publication September 15, 1999.

Address reprint requests to Dr. Barry R. Stripp, Department of Environmental Medicine, University of Rochester, Box EHSC, 575 Elmwood Avenue, Rochester, NY 14642. E-mail: stripp@envmed.rochester.edu.

The progenitor role of Clara cells makes them a likely target for deregulated growth control and neoplastic progression. In contrast, PNE cells are not considered to be a progenitor population in the adult lung and are generally thought to be terminally differentiated and mitotically inert.[22] In the absence of demonstrable PNE proliferation, it has been suggested that maintenance of PNE cells in the normal lung[23] and PNE cell hyperplasia during repair[24,25] result from proliferation and differentiation of a progenitor population with multipotent differentiation capacity. However, this notion has been challenged by the finding that repair from airway injury is associated with PNE cell hyperplasia and that proliferation contributes to this hyperplastic response.[23,26,27] It is clear that additional studies are necessary to characterize the progenitor for the PNE cell lineage and to determine whether PNE hyperplasia contributes productively to airway repair.

The suggestion that pulmonary epithelial cells of the developing lung coexpress genes normally expressed by epithelial cells that are considered to be of distinct lineages raises the possibility that PNE cells may serve as a pluripotent progenitor cell.[28,29] In addition, studies investigating the contribution of PNE cell-derived neuropeptides in lung development suggest a role for PNE cells in regulation of epithelial cell renewal through elaboration of paracrine regulatory factors.[30] This possibility is supported by studies in developing pulmonary airways that show an inverse relationship between epithelial mitotic index and distance from clusters of PNE cells, termed neuroepithelial bodies (NEBs)[31] and the observation that neuropeptides such as gastrin-releasing peptide (GRP) have been shown to function as potent epithelial cell mitogens and contribute to lung branching.[32–34]

We and others have shown that parenteral administration of naphthalene to mice results in acute Clara cell toxicity.[35–37] Naphthalene is metabolized by cytochrome P450–2F2 (CYP-2F2) and -2B2 isoenzymes, expressed principally within Clara cells of the mouse lung, resulting in the production of the highly toxic naphthalene 1R, 2S epoxide.[38] Renewal of the injured airway involves focal-cell proliferation that leads to restoration of Clara cell secretory protein (CCSP)-expressing cells and hyperplasia of calcitonin gene-related peptide (CGRP)-immunopositive (IP) PNE cells.[27,35] The goal of the current study was to investigate the relationship between Clara cells and PNE cells in repairing airways of adult naphthalene-exposed mice. These studies test the hypothesis that the NEB microenvironment is critical for the maintenance of a "reserve" progenitor-cell population in the mature lung that participates in airway repair after acute airway injury involving an abundant progenitor cell (the Clara cell). We demonstrate that both Clara cells and PNE cells proliferate during airway repair and that both populations colocalize to the neuroepithelial body. Moreover, we demonstrate that the NEB microenvironment of the normal and injured lung supports the maintenance of at least two epithelial cell variants, one with a phenotype intermediate between Clara and PNE cells, and a Clara cell variant with little or no immunoreactive CYP-2F2 protein. These studies suggest a novel role for NEB in maintenance of multiple progenitor-cell populations in the mature airway.

Materials and Methods

Animals and Treatments

Wild-type male FVB/n mice (2–4 months old) used in this study were maintained as a specific pathogen-free in-house colony. They were allowed food and water *ad libitum* and maintained on a 12-hour/day light-dark cycle. Representative animals from the colony were screened quarterly using a comprehensive 16-agent serological panel, and for the absence of ecto- and endoparasites (Microbiological Associates, Rockville, MD). Naphthalene was obtained from Sigma Chemical Co. (St. Louis, MO). Naphthalene was dissolved in Mazola corn oil at a concentration of 30 mg/ml and administered intraperitoneally at 10 ml/kg body weight. One hour before sacrifice, animals were injected intraperitoneally with 2.5 mCi [^{3}H]-thymidine/kg. Groups of three mice were exposed to 1.0 part per million (ppm) O_3 for 48 or 72 hours as previously described.[39] Control mice for naphthalene and ozone exposures received either 10 ml of corn oil/kg body weight or were exposed to filtered room air, respectively.

Tissue Collection

Mice were sacrificed by intraperitoneal injection of 100 mg/kg sodium pentobarbital and were exsanguinated. Lungs were inflation fixed through a tracheal cannula at 10 cm water pressure with neutral buffered formalin for 10 minutes and immersion fixed in the same fixative overnight, followed by 24 hours in phosphate-buffered saline (PBS). The infracardiac lobe was dehydrated and embedded in paraffin. Adjacent serial 5 μm sections were cut to reveal the major axial pathway, three to five minor daughter airways, and numerous terminal bronchioles.

Immunohistochemistry

Sections were baked at 60°C overnight, cleared with three changes of xylene, and hydrated in a graded ethanol series to water. Endogenous peroxidase activity was quenched with 3% aqueous hydrogen peroxide for 10 minutes, and the tissue was blocked with PBS/0.5% bovine serum albumin (BSA)/5% normal goat serum for 30 minutes. Rabbit anti-CCSP was obtained from G. Singh (University of Pittsburgh, Pittsburgh, PA) and used at a dilution of 1:12,000. CCSP is abundant, is expressed in both immature and mature Clara cells, and serves as a reliable immunohistochemical marker for this cell type (28 and references therein). Previous *in situ* hybridization analysis has demonstrated that nascent Clara cells of the regenerating naphthalene-exposed airway express CCSP mRNA.[35] Rabbit anti-CGRP was purchased from Sigma and used at a dilution of 1:5000. CGRP is a neuropeptide found in pulmonary nerves and specifically expressed in PNE cells and NEBs of the airway epithelium. It is the most commonly used and ubiquitous

immunohistochemical marker for cells of the PNE lineage.[24,28,40,41] Rabbit anti-CYP-2F2 was generated by Biosynthesis (Louisville, TX), using a synthetic peptide for amino acids 397–407. This polyclonal antibody was affinity purified using a peptide affinity column generated by the manufacture's directions (Pierce, Rockville, IL) and preadsorbed with spleen lysate. The affinity purified/preadsorbed CYP-2F2 antibody detects a single 53-kd protein on Western blots of both lung and liver lysates, two tissues known to express abundant CYP-2F2. No bands were detected on heart or spleen lysates (data not shown). This antibody was used at a dilution of 1:7000 for immunohistochemistry. Primary antibodies were diluted in PBS/0.5% BSA/5% normal goat serum. After an overnight incubation, the tissue was washed with PBS and incubated for 30 minutes at room temperature with biotinylated goat anti-rabbit secondary antibody (Sigma) diluted 1:2000 in PBS/0.5% BSA/5% normal goat serum. Tissue was washed with PBS and incubated at room temperature for 30 minutes with streptavidin-HRP (Zymed, South San Francisco, CA) diluted 1:250 in PBS. Antigen-antibody complexes were detected with diaminobenzidine (DAB). Analysis was carried out on an Olympus Provis AX70 microscope equipped with a Sony 9000 CCD video capture system linked to a Pentium processor PC running Image-Pro Plus (Media Cybernetics, Silver Spring, MD). Images from CGRP- and CCSP-immunostained tissue were acquired using similar microscope and camera settings.

The specificity of the rabbit and goat anti-CCSP primary antibody[42,43] was verified by immunohistochemical analysis of lung tissue from CCSP-null mice.[44,45] The absence of immunoreactivity on this tissue indicated that the rabbit and goat anti-CCSP antibodies are specific to CCSP and that there are no antigens within neuroepithelial cells that cross-react with this antibody. The rabbit anti-CGRP primary antibodies detect rare clusters of cells located primarily at branch points and walls opposite bifurcations and scattered single cells in untreated lung tissue. No reaction was observed on Clara cells or ciliated cells identified by morphology and position. Immunoreactivity was not observed in the absence of primary antibody.

For dual immunohistochemistry, NEBs were identified by staining the middle of three adjacent serial sections for the PNE-specific antigen CGRP. Flanking sections were stained for the Clara cell marker CYP-2F2. Methods were as detailed above except that antibodies were diluted in PBS/3% BSA and amine-ethyl carbizol was used as the chromagen. Digital images of CGRP-stained NEB and NEB-associated CYP-2F2-positive cells were captured. The chromagen was removed with ethanol, and all sections were restained for the Clara cell marker CCSP as detailed above, using a goat anti-CCSP antibody (1:5000; G. Singh, University of Pittsburgh). Images of the same regions were overlaid by using Adobe Photoshop.

Autoradiography

Adjacent serial sections analyzed for proliferation were stained for CCSP or CGRP as detailed above. After immunohistochemistry, slides were washed overnight in PBS and dehydrated through ethanol. Dried slides were dipped in NTB-2 emulsion (Kodak; Rochester, NY), redried, and stored at 4°C for 1 week. Slides were developed by the manufacture's directions and counterstained with hematoxylin.

Proliferative Index

Proliferative cells were defined as those containing five or more grains over the nucleus. All airway epithelial cells with a nuclear profile were counted for determination of the labeling index. Various labeling indices were calculated as follows: epithelial labeling index, 100 (number labeled cells/number airway epithelial cells); PNE labeling index, 100 (number labeled PNE/number PNE cells); % PNE, 100 (number PNE/number airway epithelial cells); PNE proliferative fraction, 100 (number labeled PNE/number labeled airway epithelial cells); and Clara cell proliferative fraction, 100 (number labeled Clara cells/number labeled airway epithelial cells).

Dual-Labeling Confocal Microscopy

Paraffin sections were stained by the double indirect immunofluorescence method, using a combination of goat anti-CCSP and rabbit anti-CGRP. Sections were cleared with xylene, blocked with 20% normal horse serum in Tris-buffered saline for 45 minutes, and incubated overnight with primary antibody diluted 1:1000 in Tris-buffered saline/1% normal horse serum. Antigen antibody complexes were detected with Cy2-conjugated donkey anti-goat immunoglobulin (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) and Cy5-conjugated donkey anti-rabbit immunoglobulin (Jackson ImmunoResearch) diluted 1:100 in Tris-buffered saline/1% normal horse serum and incubated for 3 hours. All secondary antibodies are affinity purified and preadsorbed to minimize interspecies cross-reactivity. Sections were mounted in buffered glycerol and examined immediately using a Bethesda Research Laboratories (Bethesda, MD) confocal microscope equipped with argon and HeNe laser sources. Negative controls included incubation of sections with the inappropriate secondary antibody and omission of one or both primary antibodies from the staining protocol. The green and red images were overlaid with Adobe Photoshop.

Results

Neuroepithelial Bodies and Nascent Clara Cells Localize to the Same Spatial Domain

To define the spatial relationship between regenerating Clara cells and hyperplastic PNE cells, each population was localized in lung tissue of naphthalene-exposed mice by immunostaining adjacent serial tissue sections for CGRP and CCSP, respectively. Seventy-two hours after acute naphthalene exposure, there was a striking colocalization of CGRP- and CCSP-immunopositive (IP)

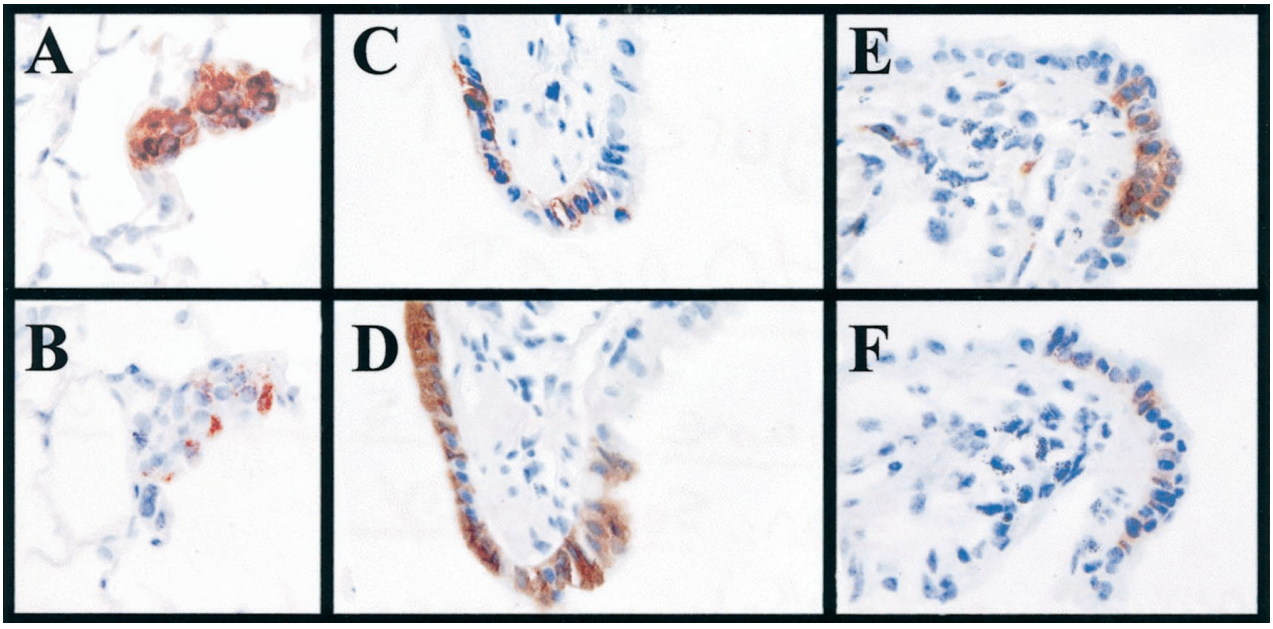

Figure 1. NEBs and nascent Clara cells localize to the same spatial domain. Adjacent serial sections (**A** and **B**, **C** and **D**, **E** and **F**) from animals 72 hours postnaphthalene treatment were stained for the neuroepithelial cell marker CGRP (**A, C, E**) or CCSP (**B, D, F**), using standard immunohistochemical techniques and DAB detection of the antigen-antibody complexes. A terminal bronchiole is represented in **A** and **B**, whereas **C–F** represent two different branch points. The frequency of NEBs associated with CCSP-IP cells was determined to be 87%. Original magnification, ×400.

cells, regardless of their anatomical location but including terminal bronchiole (Figure 1, A and B) and branch point (Figure 1, C–F) locations. Morphometric analysis indicated that 87% of CGRP-IP regions were associated with CCSP-IP cells. In all sections analyzed, CGRP domains represented a subset of CCSP-IP regions. NEBs, defined by clusters of CGRP-IP cells, were of consistent size throughout conducting airways of the repairing lung, with each NEB generally including 10–20 CGRP-IP cells that were in the plane of the section being analyzed. In contrast, the extent of the CCSP-positive region varied both in cell number and staining intensity (compare Figure 1, B and F, with D). Spatial colocalization of CGRP-IP and CCSP-IP cells indicates an intimate association between neuroepithelial bodies and nascent Clara cells and defines the branch point as a unique microenvironment for regeneration of the airway epithelium.

Two Mitotic-Cell Populations Are Localized to NEBs

A combination of immunostaining and autoradiographic detection of incorporated [^{3}H]-thymidine was used to

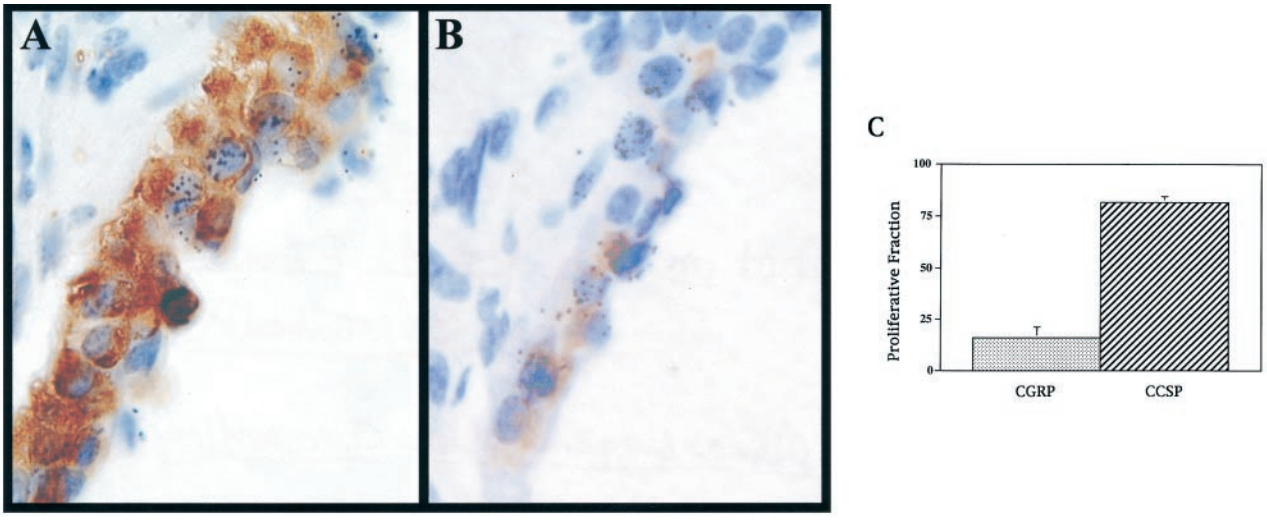

Figure 2. Two mitotic cell populations are localized to branchpoints. Adjacent serial sections from animals 72 hours postnaphthalene treatment were stained for CCSP (**A**) or for CGRP (**B**), using standard immunohistochemical techniques and DAB detection of the antigen-antibody complexes. Slides were then dipped in emulsion, and grains (black dots over nuclei) developed after 1 week. Original magnification, ×1000. **C:** The percent of all mitotic airway cells expressing either CGRP or CCSP was 16.06 ± 5.53 or 81.47 ± 3.00%, respectively.

Table 1. Labeling Index of Airway Epithelial Cells Exposed to Naphthalene or Ozone

Exposure	Epithelial labeling index*	PNE labeling index†	% PNE‡	PNE proliferative fraction§
Control	0.06 ± 0.02	0.00 ± 0.00	1.28 ± 0.32	0.00 ± 0.00
Naphthalene	3.51 ± 0.54	10.13 ± 1.82	4.92 ± 0.91	16.06 ± 5.53
Ozone	1.91 ± 0.63	1.37 ± 0.67	1.55 ± 0.29	1.42 ± 0.50

Four mice were injected with 300 mg/kg naphthalene in coin oil intraperitoneally and with 2.5 mCi [^{3}H]-TdR/kg 1 hour before sacrifice. Three mice were exposed to either room air or 1.0 ppm ozone for 48 or 72 hours and injected with 2.5 mCi [^{3}H]-TdR 1 hour before sacrifice. Lung tissue was processed by standard techniques. PNE were identified by CGRP immunohistochemistry and proliferating cells by sequential autoradiography.

*Epithelial labeling index = 100 (labeled cells/airway epithelial cells).

†PNE labeling index = 100 (labeled PNE/PNE).

‡% PNE = 100 (PNE/airway epithelial cells).

§PNE proliferative fraction = 100 (labeled PNE/labeled airway epithelial cells).

define progenitor-cell populations participating in repair of the naphthalene-injured airway epithelium at the 72-hour postexposure time point. Greater than 95% of labeled cells occurred within areas of clustered CGRP-IP cells (indicative of NEBs) that were associated with CCSP-IP cells. Within these regions, both CCSP- and CGRP-IP cell populations were proliferative, as evidenced by colocalization of immunostaining (CCSP or CGRP) and [^{3}H]-thymidine incorporation (Figure 2, A and B, respectively). These data indicate that normally quiescent PNE cells contained within a NEB enter the cell cycle after naphthalene treatment, and the data also suggest that PNE cell proliferation contributes to the development of NEB hyperplasia. Furthermore, these findings define the PNE cell as a second proliferative population at sites of airway regeneration after naphthalene injury. The relative contribution of CGRP- and CCSP-IP cells to the total epithelial proliferative index is quantified in Figure 2C. CGRP-IP cells compose 16.06% ± 5.53% of all proliferating epithelial cells whereas CCSP-IP cells are 81.47% ± 3.00% of all proliferating epithelial cells. Thus, two epithelial cell populations proliferate in response to ablation of Clara cells by naphthalene.

PNE and Clara Cells Exhibit a Hierarchical Contribution to the Repair Process

The data presented above indicate that PNE cells are a major contributor to the mitotic cell population after naphthalene injury of mature Clara cells. To determine whether the signals leading to PNE cell proliferation after naphthalene injury are unique to regeneration of the Clara cell-depleted airway, we have compared the proportion of labeled cells expressing CGRP after either acute naphthalene or ozone exposure. The epithelial labeling index at 72 hours postnaphthalene exposure is 3.51% ± 0.54%, whereas the labeling index of CGRP-IP cells (PNE labeling index) is 10.13% ± 1.82% (Table 1). Thus, in the naphthalene-injured lung, PNE cells are three times more likely to proliferate than epithelial cells in general. The frequency of PNE cells at 72 hours postnaphthalene treatment is 4.92% ± 0.91%, whereas their frequency in the mitotic population (as mentioned above) is 16.06% ± 5.53%. These data indicate that PNE cells contribute preferentially to the proliferative population after naphthalene injury.

Exposure of mice to 1.0 ppm ozone for 48 or 72 hours results in oxidative damage, with ciliated cells representing the principal target in a distal-to-proximal gradient (data not shown). The overall epithelial labeling index after ozone exposure is 1.91% ± 0.63% (Table 1). Similarly, the labeling index of CGRP-IP cells is 1.37% ± 0.67% after ozone exposure. These data indicate that the proliferative response of PNE cells is similar to that of all epithelial cells after exposure to ozone. To determine whether PNE cells contribute preferentially to the mitotic cell pool after ozone injury, the frequency of CGRP-IP cells in the airway epithelium was compared with the labeling index for CGRP-IP cells. The fraction of epithelial cells that express CGRP does not vary significantly between control and exposed groups and is 1.28 ± 0.32% and 1.55 ± 0.29%, respectively (Table 1). The representation of PNE cells within both the epithelium (1.55 ± 0.29%) and among mitotic cells (1.42 ± 0.5%) is also constant after ozone exposure. These data suggest that the contribution of PNE cells to the mitotic pool is reflective of their frequency in the epithelium and support the conclusion that PNE cells do not preferentially contribute to the mitotic cell population after ozone injury. Previously published results indicate that Clara cells function as the predominant stem cell population for repair of the ozone-injured airway epithelium.[18] The current data indicate that repair from ozone-induced airway injury requires minimal PNE cell participation. In contrast with oxidative injury, PNE cells compose one sixth of all mitotic cells in the repairing naphthalene-injured lung. We suggest that severe injury to the preferred progenitor cell of the mature conducting airway, the Clara cell, results in recruitment of a novel progenitor cell, the PNE cell, to participate in the repair process.

CGRP- and CCSP-IP Cells Are Distinct Populations

To determine whether CGRP-IP and CCSP-IP cells included an overlapping subset of intermediate cells, the

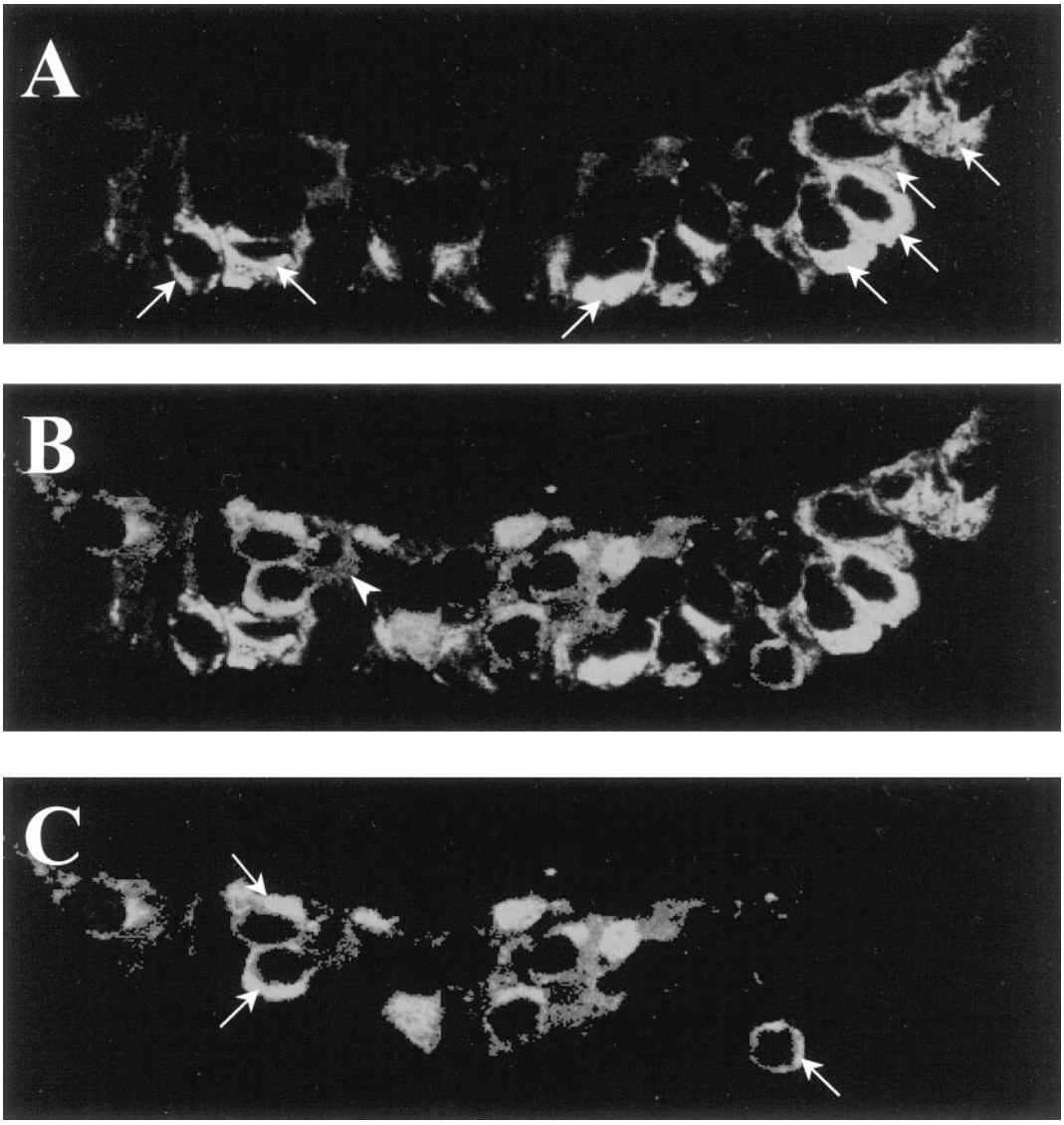

Figure 3. CCSP- and CGRP-IP cells are distinct populations. Dual immunofluorescence and laser scanning confocal microscopy were used to detect CCSP-IP cells (**A**, Cy2-green epifluorescence) and CGRP-IP cells (**C**, Cy5-red epifluorescence) in the same region of the same section. **B:** Image generated by merging **A** and **C**. The majority of cells in this section expressed either CCSP or CGRP (**arrows**). This pattern is representative of the majority of NEBs studied. The **arrowhead** indicates cells that may express both lineage markers simultaneously. Original magnification, ×630.

two antigens were colocalized in lung tissue recovered from mice 72 hours postnaphthalene exposure, using dual immunofluorescence and LASER scanning confocal microscopy. Figure 3, A-C, shows the same airway location in which CCSP and CGRP immunoreactivity is indicated by green or red fluorescence, respectively. Overlay of CCSP immunofluorescence (Figure 3A) and CGRP immunofluorescence (Figure 3C) demonstrates that the majority of cells in this section express either CCSP or CGRP (arrows), a pattern that is representative of the majority of NEBs studied. In contrast with NEB of the normal or dysplastic lung, where Clara cells are restricted to the apical portion of the NEB,[46] CCSP- and CGRP-expressing cells intermingle within the NEBs of the repairing adult airway. These data indicate that distinct CGRP- and CCSP-immunopositive cell populations are localized to NEBs 72 hours after acute naphthalene exposure.

A Rare Population of Dual-Positive Cells Is Localized to NEBs

The faint yellow fluorescence observed in Figure 3B (arrowhead) suggests a small population of cells that express both CGRP and CCSP. An additional example of such dual-expressing cells is shown in Figure 4, A-C. Approximately 10% of cells associated with NEBs express both CGRP and CCSP. Interestingly, the normal basal distribution of CGRP and the apical enrichment of CCSP were maintained in the coexpressing cells, suggesting that both of these antigens are produced within

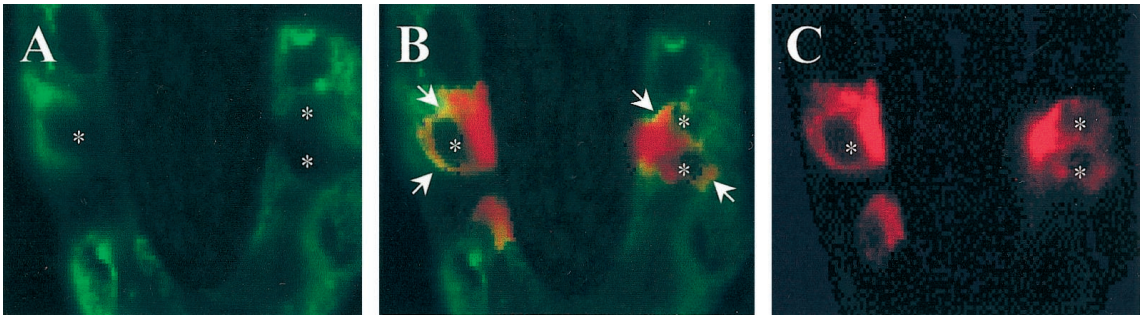

Figure 4. A rare population of dual-positive cells is localized to NEBs. Dual immunofluorescence and laser scanning confocal microscopy were used to detect CCSP-IP cells (**A**, Cy2-green epifluorescence) and CGRP-IP cells (**C**, Cy5-red epifluorescence) in the same region of the same section. **B:** Image generated by merging **A** and **C**. Three cells (*) that express both antigens are located within this branch point NEB. Original magnification, ×630.

the dual-expressing cell. Similar dual immunopositive cells were also detected in NEBs of the normal lung. Figure 5A depicts a CGRP-IP NEB (arrows and arrowheads). Sequential staining of this region for CCSP is illustrated in Figure 5B. Numerous CCSP-IP cells are found adjacent to the NEBs, whereas rare CCSP-IP cells are localized to the basal portion of the NEBs (arrowheads). Overlay of these images (Figure 5C) demonstrates a population of CCSP/CGRP dual immunopositive cells (arrowheads). In both normal and repairing epithelium, dual-immunopositive cells were preferentially located in the basal region of the NEBs.

Variant Clara Cell Subpopulations Are Associated with NEBs in the Normal Lung

To test the hypothesis that NEBs provide an environment for the maintenance of naphthalene-resistant CCSP-immunopositive cells (variant Clara cells) in the normal airway, we used dual immunohistochemistry to determine the cellular colocalization of CCSP and CYP-2F2 antigens in the NEB microenvironment. Figure 5, D–F, demonstrates the existence of cells that expresses the pan Clara cell marker CCSP but lack detectable levels of the phase I metabolizing enzyme CYP-2F2. CYP-2F2-IP cells were found adjacent to but not within NEBs (Figure 5, D and F, arrowheads). In contrast, CCSP-IP cells are located both adjacent to and within the NEB microenvironment (Figure 5E, arrowheads and arrows).

Discussion

The NEB Microenvironment Serves as a Reservoir for Airway Progenitor Cells

Our previous studies investigating mechanisms of repair after acute naphthalene-induced injury demonstrated that focal groups of pollutant-resistant progenitor cells localizing primarily to airway bifurcations served an im-

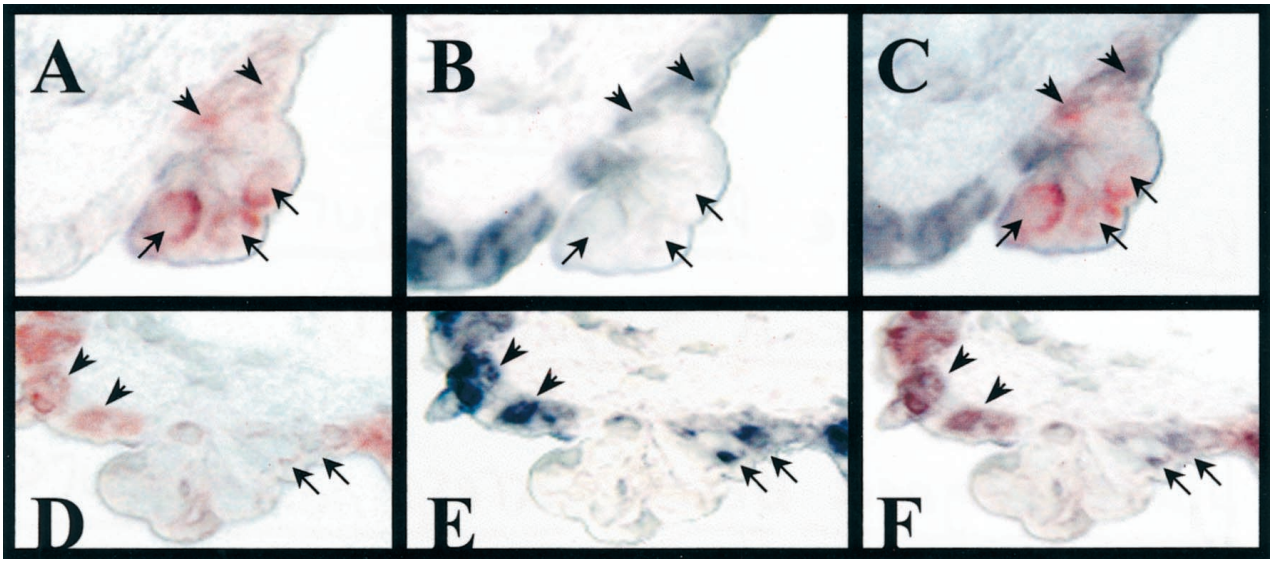

Figure 5. Two variant Clara cell subpopulations are associated with NEBs in the normal lung. Sequential immunohistochemistry (Materials and Methods) was used to detect CGRP (**A**) and CCSP (**B**) on the same section. Amine-ethyl carbizol (pink-red) was used to detect CGRP, and DAB (gray) was used to detect CCSP immunocomplexes. **C:** Image generated by overlaying **A** and **B**. **Long arrows** in **A–C** indicate CGRP-IP/CCSP-immunonegative cells, whereas **short arrows** mark cells expressing both CGRP and CCSP. Similar methods were used to detect the Clara cell markers P450–2F2 (**D**, amine-ethyl carbizol detection) and CCSP (**E**, DAB detection) on the same section. **F:** Image generated by overlaying **D** and **E**. **Short arrows** in **D–F** indicate P450–2F2-IP/CCSP-IP cells, and **long arrows** mark P450–2F2 immunonegative/CCSP-IP cells. Original magnification, ×1000.

portant role in epithelial regeneration.[35] In this model, repair of the Clara cell-depleted airway epithelium is associated with hyperplasia of NEBs (27). Whole-mount immunohistochemical analysis presented in our companion paper demonstrated that NEBs located at both branch points and segmental locations throughout the major axial pathway exhibit altered morphology characterized by a loss of corpuscular structure and intermingling of cuboidal CGRP-IP and squamous CGRP-immunonegative cells.[47] The current study was undertaken to investigate the hypothesis that PNE cells play a central role in airway regeneration after acute injury to an abundant airway epithelial progenitor-cell population. We demonstrate that regenerating Clara (CCSP-IP) cells colocalize to the same airway microenvironment as clusters of PNE (CGRP-IP) cells in the repairing lung. Both Clara and PNE cells proliferate in the repairing lung, indicating that they both serve as progenitor cell populations after Clara cell injury. Spatial colocalization of these cell types suggests that the NEB represents a unique microenvironment that harbors progenitor cells capable of restoring the injured airway epithelium after injury of pollutant-sensitive airway progenitor cells.

The Mitotic Activity of PNE Cells Is Modulated by Environmental Factors

The finding that the NEB microenvironment of repairing airways includes two mitotic populations 72 hours postnaphthalene injury can be explained by the existence of one or more naphthalene-resistant progenitor cells within this domain at the time of injury. A logical candidate based on these findings is the PNE cell. However, it has previously been demonstrated that PNE cells have a very low mitotic index in the normal lung and that, in certain model systems, their proliferation is not increased by agents known to cause PNE cell hyperplasia.[24,48,49] This has led to speculation that the PNE cell cannot function as a progenitor cell and that maintenance of PNE cells in the normal lung and PNE cell hyperplasia that accompanies certain types of lung pathology are accomplished through proliferation of a non-PNE progenitor cell that subsequently differentiates into a PNE cell (reviewed in 22). The observed increases in the PNE proliferative fraction during repair from naphthalene-induced Clara cell injury demonstrate that PNE cells do possess the capacity to proliferate and suggest that their mitotic activity can be modulated to various degrees by environmental factors. This is further supported by the demonstration of differences in progenitor-cell use that were dependent on the type of pollutant and target cells for injury. Qualitatively, the same proliferating-cell populations, CCSP-IP Clara and CGRP-IP PNE cells, were observed in repairing airways after either naphthalene- or ozone-induced injury, despite differences between these pollutants in their target cell selectivity. However, the PNE proliferative fraction after ozone-induced airway injury was only 8% of that observed during repair from naphthalene exposure. We show that the proliferative fraction of PNE cells increases as a result of compromised repair that is secondary to ablation of a principal progenitor-cell population. These data suggest that the NEB-associated regenerative cell population is distinct from previously identified airway progenitors. The demonstration of PNE cell hyperplasia in response to naphthalene-induced Clara cell injury, but not in response to ozone-induced ciliated cell injury, reveals an important mechanism in the pathogenesis of this airway anomaly that is common to many types of chronic human lung disease.[50–57]

Origin of Regenerating CCSP-Positive Cells

As mentioned above, the finding that regenerating Clara cells colocalize with NEB-associated PNE cells in the repairing lung suggests that a naphthalene-resistant progenitor with the capacity to differentiate into a Clara cell must exist in the NEB microenvironment. If so, there are two possible explanations for this finding: 1) PNE cells serve as a pluripotent stem cell population capable of regenerating Clara cell progenitors, or 2) a naphthalene-resistant progenitor cell population, distinct from PNE cells, must exist in the NEB microenvironment. Our finding of intermediate Clara/PNE cells within the NEB support the first hypothesis. These cells may represent a pluripotent stem cell similar to that described in the developing lung epithelium.[28] These data are also consistent with the known plasticity of tumor phenotypes observed between small-cell (neuroendocrine-derived) and non-small-cell (Clara/type-II cell-derived) lung cancers.[19] However, an argument can also be made that a non-PNE progenitor cell may be responsible for regeneration of Clara cells postnaphthalene injury. Two candidates for a naphthalene-resistant non-PNE progenitor are basal cells and modified Clara cells. Exclusion of basal cells from the NEB microenvironment[58,59] and preferential distribution of these cells to the trachea and the most proximal regions of the airway[14,60] diminish the possibility that basal cells function as progenitor cells for repopulation of the airway after acute naphthalene exposure. In contrast, studies investigating ultrastructural characteristics of cells with the NEB have revealed the existence of nonciliated epithelial cells termed “modified Clara cells” that are closely associated with PNE cells in a number of mammalian species.[58,59] Modified Clara cells are nonciliated epithelial cells that lack features typically associated with Clara, serous, or goblet cells of conducting airway epithelia. It is therefore possible that modified Clara cells that are associated with NEBs represent a morphologically variant nonciliated cell population that is resistant to naphthalene toxicity. In support of this hypothesis we demonstrate the existence of variant CCSP-immunopositive cell populations that localize to the same spatial domain as PNE cells. Variant Clara cells were distinguished from typical Clara cells by their lack of detectable CYP-2F2. We propose that variant Clara cells deficient in CYP-2F2 would be unable to metabolize naphthalene to the highly cytotoxic 1R, 2S-naphthalene epoxide and would therefore be resistant to the direct cytotoxicity of parenterally administered naphthalene. Therefore, it is possible that variant Clara cells function as

a naphthalene-resistant progenitor population, a property that accounts for the abundance of regenerating Clara cells in close proximity to NEBs. A similar deficiency in phase-I metabolism has been proposed to afford selective protection of hepatic oval cells from pollutant injury.[61,62] Mechanisms involved in maintenance and activation of airway progenitor cells are currently unknown but represent a focus for future studies.

Acknowledgment

We thank Dr. Jacob Finkelstein, Dr. Michael O'Reilly, Gregory Mango, and Kyung Hong for critical reading of the manuscript.

References

1. Rennard SI, Beckmann JD, Robbins RA: Biology of airway epithelial cells. The Lung: Scientific Foundations. Edited by RG Crystal, JB West. New York, Raven Press, pp 157–167
2. Carter PM, Heinly TL, Yates SW, Lieberman PL: Asthma: the irreversible airways disease. J Invest Allergol Clin Immunol 1997, 7:566–571
3. Frampton MW, Morrow PE, Torres A, Voter KZ, Whitin JC, Cox C, Speers DM, Tsai Y, Utell MJ: Effects of ozone on normal and potentially sensitive human subjects. Part II. Airway inflammation and responsiveness to ozone in nonsmokers and smokers. Res Rep Health Effects Inst 1997, 78:39–72
4. Bates, DV: The effects of air pollution on children. Environ Health Perspect 1995, 103(Suppl 6):49–53
5. Leikauf GD, Kline S, Albert RE, Baxter CS, Bernstein DI, Buncher CR: Evaluation of a possible association of urban air toxics and asthma. Environ Health Perspect 1995, 102(Suppl 6):253–271
6. Nakanishi K, Hiroi S, Kawai T, Suzuki M, Torikata C: Argyrophilic nucleolar-organizer region counts and DNA status in bronchioloalveolar epithelial hyperplasia and adenocarcinona of the lung. Hum Pathol 1998, 29:235–239
7. Betticher DC, Heighway J, Thatcher N, Hasleton PS: Abnormal expression of CCND1 and RB1 in resection margin epithelia of lung cancer patients. Br J Cancer 1997, 75:1761–1768
8. Smith AL, Hung J, Walker L, Rogers TE, Vuitch F, Lee E, Gazear AF: Extensive areas of aneuploidy are present in the respiratory epithelium of lung cancer patients. Br J Cancer 1996, 73:203–209
9. Agapitos E. Molo F, Tomatis L, Katsouyanni K, Lipworth L, Delsedime L, Kalandidi A, Karakatsani A, Riboli E, Saracci R, Trichopoulos D: Epithelial, possibly precancerous, lesions of the lung in relation to smoking, passive smoking, and socio-demographic variables. Scan J Soc Med 1996, 24:259–263
10. Crowell RE, Gilliland FD, Temes RT, Harms HJ, Neft RE, Heaphy E, Auckley DH, Crooks LA, Jordan SW, Samet JM, Lechner JF, Belinsky SA: Detection of trisomy 7 in nonmalignant bronchial epithelium from lung cancer patients and individuals at risk for lung cancer. Cancer Epidemiol Biomarkers Prevent 1996, 5:631–637
11. Trichopoulos D, Mollo F, Tomatis L, Agapitos E, Delsedime L, Zavitsanos X, Kalandidi A, Katsouyanni K, Riboli E, Saracci R: Active and passive smoking and pathological indicators of lung cancer risk in an autopsy study. J Am Med Assoc 1992, 268:1697–1701
12. Alavanja MC, Brownson RC, Boice JD, Hock E: Preexisting lung disease and lung cancer among nonsmoking women. Am J Epidemiol 1992, 136:623–632
13. Osann KE: Lung cancer in women: the importance of smoking, family history of cancer and medical history of respiratory disease. Cancer Res 1991, 51:4893–4897
14. Plopper CG, Mariassy AT, Wilson DW, Alley JL, Nishio SJ, Nettesheim P: Comparison of nonciliated trachial epithelial cells in six mammalian species: ultrastructure and population densities. Exp Lung Res 1983, 5:281–294
15. Plopper CG, Dungworth DL: Structure, function, cell injury, and cell renewal of bronchiolar, and alveolar epithelium. Lung Carcinoma. Edited by EM McDowell. London, Churchill Livingstone. 1987, pp 29–44
16. Devereux TR, Donim BA, Philpot RM: Xenobiotic metabolism by isolated pulmonary cells. Pharmacol Ther 1989, 41:243–256
17. Evans MJ, Johnson LV, Stephens RJ, Freeman G: Renewal of the terminal bronchiolar epithelium in the rat following exposure to NO_2 or O_3. Lab Invest 1976, 35:246–257
18. Evans MJ, Cabral-Anderson LJ, Freeman G: Role of the Clara cell in renewal of the bronchiolar epithelium. Lab Invest 1978, 38:648–653
19. Linnoila RI, Mulshine JL, Steinberg SM, Funa K, Matthews MJ, Cotelingam JD, Gazdar AF: Neuroendocrine differentiation in endocrine and nonendocrine lung carcinomas. Am J Clin Pathol 1988, 90:641–652
20. Linnoila RI, Jensen SM, Steinberg SM, Mulshine JL, Eggleston JC, Gazdar AF: Peripheral airway cell marker expression in non-small cell lung carcinoma. Am J Clin Pathol 1992, 97:233–243
21. Linnoila RI, Aisner SC: Pathology of lung cancer: exercise in classification. Lung Cancer. Current Clinical Oncology. Edited by BE Johnson, DH Johnson. New York, John Wiley & Sons. 1994, pp 73–95
22. Gosney JR: Pulmonary neuroendocrine cell system in pediatric and adult lung disease. Microsc Res Tech 1997, 37:107–113
23. Linnoila RI: Effects of diethylnitrosamine of lung neuroepithelial cells. Exp Lung Res 1982, 3:225–236
24. Sunday ME, Willett CG, Patidar K, Graham SA: Modulation of oncogene and tumor suppressor gene expression in a hamster model of chronic lung injury with varying degrees of pulmonary neuroendocrine cell hyperplasia. Lab Invest 1994, 70:875–888
25. Sunday ME, Willett CG: Induction and spontaneous regression of intense pulmonary neuroendocrine cell differentiation in a model of preneoplastic lung injury. Cancer Res 1992, 52(Suppl):2677S–2686S
26. Ito T, Ikemi Y, Ohmori K, Kiamura H, Kanisawa M: Airway epithelial cell changes in rats exposed to 0.25 ppm ozone for 20 months. Exp Toxic Pathol 1994, 46:1–6
27. Stevens T, McBride JT, Peake JL, Pinkerton KE, Stripp BR: Cell proliferation contributes to PNEC hyperplasia following acute airway injury in adult mice. Am J Physiol 1997, 272:L486–L493
28. Wuenschell CW, Sunday ME, Singh G, Minoo P, Slavkin HD, Warburton D: Embryonic mouse lung epithelial progenitor cells co-express immunohistochemial markers of diverse mature cell lineages. J Histochem Cytochem 1996, 44:113–123
29. Wert SE, Glasser SW, Korfhagen TR, Whitsett JA: Transcriptional elements from the human SP-C gene direct expression in the primordial respiratory epithelium of transgenic mice. Dev Biol 1993, 156: 426–443
30. Cutz E, Gillan JE, Perrin DG: Pulmonary neuroendocrine cell system: an overview of cell biology and pathology with emphasis of pediatric lung disease. Pulmonary Disease: Perspectives in Pediatric Pathology, vol. 18. Edited by FB Askin , FC Langston, J Bernstein. Basel, Kager, 1995, pp 32–70
31. Holt RF, McNelly NA, Sorokin SP: Dynamics of neuroepithelial body (NEB) formation in developing hamster lung: light microscopic autoradiography after ^{3}H-thymidine labeling in vivo. Anat Rec 1990, 227: 340–350
32. Sunday ME, Hua J, Dai HB, Nusrat A, Torday JS: Bombesin increases fetal lung growth and maturation in utero and in organ cultures. Am J Respir Cell Mol Biol 1990, 3:199–205
33. Sunday ME, Choi N, Spindel ER, Chin WW, Mark E: Gastrin-releasing peptide gene expression in small cell and large cell undifferentiated lung carcinomas. Hum Pathol 1991, 22:1030–1039
34. Sunday ME, Hua J, Reyes B, Masui H, Torday JS: Anti-bombesin antibodies modulate fetal murine lung growth and maturation in utero and in organ cultures. Anat Rec 1993, 236:25–32
35. Stripp BR, Maxson K, Mera R, Singh G: Plasticity of airway cell proliferation and gene expression after acute naphthalene injury. Am J Physiol 1995, 269:L791–L799
36. Plopper CG, Suverkropp C, Morin D, Nishio S, Buckpitt A: Relationship of cytochrome P-450 activity to Clara cell cytotoxicity. I. Histopathologic comparison of the respiratory tract of mice, rats and hamsters after parenteral administration of naphthalene. J Pharmacol Exp Ther 1992, 26:353–363
37. Mahvi D, Bank H, Harley R: Morphology of a naphthalene-induced bronchiolar lesion. Am J Pathol 1977, 86:559–572
38. Buckpitt AR, Buonarati M, Avey LB, Chang AM, Morin D, Plopper C: Relationship of cytochrome P450 activity to Clara cell cytotoxicity. II.

Comparison of stereoselectivity of naphthalene epoxidation in lung and nasal mucosa of mouse, hamster, rat and rhesus monkey. J Pharmacol Exp Ther 1992, 261:364–372
39. Mango GW, Johnston CJ, Reynolds SD, Finkelstein JN, Plopper CG, Stripp BR: Clara cell secretory protein deficiency increases oxidant stress response in conducting airways. Am J Physiol 1998, 275: L348–356
40. Willett CG, Shahsafei A, Graham SA, Sunday ME: CD10/neutral endopeptidase inhibition augments pulmonary neuroendocrine cell hyperplasia in hamsters treated with diethylmitrosamine, and hyperoxia. Am J Respir Cell Mol Biol 1999, 21:13–20
41. Borges, M, Linnoila RI, van de Velde HJK, Chen H, Nelkin BD, Mabry M, Baylin SB, Ball DW: An achaete-scute holologue essential for neuroendocrine differentiation in the lung. Nature 1997, 386:852–855
42. Cardoso WV, Stewart LG, Pinkerton KE, Ji C, Hook GE, Singh G, Katyal SL, Thurlbeck WM, Plopper CG: Secretory product expression during Clara cell differentiation in the rabbit and rat. Am J Physiol 1993, 264:L543–L552
43. Ten Have-Opbroek AA, De Vries EC: Clara cell differentiation in the mouse: ultrastructural morphology and cytochemistry for surfactant protein A and Clara cell 10 kD protein. Microsc Res Tech 1993, 26:400–411
44. Stripp BR, Lund J, Mango GW, Doyan KC, Johnston C, Juntenby K, Nord M, Whitsett JA: Clara cell secretory protein: a determinant of PCB bioaccumulation in mammals. Am J Physiol 1996, 271:L656–L664
45. Reynolds SD, Mango GW, Gelein R, Boe I-M, Lund J, Stripp BR: Normal function and lack of fibronectin accumulation in kidneys of Clara cell secretory protein/uteroglobin deficient mice. Am J Kidney Dis 1999, 33:541–551
46. Khoor A, Gray ME, Singh G, Stahlman MT: Ontogeny of Clara cell-specific protein and its mRNA: their association with neuroepithelial bodies in human fetal lung and in bronchopulmonary dysplasia. J Histochem Cytochem 1996, 44:1429–1438
47. Peake JL, Reynolds SD, Stripp BR, Stephens KE, Pinkerton KE: Alteration of pulmonary neuroendocrine cells during repair of naphthalene-induced airway injury. Am J Pathol 2000, 156:279–286
48. Hoyt RF, McNelly NA, McDowell EM, Sorokin SP: Neuroepithelial bodies stimulate proliferation of airway epithelium in fetal hamster lung. Am J Physiol 1991, 260:L234–L240
49. Montuenga LM, Springall DR, Gaer J, Winter FJ, Zhao L, McBride JT, Taylor KM, Barer G, Polak JM: CGRP-immunoreactive endocrine cell proliferation in normal, and hypoxic rat lung studied by immunocytochemical detection of incorporation of 5′-bromodeoxyuridine: Cell Tissue Res 1992, 268:9–15
50. Alshehri M, Cutz E, Banzhoff A, Canny G: Hyperplasia of pulmonary neuroendocrine cells in a case of childhood pulmonary emphysema. Chest 1997, 112:553–556
51. Aguayo SM: Determinants of susceptibility to cigarette smoke: potential roles for neuroendocrine cells and neuropeptides in airway inflammation, airway wall remodeling, and chronic airflow obstruction. Am J Respir Crit Care Med 1994, 149:1692–1698
52. Aguayo SM, King TE, Waldron JA, Sherritt KM, Kane MA, Miller YE: Increased pulmonary neuroendocrine cells with bombesin-like immunoreactivity in adult patients with eosinophilic granuloma. J Clin Invest 1990, 86:838–844
53. Dovey M, Wisseman CL, Roggli VL, Romans GM, Shelburne JD, Spock A: Ultrastructural morphology of the lung in cystic fibrosis. J Submicrosc Cytol Pathol 1989, 21:521–534
54. Gould VE, Linnoila RI, Memoli VA, Warren WH: Neuroendocrine components of the bronchopulmonary tract: hyperplasias, dysplasias, and neoplasms. Lab Invest 1983, 49:519–537
55. Cutz E, Perrin DG, Hackman R, Czegledy-Nagy EN: Maternal smoking and pulmonary neuroendocrine cells in sudden infant death syndrome. Pediatrics 1996, 98:668–672
56. Gillan JE, Cutz E: Abnormal pulmonary bombesin immunoreactive cells in Wilson-Mikity syndrome (pulmonary dysmaturity) and bronchopulmonary dysplasia. Pediatr Pathol 1993, 13:165–180
57. Johnson DE, Lock JE, Elde RP, Thompson TR: Pulmonary neuroendocrine cells in hyaline membrane disease and bronchopulmonary dysplasia. Pediatr Res 1982, 16:446–454
58. van Lommel A, Lauweryns JM: Neuroepithelial bodies in the fawn hooded rat lung: morphological and neuroanatomical evidence for a sensory innervation. J Anat 1993, 183:553–566
59. Hung KS, Loosli CG: Bronchiolar neuro-epithelial bodies in the neonatal mouse lungs. Am J Anat 1974, 140:191–199
60. Plopper CG, Halsebo JE, Berger WJ, Sonstegard KS, Nettesheim P: Distribution of non-ciliated bronchiolar epithelial (Clara) cells in the intra- and extrapulmonary airways of the rabbit. Exp Lung Res 1983, 5:79–98
61. Mathis GA, Walls SA, D'Amico P, Gengo TF, Sirica AE: Enzyme profile of rat bile ductular epithelial cells in reference to the resistance phenotype in hepatocarcinogenesis. Hepatology 1989, 9:477–485
62. Sirica AE, Mathis GA, Sano N, Elmore LW: Isolation, culture, and transplantation of intrahepatic biliary epithelial cells, and oval cells. Pathobiology 1990, 58:44–64