

Cell proliferation contributes to PNEC hyperplasia after acute airway injury

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Stevens, Timothy P., John T. McBride, Janice L. Peake, Kent E. Pinkerton, and Barry R. Stripp. Cell proliferation contributes to PNEC hyperplasia after acute airway injury. *Am. J. Physiol.* 272 (Lung Cell. Mol. Physiol. 16): L486–L493, 1997.—Pulmonary neuroendocrine cells (PNECs) are airway epithelial cells that are capable of secreting a variety of neuropeptides. PNECs are scattered throughout the bronchial tree either as individual cells or clusters of cells termed neuroepithelial bodies (NEBs). PNECs and their secretory peptides have been considered to play a role in fetal lung development. Although the normal physiological function of PNECs and neuropeptides in normal adult lungs and in repair from lung injury is not known, PNEC hyperplasia has been associated with chronic lung diseases, such as bronchopulmonary dysplasia, and with chronic exposures, such as hypoxia, tobacco smoke, nitrosamines, and ozone. To evaluate changes in PNEC number and distribution after acute airway injury, FVB/n mice were treated with either naphthalene or vehicle. Naphthalene is an aromatic hydrocarbon that, at the dose used in this study, selectively destroys nonciliated bronchial epithelial cells (Clara cells) through cytochrome *P*-450-mediated metabolic activation into cytotoxic epoxides. PNECs were identified by immunohistochemical analysis of calcitonin gene-related peptide-like immunoreactivity (CGRP-IR). Proliferating cells were marked with [³H]thymidine incorporation. Acute naphthalene toxicity results in PNEC hyperplasia that is detectable after 5 days of recovery. PNEC hyperplasia is characterized by increased numbers of NEBs without significant changes in the number of isolated PNECs and by increased [³H]thymidine labeling of CGRP-IR cells. These data show that cell proliferation contributes to PNEC hyperplasia after acute airway injury and suggest that PNECs may be capable of more rapidly increasing their number in response to injury than previously recognized.

calcitonin gene-related peptide; lung; naphthalene; neuroepithelial body; pulmonary neuroendocrine cells

PULMONARY NEUROENDOCRINE CELLS (PNECs) are sparsely distributed airway epithelial cells that have been implicated in fetal lung growth and differentiation. PNECs secrete several neuropeptides, including gastrin-releasing peptide (GRP, a mammalian homologue of the amphibian peptide bombesin), calcitonin gene-related peptide (CGRP), and chromogranin A (CGA) (37). In fetal mouse lung, GRP has been shown to enhance [³H]choline incorporation into saturated phosphatidylcholine, [³H]leucine incorporation into protein, [³H]thymidine incorporation into DNA, and to increase the relative number of type II pneumocytes (35, 36). In murine lung explants, bombesin enhances branching morphogenesis but does not increase cell proliferation

(1, 15). GRP is mitogenic for Swiss 3T3 fibroblasts (27), PNECs (31), and bronchial epithelial cells (45). In airway development, PNECs have paracrine effects on cell proliferation. Hoyt et al. (8) documented an increase in proliferation of non-PNECs in the immediate vicinity of neuroepithelial bodies (NEBs) compared with more distant regions.

PNEC hyperplasia has been associated with chronic lung diseases, such as bronchopulmonary dysplasia (13), and with pollutant exposures, such as tobacco smoke (37), ozone (11), and nitrosamines (18, 38, 40). The role of PNECs and their secretory neuropeptides during repair from injury is not clear. Classically, NEBs have been considered to be chemoreceptors responding to airway hypoxia (17, 47). GRP is both a vasoconstrictor and bronchoconstrictor (10, 16, 28), whereas CGRP is a pulmonary vasodilator (20, 43) and a bronchoconstrictor (5). Consequently, PNECs and their secretory peptides GRP and CGRP have been implicated in regulating airway tone and pulmonary blood flow during respiration.

PNECs can be isolated individual airway epithelial cells or innervated clusters of cells termed NEBs. NEBs are located exclusively within the conducting airways and are commonly found at airway branch points (4, 9, 30). Studies investigating the mechanism for increased PNEC numbers observed during lung development have detected few mitotic figures or little [³H]thymidine labeling, suggesting that mature PNECs have a reduced capacity to undergo mitosis (7, 8). PNEC hyperplasia, defined as increased numbers of PNECs, may then be due to differentiation of progenitor cells (8, 31, 40). Alternatively, an apparent PNEC hyperplasia may result from enhanced PNEC detection when intracellular levels of neuropeptides are increased to levels detectable by immunohistochemistry such as would occur with changes in gene transcription or peptide secretion. Sunday and Willet (38) suggested that altered neuropeptide gene transcription contributes to the induction of PNEC hyperplasia and later spontaneous regression after diethylnitrosamine (DEN) injections and continuous hyperoxia (38). Reduced CGRP secretion during acute hypoxia has been proposed by Roncalli et al. (26) to explain observed increases in CGRP-like immunoreactivity (CGRP-IR) within PNECs as early as 4 h after exposure to hypoxia.

Studies investigating the mechanism of PNEC hyperplasia after injury are difficult because of the chronic nature of the injury needed to observe hyperplasia. In a hamster model, Linnoila (18) described PNEC hyperplasia and increased neuroendocrine cell (NEC) prolifera-

tion after a 4-wk exposure to twice weekly injections of DEN. The greatest changes were seen after a 3-mo exposure. Studies combining DEN and hyperoxia or hyperoxia alone have shown increased lung neuropeptide levels in hamsters 3 wk or more after exposure (22, 38, 40), although these changes may not be sustained (38). For tobacco smoke, PNEC hyperplasia was shown in hamsters after a 3-wk exposure (41). PNEC hyperplasia has also been described in lungs from neonatal rabbits adapted to high altitude and chronic hypoxia (14, 42) but not in adult rats exposed to 7 days of hypoxia (21).

In the present study, we have used naphthalene injury to the conducting airway epithelium to evoke a repair response. This model was chosen to understand how selective conducting airway injury participates in the establishment of PNEC hyperplasia. Naphthalene is a component of cigarette smoke (29) that, in the mouse, causes selective airway cell injury due to activation to cytotoxic epoxides by cytochrome *P*-450 2F2 and 2B within nonciliated (Clara) cells (25). We have previously shown that airway branch points are sites of early nonciliated bronchial epithelial cell (Clara cell) differentiation after injury and a preferential site of cell proliferation at the 72-h postnaphthalene time point (34). This pattern of differentiation and proliferation is similar to the spatial distribution of NEBs in the lung. We investigated the hypothesis that PNECs respond to acute conducting airway cell injury by increasing their numbers through a mechanism involving both cell proliferation and differentiation.

MATERIALS AND METHODS

Animals and treatments. Naphthalene (300 mg/kg) in corn oil vehicle or vehicle alone was administered intraperitoneally to 7- to 9-wk-old male FVB/n mice (6 mice/group). Animals were obtained from Taconic Farms Animal Breeders (Germantown, NY) and were housed in a pathogen-free environment during a 5-day acclimatization and observation period before experimental treatments. [³H]thymidine (20 Ci/mmol; New England Nuclear, Boston, MA) was administered intraperitoneally at a dose of 2.5 μ Ci/kg between 36 and 72 h after naphthalene or corn oil treatments. Lungs were harvested at 5 days after injury and inflation fixed in 10% neutral buffered Formalin at 20 cm pressure. The right lower lobe was embedded in paraffin, and 5- μ m sections were taken parallel to the lobar bronchus to include the major airway branches leading to terminal bronchioles.

Immunohistochemistry and tissue autoradiography. Tissues were deparaffinized in xylene and hydrated in graded alcohol washes. Each section was blocked by incubation with 10% fetal bovine serum (FBS) in phosphate-buffered saline (PBS). Polyclonal rabbit anti-rat CGRP (Sigma, St. Louis, MO) was used as primary antibody at a dilution of 1:1,000 in 10% FBS. Slides were washed in PBS, and an alkaline phosphatase conjugated monoclonal mouse anti-rabbit antibody (Sigma) was used as a secondary antibody at a 1:150 dilution in 10% FBS. After three PBS washes, alkaline phosphatase was detected using a BCIP/NBT kit according to the manufacturers instructions (Sigma).

For [³H]thymidine labeling studies, biotinylated secondary antibody with a horseradish peroxidase 3,3'-diaminobenzidine (DAB) color development system was used. The peroxidase detection system was used because it permitted tritium

detection. Tissues were processed for immunohistochemical staining as outlined above, except endogenous peroxidases were quenched with 0.03% hydrogen peroxide in methanol for 15 min before blocking. Biotinylated goat anti-rabbit antiserum (Cappel Laboratories, Oganon Teknika, West Chester, PA) was used at a dilution of 1:1,000 in 10% FBS. Secondary antibody was detected using a streptavidin solution (Vectastain ABC; Vector Laboratories, Burlingame, CA) prepared according to the manufacturer's instructions, followed by a DAB color development solution (Vector). Tissues were counterstained with hematoxylin. As a second marker for PNECs, CGA immunostaining was performed using polyclonal rabbit anti-bovine SP-1/CGA (Incstar, Stillwater, MN) at a dilution of 1:2,000. This primary antibody was incubated with the tissue overnight at 4°C. The secondary antibody was biotinylated goat anti-rabbit antibody with avidin-biotin horseradish peroxidase (Vector) used with a DAB color development system.

Tritium labeling of lung tissue was detected using autoradiography. Tissues were double dipped in NBT II photoemulsion (Kodak, Rochester, NY), exposed for 7 days, and developed with D-19 (Kodak) according to the manufacturers instructions. Tissues were dehydrated in graded alcohols, mounted using a xylene-based mounting media, and covered with coverslips. To assess the adequacy and consistency of [³H]thymidine labeling, each animal's right testis was taken and digested, and total DNA was isolated. [³H]thymidine incorporation into total DNA was then measured. There was comparable tritium incorporation into all animals, indicating consistent and reliable delivery of the [³H]thymidine.

Morphometry and statistical analysis. Five sections at 10- μ m intervals cut through regions of optimal airway branching were immunostained to localize CGRP. The middle (third) section was used as the reference slide from which all measurements were based. A digital image that included all conducting airway epithelium observed in this section was made. The total airway epithelial basement membrane (BM) length and the length of BM subtending CGRP-IR regions were traced and measured using Metamorph image analysis software. To discriminate isolated immunoreactive cells from larger groups, each CGRP-IR region was followed through two serial sections on each side of the reference section (total span = 45 μ m). An isolated PNEC was defined as a CGRP-IR region containing only one nucleus or $\leq 15 \mu\text{m}^2$. Collections of PNECs, i.e., NEBs, were defined as CGRP-IR regions $> 15 \mu\text{m}^2$. Data were collected from each animal and analyzed using the Student's *t*-test for unpaired samples.

The length of each CGRP-IR region was determined immunohistochemically using an alkaline phosphatase detection system. For CGRP-IR cell nuclei counts and autoradiography, one section from each animal was subjected to analysis. Counts for CGRP-IR nuclei were determined in tissues stained for CGRP-IR cells using horseradish peroxidase and DAB color development counterstained with hematoxylin. Total BM was traced and measured as above. CGRP-IR cell nuclei were then counted and expressed per millimeter BM. Autoradiography was performed on these tissues to determine the thymidine labeling index of CGRP-IR cells. To quantitate [³H]thymidine labeling of CGRP-IR cells, the number of cells that were both CGRP immunoreactive and that had at least five silver grains overlying the nucleus was expressed as a percentage of all CGRP-IR cells. An effort was made to count similar numbers of CGRP-IR cells. An average of 55 CGRP-IR cells was counted from naphthalene-treated animals ($n = 6$ animals; 1 tissue section from each animal), and 30 CGRP-IR cells were counted from vehicle only animals ($n = 6$ animals; 2 sections from each animal). The presence of

tritium in a cell nucleus was indicated by silver grains overlying the nucleus. We chose five silver grains overlying the nucleus as indicative of cell proliferation based on a comparison between the number of silver grains overlying intestinal villous crypt and apical enterocytes. The background signal for thymidine labeling was extremely low. Data were collected from each animal and analyzed using the Student's *t*-test for unpaired samples.

RESULTS

Airway CGRP-IR is increased after acute epithelial injury. Airway epithelium from mice treated with either a single dose of naphthalene or corn oil (vehicle) were analyzed at 5 days recovery for CGRP-IR using immunohistochemistry for the PNEC marker CGRP. Figure 1 shows a representative photomicrograph demonstrating increased CGRP immunostaining of tissue prepared from mice 5 days postnaphthalene treatment vs. mice treated with corn oil vehicle alone. Quantitative measurements demonstrated a fourfold increase in areas of CGRP immunoreactivity, as shown in Fig. 2. The percentage of BM subtending CGRP-positive cells was $2.32 \pm 0.25\%$ 5 days after naphthalene treatment compared with $0.38 \pm 0.11\%$ in vehicle-treated animals ($P < 0.001$). Similar increases were observed in the number of CGRP-IR cell nuclei per millimeter

BM (2.68 ± 0.21 vs. 0.63 ± 0.27 , $P < 0.001$), representing a similar fourfold increase after naphthalene treatment.

Acute airway injury is associated with increased [³H]thymidine incorporation into CGRP-IR cells. To help understand the basis for the increased number of PNECs, CGRP immunoreactive cells were evaluated for proliferation by [³H]thymidine labeling. Cells actively synthesizing DNA at the time of [³H]thymidine injection were detected by the presence of black silver grains. Cells were considered to have significant labeling if at least five silver grains were overlying the nucleus (Fig. 3). The number of proliferating CGRP-IR cells per millimeter BM was significantly greater in naphthalene-treated animals (0.324 ± 0.052) than in vehicle only controls (0.025 ± 0.025 , $P < 0.001$). To further characterize differences in cell proliferation, the proliferative index of CGRP-IR cells was determined. A significantly greater percentage of CGRP-IR cells were tritium labeled in naphthalene-treated animals ($12.0 \pm 1.4\%$) than in vehicle only animals ($3.3 \pm 3.3\%$, $P = 0.04$; Table 1).

Analysis of CGRP-IR region size, number, and cellularity. Because analysis of CGRP immunostained airways demonstrated PNEC hyperplasia, we sought to

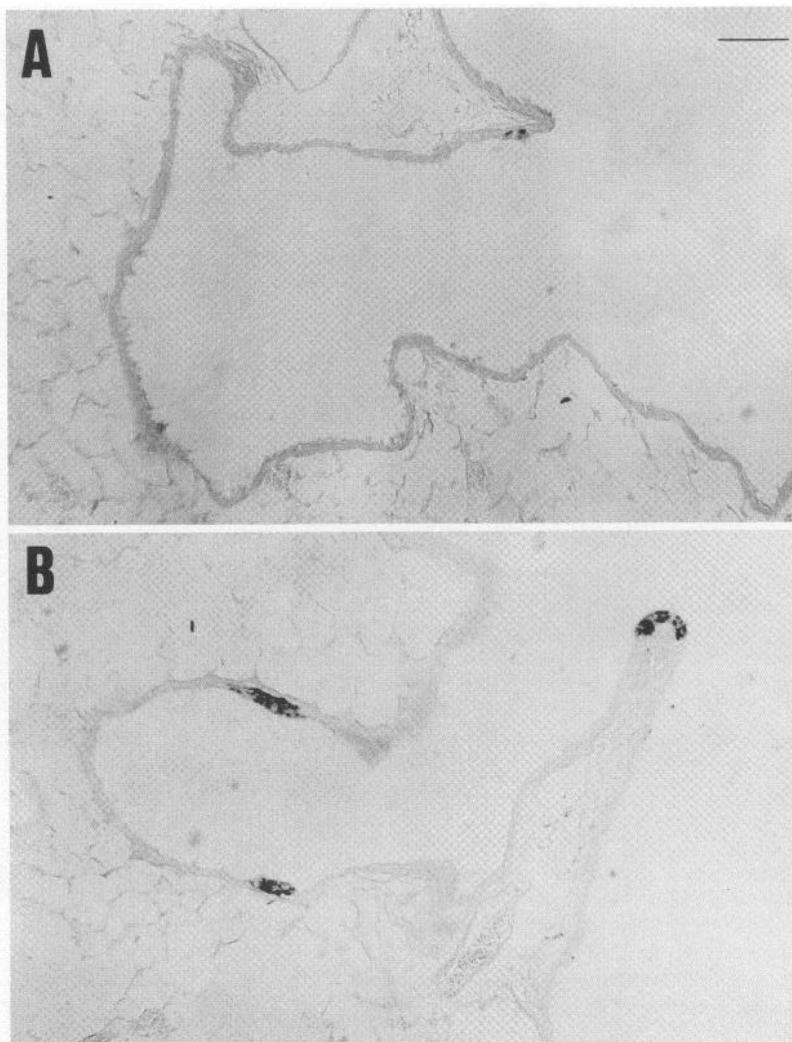


Fig. 1. Increased calcitonin gene-related peptide (CGRP) immunostaining. Increased CGRP-like immunoreactivity (CGRP-IR) is demonstrated in lung tissue from a naphthalene-treated animal (B) compared with a corn oil-treated control animal (A). Bar = 50 μ m.

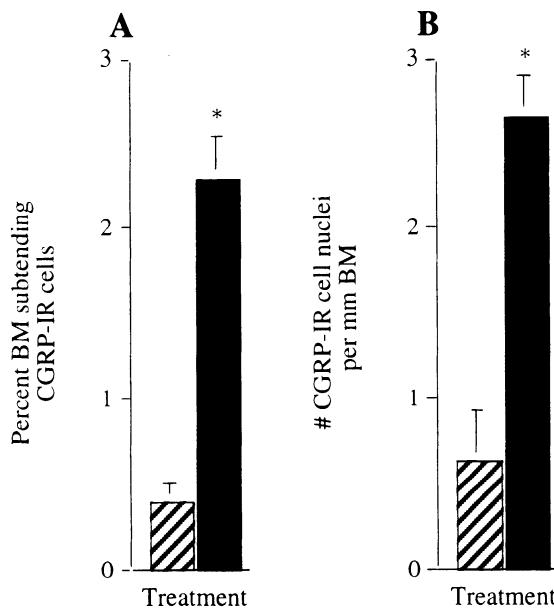


Fig. 2. Pulmonary neuroendocrine cell (PNEC) hyperplasia. A: increased area of CGRP immunoreactivity is expressed as the percentage of total airway basement membrane (BM) covered with CGRP-IR staining in vehicle only (hatched bars) vs. naphthalene (filled bars)-treated animals. B: increased numbers of CGRP-IR cells (PNECs) are represented/mm BM. Results are presented as means + SE for each treatment condition; * $P < 0.01$.

characterize the spatial pattern of this increase using several morphometric measurements. An increase in the number of CGRP-IR regions per millimeter BM was observed in naphthalene-treated animals (0.102 ± 0.024) vs. controls (0.058 ± 0.074 , $P < 0.001$). To determine the surface area of each NEB, each CGRP-IR region present on the reference slide was followed on adjacent serial sections. Analysis revealed a roughly sixfold increase in the number of CGRP-IR regions $>15 \mu\text{m}^2/\text{mm BM}$ in naphthalene-treated animals (0.529 ± 0.068) compared with vehicle-treated animals (0.089 ± 0.024 , $P < 0.001$; see Fig. 4). Analysis of the number of CGRP-IR regions $\leq 15 \mu\text{m}^2/\text{mm BM}$ did not reveal a significant difference in naphthalene-treated (0.033 ± 0.012) vs. vehicle-treated (0.014 ± 0.0089 , $P = 0.22$) animals. In separate sections from the same animals, the number of CGRP-IR regions per millimeter BM was analyzed by the number of CGRP-IR cell nuclei within each CGRP-IR region. The number of regions containing more than one CGRP-IR cell nucleus was $0.463 \pm 0.055/\text{mm BM}$ in naphthalene-treated animals vs. $0.110 \pm 0.056/\text{mm BM}$ in vehicle-treated animals ($P = 0.001$), indicating preferential induction of increased numbers of CGRP-IR PNECs in NEBs. The number of isolated PNECs per millimeter BM was not significantly different in naphthalene-treated (0.064 ± 0.024) vs. vehicle-treated (0.038 ± 0.019 , $P = 0.41$) animals. The significantly increased number of CGRP-IR regions $>15 \mu\text{m}^2/\text{mm BM}$ and CGRP-IR regions with more than one CGRP-IR nucleus per millimeter BM suggests an increased number of NEBs rather than an increased number of isolated PNECs. Although not morphometrically studied, CGRP-IR nerve fibers were frequently observed innervating NEBs. Of note, few

inflammatory cells were seen in any of the tissue sections.

Immunohistochemistry for CGA was used to confirm regions containing PNECs. There was excellent correlation between regions of CGA-like immunoreactivity and CGRP-IR on adjacent serial sections. However, the CGA antibody reaction was considerably less intense than that for CGRP. This may be due to low cross-reactivity between anti-bovine CGA antibody and mouse CGA.

Measurements of the size of each NEB did not reveal significant differences. The average length of a CGRP-IR region in control animals compared with naphthalene-treated animals was 34.4 ± 3.9 and $42.9 \pm 4.0 \mu\text{m}$, respectively ($P = 0.16$). The number of CGRP-IR cell nuclei per CGRP-IR region in control vs. treated animals was 4.4 ± 1.1 vs. 5.25 ± 0.4 ($P = 0.45$). Together, these data indicate that recovery from acute naphthalene injury results in increased numbers of NEBs but no change in the average size of each NEB and no change in the number of isolated PNECs.

DISCUSSION

Acute airway injury results in rapid PNEC hyperplasia. We have demonstrated that repair from acute injury to the conducting airway epithelium is associated with increased CGRP-IR characterized by a significant increase in the proportion of airway BM subtending CGRP-IR cells. In other studies, increased CGRP-IR has been attributed to cell hypertrophy (32) and increased intracellular CGRP content (19, 26) rather than cell hyperplasia. In our model, we have shown an increased number of CGRP-IR cells per millimeter BM. Furthermore, the mechanism responsible for increased numbers of CGRP-IR cells includes cell proliferation, as indicated by an increased [^3H]thymidine labeling index. By our current methods, the proportion of PNEC hyperplasia directly attributable to cell proliferation cannot be determined. Consequently, mechanisms in addition to cell proliferation may be contributing to the observed PNEC hyperplasia in our study. In models of PNEC hyperplasia in which little cell proliferation is seen, it has been suggested that PNEC hyperplasia may be the result of differentiation of mature PNECs from epithelial stem cells, undifferentiated but committed PNECs, or immature PNECs (38). Our data are consistent with the possibility that PNEC differentiation along with cell proliferation contribute to PNEC hyperplasia after acute airway injury.

PNEC hyperplasia has been described after chronic exposures to several classes of lung toxicants. Oxidant stress, such as continuous exposure to ozone (11) or hyperoxia (23), has been associated with PNEC hyperplasia as have hypoxia (14) and living at high altitude (42). After acute inhalation burn injury in humans, elevated serum calcitonin persisting for up to eight years has been described. However, changes in the NEC population were not able to be assessed (2). Both mainstream tobacco smoke (41) and sidestream tobacco smoke (12) are associated with PNEC hyperplasia. With tobacco smoke, the effect on PNEC number has been attributed to the following two smoke subfrac-

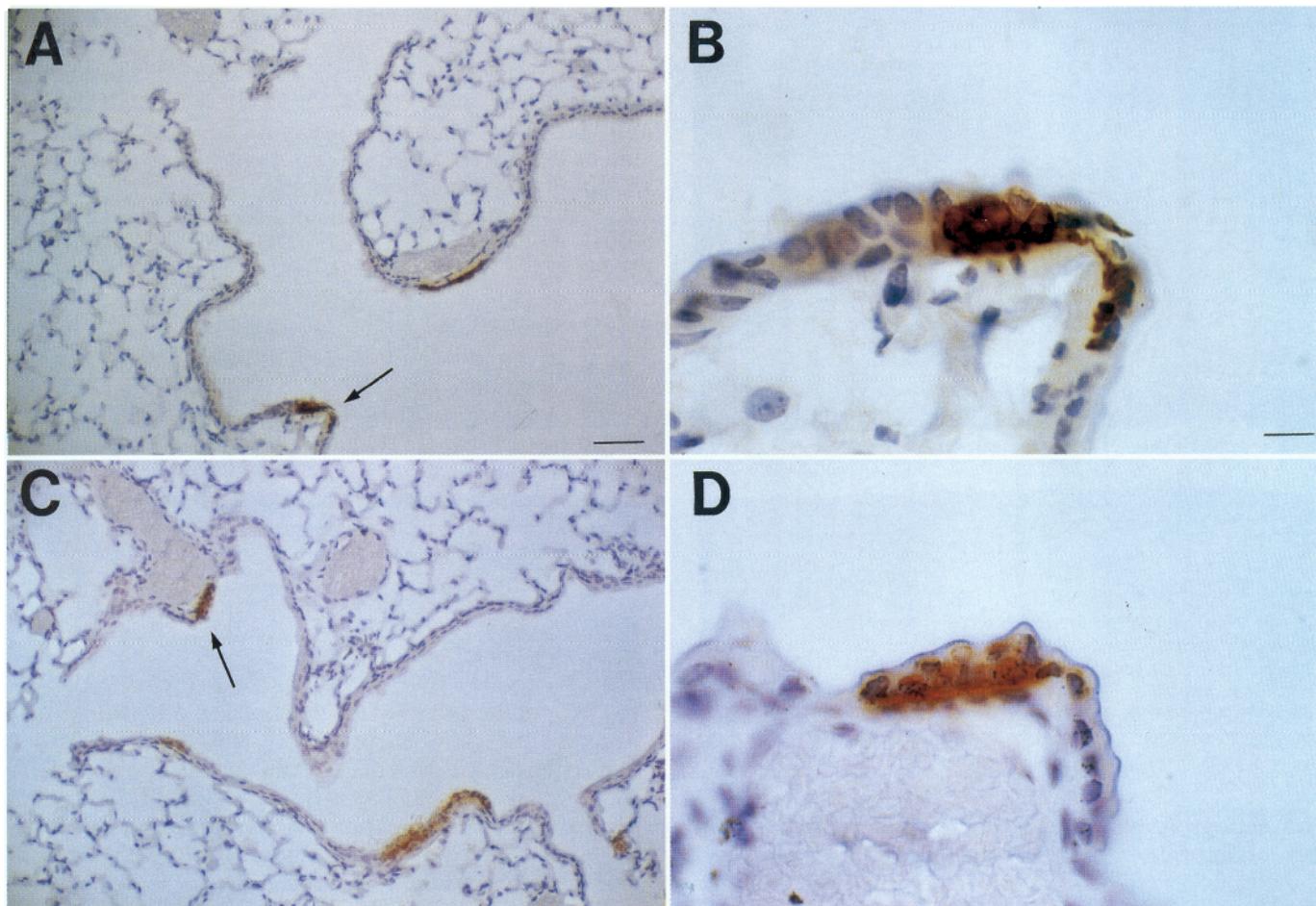


Fig. 3. $[^3\text{H}]$ thymidine labeling is increased during repair from naphthalene injury. Increased cell proliferation within and adjacent to a neuroepithelial body is demonstrated comparing lung tissue from a corn oil-treated control animal (*A* and *B*) with a naphthalene-treated animal (*C* and *D*). Tissues were immunostained for CGRP and labeled with $[^3\text{H}]$ thymidine (silver grains). Arrows in *A* and *C* identify CGRP-IR regions shown in *B* and *D*. *A* and *C*: bar = 50 μm ; *B* and *D*: bar = 10 μm .

tions: nitrosamines (18, 23, 38–40), which cause PNEC hyperplasia *in vivo*, and nicotine (24), which stimulates PNEC proliferation *in vitro*. We now report PNEC hyperplasia after a single acute insult with naphthalene, a component of the lipophilic subfraction of cigarette smoke. Naphthalene and its related compounds, 2-methylnaphthalene and 1-methylnaphthalene, are found in small quantities in cigarette smoke (29). Although the effect of inhaled naphthalene in human airways is not known, primate Clara cells are less able

to metabolize naphthalene to cytotoxic derivatives than are mice, due to differences in cytochrome *P*-450 isoenzymes (3). Parenterally administered naphthalene does, however, appear to a useful model to study the effects of acute, selective Clara cell injury by lipophilic cytotoxins.

PNEC hyperplasia after acute airway injury is restricted to NEBs through a mechanism including increased cell proliferation. The spatial pattern of PNEC hyperplasia is characterized by an increased number of PNEC clusters or NEBs. However, the number of PNECs per NEB and the number of isolated PNECs are not significantly different between treated and control groups. This pattern of hyperplasia is consistent with the findings of Joad et al. (12) in a study of rats exposed to sidestream tobacco smoke *in utero* and/or postnatally. They also found increased numbers of NEBs but found that the average size of each NEB was similar, suggesting that the increased number of NEBs is not simply due to the enlargement of existing NEBs. In contrast, Sunday and Willet (38), in a model of preneoplastic lung injury, found increased numbers of PNECs within NEBs at 6–7 wk of continuous exposure to DEN

Table 1. Increased $[^3\text{H}]$ thymidine labeling of CGRP-IR cells

	Vehicle	Naphthalene	<i>P</i> Value
No. of $[^3\text{H}]$ thymidine-labeled CGRP-IR cells/mm BM	0.025 ± 0.025	0.324 ± 0.052	<0.001
CGRP-IR cells labeled with $[^3\text{H}]$ thymidine, %	3.3 ± 3.3	12.0 ± 1.4	0.04

Data are expressed as means \pm SE and are expressed as percent $[^3\text{H}]$ thymidine-labeled calcitonin gene-related peptide-like immunoreactivity (CGRP-IR) from naphthalene-treated and vehicle only animals. BM, basement membrane. Data were analyzed by Student's *t*-test for unpaired variables.

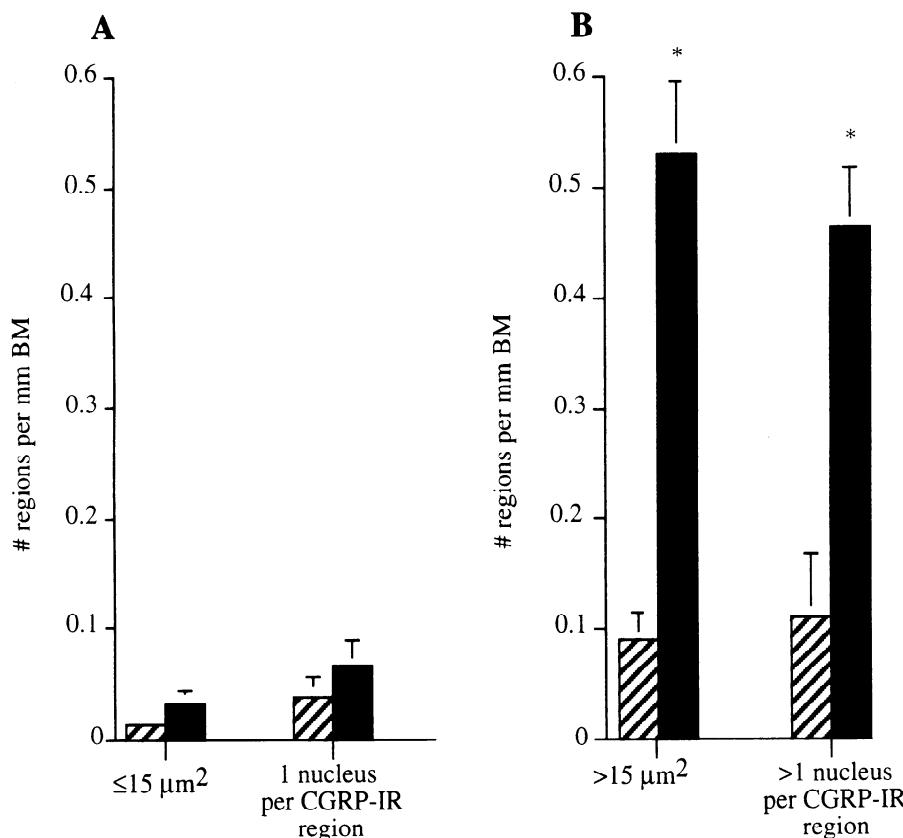


Fig. 4. Spatial pattern of PNEC hyperplasia. *A*: similar numbers of isolated PNECs/mm epithelial BM are seen in naphthalene-treated (filled bars) and vehicle only (hatched bars) animals. Isolated PNECs are defined as CGRP-IR regions $\leq 15 \mu\text{m}^2$ or consisting of only one CGRP-IR cell nucleus per CGRP-IR region. *B*: significantly increased numbers of clustered neuroendocrine cells [neuroepithelial bodies (NEBs)]/mm BM are seen in naphthalene-treated vs. vehicle only animals. NEBs are defined as CGRP-IR regions $> 15 \mu\text{m}^2$ or consisting of > 1 cell nucleus/CGRP-IR region. Results are presented as means \pm SE for each treatment condition; * $P < 0.01$.

and hyperoxia. Increases in both PNECs and NEBs have been described in a model of ozone-induced PNEC hyperplasia (11).

Based on previous observations of spatially controlled airway cell proliferation and redifferentiation after acute naphthalene injury (34), we chose to investigate changes in the PNEC population at 5 days into the repair response. After acute injury, Clara cell death is evident by marked reductions in the number of cells with Clara cell secretory protein (CCSP) immunoreactivity. Airways are initially repopulated with an immature (non-CCSP expressing) cell population that then is replaced with mature Clara cells in a process that begins preferentially at airway branch points (34). Maximal cell proliferation is seen during a 36-h window beginning at ~ 36 h after injury and ending at 72 h. By 72 h after the insult, airway epithelial cell proliferation is primarily restricted to airway branch points. In this study, animals were injected with [^3H]thymidine during the 36-h period of maximal cell proliferation. At 5 days recovery, a significant increase in the number of ^3H -labeled CGRP-IR cells was seen in naphthalene-treated animals vs. vehicle only controls. Montuenga et al. (21) demonstrated proliferation of PNECs in the lungs of both normal and hypoxic rats. The PNEC proliferation rate was similar in rats exposed to 7 days of hypoxia compared with controls. This may be because 7 days of hypoxia did not generate PNEC hyperplasia. In a hamster model of airway injury, Linnoila (18) described NEC hyperplasia and increased NEC proliferation after a 3- to 4-wk exposure to twice weekly injections of DEN.

In our study, PNEC hyperplasia developed rapidly (by 5 days after injury), suggesting that NECs may be capable of more rapidly increasing their number in response to stimuli than previously appreciated. We have not demonstrated that fully differentiated PNECs proliferate but rather that cell proliferation contributes to a significant increase in PNEC number. The phenotype and lineage of the proliferating cells at the time that they proliferate is not clear. The observation that similar or increased numbers of PNECs exist along with increased numbers of NEBs suggests differentiation of progenitor cells into PNECs or de novo formation of NEBs. If clonal expansion of the existing PNECs into NEBs is the only mechanism for increased numbers of NEBs, a depleted number of mature, isolated PNECs would result. Little is known of the progenitor relationships for PNECs in lung repair. In lung development, recent work has suggested coexpression of the immunohistochemical markers CGRP, CCSP, and surfactant protein A on serial sections during mouse embryogenesis, embryonic days 13–15 (46). This suggests a common lineage for such diverse cell types as Clara cells, PNECs, and alveolar type II cells. It is known that alveolar type II cells may transiently express neuropeptide markers. Isolated rat alveolar type II cells secrete CGRP in culture (6), whereas hamster type II cells in vivo transiently immunostain for bombesin after combined hyperoxia and DEN exposure (23).

The physiological significance of PNEC hyperplasia during repair from injury is not clear. NEBs have paracrine effects on cell proliferation during lung development. In fetal Syrian golden hamsters, Hoyt et al. (8)

carefully documented a statistical increase in non-PNEC proliferation immediately adjacent to NEBs compared with more distant regions and suggested that a few nonneuroendocrine daughter cells may enter NEBs. This agrees with our impression (suggested in Fig. 3) that cell proliferation is increased in non-PNECs adjacent to NEBs after naphthalene toxicity. Two PNEC secretory neuropeptides, GRP and CGRP, are mitogens for airway epithelial cells (44, 45) and therefore may play a role in stimulating cell proliferation to initiate repopulation of injured epithelium. Classically, PNECs have been considered airway chemoreceptors responding to changes in airway oxygen tension (17, 47). GRP is a bronchoconstrictor (10, 28), whereas CGRP is a vasodilator (20, 43). One may speculate that NEBs containing GRP and CGRP and located at airway branch points are uniquely positioned to regulate regional ventilation and perfusion by regulating bronchial and vascular smooth muscle tone. In this way, in normal lung, oxygenation may be optimized through matching ventilation and perfusion, whereas lung injury by inhaled toxicants may be limited by regulating regional ventilation and perfusion. Consistent with this theory, the major sensory innervation of NEBs, C-nerve fibers containing CGRP, has been suggested to play a protective role in inhaled ozone exposure by inducing bronchospasm and by altering respiratory reflexes such as cough and rapid shallow breathing (33). Further studies are required to understand the role of secretory neuropeptides in lung repair and in exploring their role in regulating pulmonary physiology.

In summary, we have shown that repair of the murine airway after single-dose, acute naphthalene toxicity is associated with PNEC hyperplasia in a pattern that is restricted to NEBs as early as 5 days after injury. The mechanism responsible for PNEC hyperplasia is not clear but includes cell proliferation. Furthermore, the mechanism appears to include differentiation of both an existing progenitor population and newly proliferated cells into NECs. Whether hyperplastic PNECs and their secretory neuropeptides participate in regulating repair responses, physiological parameters, or are nonspecifically generated during epithelial renewal remains to be determined.

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REFERENCES

- Aguayo, S. M., W. E. Schuyler, J. J. Murtagh, and J. Roman.** Regulation of lung branching morphogenesis by bombesin-like peptides and neutral endopeptidase. *Am. J. Respir. Cell Mol. Biol.* 10: 635–642, 1994.
- Becker, K. L., J. O'Neill, R. H. Snider, E. S. Nylen, C. F. Moore, J. Jeng, O. L. Silva, M. S. Lewis, and M. H. Jordan.** Hypercalcitonemia in inhalation burn injury: a response of the pulmonary neuroendocrine cell? *Anat. Rec.* 236: 136–138, 1993.
- Buckpitt, A., M. Buonarati, L. B. Avey, A. M. Chang, D. Morin, and C. G. Plopper.** Relationship of cytochrome P450 activity to Clara cell cytotoxicity. II. Comparison of stereoselectivity of naphthalene epoxidation in lung and nasal mucosa of the mouse, hamster, rat and rhesus monkey. *J. Pharmacol. Exp. Ther.* 261: 364–372, 1992.
- Cutz, E., W. Chan, and K. S. Sonstegard.** Identification of neuroepithelial bodies in rabbit fetal lungs by scanning electron microscopy: a correlative light, transmission and scanning electron microscopic study. *Anat. Rec.* 192: 459–466, 1978.
- Gatto, C., R. C. Lussky, L. W. Erickson, K. J. Berg, J. D. Wobken, and D. E. Johnson.** Calcitonin and CGRP block bombesin- and substance P-induced increases in airway tone. *J. Appl. Physiol.* 66: 573–577, 1989.
- Hastings, R. H., and X. Y. Hua.** Expression of calcitonin gene-related peptide by cultured rat alveolar type II cells. *Am. J. Respir. Cell Mol. Biol.* 13: 563–569, 1995.
- Hernandez-Vasquez, A., J. A. Will, and W. B. Quay.** A radioautographic study of the neuroepithelial bodies of lungs in fetal and neonatal rabbits. *Cell Tissue Res.* 186: 203–207, 1978.
- Hoyt, R. F., N. A. McNelly, E. M. McDowell, and S. P. Sorokin.** Neuroepithelial bodies stimulate proliferation of airway epithelium in fetal hamster lung. *Am. J. Physiol.* 260 (Lung Cell. Mol. Physiol. 4): L234–L240, 1991.
- Hung, K.-S., A. L. Chapman, and M. A. Mestemcher.** Scanning electron microscopy of bronchiolar neuroepithelial bodies in neonatal mouse lung. *Anat. Rec.* 193: 913–926, 1979.
- Impicciatore, M., and G. Bertaccini.** The bronchoconstrictor action of the tetradecapeptide bombesin in the guinea pig. *J. Pharm. Pharmacol.* 25: 872–875, 1973.
- Ito, T., Y. Ikemi, K. Ohmori, H. Kitamura, and M. Kanisawa.** Airway epithelial cell changes in rats exposed to 0.25 ppm ozone for 20 months. *Exp. Toxic Pathol.* 46: 1–6, 1994.
- Joad, J. P., C. Ji, K. S. Kott, J. M. Bric, and K. E. Pinkerton.** In utero and postnatal effects of sidestream cigarette smoke exposure on lung function, hyperresponsiveness, and neuroendocrine cells in rats. *Toxicol. Appl. Pharmacol.* 132: 63–71, 1995.
- Johnson, D. E., W. R. Anderson, and B. A. Burke.** Pulmonary neuroendocrine cells in pediatric lung disease: alterations in airway structure in infants with bronchopulmonary dysplasia. *Anat. Rec.* 236: 115–119, 1993.
- Keith, I. M., and J. A. Will.** Dynamics of the neuroendocrine cell-regulatory peptide system in the lung (specific overview and new results). *Exp. Lung Res.* 3: 387–402, 1982.
- King, K. A., J. S. Torday, and M. E. Sunday.** Bombesin and [Leu^8]phyllo-ltorin promote fetal mouse lung branching morphogenesis via a receptor-mediated mechanism. *Proc. Natl. Acad. Sci. USA* 92: 4357–4361, 1995.
- Lach, E., E. Haddad, and J. Gies.** Contractile effect of bombesin on guinea pig lung in vitro: involvement of gastrin-releasing peptide-preferring receptors. *Am. J. Physiol.* 264 (Lung Cell. Mol. Physiol. 8): L80–L86, 1993.
- Lauweryns, J. M., M. Cokelaere, and T. Lerut.** Cross circulation studies on the influence of hypoxia and hypoxemia on neuro-epithelial bodies in young rabbits. *Cell Tissue Res.* 193: 373–386, 1978.
- Linnola, R. I.** Effects of diethylnitrosamine on lung neuroendocrine cells. *Exp. Lung Res.* 3: 225–236, 1982.
- McBride, J. T., D. R. Springall, R. J. D. Winter, and J. M. Polak.** CGRP-like immunoreactivity in lung endocrine cells in hypoxic rats measured by quantitative immunocytochemistry. *Am. J. Respir. Cell Mol. Biol.* 3: 587–593, 1990.
- McCormack, D. G., R. O. Salonen, and P. J. Barnes.** Effect of sensory neuropeptides on canine bronchial and pulmonary vessels in vitro. *Life Sci.* 45: 2405–2412, 1989.
- Montuenga, L. M., D. R. Springall, J. Gaer, R. J. D. Winter, L. Zhao, J. T. McBride, K. M. Taylor, G. Barer, and J. M. Polak.** CGRP-immunoreactive endocrine cell proliferation in normal, and hypoxic rat lung studied by immunocytochemical

- detection of incorporation of 5'-bromodeoxyuridine. *Cell Tissue Res.* 268: 9–15, 1992.
22. **Nylen, E. S., and K. L. Becker.** Chronic hyperoxia, and pulmonary neuroendocrine cell bombesin and calcitonin. *Anat. Rec.* 236: 248–252, 1993.
 23. **Nylen, E. S., K. L. Becker, P. A. Joshi, R. H. Snider, and H. M. Schuller.** Pulmonary bombesin and calcitonin hamsters during exposure to hyperoxia and diethylnitrosamine. *Am. J. Respir. Cell Mol. Biol.* 2: 25–31, 1990.
 24. **Nylen, E. S., K. L. Becker, R. H. Snider, A. R. Tabassian, M. M. Cassidy, and R. I. Linnoila.** Cholinergic-nicotinic control of growth and secretion of cultured pulmonary neuroendocrine cells. *Anat. Rec.* 236: 129–135, 1993.
 25. **Plopper, C. G., J. Macklin, S. J. Nishio, D. M. Hyde, and A. R. Buckpitt.** Relationship of cytochrome P-450 activity to Clara cell cytotoxicity. III. Morphometric comparison of changes in the epithelial populations of terminal and lobar bronchi in mice, hamsters and rats after parenteral administration of naphthalene. *Lab. Invest.* 67: 553–565, 1992.
 26. **Roncalli, M., D. R. Springall, M. Maggioni, A. Moradoghli-Haftvani, R. J. D. Winter, L. Zhao, G. Coggi, and J. M. Polak.** Early changes in the calcitonin gene-related peptide (CGRP) content of pulmonary endocrine cells concomitant with vascular remodeling in the hypoxic rat. *Am. J. Respir. Cell Mol. Biol.* 9: 467–474, 1993.
 27. **Rosengurt, E., and J. Sinnett-Smith.** Bombesin stimulation of fibroblast mitogenesis: specific receptors, signal transduction and early events. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 327: 209–221, 1990.
 28. **Salonen, R. O., S. E. Webber, and J. G. Widdicombe.** Effects of neuropeptides and capsaicin on the canine vasculature in vivo. *Br. J. Pharmacol.* 95: 1262–1270, 1988.
 29. **Schmeltz, I., J. Tosk, and D. Hoffmann.** Formation and determination of naphthalenes in cigarette smoke. *Anal. Chem.* 48: 645–650, 1976.
 30. **Sorokin, S. P., and R. F. Hoyt.** Neuroepithelial bodies and solitary small-granule cells. In: *Lung Cell Biology (Lung Biology in Health and Disease)*, edited by D. Massaro. New York: Dekker, 1989, vol. 41, p. 191–344.
 31. **Speirs, V., E. Bienkowski, V. Wong, and E. Cutz.** Paracrine effects of bombesin/gastrin-releasing peptide and other growth factors on pulmonary neuroendocrine cells in vitro. *Anat. Rec.* 236: 53–61, 1993.
 32. **Springall, D. R., G. Collina, G. Barer, A. J. Suggett, D. Bee, and J. M. Polak.** Increased intracellular levels of calcitonin-gene related peptide-like immunoreactivity in pulmonary endocrine cells of hypoxic rats. *J. Pathol.* 155: 259–267, 1988.
 33. **Sterner-Kock, A., K. R. Vesley, M. Y. Stovall, E. S. Schelegle, J. F. Green, and D. M. Hyde.** Neonatal capsaicin treatment increases the severity of ozone-induced lung injury. *Am. J. Respir. Crit. Care Med.* 153: 436–443, 1996.
 34. **Stripp, B. R., K. Maxson, R. Mera, and G. Singh.** Plasticity of airway cell proliferation and gene expression after acute naphthalene injury. *Am. J. Physiol.* 269 (Lung Cell. Mol. Physiol. 13): L791–L799, 1995.
 35. **Sunday, M. E., J. Hua, H. B. Dai, A. Nusrat, and J. S. Torday.** Bombesin increases lung growth and maturation in utero and in organ culture. *Am. J. Respir. Cell Mol. Biol.* 3: 199–205, 1990.
 36. **Sunday, M. E., J. Hua, B. Reyes, H. Masui, and J. S. Torday.** Anti bombesin monoclonal antibodies modulate fetal mouse lung growth and maturation in utero and in organ cultures. *Anat. Rec.* 236: 25–32, 1993.
 37. **Sunday, M. E., L. M. Kaplan, E. Motoyama, and W. W. Chin.** Biology of disease: gastrin-releasing peptide (mammalian bombesin) gene expression in health and disease. *Lab. Invest.* 59: 5–24, 1988.
 38. **Sunday, M. E., and C. G. Willet.** Induction and spontaneous regression of intense pulmonary neuroendocrine cell differentiation in a model of preneoplastic lung injury. *Cancer Res. Suppl.* 52: 2677S–2686S, 1992.
 39. **Sunday, M. E., G. C. Willet, S. A. Graham, V. I. Oreffo, R. I. Linnoila, and H. Witschi.** Histochemical characterization of non-neuroendocrine tumors and neuroendocrine cell hyperplasia induced in hamster lung by 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanolone with or without hyperoxia. *Am. J. Pathol.* 147: 740–752, 1995.
 40. **Sunday, M. E., C. G. Willett, K. Patidar, and S. A. Graham.** 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.* 70: 875–888, 1994.
 41. **Tabassian, A. R., E. S. Nylen, I. Linnoila, R. H. Snider, M. M. Cassidy, and K. L. Becker.** Stimulation of hamster pulmonary neuroendocrine cells and associated peptides by exposure to cigarette smoke. *Am. Rev. Respir. Dis.* 140: 436–440, 1989.
 42. **Taylor, W.** Pulmonary argyrophil cells at high altitude. *J. Pathol.* 122: 137–144, 1977.
 43. **Tjen-A-Looi, S., R. Ekman, H. Lippton, J. Cary, and I. Keith.** CGRP and somatostatin modulate chronic hypoxic pulmonary hypertension. *Am. J. Physiol.* 263 (Heart Circ. Physiol. 32): H681–H690, 1992.
 44. **White, S. R., M. B. Hershenson, K. S. Sigrist, A. Zimmerman, and J. Solway.** Proliferation of guinea pig tracheal epithelial cells induced by calcitonin gene-related peptide. *Am. J. Respir. Cell Mol. Biol.* 8: 592–596, 1993.
 45. **Willey, J. C., J. F. Lechner, and C. C. Harris.** Bombesin and the C-terminal tetradecapeptide of gastrin-releasing peptide are growth factors for normal human bronchial epithelial cells. *Exp. Cell Res.* 153: 245–248, 1984.
 46. **Wuenschell, C. W., M. E. Sunday, G. Singh, P. Minoo, H. C. Slavkin, and D. Warburton.** Embryonic mouse lung epithelial progenitor cells co-express immunohistochemical markers of diverse mature cell lineages. *J. Histochem. Cytochem.* 44: 113–123, 1996.
 47. **Youngson, C., C. Nurse, H. Yeger, and E. Cutz.** Oxygen sensing in airway chemoreceptors. *Nature* 365: 153–155, 1993.