Induction and Spontaneous Regression of Intense Pulmonary Neuroendocrine Cell Differentiation in a Model of Preneoplastic Lung Injury

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Abstract

Pulmonary neuroendocrine cell (PNEC) hyperplasia is associated with chronic lung diseases in humans, where it is thought to play a role in reparative responses to lung injury. To investigate the kinetics of strongly induced PNEC hyperplasia in an animal model, we exposed hamsters to a combination of hyperoxia (60% O₂) and diethylnitrosamine (DEN) for up to 20 weeks. We thus demonstrate not only the induction but also spontaneous regression of intense PNEC differentiation and growth, which are much more intense than those observed with DEN alone. Lung tissues were immunostained for serotonin, calcitonin gene-related peptide (CGRP), calcitonin (CT), and gastrin-releasing peptide (GRP) (mammalian bombesin). Between 9 and 12 weeks of treatment, the number of neuroepithelial bodies immunostained for CGRP, serotonin, and CT peaked at 12-14 weeks of treatment, thereafter regressing to near-control levels by 20 weeks, in spite of continued DEN/O₂ treatment. Simultaneously, by 6-7 weeks of treatment, there was a significant increase in the mean number of CGRP-positive cells per neuroepithelial body, which continued to rise up to double control levels, with a plateau at 13-20 weeks. GRP and pro-GRP immunostaining were not detectable at any time point. Polymerase chain reaction analyses of neuroendocrine-specific mRNAs demonstrated that CGRP, CT, and GRP mRNAs (normalized for β-actin) peaked in lung tissues from most animals at 9-14 weeks after the beginning of DEN/O₂ treatment, with decreased expression at 16-20 weeks. These data suggest that regulation of levels of these neuropeptides may be primarily transcriptional. This model may be a valuable system for analyzing mechanisms of induction and regression of normal PNEC differentiation and growth.

Introduction

PNECs are numerous within human fetal lung (1, 2), where they may play a significant role in normal lung growth and maturation (3, 4). These epithelial cells are also generally believed to be the precursors of human lung carcinoid tumors and small cell lung carcinomas (5-8). However, this hypothesis has been untested, due to the lack of a suitable animal model for studying PNEC differentiation or tumorigenesis.

PNECs occur normally in relatively small numbers in rodent lung, with highest cell densities present in neonatal animals (9-13). DEN and other carcinogenic nitrosamines are known to induce mild PNEC hyperplasia in hamsters with short term continuous treatment (11, 14-18). The acute histopathological change has been reported as up to a 2-fold increase in the number of PNECs per NEB and also about a 2-fold increase in the number of NEBs, both of which occur after 2-3 months of treatment with DEN alone. However, nitrosamines have typically led to only non-neuroendocrine lung tumors, predominantly adenocarcinomas, after treatment of hamsters for 6 months or more (19-21). Similar changes have been observed in rats (22) and rabbits (23). The tissue and cellular specificity of these compounds appears to be mediated, at least in part, by preferential binding and metabolism of nitrosamines into their active carcinogenic forms by PNECs and Clara cells (24, 25).

Recently, Schuller et al. (26) reported a model for inducing "neuroendocrine lung cancers" in 100% of hamsters after 8 weeks of treatment with DEN and continuous hyperoxia (70% O₂). In other papers, the same group found that 62.5% of hamsters treated with DEN and 60% O₂ developed similar lung cancers within 8 weeks (27) or an unknown percentage of animals within 3-6 weeks (28). It was suggested that this was the first animal model of pulmonary carcinoid tumors and/or small cell lung carcinoma, which may be termed well differentiated and poorly or undifferentiated neuroendocrine carcinoma, respectively (29).

The present study began as an effort to confirm the induction of lung neuroendocrine tumors, either benign or malignant, by 8-12 weeks of DEN/O₂ treatment of hamsters. Although we used essentially the same experimental protocols as did Schuller and co-workers (27, 28), we have been unable to detect any PNEC neoplasia in hamster lungs up to 20 weeks after the initiation of DEN/O₂ treatment. However, we do demonstrate induction of intense PNEC hyperplasia at 9-14 weeks with subsequent spontaneous regression from 14-20 weeks, making this a valuable model for analyzing regulation of normal PNEC development.

Materials and Methods

Care and Treatment of Hamsters. Outbred female Syrian golden hamsters (virus and pathogen free) were received from Charles River Laboratories (Wilmington, MA), at 6 weeks of age, and were rested for 2 weeks before the beginning of DEN injections and exposure to continuous hyperoxia. Hamsters were maintained under standard laboratory conditions, in compliance with Interdisciplinary Principles and Guidelines for the Use of Animals in Research, Testing, and Education issued by the New York Academy of Sciences’ Ad Hoc Committee on Animal Research. They were housed at 20-25°C with five animals/cage, fed standard Purina rodent chow and water ad libitum, and exposed to a 12-h light/12-h dark cycle. Animals were exposed to 100% O₂ in a vented Plexiglas chamber, by ventilation with a mixture of 100% O₂ and compressed air at a flow rate of 20-25 liters/min, with relative humidity maintained at 45-60%. This same chamber had been used successfully by another group for hyperoxia studies (30). Oxygen levels were continuously monitored with a digital oxygen analyzer (Hudson; RCI). The animals were removed from this environment only for brief (<15 min) intervals required for s.c. injections with DEN and/or PBS. Oxygen-exposed hamsters were treated with DEN (20 mg/kg in 0.1-0.4 ml of PBS) (Sigma Chemical Co., St. Louis, MO) or with PBS alone, injected s.c. in the flank on Tuesdays and Thursdays at 7:30 a.m. Other controls included hamsters receiving injections of DEN.
of the thoracic and abdominal viscera was carried out. All lung lobes at ambient oxygen levels and hamsters from 8 to 24 weeks of age were conserved in rodents and humans) antisera were raised as described working 5HT antibody dilution of 1/80 for immunoperoxidase analyses. Biotinylated affinity-purified goat anti-rat IgG were both obtained from other half was snap-frozen in liquid nitrogen for RNA analyses. paraformaldehyde for immunohistochemistry and histology and the other half was snap-frozen in light mineral oil (Sigma) and subjected to 35 cycles (except for actin, which was subjected to 25 cycles) of denaturation (0.5 min, 93°C), annealing (1.0 min, 50°C), and extension (1.5 min, 72°C), using a programmable thermal cycler (Gene Machine II; USA/Scientific Plastics, Ocala, FL). The products of the reverse transcription reaction mixture was added to 2.5 μl of 20× reaction buffer (20× buffer = 1.0 M Tris-HCl, pH 9.0, 400 mM ammonium sulfate, 30 mM MgCl2, 1.25 μl of 2 mM dATP, dGTP, dCTP, and dTTP (each), 1 μl of each primer (27 base pairs each, 20 μM), 28-116) (37, 38). Synthetic oligodeoxynucleotides were made by the phosphoramidite method, using an Applied Biosystems 381A DNA synthesizer, using sequences known to be conserved between rodents and humans, corresponding to rat GRP (precursor peptide amino acids 20–116) (34), rat a-CGRP (precursor peptide amino acids 28–324) (35), rat CT (precursor peptide amino acids 28–138) (36), and mouse β-actin (amino acids 28–116) (37, 38). PCRs were carried out using Replinase thermostable DNA polymerase (New England Nuclear Research Products, Boston, MA), as specified by the manufacturer. Briefly, 1 μl of the reverse-transcribed RNA mixture was added to 2.5 μl of 20× reaction buffer (20× buffer = 1.0 M Tris-HCl, pH 9.0, 400 mM ammonium sulfate, 30 mM MgCl2, 1.25 μl of 2 mM dATP, dGTP, dCTP, and dTTP (each), 1 μl of each primer (27 base pairs each, 20 μM), 1 μl (1 unit) of Replinase, and 38.5 μl of deionized water, in 96-well plates; each well was overlaid with 30 μl of light mineral oil (Sigma) and subjected to 35 cycles (except for actin, which was subjected to 25 cycles) of denaturation (0.5 min, 93°C), annealing (1.0 min, 50°C), and extension (1.5 min, 72°C), using a programmable thermal cycler (Gene Machine II; USA/Scientific Plastics, Ocala, FL). The products of the reaction were analyzed on 4% agarose gels, containing 0.5 μg/ml ethidium bromide, run in 1× TBE buffer (10 mM Tris base, 10 mM boric acid, 40 mM EDTA, pH 8.0, with final pH 7.6). Southern blots were then carried out onto nitrocellulose, according to standard protocols (39). T4 kinase end-labeled probes were made as described previously (39), using 30-base pair primers corresponding to the internal cDNA sequence of each gene analyzed, and were hybridized at 42°C with the appropriate blot for 18 h in 6× SSC (1× SSC = 0.15 M NaCl, 0.015 M sodium citrate), 5× Denhardt’s (5× Denhardt’s = 20 mg/ml Ficoll-400, 20 mg/ml polyvinylpyrrolidone, 20 mg/ml bovine serum albumin), 0.1% sodium dodecyl sulfate, 250 μg/ml salmon sperm DNA. Blots were washed in 2× SSC, 0.1% sodium dodecyl sulfate, at 42°C, before exposure to Kodak XAR film at 20°C for 2–4 h. These conditions allowed a general linear correlation between the relative amounts of given mRNAs present in the products of the same reverse transcription reaction, which could then be subsequently normalized to actin as a control reference mRNA (40). Densitometry was carried out by three-dimensional integration of the area of whole bands on autoradiograms, using a Molecular Dynamics model 300A densitometer.

Results

PNEC Histochemistry. The results of immunoperoxidase analyses are given in Tables 1 and 2 and Figs. 1–4. We will first consider data related to PNEC differentiation, manifested as the number of foci immunostained for CGRP, 5HT, and/or calcitonin. The most prevalent immunostaining was for CGRP, with 17 of 23 normal hamster lungs (8 weeks old) containing CGRP-positive PNEC foci, which were predominantly (>95%) NEBs (Fig. 1A). CGRP positivity was abolished by preabsorption of anti-CGRP antiserum with CGRP (5 μg/ml) (Fig. 1B). A subset of these CGRP-positive foci (as in Fig. 1C) also stained for 5HT in serial sections (Fig. 1D) in 9 of the 23 controls. Normal hamster lung at 12–30 weeks of age contained a smaller number of CGRP-positive foci (Fig. 2B). The untreated control animals generally had only weak to moderate immunostaining of most NEBs (Fig. 1, C and D). There was no staining of 8–30-week-old normal adult hamster lung for either mammalian bombesin or calcitonin (data not shown). Only 1 of 23 control hamsters had >70 CGRP-positive or >50 5HT-positive foci/cm airway epithelium.

Morphometric analyses of PNEC marker expression in DEN/O2-treated hamsters are summarized in Table 1 and Fig. 2A. After only 1 week of exposure to DEN/O2, there was no...
change in the number of CGRP- or 5HT-positive foci. However, at 2–4 weeks of treatment, there was a significant doubling of the mean number of CGRP-positive foci (P < 0.017), compared to the 8-week controls, which was the negative control group with the highest density of NEBs. These higher mean levels were primarily due to marked elevations in 6 of 15 animals at 2 weeks and 7 of 10 animals at 4 weeks of treatment (each of these with >70 CGRP-positive foci/cm). Similarly, a significant elevation of the mean number of 5HT-positive foci at 6 weeks of treatment reflects marked increases in 4 of 9 animals (>50 foci/cm). This early multifocal PNEC hyperplasia suggests a triggering of early PNEC differentiation in a subset of hamsters.

By 9–13 weeks of treatment, all animals had markedly increased numbers of CGRP-positive foci, >98% of which were NEBs (Figs. 2 and 3, A–C). This sharp rise in the number of differentiated neuroendocrine foci occurred in all animals after 9–12 weeks of DEN/O2 treatment, with a >10-fold increase in the mean number of CGRP-positive PNEC foci. These CGRP-positive NEBs were composed of larger numbers of PNECs, compared to those in hamsters at earlier time points, consistent with simultaneous proliferation (see Fig. 3A and below), and these NEBs at 12 weeks or more tended to bulge into the airway lumen (Fig. 3B). CGRP positivity was abolished by preabsorption of anti-CGRP antiserum with CGRP (data not shown), similar to Fig. 1D. At 13 weeks the numbers of SHT- (Fig. 3D) and CT-positive foci (Fig. 3F) were also elevated in essentially all animals (Table 1).

In spite of continued DEN/O2 treatment, from 14 to 20 weeks of treatment there was a spontaneous decline in the number of PNEC foci stainable for CGRP, 5HT, and CT (Table 1; Figs. 2–4). Associated with this phase of lung injury were scattered foci of epithelial hyperplasia (Fig. 4A), some of which were CGRP-positive (Fig. 4B). There were random foci of cell death, with cell vacuolation, karyorrhexis, karyolysis, and calcification (Fig. 4D), which were sometimes identified in NEBs (Fig. 4B). Immunostaining of NEBs was generally only weak to moderate in intensity, with maintenance of the same number of nuclei per NEB (Table 2). Areas of frank squamous metaplasia of bronchial and bronchiolar epithelium were relatively infrequent, did not immunostain for any CGRP, CT, or SHT immunoreactivity (Fig. 4C), and were not observed in the immediate vicinity of immunostainable PNECs. There was no evidence of infiltration of lymphocytes or granulocytes in regions in or around NEBs.

In addition to the evidence described above for PNEC differentiation, proliferation of PNECs was assessed by counting the number of CGRP-positive cells per NEB at different time points (Table 2). The mean number of CGRP-positive cells per NEB became significantly elevated, from 8.6 in normal controls to 11 ± 1.1, at 6 weeks of DEN/O2 treatment (P < 0.04) and continued to rise, up to 16–20 cells/NEB, at 13–20 weeks (P < 0.001). There was no significant difference between values in different animals at any given time point. This doubling of the number of cells during a 13-week period was greater than that induced by DEN alone (Table 2), where a significant elevation in mean cell numbers per NEB was apparent only after 16 weeks of DEN treatment (P < 0.005).

Control animals treated with 60% hyperoxia and biweekly injections of PBS alone or with DEN at ambient O2 levels had only about a 2-fold increase in the number of NEBs per cm of airway epithelium at any time point up to and including 12 weeks of treatment (Fig. 2F; note smaller scale). There was also spontaneous regression of the mild PNEC hyperplasia in these controls from 12–20 weeks of treatment but, because the magnitude of the hyperplasia was small, this decline was less dramatic.

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### Table 1 Immunostaining of DEN/O2-treated hamster lungs for CGRP, 5HT, and CT

<table>
<thead>
<tr>
<th>Weeks</th>
<th>No.</th>
<th>Immunostaining (no. of positive foci/cm airway epithelium)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>DEN/O2</td>
</tr>
<tr>
<td>0</td>
<td>23</td>
<td>44 ± 9 (17, 1)</td>
</tr>
<tr>
<td>1</td>
<td>7</td>
<td>27 ± 14 (5, 1)</td>
</tr>
<tr>
<td>2</td>
<td>15</td>
<td>80 ± 10 (15, 7)</td>
</tr>
<tr>
<td>4</td>
<td>10</td>
<td>89 ± 17 (9, 6)</td>
</tr>
<tr>
<td>6</td>
<td>9</td>
<td>51 ± 20 (7, 3)</td>
</tr>
<tr>
<td>7</td>
<td>9</td>
<td>46 ± 7 (9, 2)</td>
</tr>
<tr>
<td>9</td>
<td>9</td>
<td>153 ± 37 (9, 8)</td>
</tr>
<tr>
<td>12</td>
<td>9</td>
<td>515 ± 134 (9, 9)</td>
</tr>
<tr>
<td>13</td>
<td>8</td>
<td>300 ± 55 (8, 8)</td>
</tr>
<tr>
<td>14</td>
<td>8</td>
<td>269 ± 49 (5, 5)</td>
</tr>
<tr>
<td>16</td>
<td>4</td>
<td>131 ± 26 (4, 3)</td>
</tr>
<tr>
<td>20</td>
<td>4</td>
<td>44 ± 13 (4, 1)</td>
</tr>
</tbody>
</table>

* Adult female hamsters (8–21 weeks old) were treated with biweekly injections of DEN and continuous 60% O2, as detailed in “Materials and Methods.” Lungs were harvested at 0 and 1, 2, 4, 6, 7, 9, 12, and 13 weeks.

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### Table 2 Number of CGRP-positive cells per NEB in DEN/O2-treated and DEN alone-treated hamster lungs

<table>
<thead>
<tr>
<th>Weeks</th>
<th>No. of positive cells/NEB</th>
</tr>
</thead>
<tbody>
<tr>
<td>DEN/O2</td>
<td>DEN alone</td>
</tr>
<tr>
<td>0</td>
<td>8.6 ± 0.5 (125)</td>
</tr>
<tr>
<td>1</td>
<td>9.0 ± 0.7 (67)</td>
</tr>
<tr>
<td>2</td>
<td>9.1 ± 0.9 (76)</td>
</tr>
<tr>
<td>6</td>
<td>11.1 ± 1.4 (42)</td>
</tr>
<tr>
<td>7</td>
<td>13.4 ± 1.7 (55)</td>
</tr>
<tr>
<td>9</td>
<td>12.8 ± 0.8 (69)</td>
</tr>
<tr>
<td>12</td>
<td>15.1 ± 1.1 (55)</td>
</tr>
<tr>
<td>13</td>
<td>18.1 ± 1.2 (78)</td>
</tr>
<tr>
<td>14</td>
<td>19.6 ± 1.5 (76)</td>
</tr>
<tr>
<td>16</td>
<td>15.9 ± 1.2 (56)</td>
</tr>
<tr>
<td>20</td>
<td>18.0 ± 1.6 (41)</td>
</tr>
</tbody>
</table>

* Hamsters were treated with biweekly injections of DEN and continuous 60% O2, as detailed in “Materials and Methods.” Lungs were harvested at 0–13 weeks after the beginning of DEN/O2 treatment.

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### Notes and Abbreviations

- ND, not determined.
- P < 0.05.
- P < 0.001.
- P < 0.005.
Neuroendocrine cell differentiation

Fig. 1. Immunostaining of normal hamster lungs for CGRP and serotonin. A, normal 8-week hamster lung stained using the immunoperoxidase technique, demonstrating a CGRP-positive neuroepithelial body (arrow) in a bronchiole. ×400. B, serial section to A, run in parallel using anti-CGRP antiserum preabsorbed with CGRP (5 ng/ml), has lost CGRP-specific immunostaining (arrow). ×400. C, normal 8-week hamster lung stained for CGRP immunoreactivity in a bronchiole. Two positive NEBs are indicated by arrows. ×100. D, serial section to C, with the same two NEBs stained for 5HT immunoreactivity (arrows). ×100.

We were unable to demonstrate immunostaining of NEBs for mammalian bombesin (GRP) at any time point in serial sections (Fig. 3E). This negativity for GRP held even when the immunoperoxidase analyses were carried out with a variety of different antibombesin antisera (26, 31, 41), some of which have been reported elsewhere to stain positively in DEN/O₂-treated hamsters (26). These results were obtained in spite of optimal control immunostaining of human fetal lung and normal hamster stomach, in parallel, using any of these antibombesin antisera. However, one hamster did demonstrate specific GRP immunostaining of pulmonary macrophages in a foreign body giant cell reaction (see below), similar to previous reports (3), suggesting ingestion and concentration of GRP secreted into the airway lumen.

Histopathology. In addition to the aforementioned changes in pulmonary neuroendocrine cells, progressive histopathological changes were observed that were consistent with lung damage due to DEN and/or hyperoxia.

As early as 1–2 weeks into treatment, epithelial cell dysplasia was apparent, consistent with DEN exposure (19). By 4 weeks of treatment areas of focal hyperplasia of bronchial and bronchiolar epithelium were occasionally apparent, and they became frequent by 9–12 weeks. Less than one third of these hyperplastic foci immunostained for CGRP, CT, or 5HT (Fig. 4B). Microscopic clusters (<1 mm in diameter) of clear epithelial cells were observed in two animals, one at 14 weeks of treatment and one at 20 weeks, consistent with Clara cell hyperplasia, as previously described (21).

Changes related to hyperoxic lung injury included peripheral nodules composed of fibroblasts and collagen, with or without hemosiderin deposits, occurring as early as 4 weeks (Fig. 5A). By 13 weeks of treatment, all hamsters had many of these same nodules containing hemosiderin-laden macrophages (Fig. 5B), consistent with a fibrotic response to chronic injury with hemorrhage. These small areas of scarring were not connected to airways and were frequently pleural-based. Enlarged airspaces were demonstrated at 12–20 weeks of treatment, associated with plugging of small conducting bronchioles with mucin and necrotic debris. Squamous metaplasia occurred, as described above (Fig. 4C). In addition, one hamster treated with DEN/O₂ for 13 weeks demonstrated a diffusely infiltrative lesion composed of atypical cells (Fig. 4C), giving the appearance of...
undetectable levels of these neuropeptide mRNAs. Between 2 and 4 weeks of DEN/O₂ treatment 33–67% of hamsters (three/time point) had elevated CGRP, CT, and/or GRP mRNAs significantly above the 8-week-old untreated control levels. At 6–7 weeks of treatment levels were not significantly elevated above the 8-week-old controls. From 9 to 12 weeks of treatment there was a sharp increase in the mean levels of all three mRNAs; 50% of hamsters at 9 weeks and 100% of hamsters at

an invasive tumor, but this proved to be a foreign body giant cell reaction to aspirated food matter.

Finally, as described above, at 14–20 weeks of treatment epithelial cell death was apparent in about 5–10% of epithelial cells, with associated calcification and Psammoma body formation (Fig. 4A). In some animals, goblet cell hyperplasia occurred between 14 and 20 weeks.

On general postmortem examination of the hamster organs, we observed no liver tumors. Multiple tracheal papillomas were observed in each of two hamsters treated with DEN/O₂ at 16 weeks (50%) and in three at 20 weeks (75%). Animals treated with DEN alone had similar tracheal papillomas detected in a few animals at 4–9 weeks and in 100% of animals at 12–24 weeks.

PCR Analyses of Neuroendocrine Cell-specific mRNAs. PCR analyses of reverse-transcribed total RNA from hamster lung were carried out for sensitive and specific detection of the expression of neuroendocrine cell-specific genes. In particular, neither CGRP, CT, nor GRP mRNAs were detectable on Northern blots of total lung RNA; tissue samples were too small to permit sufficient poly(A)⁺ RNA recovery for blot analyses. Pooled results of Southern blot analyses of PCR data are given in Fig. 6, where CGRP, CT, and GRP mRNAs have been normalized for the relative amount of β-actin mRNA present in the same reverse transcription reaction mixture (40). The data points represent the mean values for each time point, with three or four animals/time point. Most 8-week-old untreated control hamsters had low but detectable levels of CGRP, CT, and GRP mRNAs. By 20 weeks of age (corresponding to 12 weeks of DEN/O₂ treatment) untreated control animals had
12 weeks of DEN/O2 treatment had elevated CGRP, CT, and GRP transcripts. These data are in contrast to the age-matched negative untreated controls corresponding to the 12-week treatment group (20 weeks old). At 13–14 weeks, all animals had high levels of CGRP mRNA and 75% had high CT mRNA, but only 25% had significantly elevated levels of GRP mRNA. At 14–20 weeks of DEN/O2 treatment, GRP mRNAs were undetectable. Mean levels of CGRP and CT mRNAs declined but were still high from 16 to 20 weeks of DEN/O2 treatment. Of note, one animal at 16 weeks of treatment had undetectable CGRP and CT mRNAs.

Discussion

The present study demonstrates intensive induction of pulmonary neuroendocrine cell differentiation and growth in hamsters treated with DEN and 60% hyperoxia over a 12–14-week period, with subsequent spontaneous disappearance of differentiated foci between 14 and 20 weeks. Our data were collected and pooled from six continuous cohorts over a 2-year period. To clarify the dynamics of cell differentiation and growth, we have carried out careful quantification of both the number of differentiated PNEC foci (>95% NEBs) and the number of cells per focus, to ensure reproducibility of our observations. Some heterogeneity is expected to occur in these outbred hamsters. Significant differences in susceptibility to bronchial chemical carcinogenesis are known to occur even between syngeneic hamsters (42). At early time points (at 2–4 weeks of exposure), there appears to be a small proportion of “responder” animals that develop PNEC hyperplasia in response to DEN/O2 treatment. Nonetheless, 9–12 weeks appears to be the critical time period during which intense neuroendocrine differentiation, together with growth, is induced in all animals. This peak in PNEC hyperplasia, reflecting an increase in both neuropeptide mRNAs and immunostainable peptides, is followed by spontaneous disappearance of NEBs from 14 to 20 weeks (Figs. 2A and 6). These data suggest that regulation of neuropeptide content of PNECs may be primarily transcriptional rather than post-transcriptional (43, 44).

The mechanism of induction of PNEC differentiation and growth is unknown but may represent part of a regenerative response to chronic lung injury. DEN has been shown to bind to and be metabolized preferentially into its active chemical form by PNECs, in addition to Clara cells (24, 25). This cell-specific effect may be combined with nonspecific oxygen toxicity mediated by free oxygen radicals (45, 46), leading to lethal and sublethal damage to DNA and resultant chronic lung injury. In support of this hypothesis, multifocal PNEC hyperplasia is observed in a variety of chronic inflammatory lung diseases (7), including bronchopulmonary dysplasia (47) and bronchiolitis (48). Rats with experimental asbestosis (49) have elevated lung tissue levels of bombesin-like immunoreactivity late in the course of disease. Thus, lung injury might be a critical factor in inducing pathological PNEC differentiation and growth.

Further, one PNEC-derived peptide hormone, GRP/mammalian bombesin, has been demonstrated to induce increased growth of mesenchymal and epithelial cells as well as lung maturation in mouse and human fetal lung (4). We were able to demonstrate the induction of GRP mRNAs with a peak at 12–13 weeks of DEN/O2 treatment, which coincides with the timing of induction of PNEC growth. Our inability to demonstrate GRP immunoreactivity in spite of positive human fetal lung and hamster stomach controls is likely to be consistent with rapid secretion of this neuropeptide during a critical physiological period. One could speculate that GRP may be an important growth factor during the induction phase of intense PNEC growth and differentiation.

Mechanisms for the subsequent unexpected PNEC regression...
include PNEC dedifferentiation and/or repression of hormone
gene expression, cell death or apoptosis (50), or metaplasia to
other epithelial cell types. It is possible that there is a direct
transition from PNECs to squamous cells as a later “protective”
response with more sustained ongoing lung damage, as sug-
gested by classic ultrastructural studies by Reznik-Schuller in
1977 (51). However, neither we nor Linnoila et al. (14) have
observed squamous metaplasia in proximity to immunostaina-
NEUROENDOCRINE CELL DIFFERENTIATION

Fig. 6. PCR analyses for CGRP, CT, and GRP mRNAs in DEN/O2-treated hamster lung. Total RNA from hamster lung tissues was subjected to reverse transcription and PCR protocols for actin, CGRP, CT, and GRP mRNAs, as detailed in “Materials and Methods.” Densitometry was carried out on autoradiograms of Southern blots, to determine relative amounts of the different mRNAs in a given reverse transcription reaction mixture, and results for CGRP, CT, and GRP were normalized to actin. Mean values of the final data per time point are plotted as relative amounts of given mRNAs per unit actin mRNA.

generally thought to represent excessive hyperplasia (7, 48, 63), they may be associated with true PNEC neoplasms in rare patients (80). Support for a diagnosis of hyperplasia would be the multifocality and speed of development of the neuroendocrine lung tumors in genetically normal hamsters (26). In one transgenic mouse model of pancreatic islet cell carcinogenesis by the highly oncogenic simian virus 40 large T antigen (62), conversion of 1–2% of hyperplastic foci into true neoplasms took 11–12 weeks. In a classic rat model of hepatocarcinogenesis, most hyperplastic liver nodules regress completely, with only rare foci undergoing neoplastic conversion after months to years (52). Evidence to confirm a diagnosis of neoplasia in the DEN/O2 model (26) could be simply obtained by demonstrating continued autonomous growth of the tumors in hamsters after the withdrawal of DEN/O2 treatment (61).

In summary, our observation of PNEC hyperplasia appears to reflect a combination of growth of pre-existing mature PNECs and differentiation of mature PNECs from either totipotential epithelial stem cells, committed but undifferentiated PNECs, or immature PNECs not yet expressing the full PNEC phenotype. Molecular analyses of mechanisms of cellular differentiation have been explored only in recent years, in part due to the recognition of tumor suppressor genes (71, 72). Myocyte (73) and neuronal differentiation (74) may be triggered by the expression of single regulatory genes. Neuroendocrine differentiation has been reported to be induced by ectopic expression of ras oncones transfected into medullary thyroid carcinomas (75), PC-12 pheochromocytoma cells (76), or small cell lung cancer cell lines (77). Peptides may be responsible for cell differentiation during embryogenesis, such as mesodermal induction (78, 79). The DEN/O2 model should allow a more precise approach to understanding of mechanisms of PNEC differentiation, which in turn may lead to greater understanding of deregulated differentiation in small cell carcinoma.

Acknowledgments

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