Pathobiology of Lung Tumors Induced in Hamsters by 4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone and the Modulating Effect of Hyperoxia

Hildegard M. Schuller, Hans-Peter Witschi, Eric Nylen, Priyavadan A. Joshi, Enrique Correa, and Kenneth L. Becker

ABSTRACT

Neuroendocrine lung cancer is among the most common types of lung cancers in smokers. We have recently shown that exposure of hamsters to N-nitrosodietyamine and hyperoxia causes a high incidence of this tumor type.

In this study, we show that the tobacco-specific nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone also causes neuroendocrine lung tumors in hyperoxic hamsters. Animals maintained in ambient air while being treated with 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone developed pulmonary adenomas composed of Clara cells and alveolar type II cells. Pathogenesis experiments provide evidence for the tumors caused by 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone in ambient air being derived from Clara cells. In the hyperoxic hamsters, the neuroendocrine carcinogenesis appears to involve two stages: (a) transformation of focal alveolar type II cells into neuroendocrine cells and (b) development of neuroendocrine lung tumors from such foci.

INTRODUCTION

The tobacco-specific nitrosamine NNK is formed by N-nitrosation of nicotine during tobacco processing and storage. Significant amounts (0.2 to 4 µg/cigarette) of NNK are present in American cigarettes (1). NNK is a potent lung carcinogen in laboratory rodents (1–3). In hamsters, NNK causes pulmonary adenomas and adenocarcinomas, some of which contain areas of squamous cell differentiation (1). The cellular composition and pathogenesis of such neoplasms has not been studied. On the other hand, DEN, which is not a tobacco-specific nitrosamine, causes hamster lung tumors similar to histopathology (4, 5) to the NNK-induced neoplasms. The DEN-induced tumors have been shown to be derived from Clara cells (4) and to exhibit differentiation into Clara cells and alveolar type II cells (4, 5). Moreover, DEN caused hyperplasia and squamous metaplasia of pulmonary neuroendocrine cells (6, 7). However, only in hamsters exposed simultaneously to hyperoxia did DEN cause the development of neuroendocrine lung tumors (8). The most malignant subtype (small cell cancer) of this cancer category is among the most common lung cancers in smokers (9) but had never before been induced experimentally. Its putative cell type of origin (pulmonary neuroendocrine cell) is sparse in the lungs of healthy adult mammals (10) but responds rapidly with numerical increases to conditions resulting in reduction (11, 12) or increase (10, 13) in preexisting normal pulmonary oxygen levels. We therefore assumed that the development of neuroendocrine lung cancer involves at least two stages, Stage 1, providing increased numbers of pulmonary neuroendocrine cells in response to altered pulmonary oxygen levels, and Stage 2, involving neoplastic transformation of the now abundant neuroendocrine cells by nitrosamine.

With respect to the prevalence of small cell lung cancer in smokers (9), the tobacco-specific nitrosamines are likely to cause cancers belonging to this category if administered under conditions (such as hyperoxia) which increase the number of neuroendocrine lung cells. In keeping with this assumption, hamsters exposed simultaneously to hyperoxia and NNK developed neuroendocrine lung tumors, whereas animals maintained in ambient air while receiving NNK developed adenomas and adenocarcinomas of Clara cell origin.

MATERIALS AND METHODS

Eight-week-old male outbred Syrian golden hamsters (Charles River, Wilmington, DE) were maintained five per cage, with food (Standard Purina Rodent Chow, Agri-Feed, Knoxville, TN) and water ad libitum. Some of the animals were maintained under ambient air conditions while some were maintained in their own cages in the previously described (8) hyperoxia chambers (O2 concentration, 70%). The individual treatment groups and experimental design are listed in Table 1. NNK was purchased from Chemscie Science Laboratories (Lennexa, KY). The vehicle for NNK was trioctanoin (C17H35O3; Kodak, Rochester, NY). Control groups were given injections of trioctanoin alone (Table 1). For pathology evaluation, complete necropsies were conducted and all major organs were processed for histopathology. Selected tissue samples of lung and lung tumors were processed for transmission electron microscopy. Immunocytochemical stains for mammalian bombesin and calcitonin were performed using the Vectastain ABC-Kit (Vector, Burlingame, CA), after incubation of deparaffinized lung sections with primary antibody (1:1000) for 48 h at 4°C. The primary antibodies were supplied by Dr. K. L. Becker and are specific for the carboxyl terminals of bombesin (14) and calcitonin (14, 15). Controls included sections without primary antisera, sections treated with excess antigen, and, for calcitonin, sections of thyroid gland.

The incorporation of [methyl-3H]thymidine (250 µCi/animal) into the DNA of lung cells was determined by autoradiography using NTB-2 (Kodak) emulsion, 10-day exposure time, and methylene blue-fuchsin as counterstain. Cell counts were done on 3 or 4 lung sections/animal (3 hamsters/time point, 3000 parenchymal cells and 1000 bronchial/bronchiolar cells/animal). No efforts were made to count individual cell types of the bronchi/bronchioles separately because some of them are very scanty while others require additional diagnostic techniques for identification. For the pathogenesis studies, the animals (3/time point) were sacrificed every 2 weeks when exposed to NNK and hyperoxia, while they were sacrificed every 4 weeks when receiving NNK under ambient air conditions. All five pulmonary lobes of each hamster were sectioned sagittally along the longitudinal axis of their lobar bronchi with a razor blade, to expose the bronchial tree for fixation (10% buffered formalin) and embedding (paraffin). For the semiquantitative analysis of pathological changes (Table 4), pairs of two serial sections were sectioned at five steps each, 10 µm apart, from each lung lobe. One of each pair of sections was stained with hematoxylin and eosin while the corresponding section was subjected to the immunocytochemical staining reaction for mammalian bombesin. The lesions

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3 To whom requests for reprints should be addressed.

4 The abbreviations used are: NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; DEN, N-nitrosodietyamine.

1960
NNK-INDUCED LUNG TUMORS AND HYPEROXIA

Table 1 Experimental design

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Treatment</th>
<th>Sacrifice</th>
<th>Analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>15</td>
<td>NNK + 70% O₂</td>
<td>When moribund (12-16 wk)</td>
<td>Pathology</td>
</tr>
<tr>
<td>2</td>
<td>15</td>
<td>NNK + 70% O₂</td>
<td>Serial sacrifice</td>
<td>Pathology</td>
</tr>
<tr>
<td>3</td>
<td>15</td>
<td>Triocanoin + 70% O₂</td>
<td>At same time as last animals of group 1</td>
<td>Pathology</td>
</tr>
<tr>
<td>4</td>
<td>15</td>
<td>NNK + ambient air</td>
<td>When moribund (8-12 mos)</td>
<td>Pathology</td>
</tr>
<tr>
<td>5</td>
<td>15</td>
<td>NNK + ambient air</td>
<td>Serial sacrifice</td>
<td>Pathogenesis</td>
</tr>
<tr>
<td>6</td>
<td>15</td>
<td>Triocanoin + ambient air</td>
<td>At same time as last animals of group 4</td>
<td>Pathology</td>
</tr>
<tr>
<td>7</td>
<td>24</td>
<td>70% O₂ + [methyl-³H]thymidine</td>
<td>Serial sacrifice</td>
<td>Autoradiography</td>
</tr>
</tbody>
</table>

Table 2 Pathobiology of NNK-induced lung tumors

<table>
<thead>
<tr>
<th>Tumor type</th>
<th>NNK + 70% O₂</th>
<th>NNK + ambient air</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neuroendocrine; mixed neuroendocrine/squamous</td>
<td></td>
<td>Adenomatous, mixed adenosquamous</td>
</tr>
<tr>
<td>Tumor incidence</td>
<td>70% (10)⁺</td>
<td>80% (12)</td>
</tr>
<tr>
<td>Tumor latency⁶</td>
<td>8 weeks</td>
<td>16 weeks</td>
</tr>
<tr>
<td>Survival time</td>
<td>12-16 weeks</td>
<td>8-12 months</td>
</tr>
<tr>
<td>Pathogenesis</td>
<td>Type II cell → neuroendocrine cell → neuroendocrine tumor</td>
<td>Clara cell → mixed Clara cell/alveolar type II cell tumor</td>
</tr>
</tbody>
</table>

⁺ Numbers in parentheses, number of tumor-bearing animals.
⁶ Time at which the first microscopically detectable tumors were found is defined as “tumor latency” in this study.

RESULTS

As has been previously reported (1) NNK caused a significant incidence of adenomatous and adenosquamous lung tumors in hamsters maintained under ambient air conditions (Table 2, Fig. 1). These tumors were microscopically detectable after 16 weeks of NNK treatment, although the animals did not show clinical symptoms of lung tumors before 8 months after the start of the experiment (Table 2). The tumor morphology was identical to that of DEN-induced adenocarcinomas previously reported (4, 5). Briefly, they demonstrated a glandular growth pattern (Fig. 1) and were composed of Clara cells and alveolar type II cells. The tumors were multiple, sometimes confluent, and occupied large portions, up to entire lobes of the lungs. As is common for all rodent lung tumors, metastasis was not observed and mitotic activity was low. However, because of their considerable size and confluency, these tumors were diagnosed as adenocarcinomas.

The serial sacrifice experiment conducted under ambient air conditions revealed a pathogenesis identical to that reported for diethylnitrosamine (4, 6, 7) and nitrosomorpholine (4, 16). Briefly, hypertrophy of smooth endoplasmic reticulum in Clara cells was the earliest detectable change in such animals. This was followed by the development of alveolar type II cell characteristics (production of phospholipid) (Fig. 2) in numerous
Fig. 4. Histopathology of neuroendocrine lung tumor induced by NNK and hyperoxia. The tumor is compact and highly cellular, and gland-like cell growth is rare. Hematoxylin and eosin, × 160.

Fig. 5. Immunocytochemical stain for mammalian bombesin of neuroendocrine lung tumor induced by NNK and hyperoxia. Note large patchy (dark stained) areas of positive immunoreactivity. Primary antibody (1:1000) for 48 h. Vectastain ABC kit, Meyer's hematoxylin counterstain. × 240.

Clara cells. Subsequently developing small adenomatous lesions were composed of a mixture of both these cell types. Throughout the treatment period, multiple focal hyperplasias of bronchial and bronchiolar neuroendocrine cells (Fig. 3) were found, some of which demonstrated delicate bundles of tonofilaments by electron microscopy. Squamous foci in the adenomatous tumors which had developed by the end of the experiment occasionally contained neuroendocrine granules as well as tonofilaments by electron microscopy, as previously reported (7).

Hamsters which had been maintained in an atmosphere of 70% O₂ while receiving NNK developed neuroendocrine lung tumors (Figs. 4–6). The neoplasms were always multiple, compact, and highly cellular (Fig. 4) with only occasionally gland-like patterns. Large areas of the tumors demonstrated positive immunoreactivity to mammalian bombesin (Fig. 5) and calcitonin, and the majority of tumor cells contained numerous dense-cored granules of the neuroendocrine type (Fig. 6), while some also exhibited early squamous metaplasia. Moreover, all animals in this group lost substantially more weight (average body weight at sacrifice, 105 ± 15 g) than the animals given NNK under ambient air conditions (average body weight at sacrifice, 153 ± 18 g). Mitosis was more frequent than in the adenocarcinomas. Peripheral tumor areas exhibited infiltrative growth into the surrounding lung parenchyma and occasionally into pulmonary blood vessels. Because they did not demonstrate the oat-like cellular morphology typical of small cell cancer in humans but, in contrast to carcinoids and tumorlets, showed distinct signs of malignancy, these tumors were classified as neuroendocrine carcinomas.

The serial sacrifice experiment conducted under such conditions revealed, 2 weeks after the start of the experiment, numerous foci of alveolar cells with a weak but reproducible (stain was repeated 3 times) positive immunoreactivity to mammalian bombesin (Fig. 7, Table 4). Such foci were, however, negative when exposed to immunostains for calcitonin. By electron mi-
and neuroendocrine cells (dense-cored secretion granules). Their ultrastructure demonstrated dual differentiation into alveolar type II cells (lamellar bodies) and neuroendocrine cells (secretion granules). Their ultrastructure thus supports the interpretation of the immunostain illustrated in Fig. 7 that alveolar type II cells change into bombesin-producing neuroendocrine cells during initial O2-NNK exposure. Uranyl acetate, lead citrate, × 17,500.

Table 3 Labeling index of lung cells in hyperoxia-exposed hamsters given injections of [methyl-3H]thymidine

<table>
<thead>
<tr>
<th>Oxygen exposure</th>
<th>Alveolar type II cells</th>
<th>Bronchial and bronchiolar cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>None (controls)</td>
<td>0.54 ± 0.16</td>
<td>0.18 ± 0.09</td>
</tr>
<tr>
<td>4 h</td>
<td>0.46 ± 0.18</td>
<td>0.16 ± 0.11</td>
</tr>
<tr>
<td>12 h</td>
<td>0.35 ± 0.11</td>
<td>0.12 ± 0.12</td>
</tr>
<tr>
<td>24 h</td>
<td>1.31 ± 0.05*</td>
<td>0.50 ± 0.05*</td>
</tr>
<tr>
<td>2 days</td>
<td>1.55 ± 0.40*</td>
<td>1.36 ± 0.55*</td>
</tr>
<tr>
<td>3 days</td>
<td>2.28 ± 0.62*</td>
<td>0.67 ± 0.15*</td>
</tr>
<tr>
<td>4 days</td>
<td>2.38 ± 0.08*</td>
<td>0.79 ± 0.20*</td>
</tr>
<tr>
<td>7 days</td>
<td>2.84 ± 0.25*</td>
<td>0.78 ± 0.11*</td>
</tr>
<tr>
<td>14 days</td>
<td>1.86 ± 0.68*</td>
<td>0.55 ± 0.14*</td>
</tr>
</tbody>
</table>

* Significantly higher (P < 0.01) than ambient air controls.

The corresponding ambient air groups (Table 2).

 Autoradiograms from lungs of hyperoxia-exposed hamsters after injection of [methyl-3H]thymidine were prepared in an attempt to clarify the controversial question of whether proliferation of pulmonary neuroendocrine cells arises from the few preexisting neuroendocrine cells present in the adult mammalian lung or from other lung cell types. Labeling of bronchial/bronchiolar cells was low but significant (P < 0.01; Table 3). Moreover, a highly specific and significant (P < 0.01) increase in labeling index was observed in alveolar type II cells, while alveolar type I cells, endothelia, and connective tissue elements remained unlabeled.

**DISCUSSION**

Our data demonstrate that the most potent carcinogenic nitrosamine contained in cigarette smoke, NNK, causes a high incidence of neuroendocrine lung tumors when administered in conjunction with hyperoxia. It is possible that a direct effect of O2 on nitrosamine metabolism is partially responsible for this effect. However, there is ample documentation that the normally sparse pulmonary neuroendocrine cells increase rapidly in number in response to alterations (decreases as well as increases) in pulmonary oxygen levels (10, 12, 13). In fact the only physiological stimulus for this response is the drastic increase in pulmonary oxygen levels concomitant with birth (10, 12, 13). Studies in rabbits and rats have demonstrated that this reaction is mediated by oxygen receptors which detect deviations from preexisting normal oxygen levels in the ventilated air of the lung (17, 18). In our model, such rapid numerical increase in pulmonary neuroendocrine cells appears to be initially accomplished through proliferation and differentiation of alveolar type II cells into neuroendocrine cells. The study with [3H]thymidine shows that exposure to hyperoxia by itself has a strong mitogenic effect on alveolar type II cells. In contrast, bronchial/bronchiolar epithelial cells including preexisting neuroendocrine cells had a much lower labeling index. The immunocytochemical stains for mammalian bombesin and calcitonin revealed multiple foci of alveolar cells which demonstrated a weak positive reaction to bombesin but not to calcitonin after 2 weeks of exposure to NNK and hyperoxia (Table 4). Because the positive immunoreaction was only weak (Fig. 7), although reproducible, such areas were additionally studied by electron microscopy. In support of our interpretation of the immunocytochemical data (that alveolar type II cells in such foci were producing mammalian bombesin), we found neuroendocrine secretion granules in such cells. Such granules are the storage site of polypeptides such as mammalian bombesin (10, 13). Moreover, the fact that only moderate to low numbers of these granules were present in the cells explained why the immunoreaction was only weak. These findings support the theory that pluripotent lung cells can give rise to a variety of phenotypes (19), which in turn explains the well known heterogenicity of human lung tumors (19).

As evidenced by unsuccessful efforts to induce neuroendocrine lung tumors with chemical carcinogens including nitrosamines (20), the altered pulmonary oxygen levels are an essential factor for the induction of this tumor type. Accordingly, in our experiments NNK induced non-neuroendocrine tumors of Clara cell origin when administered to hamsters maintained in ambient air conditions. Similarly, DEN causes Clara cell-derived lung adenomas in hamsters maintained in ambient air (4, 5, 8), while inducing neuroendocrine lung tumors when given to animals exposed to hyperoxia (8). The hyperoxia-nitrosa-
mine lung tumors in the hamster represent a good model for human neuroendocrine neoplasia, including small cell lung cancer. Diagnostic difficulties in a subclassification of human neuroendocrine lung tumors into carcinoids, atypical carcinoids, and small cell lung cancer has prompted the suggestion of using the term "neuroendocrine carcinoma" instead (10, 19–21).

With respect to their malignant biological behavior (local invasiveness, impairment of pulmonary function, cancer cachexia), our hamster tumors are distinctly different from human central and peripheral carcinoids and most closely resemble the borderline cases of human atypical carcinoid/small cell lung cancer, without expressing all the typical features of only one or the other. As in many cases of human lung tumors, it is therefore most appropriate to classify our hamster tumors as neuroendocrine carcinomas. Because of humane considerations, the hamsters in our study were sacrificed upon occurrence of clinical symptoms. This contrasts sharply with the situation in human cancer patients, who generally do not consult a doctor unless such clinical symptoms have been persistent for some time and are then kept alive as long as possible. Consequently, the pathology of our hamster tumors reflects a relatively early stage of cancer development, which in human patients may remain undetected in most cases. Moreover, the above may well explain why the epidemiological association between cigarette smoking and small cell lung cancer is strong (9), whereas no such link has been established with less malignant tumors of this category.

With both nitrosamines, tumor latency periods and survival times were substantially shorter in animals developing neuroendocrine tumors than in hamsters developing Clara cell-derived adenomas. This is in accord with the aggressive biological behavior of human neuroendocrine lung cancer (16). A possible explanation for this phenomenon is the recently documented receptor-mediated uptake mechanism of nitrosamines by pulmonary neuroendocrine cells (18, 19), which results in a more than 1000-fold higher rate of DEN metabolism in pulmonary neuroendocrine cells than in Clara cells (20–22). This in turn may yield higher levels of carcinogenic metabolite(s) in individuals with greater than normal numbers of pulmonary neuroendocrine cells or neuroendocrine tumors. Moreover, our data demonstrate that the neuroendocrine tumors induced by hyperoxia and DEN (8) or NNK produce mammalian bombesin and that detectable levels of this polypeptide are already present at an early precancerous stage of tumor development. It has been amply documented that mammalian bombesin acts as an autocrine growth factor on neuroendocrine lung tumors (16). It seems likely therefore that, in our model, mammalian bombesin produced by hyperplastic pulmonary neuroendocrine cells and tumors derived therefrom contributes to the rapid tumor growth. In this context it is of interest that the secretion of mammalian bombesin is stimulated by acetylcholine and cholinergic agonists (27). Recent experiments have shown that nicotine as well as DEN and NNK are taken up by nicotinic cholinergic receptors in pulmonary neuroendocrine cells (22, 23) and stimulate secretion of neuroendocrine peptides (28-30) and cell proliferation (22, 23), thus mimicking the proliferative and secretagogue effects of the neurotransmitter acetylcholine. Our current working hypothesis, is, therefore (a) unphysiological pulmonary oxygen levels cause a change of differentiation of alveolar type II cells into neuroendocrine cells; (b) these new numerous neuroendocrine cells take up nitrosamines via cholinergic receptors, thus acting as agonists of acetylcholine; (c) the neuroendocrine cells respond to the cholinergic agonists by secretion of peptides such as mammalian bombesin and produce carcinogenic metabolites of nitrosamines; and (d) the combined effects of the continuously secreted growth factor bombesin and produced nitrosamine metabolites result in uncontrolled and rapid growth of neuroendocrine cells, culminating in the development of neuroendocrine lung cancer.
REFERENCES


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