Role of the Alveolar Type II Cell in the Development and Progression of Pulmonary Tumors Induced by 4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone in the A/J Mouse

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ABSTRACT

The role of the type II cell in the development of pulmonary tumors induced in the adult A/J mouse (6 weeks of age) by treatment with a single dose (100 mg/kg, i.p.) of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) was investigated. Twenty-four h following treatment with NNK, the concentration of O6-methylguanine was similar in Clara and type II cells. However, hyperplasias were detected only along the alveolar septa in lungs 14 weeks after carcinogen treatment. Examination of the ultrastructure of several hyperplasias revealed that the proliferating cells resembled type II pneumocytes. The proliferating cells were cuboidal in shape, with centrally localized ovoid nuclei characterized by minor indentations. Lamellar bodies, one of the major hallmarks of the type II cell, were present in the cytoplasm. The progression of pulmonary lesions was followed by sacrificing mice at 4-week intervals from 14 to 54 weeks after treatment with NNK. From 34 to 42 weeks after treatment, progression to neoplasia was demonstrated by a decline in the frequency of hyperplasias and an increase in the frequency of adenomas. Approximately 50% of the adenomas were observed arising within hyperplasias. Carcinomas appeared to increase in frequency 34 weeks after carcinogen treatment and comprised greater than 50% of the pulmonary lesions by 54 weeks. Approximately 30% of the carcinomas were observed arising within adenomas. The growth pattern of carcinomas began to change from solid to mixed (solid and papillary) 42 weeks after NNK. Moreover, electron micrographic analysis demonstrated that, within a hyperplasia, proliferating type II cells could change from cuboidal to columnar in shape and could also exhibit nuclear indentations, both characteristics displayed by the Clara cell. Thus, this divergence of the type II cell from its well characterized morphological features indicates that the selective growth advantage which these initiated cells possess can result in changes to the normal ultrastructure of this cell as it progresses toward malignancy. DNA was isolated from 20 hyperplasias and screened for the presence of an activated K-ras gene. This gene was activated in 17 of 20 lesions, with 85% of the mutations involving a GC to AT transition within codon 12 (GGT to GAT), a mutation consistent with base mispairing produced by the formation of the O6-methylguanine adduct. This specificity for activation of the K-ras gene was identical to that observed previously in adenocarcinomas induced by NNK. These results indicate that the genesis of pulmonary neoplasia induced in the A/J mouse by NNK involves formation of the O6-methylguanine adduct within type II cells, which leads to the activation of the K-ras gene, followed by proliferation of type II cells and subsequent progression to a malignant tumor.

INTRODUCTION

The tobacco-specific nitrosamines, in particular NNK,1 have been identified as potent carcinogens in laboratory animals. Treatment of rats with NNK results in the induction of tumors in the nasal cavity, lung, liver, and pancreas (1, 2). Pulmonary tumors are also induced in mice and hamsters following either acute or chronic exposure to this carcinogen (3, 4). NNK is a major constituent of tobacco products, being present in both mainstream and sidestream smoke and in unburned tobacco (5). The activation of NNK may occur via α-hydroxylation, resulting in the formation of the promutagenic adduct O6MG (6), or via hydroxylation at the N-methyl carbon, yielding, upon hydrolysis, a pyridyloxobutyl diazohydroxide (7), which could react with DNA to form a bulky adduct. The involvement of the O6MG adduct in the induction of pulmonary tumors by NNK was substantiated in studies with A/J mice. Lung tumors induced in A/J mice by treatment with NNK were evaluated for the presence of activated oncoproteins (8). Activation of the K-ras proto-oncogene was detected in 100% of the tumors evaluated. The majority of activating mutations were localized to codon 12 of this gene, and the GC to AT mutation observed was consistent with the base mispairing associated with formation of the O6MG adduct (8).

All of the pulmonary tumors examined in the previous study (8) were classified as papillary adenocarcinomas, a tumor type that has some morphological properties similar to those of the nonciliated bronchiolar epithelial cell (Clara cell) (9, 10). The role of the Clara cell in the development of pulmonary tumors in A/J mice seemed consistent with previous studies in rats, where the O6MG adduct had been found to accumulate and to exhibit cell specificity for formation in Clara cells from F344 rats during multiple administrations of NNK (11–13). In addition, the rate of repair for this promutagenic lesion was very low in Clara cells, compared to other pulmonary cell types (14). A recent study (15) also examined the relationship between the formation of the O6MG adduct and the induction of pulmonary tumors in the F344 rat. A linear relationship was observed when the concentration of O6MG in Clara cells as a function of dose was plotted against the corresponding tumor incidence. This relationship was not observed using DNA adduct concentrations in type II cells or whole lung. Thus, these results indicate that the concentration of O6MG in the Clara cell is an excellent indicator of the carcinogenic potency of NNK in the lung and, based on biochemical data, also support the involvement of the Clara cell in the development of pulmonary neoplasms induced by NNK in rats. However, morphological studies failed to support the Clara cell being the site for development of NNK-induced neoplasms in the rat. The early proliferative changes did not involve the bronchiolar airways, the anatomical location for the Clara cells; rather, the hyperplasias detected all arose within the alveolar area. These lesions appeared to progress to solid adenomas, tumors which in mice are thought to derive from type II cells (9). Differentiation to the malignant state was associated with the appearance of a papillary growth pattern, which has frequently been ascribed to tumors of bronchiolar origin (9, 10). However, ultrastructural examination of hyperplasias, adenomas, and carcinomas in this study revealed morphological features characteristic of the type II cell (15).
The dichotomy between biochemical and morphological studies in F344 rats makes it difficult to hypothesize a cell of origin for the observed pulmonary neoplasms. The purpose of the present study is to determine the role of the type II cell and the Clara cell in the induction of pulmonary tumors in A/J mice by NNK. The development and progression of pulmonary tumors will be followed by histological examination of lungs from mice sacrificed over a 12-month period following treatment with NNK. Tumor localization and development will also be compared to the distribution of the O6MG adduct in pulmonary cells and the mutation spectrum within the K-ras gene in preneoplastic lesions induced following carcinogen treatment.

MATERIALS AND METHODS

Carcinogen Treatment. NNK was synthesized (16,17) by Chemsyn Science Laboratories (Lenexa, KS). Purity was >99%, according to nuclear magnetic resonance, UV, and IR spectroscopy and thin layer chromatography. Female strain A mice (6 weeks old; The Jackson Laboratory) were treated with a single dose of NNK (100 mg/kg, i.p.) dissolved in trioctanoin. Animals were fed NIH 07 diet and water ad libitum. Mice used for the quantitation of the O6MG adduct in pulmonary cells and whole lung were sacrificed 24 h after carcinogen treatment. The time course for the development and progression of proliferative lesions in animals treated with NNK was determined by sacrificing mice starting 14 weeks after treatment and every 4 weeks thereafter, with the final time point being 54 weeks after carcinogen exposure. Fifteen mice were sacrificed at each time point.

Histological Procedures. Lungs were inflated and fixed with 4% buffered paraformaldehyde for 18–24 h and were then transferred to 70% ethanol for routine histological processing and staining of paraffin sections with hematoxylin and eosin. A single standardized section was prepared from all lungs, by first facing the tissue block and then cutting a full-face 5-μm section which included all five lobes (18). All lungs appeared to have been inflated to a similar extent, since the area of the tissue section did not differ markedly among samples. For electron microscopic evaluation, selected pieces of tissue macroscopically visible lung lesions were excised, cut into approximately 1- x 1-mm blocks, and fixed at 4°C overnight in 2% glutaraldehyde. The tumors were then postfixed in 1% osmium tetroxide in 0.1 M cacodylate buffer (pH 7.4) for 1 h, dehydrated in graded ethanol, and embedded in epoxy resin. Sections were prepared on an ultramicrotome and stained with 1% toluidine blue for light microscopic evaluation and orientation of lungs from mice sacrificed over a 12-month period. The development and progression of pulmonary tumors will be followed by histological examination of lungs from mice sacrificed over a 12-month period following treatment with NNK. Tumor localization and development will also be compared to the distribution of the O6MG adduct in pulmonary cells and the mutation spectrum within the K-ras gene in preneoplastic lesions induced following carcinogen treatment.

Cell Separation. Eighteen mice were required to generate sufficient numbers of pulmonary cells to facilitate DNA isolation and the quantitation of O6MG. Three sets of mice were used, to allow triplicate analysis of the O6MG adduct. Lung cells were isolated as described previously (12), with the following modifications. The lung cell suspension was layered onto 8 ml fetal bovine serum and centrifuged for 12 min at 200 x g (19). The supernatants were discarded, and the cell pellets were resuspended in elution buffer [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid-buffered salt solution: F12K, 2.1 containing 0.05% Dnase I. Fractions of pulmonary small cells (primarily endothelial cells and lymphocytes), alveolar type II cells, and Clara cells were then isolated from the lung cell digest by centrifugal elutriation. The first fraction, which consisted largely of debris and RBC, was collected with a rotor speed of 2500 rpm and a flow rate of 10 ml/min and was discarded. Small cells (135 x 10^6 cells) were then collected with a rotor speed of 2200 rpm and a flow rate of 14 ml/min. This fraction consisted primarily of small unidentified cells and lymphocytes, as well as 24 ± 3% type II cells, 10 ± 1% macrophages, and <1% Clara cells. The alveolar type II cell fraction (108 ± 4 x 10^6 cells) was then collected at a rotor speed of 1200 rpm and a flow rate of 13 ml/min. This fraction contained 51 ± 2% type II cells, 27 ± 2% macrophages, 5 ± 1% Clara cells, approximately 10% lymphocytes, and other unidentified cells. After collection of this fraction, the rotor was stopped and the remainder of the cells in the elutriation chamber were collected (34 ± 2 x 10^6 cells) and constituted the Clara cell fraction. This fraction consisted of 53 ± 4% Clara cells, 26 ± 3% macrophages, 5 ± 1% type II cells, and other unidentified cells. The cells from three experiments were counted, and the numbers are means ± SE of 18 lungs/experiment. Clara cells were identified after staining with nitroblue tetrazolium (20), and type II cells were identified by staining with the modified Papanicolaou stain (21). Macrophages could also be identified with this stain.

DNA Isolation. DNA was isolated from whole lung and lung cell fractions by digestion with Pronase (10 mg/ml; Calbiochem) in 1% sodium dodecyl sulfate in TNE buffer (10 mM Tris, 150 mM NaCl, 2 mM EDTA, pH 7.5), followed by phenol-chloroform extraction and ethanol precipitation (22). Samples were incubated with RNase A (300 units) and RNase T1 (100 units) for 3 h at 37°C, and DNA was recovered by ethanol precipitation.

Determination of O6-Methylguanine. A competitive radioimmunoassay was used for the measurement of O6MG in lungs, as described previously (12, 23). Limits of detection were 0.1 pmol/μmol of unmodified base.

Isolation of Hyperplasias. Eleven 10-μm sections were cut from each paraffin block, and the middle section was stained with hematoxylin and eosin for diagnosis of focal lesions. Photographs were taken of each stained section, so that hyperplasias could be identified and located on the unstained slides. Individual hyperplasias were scraped from each slide, and 10 serial sections from one hyperplasia were placed in a 1.5-ml Eppendorf tube. Razor blades and gloves were changed between each sample, to avoid tissue carryover. When all samples were ready to process, they were spun in an Eppendorf desktop centrifuge for 1 min, to collect the tissue in the bottom of the tubes.

Samples were deparaffinized by extracting twice with xylene and two times with 100% ethanol, followed by drying in a Speed Vac (Savant) for 30 min. The tissue precipitates were suspended in 100 μl autoclaved distilled water, boiled for 15 min, and centrifuged for 5 min in an Eppendorf desktop centrifuge. Forty μl of the supernatant were used for amplification of the first or second exons of the K-ras gene.

DNA Amplification. DNA was amplified by the polymerase chain reaction (24). Two sets of primers were used to amplify the first and second exons of the K-ras gene in DNA isolated from paraffin-embedded lung hyperplasias. First, separate amplification reactions of 35 cycles (95°C for 1 min, 45°C for 2 min, and 72°C for 2 min), using
Fig. 1. Development and progression of neoplasia in the mouse lung following treatment with NNK. A, focal hyperplasia localized with the alveolar parenchyma. Within the hyperplasia, there is proliferation of type II cells along existing alveolar walls. × 65. B, a solid adenoma (arrow) characterized by proliferating epithelial cells is arising within an existing hyperplasia. × 165. C, a large solid adenoma just below the pleural surface has effaced and replaced the alveolar parenchyma, causing compression of the adjacent alveolar tissue (arrow). × 65. D, a carcinoma composed of columnar cells (arrows) is arising at the edge of an adenoma. × 120. E, the pulmonary parenchyma has been replaced by a mixed (solid and papillary) carcinoma. A portion of this carcinoma which invaded the airway in a different plane of the section is present (arrow). × 65. F, high magnification of a microcarcinoma which has arisen within the alveolar parenchyma. Note the cellular and nuclear pleomorphism and hyperchromasia. × 325.
outer primers (0.1–0.2 μM) near the 5' and 3' ends of the exons (5'-TTACCTCTATTGAGGGCTCTGAG-3' and 5'-GCAGCTACCACTCTACTCATCGTA-3' for exon 1 and 5'-TTCCTCAGGACTCTCTACAGGA-3' and 5'-ACCCACCTATAAGGTGAAT-3' for exon 2), were used to generate a 142-base pair fragment for exon 1 or a 192-base pair fragment for exon 2. This was followed by 40 (exon 2) or 45 (exon 1) cycles of amplification (95°C for 1 min, 50–52°C for 2 min, and 72°C for 2 min) with a fresh reaction mix containing 2 μl of the first polymerase chain reaction mixture as the source of DNA template and other sets of inner primers (1 μM) (5'-AGTACGTGAGTATAAACCCTGT-3' and 5'-TCGTTACTCCCTCAAAGTG-3' for exon 1 and 5'-TACAGGAAACAGTGAAATGTTGGAGGA-3' and 5'-TAATGGTGAATCTTCAATGTTATG-3' for exon 2), to generate a fragment of 98 base pairs for exon 1 and 171 base pairs for exon 2. Incubations containing DNA from normal tissue and controls without DNA were run with all sets of reactions.

Amplified DNA was desalted, and unused primers and deoxynucleotide triphosphates were removed by spin dialysis in a Centricon 30 tube (Amicon, Danvers, MA). DNA concentrations were measured by reading the A405 through which samples were evaporated and stored at −20°C until further use.

Slot Blot Oligonucleotide Hybridization. Second cycle-amplified DNA samples (10–50 ng) were denatured in 0.4 M NaOH/3 M NaCl and applied to Nytan nylon filters (0.45-μm mesh), using a slot-blot apparatus (Schleicher & Schuell, Keene, NH). The filters were hybridized to 19-mer probes that were centered on the second base of codon 12 of the mouse K-ras gene and contained either wild-type sequence (GGT) or mutant sequence (GAT or GTT) for this codon. Nineteen-mer probes were also centered on the second base of codon 61 and contained either a wild-type (CAA) or mutant sequence (CGA) for this codon. Following hybridization, blots were washed according to the method of Saiki et al. (25).

Restriction Fragment Length Polymorphic Identification of K-ras Mutations. An additional method was employed to identify mutations in the K-ras gene from alveolar hyperplasias. This was based on restriction fragment length polymorphism in DNA containing either a GGT to GTT mismatch instead of a C in the third base from the 3' end, causing the formation of a restriction site for the enzyme HphI only if the second base of codon 12 also contains the G to A transition mutation. The inner primers used for amplification of exon 1 were 5'-AATCTGGGTAAGTGGAGGT-3' and 5'-TTACCTCTATCGTAGGGTCGTACTCATCCA-3' and generated a fragment of 104 base pairs. A restriction site for the Taq 1 enzyme is created by the presence of a A to G mutation in the second base of codon 61. Thus, amplified DNA from 20 hyperplasias was digested with HphI (1.5 μg amplified exon 1 DNA) or Taq 1 (0.5 μg amplified exon 2 DNA), in a volume of 20 μL, for 2 h at 37°C. The reaction was stopped by heating for 10 min at 65°C. The DNA was end-labeled by adding [γ-32P]ATP (1 μCi) and phosphate exchange buffer (Boehringer Mannheim end labeling kit). The reaction was allowed to proceed for 40 min at 37°C, in a final volume of 25 μL. Three μl of TBE buffer (90 mM Tris-borate, 2 mM EDTA (10X)) and 2 μl of dye mixture were added before 5 μl of the reaction mix were loaded onto an 8% nondenaturing acrylamide gel, which was run for 2–2.5 h at 600 V. Gels were dried and exposed to X-ray film overnight.

Direct Sequencing. Direct sequencing of the amplified first exon of the K-ras gene was performed as described by Tindall and Stankowski (27). The sequencing primer used for exon 1 was 5'-TGGATTCGAAT-3'. The sequencing primer (30 pmol), end-labeled with [γ-32P]ATP and T4 polyadenylate kinase, was annealed to the amplified DNA sample (0.2–0.5 μg) for 5 min on ice, following heat denaturation at 95°C for 5 min in 40 mM Tris, pH 7.5, 20 mM MgCl2, 50 mM NaCl. A 3-μl aliquot of labeled primer-DNA mixture was then added to four tubes containing 4 μl of Sequenase enzyme 1.0 (USB, Cleveland, OH), dithiothreitol, deoxynucleotide triphosphates, and either dideoxy-ATP, dideoxy-CTP, dideoxy-GTP, or dideoxy-TTP. The sequencing reactions were allowed to proceed for 2 min at 37°C and were terminated with 8 μl stop solution-dye mixture (USB). Samples were denatured at 95°C for 5 min prior to electrophoresis on an 8% acrylamide gel containing 8 M urea. Gels were dried and exposed to X-ray film for 1–2 days. The proportion of mutant allele, relative to wild-type allele, was estimated based on densitometry comparisons of band intensities for the guanine (GGT, wild-type) versus adenine (GAT, mutant) at codon 12 detected on the sequencing ladders.

RESULTS

Development and Progression of Pulmonary Tumors. The time course for the development of pulmonary tumors was determined in the A/J mouse following single dose treatment with NNK (100 mg/kg). Groups of animals were sacrificed starting 14 weeks after carcinogen treatment and every 4 weeks thereafter, with the last time point occurring 54 weeks after carcinogen exposure. The number of histological lesions in each lung was estimated from a standardized 5-μm paraffin section stained with hematoxylin and eosin. The relative lesion frequency at each sacrifice time point was determined by dividing the number of a specific lesion (e.g., hyperplasia) by the total number of lesions observed and multiplying by 100. This was done for each animal, and the mean percentage of specific lesions (compiled from 15 animals/time point) was determined at each sacrifice interval. Although examination of a single section from each lung resulted in an underestimation of the total number of lesions per lung (18), the purpose of these experiments was to determine not the absolute total number of lesions but, rather, the frequency of specific lesions and how this changed during tumor progression. However, the mean number of pulmonary lesions detected in the standardized section did appear relatively constant by 30 weeks following carcinogen treatment (Fig. 2), and the average number of lesions observed per histological section was 7.6 ± 0.3 for the remainder of the sacrifice time points. This time point also coincided with detection of the greatest number of hyperplasias per slide following carcinogen treatment. For purposes of demonstrating graphically the progression of pulmonary lesions, adenomas arising within hyperplasias were grouped with the adenomas, while carcinomas arising in adenomas were considered carcinomas arising in adenomas.
DEVELOPMENT OF TYPE II CELL TUMORS

nomas (Fig. 3). Due to the lack of histological evidence concerning the development of the small number of microcarcinomas observed in this study, this lesion was not grouped with the other carcinomas when the data were plotted in Fig. 3. The specific distribution of all categories of pulmonary lesions at each sacrifice point is presented in Table 1.

Fourteen weeks after treatment, all of the pulmonary lesions were classified as alveolar hyperplasias. The number of hyperplasias detected per histological section declined rapidly beginning 34 weeks after treatment (Fig. 2), but the relative frequency of hyperplasias began to decline 26 weeks after treatment (Fig. 3; Table 1). This latter decrease was accompanied by a marked increase in the relative frequency of adenomas (Fig. 3), with approximately 50% of these tumors detected arising within a hyperplasia (Table 1). The frequency of adenomas appeared to reach a maximum of 41% by 42 weeks after NNK treatment. At this time point, only 20% of these adenomas were detected arising within hyperplasias, an observation consistent with the progression of these preneoplastic lesions to adenomas. The relative frequency of carcinomas appeared to increase almost linearly from 34 to 50 weeks, and carcinomas ultimately comprised >50% of the pulmonary lesions 54 weeks after carcinogen treatment (Fig. 3). Approximately 30% of the carcinomas arose within adenomas (Table 1). The relative frequency of microcarcinomas ranged from 0 to 6% during the 40-week sacrifice interval.

Localization and Ultrastructure of Pulmonary Lesions. The earliest lesions detected in lungs from A/J mice treated with NNK were localized to the alveolar area and consisted of focal proliferation along the alveolar septae (Fig. 1). This lesion was further characterized by electron micrographic examination. Depicted in Fig. 4A is a toluidine blue-stained section of an alveolar hyperplasia. Electron micrographic analysis was conducted from three different areas of this lesion, the border (Fig. 4B), an area halfway to the center of this hyperplasia (Fig. 4C), and the center portion (Fig. 4D). Evaluation of the border of this lesion (Fig. 4B) revealed the presence of two type II cells adjacent to each other. These cells were identified by their cuboidal shape, with centrally localized ovoid nuclei characterized by minor indentations. Lamellar bodies, one of the major distinctive features of the type II cell, were present in the cytoplasm. Toward the center of this hyperplasia (Fig. 4C), there was thickening of the alveolar septae by multiple type II cells. In addition to the presence of abundant lamellar bodies, multivesicular bodies and dense mitochondria were present in the cytoplasm of these cells. The center of this hyperplasia was occupied by numerous confluent type II cells (Fig. 4D) that often had a columnar shape, pronounced nuclear indentations, and associated tubular myelin. At the electron microscopic level, carcinomas were composed of cells with ultrastructural features similar to those observed in hyperplasias (data not shown).

The growth pattern of carcinomas induced by NNK treatment appears to change during the progression of these lesions (Fig. 5). Seventy % of the carcinomas that were initially detected after 34 weeks of treatment exhibited a solid growth pattern. However, by 46 weeks, a significant increase in the number of carcinomas exhibiting a mixed (solid, papillary, and sometimes glandular) growth pattern was discernable. The percentage of carcinomas with a mixed growth pattern increased over time, while the frequency of tumors with a solid growth pattern declined (Fig. 5).

Cell-specific Differences in O6-Methylguanine Formation. The concentration of O6MG was determined in whole lung, small cells, type II cells, and Clara cells 24 h after treatment of mice with 100 mg/kg NNK, the same dose of carcinogen used to induce pulmonary tumors. A recent paper by Chichester et al. (28) reports the isolation of a Clara cell fraction of 75–80% purity from Swiss Webster mice. However, this method does not permit the isolation of a type II or small cell fraction. The purity of both the type II and Clara cell fractions isolated in this study was approximately 50% (Table 2), with macrophages being the major contaminant (approximately 25%) of both cell preparations. Unfortunately, the yield of macrophages from pulmonary lavage was not sufficient to enable adequate amounts of DNA to be isolated for quantitation of the O6MG adduct. However, since macrophages contaminated the Clara cell and type II fractions to a similar extent, it was possible to compare the concentration of O6MG adduct present in these two cell types. Cell specificity was observed in the lung for the formation of O6MG (Table 2). The concentration of this adduct was similar in type II and Clara cells. The concentration of O6MG in these two cell types was 2- and 5-fold greater than adduct concentrations observed in whole lung and small cells, respectively (Table 2).

Identification of Mutations in the K-ras Gene from Alveolar Hyperplasias. Twenty hyperplasias were selected from paraffin-embedded lungs obtained 26–30 weeks after NNK treatment, for analysis of mutations in the K-ras gene. DNA was amplified by the polymerase chain reaction, and mutations were identified in 17 of 20 hyperplasias (Table 3) by restriction fragment length polymorphism and oligonucleotide mismatch hybridization. DNA isolated from the hyperplasias occupies Fig. 5, lanes 1–20, while Fig. 5, lane 21, contains DNA isolated from an adenocarcinoma with a GC to AT mutation in codon 12 and Fig. 5, lane 22, contains DNA from a control lung. HphI restriction enzyme digestion of DNA containing a GGT to GAT mutation in codon 12 generates a 72-base pair fragment, which can be end-labeled with 32P and separated on an acrylamide gel (Fig. 6A). Using this method, GC to AT mutations were detected in 15 of 20 hyperplasias (Fig. 6A, lanes 1–6, 8–

Fig. 3. Progression of pulmonary lesions in the A/J mouse lung following treatment with NNK. Conditions were as described in the legend to Fig. 2. Pulmonary lesions were classified as either a hyperplasia, adenoma, or carcinoma, and the relative frequency of each lesion was plotted as a function of time after carcinogen treatment.
Table 1. Distribution of pulmonary lesions in lungs from A/J mice following treatment with NNK

A/J mice were treated with a single dose of NNK (100 mg/kg, i.p.) at 6 weeks of age and were sacrificed beginning 14 weeks after treatment. Pulmonary lesions were classified as hyperplasia, adenoma arising in a hyperplasia, adenoma, carcinoma arising in an adenoma, carcinoma, or microcarcinoma. The relative lesion frequency at each sacrifice time point was determined by dividing the number of a specific lesion by the total number of lesions per slide and multiplying by 100. Each point represents the mean ± SE from 15 lungs per time point.

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Fig. 4. Alveolar hyperplasia induced by treatment with NNK. A, toluidine blue-stained section (1 μm) of an alveolar hyperplasia. × 50. Electron micrographic analysis was conducted from three different areas of this lesion, the border (B), an area halfway to the center of this hyperplasia (C), and the center portion (D). Arrows, locations of these three regions. B, two adjacent alveolar type II cells with prominent lamellar bodies (arrows). × 1900. C, marked thickening of the alveolar septae with multiple type II cells. Lamellar bodies, multivesicular bodies, and dense mitochondria are prevalent within the cytoplasm of these cells. × 1900. D, dense area of type II cells, which appear columnar in shape, some nuclear indentations are evident in some cells and microvilli are present along the luminal surfaces. × 1900.
The results from this study indicate that the induction of lung tumors in A/J mice involves the formation of the O\textsuperscript{6}MG adduct in the alveolar type II pneumocyte. The generation of this adduct can result in base mispairing of guanine with thymidine, most likely because the O\textsuperscript{6}-position of guanine reduces the interstrand hydrogen bonding interaction, relative to a normal cysteine-guanine base pair, and makes it similar to an adenine-thymine base pair (29). This would, in turn, contribute to a loss of fidelity during replication, leading to the insertion of the adenine-thymine base pair. The proliferation of the type II cell then results in the generation of a GC to AT mutation. This mutation is detected in the K-ras gene in 85% of the alveolar hyperplasias examined. The focal proliferation of the alveolar type II cell and subsequent fixation of promutagenic damage could result in response to damage to the type I cell. Toxicity to the type I cell, followed by marked proliferation of the type II cell, has been demonstrated following exposure to bleomycin, cadmium, nickel, trypsin, and lysolysin (30). Thus, reactive intermediates (e.g., diazonium hydroxide) or metabolites (e.g., ketoaldehyde) formed from the metabolism of NNK by the type II cell (31) could migrate into the adjacent type I cell and result in irreversible damage to this alveolar lining cell. Although no overt toxicity to the type I cell was observed after NNK treatment (data not shown), damage to this specific cell type could be relatively infrequent and detected only by electron microscopic evaluation.

The role of the type II cell in the development of NNK-induced tumors is further supported by the fact that a significant number of carcinomas appear to result from the conversion of proliferative lesions along the alveolar septae to adenomas and, ultimately, to carcinomas. These carcinomas also contain the GC to AT mutation within the K-ras gene (8) that was detected in the hyperplasias. The rate of neoplastic progression appears to be heterogeneous within the A/J lung, as evidenced by the fact that 54 weeks after carcinogen treatment approximately one hyperplastic lesion was still present among benign and malignant tumors on each slide examined. Ten of the hyperplasias present after 46–50 weeks of treatment were evaluated for mutations within the K-ras gene. All of these lesions contained a GGT to GAT mutation (data not shown). These results support the hypothesis that the clonal expansion and progression of hyperplastic lesions may require multiple genetic or epigenetic alterations, in addition to the activation of the K-ras protooncogene. The microcarcinomas observed in this study may represent a small subset of tumors which either develop very rapidly or arise independently of distinct progressive stages.

Several previous studies have suggested that papillary tumors derived from Clara cells, while solid tumors originate from type II cells (9, 10). These suggestions were based on ultrastructural findings, which demonstrated the presence of dense secretory granules, nuclear indentations, a cuboidal to columnar shape, and microvilli at the luminal surface of the tumor cells. This interpretation was disputed, because it later proved impossible to demonstrate a Clara cell antigen in papillary neoplasms from B6C3F1 (C57BL/6 × C3H F\textsubscript{1}) and BALB/c mice, while several tumors proved to be immunoreactive for surfactant apoproteins of normal alveolar type II cells (32). However, histological growth patterns (solid versus papillary) were subsequently used to categorize the cellular derivation of lung tumors in sensitive and resistant strains after urethane treatment (33, 34). These earlier studies concluded that, because solid tumors appear smaller than the papillary tumors, growth of solid tumors beyond a limited size must be suppressed. In contrast, the growth of papillary tumors was thought-to-progress, suggesting
Table 3  Pattern of mutations in the K-ras gene from hyperplasias and adenocarcinomas induced in the A/J mouse by NNK

DNA was isolated from hyperplasias microdissected from paraffin-embedded sections, as described in "Materials and Methods." The polymerase chain reaction was employed to amplify the first and second exon of the K-ras gene. Mutations were identified by both restriction fragment length polymorphism and mismatch oligonucleotide hybridization.

<table>
<thead>
<tr>
<th>Lesion</th>
<th>Frequency of activated K-ras</th>
<th>Codon 12 Mutations</th>
<th>Codon 61 Mutations</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Normal, GGT (Gly)</td>
<td>GTT (Val)</td>
<td>GAT (Asp)</td>
</tr>
<tr>
<td>Hyperplasia</td>
<td>17/20</td>
<td>2</td>
<td>15</td>
</tr>
<tr>
<td>Adenocarcinoma</td>
<td>10/10</td>
<td>1</td>
<td>7</td>
</tr>
</tbody>
</table>

* One hyperplasia contained both a GAT and a GTT mutation.

* Data taken from Belinsky et al. (8).

that the Clara cell is the predominant cell of origin of pulmonary adenocarcinomas in mice (34).

Experimental evidence presented in this study and in a previous paper by Rehm et al. (35) does not support the involvement of the Clara cell in the development of pulmonary neoplasms in mice. There was no evidence that a subpopulation of tumors within A/J mice stopped growing. In contrast, evidence for progression of the majority of lesions was demonstrated by the continual decline in the frequency of hyperplasias throughout the sacrifice time points. In addition, a significant percentage of the adenomas and carcinomas were observed arising within hyperplasias and adenomas, respectively. The progression of adenomas to carcinomas has also been observed in A/J mice treated with urethane (36) and in offspring from pregnant Swiss Webster mice which had been exposed to N-nitrosoethyleneurea during gestation (34). Moreover, results from our study demonstrate that the growth pattern of tumors in the A/J lung changes from solid to mixed and, ultimately, to papillary growth patterns observed in adenocarcinomas in which activation of the K-ras gene by NNK was first detected (8). Immunofluorescence studies have also demonstrated that both solid and papillary tumors contain proteins that are specific markers for alveolar type II cells, while none of the tumors appear to be reactive for Clara cell-specific antigen (35). Examining the ultrastructure of pulmonary lesions at the light microscopic level is not sufficient to characterize the early hyperplastic foci. Electron micrographic analysis demonstrates that, even within a hyperplasia, proliferating type II cells can change from a cuboidal to a columnar shape, nuclear indentations can become apparent, and one can even detect early signs of a papillary growth pattern. Thus, this divergence of the alveolar type II cell from its well characterized morphological features during the early development of the pulmonary neoplasm indicates that the selective growth advantage which these initiated cells possess can result in marked changes in the normal ultrastructure of this cell as it progresses toward malignancy.

Previous studies in F344 rats have demonstrated cell specificity for formation of the O6MG adduct in Clara cells. Following treatment of rats with NNK (100 mg/kg), the concentration of this adduct was 6-fold greater in Clara cells than in type II cells (13, 14). Under similar treatment conditions, adduct for-
mation did not differ significantly between Clara cells and type II cells isolated from A/J mice. The reason for the difference in adduct distribution between rats and mice most likely relates to the distribution of cytochrome P450 isozymes within the lung. Cytochrome P450s (P450 IIB) is the major isozyme involved in the activation of NNK in rat lung (13). The presence of this isozyme has been demonstrated in mice, and in both species it is present constitutively but is not induced by treatment with phenobarbital. A monoclonal antibody specific for an epitope common to rat P450s is also reactive with murine cytochromes P-450 localized in the alveolar type II cells and Clara cells (37). The distribution of cytochrome P450s, as detected by immunofluorescence, was only slightly greater in Clara cells than in type II cells from mice. In contrast, immunoperoxidase staining of rat cytochrome P450b reveals significantly greater reactivity toward bronchiolar cells than toward alveolar cells (38) within the rat lung. Thus, differences in the distribution of the P450s isozyme within lungs from mice and rats are consistent with the pattern of O6MG formation observed in these species.

Activation of the K-ras gene also appears to be an early step in the induction of lung tumors by NNK in C3H mice (39), a strain resistant to spontaneous and chemically induced tumor formation (40). In spite of the fact that lung tumors can be induced in C3H mice through repeated carcinogen administration, the size, multiplicity, and latency differ considerably from those observed in the sensitive A/J mice. Hanigan et al. (41) hypothesized that differences in the susceptibility for hepatocarcinogenesis could be linked to factors that control the clonal expansion and growth of preneoplastic lesions. At present, the genetic loci responsible for controlling proliferation of pulmonary preneoplastic lesions have not been identified. The identification of the type II cell as the target for NNK-induced neoplasia should now enable studies to focus on identifying differences in the regulation of growth and differentiation of type II cell hyperplasias in sensitive and resistant mouse strains.

REFERENCES

Role of the Alveolus Type II Cell in the Development and Progression of Pulmonary Tumors Induced by 4-(Methylnitrosamino)-(3-pyridyl)-1-butanone in the A/J Mouse

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