ABSTRACT

Naturally occurring and N-nitrosomethylurea-induced lung tumors were studied in male F344/NCr rats by sequential histological, electron microscopic, and immunohistochemical methods. Rats were given one injection at 6 weeks of age of N-nitrosomethylurea at a dosage level of 41.2 mg/kg body weight i.v. Groups of rats were sacrificed at 20, 33, and 52 weeks, while some were sacrificed while moribund. Nine lung tumors from aged F344/NCr male rats were also studied. For determining localization of pulmonary antigens, sections of lungs were stained by the avidin-biotin-peroxidase complex immunocytochemical technique using antibodies to rat surfactant apoprotein or rat Clara cell antigen. At 20 weeks, in rats receiving N-nitrosomethylurea, focal alveolar type II cell hyperplasia, adenoma in focal alveolar type II cell hyperplasia, and adenoma were found in 15 (100%), 1 (7%), and 2 (13%) of 15 rats, respectively. At 33 weeks, there were 19 rats (95%) with focal alveolar type II cell hyperplasia, 10 rats (50%) with adenoma in focal alveolar type II cell hyperplasia, and 2 rats (10%) with adenomas in 20 rats. In 53 rats allowed to live up to 52 weeks, there were 10 (19%) adenomas and 3 (6%) carcinomas, as well as 49 (92%) rats with focal hyperplasia and 31 (58%) with adenomas in focal type II cell hyperplasia. Rat surfactant apoprotein was found in the cytoplasm of normal alveolar type II cells and the majority of cells in focal type II cell hyperplasia, adenomas in hyperplastic lesions, adenomas, and carcinomas. The ultrastructure of these lesions supported immunocytochemical findings with evidence of lamellar bodies. All nine naturally occurring lung tumors studied contained rat surfactant apoprotein. Rat Clara cell antigen was found, however, only focally within one adenoma induced by N-nitrosomethylurea and one adenoma in a hyperplastic lesion, and also focally in three neoplasms which occurred naturally. This study provided morphological, immunohistochemical, and ultrastructural evidence that the vast majority of pulmonary adenomas and adenocarcinomas of mice, including those associated with bronchioles, contained RSAP and not RCCA. There are no reports, however, on immunohistological analysis of surfactant apoprotein and secretory products of Clara cells in rat bronchioalveolar tumors. The present study is an attempt to clarify the origin and histogenesis of bronchioalveolar tumors induced by NMU and occurring naturally in F344 rats using sequential immunohistochemical methods with antibodies to RSAP and RCCA.

INTRODUCTION

Bronchioalveolar tumors can be induced in rats (9, 10, 13, 14, 18, 24), mice (7, 12), and hamsters (8, 17) by various carcinogens and also occur spontaneously (3, 18, 23). The histogenesis of bronchioalveolar tumors of laboratory animals and humans has been controversial (1, 2). Several authors have reported that bronchioalveolar tumors may be derived from bronchiolar epithelium, especially Clara cells on the basis of light and electron microscopic appearance (7, 8, 14), whereas other investigators have considered alveolar type II cells as the origin of these tumors (12, 18).

Specific recognition of surfactant apoprotein of the pulmonary alveolar type II cell has been accomplished by immunoperoxidase staining using specific antisera (6, 19, 23, 28). Most recently, Singh et al. (22, 23) described an antibody to the secretory products of rat Clara cells. Of 123 human cases of bronchioloalveolar carcinomas, 41 cases (33.3%) were shown to have surfactant apoprotein in the cytoplasm of tumor cells by using the immunoperoxidase technique with rabbit anti-monkey surfactant apoprotein antibody (1, 20, 21). We have recently demonstrated that the vast majority of pulmonary adenomas and adenocarcinomas of mice, including those associated with bronchioles, contained RSAP and not RCCA (26). There are no reports, however, on immunohistological analysis of surfactant apoprotein and secretory products of Clara cells in rat bronchioalveolar tumors. The present study is an attempt to clarify the origin and histogenesis of bronchioalveolar tumors induced by NMU and occurring naturally in F344 rats using sequential immunohistochemical methods with antibodies to RSAP and RCCA.

MATERIALS AND METHODS

Animals and Chemicals. A total of 160 male F344/NCr rats, 4 weeks old, were obtained from the Animal Genetics and Production Branch of the NCI-Frederick Cancer Research Facility, Frederick, MD. Findings in 148 of these rats are reported in this paper. They were housed 5 polycarbonate cage in filtered racks in a standard-barrier facility and given sterilizable Wayne Lab-Blox (Allied Mills, Inc., Chicago, IL) or iodine-adequate diet (Remington iodine-deficient test diet with added potassium iodate at 0.01 g/kg; Teklad, Madison, WI) and tap water ad libitum. The diets had no effect on the induction of lung tumors by NMU; thus, the results of both groups are combined in this study. These rats were part of a large thyroid tumor promotion study (16). NMU (Ash Stevens, Inc., Detroit, MI) was dissolved at 40 mg in sodium citrate buffer solution, pH 5.5.

Seven male F344/NCr rats, 2 to 3 years old, with naturally occurring alveolar type II cell hyperplasias.

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pulmonary proliferative lesions were originally obtained from NCI-Frederick Cancer Research Facility and maintained to 3 years of age as untreated controls in other carcinogenesis and aging experiments.

Experimental Design. The complete protocol has been reported (16). Briefly, rats were given one i.v. injection in the tail vein of 0.4 mmol (41.2 mg) of NMU per kg of body weight at 6 weeks of age and fed Wayne Lab-Blox or iodine-deficient diet for 20, 33, or up to 52 weeks. Rats were killed when clinical signs (loss of body weight, dyspnea, moribund) indicated or at Week 52 after NMU injection. Groups of 10 untreated controls were sacrificed at 20 and 33 weeks, and groups of 20 were at sacrificed at 52 weeks.

Pathology. A complete necropsy was performed on each rat. All gross lesions as well as the lung, thyroid, pituitary, adrenal gland, and thymus were fixed in 10% buffered formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin. The lungs were perfused to complete expansion with formalin via the trachea. Using representative sections (one section from each lobe, 5 sections/rat) for each lung, the number of focal proliferative lesions including focal alveolar hyperplasias, adenomas, and carcinomas were quantitated using an automated image analysis system (Videoplan; Carl Zeiss, Inc., New York, NY). The mean numbers (±SD) of pulmonary lesions per sq cm of lung were determined. Selected pieces of representative macroscopically visible lung lesions were excised, cut into about 1- x 1-mm blocks, and fixed with 2% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) for 2 h. They 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 selection and orientation of tissue. For electron microscopy, such selected areas were thin sectioned, mounted on copper grids, stained with uranyl acetate and lead citrate, and examined with an electron microscope. For localizing RSAP and RCCA, formalin-fixed lung sections were stained by the avidin-biotin-peroxidase complex immunocytochemical technique (5) using the Vectastain ABC Kit (Vector Laboratories, Burlingame, CA) and rabbit antiserum to RCCA (22, 23) at dilutions of 1:100, 1:200, or 1:400 with 0.9% buffered NaCl solution or rabbit antiserum to RSAP (6, 23) at dilutions of 1:100, 1:200, or 1:400 with 0.9% buffered NaCl solution or rabbit antiserum to RCCA (22, 23) at dilutions of 1:100, 1:200, or 1:400. The specificities of RSAP for alveolar type II cells and of RCCA for Clara cells of rats and mice have been described (6, 23, 27). Controls included staining lungs with normal rabbit serum at dilutions of 1:100 to 1:1600 and at the same protein concentrations. The chromagen used was 3,3′-diaminobenzidine tetrahydrochloride as a 0.06% solution with 0.01% hydrogen peroxide.

RESULTS

NMU-induced Lung Tumors. Multiple tan and pale white nodules appeared on the lung surface of the animals treated with NMU. Nodules were generally soft, were relatively sharply demarcated from the surrounding tissues, and measured up to 15 mm in diameter. Usually, only the largest lesions were firm in consistency. The lungs in the 60 control rats did not show any gross lesions.

The incidences and quantitative analyses of focal alveolar hyperplasias, adenomas within focal hyperplasias, adenomas, and carcinomas in the lung of Group 1 (NMU) at each period are summarized in Tables 1 and 2, respectively. None of these lesions were found in control rats. At Week 20, focal alveolar hyperplasia, adenoma within focal hyperplasia, and adenoma were found in 15 (100%), 1 (7%), and 2 (13%) of 15 rats, respectively. Alveolar hyperplasias were usually multiple, were located in the periphery of the lung or in peribronchial locations, and were characterized by a focal proliferation along the alveolar walls of cuboidal vacuolated alveolar type II cells with a slightly hyperchromatic nucleus (Figs. 1 and 2). Normal alveoli contained much fewer type II cells. Ultrastructural examination of hyperplasias revealed many mature and less differentiated alveolar type II cells (Fig. 3) and some macrophages. Adenomas within focal alveolar type II cell hyperplasias contained focal solid or papillary proliferation of large atypical epithelial cells within areas of focal alveolar hyperplasia (Fig. 4). These atypical cells were identical to those in large adenomas and were larger than cells in hyperplastic lesions. Sometimes they were first seen adjacent to larger blood vessels. At Week 33, there were 19 (95%) rats with alveolar cell hyperplasias, 10 (50%) with adenomas in hyperplasias, and 2 (10%) rats with adenomas. Adenomas were usually relatively well demarcated from the surrounding tissue, compressed the surrounding tissue, and were composed of basophilic cuboidal or columnar cells which were arranged in solid or tubulopapillary structures and had atypical hyperchromatic nuclei (Figs. 5 and 6). Some of the cells had vacuoles in their cytoplasm (Fig. 7) which appeared to be lipid. Many of the large tumors were not associated with hyperplasias, but only a few small adenomas were without associated focal hyperplasias. Electron microscopically, most of the adenoma cells usually had many microvilli on the apical surface and many to few lamellar bodies in the cytoplasm. In a few cases, some of the adenoma cells had lipid droplets in the cytoplasm (Fig. 8). In 53 life span rats, there were 49 (84%) rats with focal alveolar hyperplasias, 31 (58%) with adenomas, and 10 (19%) rats with adenomas, and 3 (6%) rats with carcinomas. Carcinomas were cytologically anaplastic and invasive as compared with adenomas. Two of the carcinomas were of the solid type (Fig. 9).

Immunoperoxidase Localization of RSAP and RCCA in NMU-induced Lung Tumors. The immunohistological findings of pulmonary proliferative lesions are summarized in Table 3. The cytoplasm of alveolar type II cells in the normal alveolar wall in treated and control rats and many cells in focal alveolar hyperplasias in treated rats was usually intensely immunoreactive with antibodies to RSAP (Fig. 10) and always negative for RCCA at low dilutions where normal Clara cells were immunoreactive for RCCA. Many of the weakly or moderately positive cells or lesions may have stained in this manner because of prolonged fixation or other technical effects, since normal type II cells were also usually weaker staining in these sections than normal. The immunoreactive cytoplasm of the type II cells usually revealed vacuoles. Alveolar macrophages in the lesions occasionally had immunoreactive RSAP on the cell membrane. On the other hand, the cytoplasm of many epithelial cells of the bronchioles were intensely immunoreactive for RCCA. In contrast, the majority of adenomas within focal hyperplasias, adenomas, and carcinomas showed RSAP immunoreactive cells.
tumors of aged F344 rats were classified similarly to the NMU-induced RSAP. One adenoma diffusely immunoreactive for RSAP diffusely or in focal areas of the lesions (Fig. 11) and were usually usually

None (aged rats) Adenoma within focal alveolar hyperplasia
NMU Focal alveolar type II cell hyperplasia

The latter 4 were morphologically similar to those which were induced tumors (Table 3). Seven lesions were tubulopapillary, and 2 were solid. All 9 were immunoreactive diffusely or focally (Fig. 13) for RSAP, and 4 were immunoreactive focally for RCCA. These tumors were RSAP negative. Lipid-containing cells in tumors usually contained RSAP. One adenoma diffusely immunoreactive for RSAP showed focal immunoreactive RCCA (Fig. 12).

**Table 2**

Quantitative analysis of focal proliferative lesions in the lungs of F344/NCr rats given a single injection of NMU

<table>
<thead>
<tr>
<th>Experiment period (wk)</th>
<th>No. of rats</th>
<th>Focal alveolar type II cell hyperplasia</th>
<th>Adenoma within focal alveolar type II cell hyperplasia</th>
<th>Adenoma</th>
<th>Carcinoma</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>15</td>
<td>1.4 ± 1.8</td>
<td>0.03 ± 0.13</td>
<td>0.03 ± 0.0</td>
<td>0</td>
</tr>
<tr>
<td>33</td>
<td>20</td>
<td>2.7 ± 1.4</td>
<td>0.2 ± 0.3</td>
<td>0.04 ± 0.1</td>
<td>0</td>
</tr>
<tr>
<td>Life span</td>
<td>53</td>
<td>2.2 ± 1.4</td>
<td>0.2 ± 0.2</td>
<td>0.05 ± 0.1</td>
<td>0.04 ± 0.2</td>
</tr>
</tbody>
</table>

*Mean ± SD.

*Rats that died or were sacrificed between 27 and 52 weeks after NMU injection.

**Table 3**

Immunoperoxidase localization of RSAP and RCCA in selected proliferative pulmonary lesions of F344/NCr rats

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Histological types of lesions</th>
<th>No. of lesions</th>
<th>RSAP</th>
<th>RCCA</th>
</tr>
</thead>
<tbody>
<tr>
<td>NMU</td>
<td>Focal alveolar type II cell hyperplasia</td>
<td>93 0 93 0</td>
<td>- +</td>
<td>- +</td>
</tr>
<tr>
<td></td>
<td>Adenoma within focal alveolar type II cell hyperplasia</td>
<td>24 0 24 0</td>
<td>- +</td>
<td>- +</td>
</tr>
<tr>
<td></td>
<td>Adenoma</td>
<td>12 0 12 11 1</td>
<td>- +</td>
<td>- +</td>
</tr>
<tr>
<td></td>
<td>Carcinoma</td>
<td>2 0 2 2 0</td>
<td>- +</td>
<td>- +</td>
</tr>
<tr>
<td>None (aged rats)</td>
<td>Adenoma within focal alveolar type II cell hyperplasia</td>
<td>2 0 2 1 1</td>
<td>- +</td>
<td>- +</td>
</tr>
<tr>
<td></td>
<td>Adenoma</td>
<td>4 0 4 2 2</td>
<td>- +</td>
<td>- +</td>
</tr>
<tr>
<td></td>
<td>Carcinoma</td>
<td>3 0 3 2 1</td>
<td>- +</td>
<td>- +</td>
</tr>
</tbody>
</table>

*−, negative; +, immunoreactive for the antigen.

Naturally Occurring Lung Tumors. Naturally occurring lung tumors of aged F344 rats were classified similarly to the NMU-induced tumors (Table 3). Seven lesions were tubulopapillary, and 2 were solid. All 9 were immunoreactive diffusely or focally (Fig. 13) for RSAP, and 4 were immunoreactive focally for RCCA. The latter 4 were morphologically similar to those which were not immunoreactive for RCCA.

**DISCUSSION**

Although NMU has been demonstrated to be a lung carcinogen in hamsters (4), there have been no reports on the morphology of NMU-induced lung tumors in rats. The histopathological findings of focal alveolar hyperplasia and bronchioloalveolar adenoma and carcinoma induced by NMU were similar to those caused by other carcinogens in rats (10, 13, 24, 25). In our sequential study, focal alveolar type II cell hyperplasias occurred in 100% of the rats at Week 20, while the incidence of adenomas within hyperplastic lesions, adenomas, and carcinomas increased from 20 to 52 weeks. In addition, the quantitative analysis of focal proliferative lesions in the rat lung shows that the number of alveolar hyperplasias and adenomas within hyperplastic lesions per sq cm of lung increased clearly between Weeks 20 and 33. Although few carcinomas were seen in this experiment, these results suggested that a sequential cellular progression may occur in the development of NMU-induced lung carcinoma from focal alveolar hyperplasias and adenomas. Matsumaki (12) suggested for mice that a progression of chemically induced adenomas may begin from alveolar type II cell hyperplasias and pass through intermediate stages to adenomas and, in some cases, to carcinomas.

Lamellar bodies are a morphological marker of alveolar type II cells in healthy animals and were demonstrated in the cytoplasm of tumor cells in bronchioloalveolar neoplasms of rats (18). In our study, most of the focal proliferative lesions (hyperplasia, adenoma) in rat lung showed many lamellar bodies in the cytoplasm. Therefore, this finding supports the hypothesis that bronchioloalveolar tumors induced by NMU originate from alveolar type II cells. However, Reznik-Schüller (17) demonstrated that Clara cells also can produce mature lamellar bodies and extrude them into the bronchial lumen by means of a merocrine secretion. Thus, immunohistological procedures might be appropriate for investigation of the histogenesis of these bronchioloalveolar tumors, since morphological methods alone using light and electron microscopy have not succeeded in resolving their histogenesis.

In the present study, RSAP was demonstrated immunohistochemically in all focal bronchioloalveolar proliferative lesions of the lung induced by NMU and those which occurred spontaneously, whereas RCCA was observed focally in only one lesion induced by NMU and in 4 lesions which occurred spontaneously. It is not surprising that some alveolar type II cell tumors have immunoreactive Clara cell antigen(s), since the alveoli and bronchioles have a common embryological origin (8, 15). Therefore, we believe that alveolar type II cells are involved in the ultimate formation of the majority of induced bronchioloalveolar neoplasms for 3 reasons: (a) the sequential changes were from alveolar hyperplasia to adenoma within the same lesion; (b) the tumor cells contain typical myelin lamellar bodies; and (c) all focal bronchioloalveolar proliferative lesions were RSAP positive immuno histochemically. Thus, based on our histomorphological and immunocytochemical studies, bronchioloalveolar tumors induced by NMU in F344/NCr rats should be diagnosed as alveolar type II cell adenomas and carcinomas. The origin of the naturally occurring tumors in F344 rats is more uncertain, however. While it is conceivable that early spontaneous lesions arose from alveoli, we cannot exclude the possibility of their bronchiolar origin. The vast majority of spontaneous lung tumors that we have seen in other studies appear to arise within alveoli.

Some alveolar type II cell tumors apparently contained abundant lipid in the cytoplasm. The origin, role, and fate of the lipid-containing interstitial cell which appears in the walls of alveoli during the period of postnatal lung growth are uncertain (11). Our results suggest a possibility that the alveolar type II cell and...
the postnatal lipid-containing interstitial cell may be derived from the same stem cells, at least in NMU-induced lung tumors.

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REFERENCES


Fig. 1. Low magnification of extensive focal alveolar type II cell hyperplasia (arrows) in a F344 rat given NMU showing peribronchial location. Normal alveoli (N) on edge of lesion. H & E, x 80.
Fig. 2. High magnification of focal alveolar type II cell hyperplasia in an F344 rat given NMU showing numerous enlarged vacuolated type II cells lining alveoli. H & E, x 330.
Fig. 3. Electron micrograph of focal alveolar type II cell hyperplasia illustrating lamellar bodies in type II cells (arrows). Uranyl acetate-lead citrate, x 8000.
Fig. 4. Edge of adenoma (arrows) arising within a focal alveolar type II cell hyperplastic lesion (arrowheads) in lung of an F344 rat given NMU. H & E, x 220.
Fig. 5. Solid adenoma of lung in an F344 rat given NMU. H & E, x 330.
Fig. 6. Tubuloalveolar adenoma of lung in an F344 rat given NMU. H & E, x 220.
Fig. 7. Solid adenoma of lung in an F344 rat given NMU. Some of the tumor cells contain lipid droplets in the cytoplasm. Epon embedded, toluidine blue, x 860.
Fig. 8. Electron micrograph of adenoma of lung in an F344 rat given NMU. Some of the tumor cells exhibited abundant osmiophilic lamellar bodies in the cytoplasm and microvilli on the surface. Some tumor cells have many lipid droplets in the cytoplasm. Uranyl acetate-lead citrate, x 8000.
Fig. 9. Carcinoma of lung in an F344 rat given NMU showing poorly differentiated cells forming an occasional glandular structure. H & E, x 220.
Fig. 10. Focal alveolar type II cell hyperplasia induced by NMU. Surfactant apoprotein is seen in many cells lining the alveolar walls. Immunoperoxidase-hematoxylin, x 330.
Fig. 11. Surfactant apoprotein in adenoma of lung in an F344 rat given NMU. The prominent cytoplasmic staining of tumor cells for surfactant apoprotein is obvious in the left side of the lesion, whereas the right side of the lesion shows focal immunoreactive surfactant apoprotein. Immunoperoxidase-hematoxylin, x 130.
Fig. 12. Clara cell antigen(s) in adenoma of lung in an F344 rat given NMU. Some of the tumor cells are stained for RCCA. Immunoperoxidase and hematoxylin, x 330.
Fig. 13. Surfactant apoprotein in spontaneous adenoma of lung in an F344 rat. Tumor cells are intensely positive for surfactant apoprotein. Immunoperoxidase-hematoxylin, x 330.
ALVEOLAR TYPE II CELL NEOPLASMS
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