Mouse Papillary Lung Tumors Transplacentally Induced by N-Nitrosoethylurea: Evidence for Alveolar Type II Cell Origin by Comparative Light Microscopic, Ultrastructural, and Immunohistochemical Studies

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ABSTRACT

A histogenetic study was designed to evaluate controversial findings on the cell of origin of tubular/papillary lung tumors in mice, i.e., bronchiolar Clara cell versus alveolar type II cell. N-Nitrosoethylurea (0.5 mmol or 0.74 mmol/kg) was given to pregnant C3H (C3H/HeNCr MTV) and Swiss Webster [Tac(SW)/BRR] mice as a single i.p. injection on Day 14, 15, 16, or 18 of gestation. The offspring were studied at various ages ranging from 7 days to 52 wk.

Serial sections of the whole lung (100 to 200 sections per mouse) showed that solid/alveolar and papillary tumors arose from the pulmonary acinus, invading the bronchioles only as the tumors grew. Furthermore, a mixture of solid and papillary patterns within a single nodule did not represent a merging of two tumors but a progression from the solid to the papillary form. By use of two rabbit antisera against mouse lung surfactant apoproteins found in normal alveolar type II cells, it was shown by the avidin-biotin peroxidase complex procedure, by the peroxidase-antiperoxidase technique, and by indirect immunofluorescence that both solid and papillary tumors contained these proteins that are specific markers for alveolar type II cells. With a rabbit anti-rat Clara cell antisera, none of the tumors studied was immunoreactive while normal Clara cells were reactive. The nitroblue tetrazolium formazan stain for dehydrogenase enzymes, found particularly in Clara cells, did not reveal these enzymes in any lung tumors from either strain.

Ultrastructurally, no typical features of the mature Clara cell were detected in papillary or other pulmonary neoplasms. However, all tumors showed characteristic alveolar type II cell structures such as various stages of lamellar body formation, although these features were less well differentiated in the papillary tumors. Argentaffin dense bodies, representing lysosomes and immature forms of lamellar bodies, were commonly observed in papillary tumors. Some features of the papillary tumors such as cell shape, high glycogen content, and primary cilia were equivalent to those seen in pulmonary epithelial precursor cells during fetal development. With age, the papillary tumors became invasive, accumulated neutral lipids, and developed bizarre cleaved nuclei and lamellated nuclear pseudoinclusions.

In conclusion, the papillary lung tumors of the mouse, at least those induced transplacentally by N-nitrosoethylurea, constitute less well-differentiated or poorly differentiated alveolar type II cell adenomas or carcinomas with fetal morphological and biochemical properties.

INTRODUCTION

Until 1979, there was general agreement that both solid/alveolar and tubular/papillary lung tumors of the mouse originated from the alveolar type II cell (1–3). Morphological variations among tumors were considered to represent different stages of progression of the neoplasms (4). Studies from which this consensus developed involved spontaneous and induced tumors, light microscopy of serial sections from single tumors or lung lobes (1, 5), and ultrastructural demonstration of type II cell features (6, 7). Larger papillary tumors, however, frequently grew invasively into bronchioles, and since their columnar cell shape superficially resembled that of the bronchiolar cells, such origin was occasionally suggested (8–10).

In studies on NEU²-induced papillary lung tumors of Swiss Webster mice, ultrastructural proof was presented for a histogenetic origin of the papillary mouse lung tumor from the bronchiolar Clara cell. In C3H mice, larger papillary tumors, however, were not found. In conclusion, the papillary lung tumors of the mouse, at least those induced transplacentally by N-nitrosoethylurea, constitute less well-differentiated or poorly differentiated alveolar type II cell adenomas or carcinomas with fetal morphological and biochemical properties.
were transferred to nitrocellulose paper, and the paper was stained by
reactive with SAALS can be detected in prospective cells of the pul-
monary acinus from Day 14.2 of gestation onward (33). Histochemi-
II cell-specific antigen was obtained by absorption with cross-reacting
fraction (34). Anti-CCA was used
diluted 1:800 with phosphate-buffered saline, anti-SAP-M was diluted
and, normal rabbit sera diluted likewise were used as controls at
similar concentrations for an incubation period of 30 min. The rabbit
Vectastain ABC kit (Vector Laboratories, Burlingame, CA) was used
for the localization of CCA and SAP-M in Bouin's fixed sections of
mouse lung tumors from C3H and Swiss mice. 3,3-Diaminobenzidine
was the chromogen (16), and sections were counterstained with hema-

**Fluorescence Microscopy.** The indirect immunofluorescence tech-
nique was applied as described previously in detail (32, 36) to sections
of alcohol-fixed or fresh-frozen lung tumors from C3H or Swiss Web-
ner mice using the rabbit antiserum SAALS at a dilution of 1:40 or
1:100. Control sections were treated with preimmune rabbit serum and
FITC conjugate, or FITC conjugate only.

**PAP Technique.** The PAP technique was applied to sections of lung
tumors from C3H and Swiss mice fixed in Bouin's solution, formalin,
or alcohol to test for SAALS reactivity at dilutions of 1:40 or 1:100. The
rabbit PAP technique (Nordic Immunological Laboratories, Til-
burg, The Netherlands) was used according to the double bridge method
in combination with CuSO₄ enhancement (37). Control sections were
treated with preimmunization serum and PAP according to the double
bridge method, PAP, or diaminobenzidine only.

**Ultrastructure.** Control and tumor tissues from C3H mice were fixed
in 2.5% glutaraldehyde in 0.1 M cacodylate buffer for 2 h at 4°C and
postfixed with 1% osmium tetroxide for 1 h. Except for tumors from 6-
mo-old mice, staining with 0.5% uranyl acetate was performed prior
to embedding in epoxy resin.

Two solid and 4 papillary neoplasms from 4-wk-old mice and 6
papillary tumors from 6-mo-old mice were studied. Semithin sections
were carried out on control tissue and tumors from C3H and Swiss
mice at different ages. Sections were stained with alcian blue, and
subjected to the PAS reaction with or without prior diastase treatment
to demonstrate acid or neutral mucopolysaccharides. Frozen sections
prepared from formalin-fixed or fresh tissues were stained with Oil Red
0 for neutral lipids or tested for dehydrogenase enzymes (3-hydroxy-
butyrate dehydrogenase) by the NBT reducíase method (17, 25, 26).

Antigens and Antisera. Rabbit anti-CCA was prepared as described
before (27). Rabbit anti-SAP-M was obtained and characterized as done
previously for antiserum to rat lung surfactant apoproteins (28–31).

Briefly, mouse lung surfactant was isolated by salt gradient centrifuga-
tion and used as an immunogen in rabbits. The resulting antiserum was
absorbed with mouse serum, and the rabbit serum proteins not binding
to mouse serum were collected. A γ-globulin fraction of rabbit anti-
serum to mouse surfactant was prepared by ammonium sulfate precipi-
tation, and the precipitate was dialysed in saline. In addition to the
tissue reactivity of the antiserum, the preparation was tested by immu-

Fig. 1. Mouse surfactant was subjected to sodium dodecyl sulfate-polyacry-
lamid gel electrophoresis followed by immunoblotting using rabbit anti-SAP-M.

Antigenic form of the M, 35,000 surfactant apoprotein; the two Bands B,
the M, 35,000 apoprotein.
Table 1 Age-dependent location of lung tumors induced by NEU in two strains of mice

<table>
<thead>
<tr>
<th>Strain</th>
<th>Age (wk)</th>
<th>No. of mice</th>
<th>No. of lung tumors</th>
<th>Location</th>
<th>No connection to bronchioles or pleura</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Solid (n)</td>
<td>Papillary (n)</td>
<td>Solid (n)</td>
</tr>
<tr>
<td>C3H/HeNCr MTV-</td>
<td>1</td>
<td>6</td>
<td>1 (2)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>6</td>
<td>8 (25)</td>
<td>1 (13)</td>
<td>3 (13)</td>
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<tr>
<td></td>
<td>4</td>
<td>6</td>
<td>4 (18)</td>
<td>1 (25)</td>
<td>6 (33)</td>
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<td>8</td>
<td>6</td>
<td>22 (40)</td>
<td>9 (41)</td>
<td>19 (48)</td>
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<td></td>
<td>16</td>
<td>6</td>
<td>23 (37)</td>
<td>12 (52)</td>
<td>20 (54)</td>
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<tr>
<td></td>
<td>32</td>
<td>6</td>
<td>22 (36)</td>
<td>11 (50)</td>
<td>26 (72)</td>
</tr>
<tr>
<td>Swiss Webster [Tac: (SW)fBR]</td>
<td>4-5</td>
<td>4</td>
<td>18 (34)</td>
<td>4 (22)</td>
<td>5 (15)</td>
</tr>
</tbody>
</table>

Some tumors are both in contact with pleura and invade bronchioles.

*Includes mixed tumors from age 8 wk.

†Each of two mice treated on gestation Days 14, 16, and 18 with 0.5 mmol NEU/kg, i.p.

Numbers in parentheses, percentage.

Treated on gestation Day 15 with 0.74 mmol NEU/kg, i.p.

RESULTS

Immunoblotting of Rabbit Anti-Mouse Surfactant. Anti-SAP-M stained two bands in the M, 35,000 region representing surfactant apoprotein A (Fig. 1). A stained M, ~70,000 band represents dimers of the apoprotein A. The antiserum did not react with any serum proteins.

Histogenesis of Lung Tumors from Study of Serial Sections. From serial sections of the entire lung the localization of lung tumors in relation to surrounding structures was studied as a function of age (Table 1). No differences with respect to tumor location were observed among the various days of treatment. Lung tumors rapidly increased in size, and therefore, only very early stages (1 to 2 wk) allowed an exact observation on the site of origin. No papillary tumors were found arising directly from the bronchioles in either C3H or Swiss Webster mice. Both solid and papillary tumors were very similarly distributed (Table 1). The incidence of tumors possessing an open contact to bronchioles, i.e., not interrupted by alveolar walls as shown in Fig. 2A for a solid lung tumor, was very low early in the study but increased as the mice aged and the tumors grew in size or became invasive. However, an intimate pleural contact as shown in Fig. 2B for a papillary tumor was observed in at least 50% of both tumor types at all age periods investigated in both strains.

Both pure solid and pure papillary tumors were seen at the earliest time points of sacrifice. Mixed tumors were observed for the first time in C3H mice aged 4 wk that had been exposed to NEU on Day 14 of gestation. Serial sections of mixed tumors, particularly of early ones, indicated that papillary structures arose within originally pure solid tumors as illustrated graphically in Fig. 3. Fig. 4B gives an example of a mixed tumor with a single central papillary focus. More frequently, however, neoplasms showed multiple separate foci of columnar cells that formed small rosettes or a papillary pattern within the solid tumors, lacking contact with a papillary tumor beyond the solid area. In late stages of large mixed tumors, in which the papillary growth exceeded that of the solid area, this development could no longer be recognized.
Fig. 4. Mouse lung tumors induced transplacentally by NEU. A, mixed mouse lung tumor from C3H mouse. Early central papillary growth of columnar cells within solid tumor; compare with Fig. 3. H & E, × 100. B, frozen section of two papillary lung tumors (p) from C3H mouse lacking positive NBT formazan staining as evident in normal bronchiolar cells (arrows). a, alveolar cells. × 100. C, cystic papillary lung tumor from Swiss Webster mouse staining negative for CCA. Note immunoreactive bronchiolar cells. ABC immunohistochemistry. Hematoxylin, × 100. D, papillary lung tumor from Swiss Webster mouse with positive cytoplasmic immunostaining for SAP-M of tumor cells lining the papillary folds, free macrophages (arrow), and normal alveolar type II cells at tumor border. ABC immunohistochemistry. Hematoxylin, × 250. E and F, indirect immunofluorescence of SAALS in paraffin sections from lungs of C3H mice fixed in alcohol. E, hyperplastic alveolar walls at edge of solid lung tumor with positive cytoplasmic immunofluorescence of SAALS specific for alveolar type II cells. × 270. F, papillary
Nitroblue Tetrazolium Reductase Activity. From 4-wk-old C3H mice, unfixed frozen sections of 21 papillary tumors were studied for the staining patterns of NBT formazan in an effort to identify Clara cell dehydrogenases. None of the tumors exhibited a staining intensity exceeding that of the surrounding alveoli after formalin pretreatment (Table 2). This contrasted strongly with the dark staining of bronchiolar cells (Fig. 4B). Likewise, no significant staining was observed in 6 solid and 31 papillary tumors obtained from 6-mo-old Swiss Webster mice. The formalin pretreatment of 30 s abolishes a low level of NBT reductase activity in cells other than Clara cells (25, 26). When this pretreatment was omitted, 2 of the alveolar and 31 papillary tumors obtained from 6-mo-old Swiss Webster mice. The formalin pretreatment of 30 s abolishes a low level of NBT reductase activity in cells other than Clara cells (25, 26). When this pretreatment was omitted, 2 of the alveolar and 7 of the papillary tumors exhibited noticeably higher enzyme activity than the surrounding normal type II cells.

Alcian Blue. None of the tumors studied produced secretions of acid mucopolysaccharides.

Periodic Acid-Schiff Reaction. In fresh frozen sections of normal fetal lungs, glycogen could be detected only from Day 16 onward by the PAS reaction. In tumors, glycogen was found particularly in early papillary and mixed neoplasms. Diastase-resistant intranuclear inclusions were seen in some of the papillary tumors in mice over 2 mo of age but not in solid tumors.

Oil Red O. In normal fetal lungs, single tiny droplets were first observed on Day 18. In mice 1 wk of age, the pulmonary acini contained considerable amounts of lipids. Neutral lipids were frequently present in papillary tumors of both strains, the amounts increasing with age. Some of the larger tumors contained considerable quantities of lipids (Table 2).

Immunocytochemistry. By the ABC technique, CCA was first detected in normal fetal lung on Day 18 of gestation within the apical cytoplasm of normal bronchiolar cells. Of all papillary and mixed tumors examined for CCA, none exhibited a positive reaction. This contrasted sharply with the intense intracytoplasmic staining of normal bronchiolar Clara cells (Fig. 4C) as was shown previously (16). The antiserum used proved to be highly specific for CCA, since in all cases studied, no pulmonary cells other than Clara cells reacted positively. The numbers of tumors tested for CCA were generally comparable to those examined for SAP-M (Table 3).

SAP-M was observable for the first time in developing mouse lung parenchyma only after birth. SAP-M was localized within type II cells and also formed a superficial lining of the bronchiolar epithelial surface, but with anti SAP-M there was no staining of the cytoplasm of bronchiolar cells. All solid areas from pure or mixed tumors tested for the presence of cytoplasmic SAP-M (Table 3) were moderately to strongly positive focally or diffusely. Of the papillary tumors, 75% were immunoreactive for SAP-M in the C3H and 80% in the Swiss mice. A tumor was considered reactive if at least one-third of the cells showed a yellow-brown cytoplasmic staining that was often granular and similar to that observed in the normal type II cells. This staining pattern was distinctly different from a light gray-brown diffuse background staining. Most papillary neoplasms possessed a mild-to-moderate staining reaction for SAP-M with regard to the degree of staining intensity and size of the positive tumor area. Only a few papillary tumors contained cells that stained as intensely dark-brown as many of those in solid tumors (Fig. 4D). The degree of positive staining was similar in all age groups of both strains. However, the occurrence of intranuclear inclusions described previously (16) was observed in C3H mice only after the age of 2 mo. Characteristically, the cytoplasm of cells with intranuclear inclusions was negative for SAP-M. Many tumors also possessed intraluminal black-brown deposits, presumably representing secreted surface-
Table 4  Localization of reactivity to SAALS in mouse lung tumors by PAP technique and immunofluorescence

<table>
<thead>
<tr>
<th>Technique</th>
<th>Strain</th>
<th>Age (wk)</th>
<th>No. of mice</th>
<th>Solid</th>
<th>Mixed</th>
<th>Papillary</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAP</td>
<td>C3H*</td>
<td>4</td>
<td>5</td>
<td>6/6</td>
<td>0/0</td>
<td>19/19</td>
</tr>
<tr>
<td></td>
<td>C3H</td>
<td>42</td>
<td>3</td>
<td>0/0</td>
<td>4/4</td>
<td>11/11</td>
</tr>
<tr>
<td></td>
<td>Swiss Webster*</td>
<td>12</td>
<td>1</td>
<td>3/3</td>
<td>2/2</td>
<td>3/3</td>
</tr>
<tr>
<td>IF*</td>
<td>C3H</td>
<td>4</td>
<td>2</td>
<td>3/3</td>
<td>0/0</td>
<td>16/16</td>
</tr>
<tr>
<td></td>
<td>C3H</td>
<td>24</td>
<td>2</td>
<td>0/0</td>
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<td>5/5</td>
</tr>
<tr>
<td></td>
<td>Swiss Webster</td>
<td>24</td>
<td>2</td>
<td>1/1</td>
<td>0/0</td>
<td>10/10</td>
</tr>
</tbody>
</table>

* Treated on gestation Day 16 with 0.5 mmol NEU/kg, i.p.
* Treated on gestation Day 15 with 0.74 mmol NEU/kg, i.p.
* IF, immunofluorescence.

The cytoplasm of macrophages that were regularly present often also stained positively (Fig. 4D), probably due to phagocytized surfactant. Several papillary tumors in animals aged 52 mo showed central necrosis and possessed cholesterol clefts; the necrotic material in such tumors stained positive for SAP-M.

By the PAP technique, all solid and papillary tumors stained with SAALS (Table 4) showed a positive staining reaction. A fine granular yellow-brown pattern was present in most tumor cells that was similar to the staining of normal alveolar type II cells. The staining intensity varied among tumor cells and was generally weaker than in normal type II cells. In solid tumors the staining occurred throughout the cytoplasm, whereas in papillary tumors it was generally restricted to the apical portions of the cells. Solid tumors stained more intensely than papillary tumors. Occasionally, intranuclear positive staining was seen. No strain differences were observed (Table 4). A superficial, extracellular lining layer reactive with SAALS was observed along all air spaces, i.e., lining alveolar septa as well as the bronchioles. By CuSO₄ enhancement the intensity of the staining was improved considerably.

Fluorescence Microscopy. All solid tumors tested for SAALS reactivity were positive using immunofluorescence (Fig. 4E). Similarly, all papillary neoplasms (Fig. 4F) were stained by SAALS (Table 4). An exceptionally intense staining was found in the lumina of papillary tumors by immunofluorescence (Fig. 4F). Localization and intensity of the fluorescence in lung tumors and normal pulmonary structures were comparable to the results obtained by the PAP technique.

Ultrastructure of Fetal Lungs, Mature Bronchiolar Clara Cells, and Lung Tumors. On Day 14 of gestation the yet undifferentiated developing lung consisted of tubules lined by high columnar epithelial cells. Some cells had cytoplasmic glycogen deposits, and occasionally a single apical primary cilium was present. On Day 16 of gestation, the prospective bronchial system consisted of tubules lined by very tall and slender columnar epithelium lacking glycogen and characteristic cytoplasmic organelles. Precursor cells of alveolar type II cells lining the prospective respiratory system were cuboidal and possessed glycogen deposits as well as multivesicular and dense bodies. On Day 18, cells from both systems of the respiratory tract had
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attained more mature features. Bronchial low columnar nonciliated cells, some with large amounts of glycogen, were distinguishable from developing ciliated cells. Alveolar cells showed a pyramidal shape, some were without glycogen, and mature lamellar bodies were present and released into the fetal alveolar spaces. At all stages of development single primary cilia were seen in all epithelial cell types.

Characteristic features of the mouse bronchiolar Clara cell from 24-wk-old C3H mice are shown in Fig. 5. Thick sections stained with toluidine blue (Fig. 5A) showed dense foci beneath the luminal cell membrane of Clara cells and distributed throughout the cytoplasm. Some of these foci were also seen in ciliated cells. As observed with conventional staining procedures for electron microscopy, secretory granules often appeared dense (Fig. 5B) and could not be distinguished from lysosomes. However, using only a silver stain, a clear distinction between osmiophilic lysosomes and nonosmiophilic secretory granules was possible (Fig. 5, A and D) due to a reduction of the silver by osmium (Table 2). Furthermore, Fig. 5, B and D, shows a smooth cell surface, the typical large round or ovoid mitochondria of the mouse Clara cell with very short cristae, and an abundance of smooth endoplasmic reticulum often encircling the mitochondria.

In solid lung tumors, the cells were typically packed with large lamellar bodies (Fig. 6, A and B). Various osmiophilic stages of lamellar body formation (Fig. 6B) were observed as described for the normal type II cell (40). The cross-sectional profile of cells of solid tumors and their nuclei were generally round, and the free surfaces showed several irregular microvilli (Fig. 6A). Cells were connected by interdigitating membranes (Fig. 6B) and tight junctions. Glycogen granules were scattered throughout the cytoplasm.

An early papillary tumor with apparent transitional features is shown in Fig. 7. All cells contained irregularly shaped dense bodies, multivesicular bodies, and occasionally mature lamellar bodies (Fig. 7, inset). The cell shape was elongated, resembling fetal type II cells (Fig. 8), and the nuclei were mostly round, located basally. The surface of the tumor cells possessed numerous microvilli. Mitochondria varied largely in number, size, and shape, but always showed well-developed cristae. Cytoplasmic glycogen was distributed either randomly as single granules or as larger glycogen deposits similar to those found in cells of fetal lung (Fig. 8). Further similarities between the fetal lung and features of papillary tumors are shown in Fig. 9 with regard to cell shape, glycogen deposits, and the occurrence of primary cilia (41).

In older papillary tumors a further morphological dedifferentiation could be observed. The number of cytoplasmic dense bodies varied, and mature lamellar bodies were seen less frequently, whereas the number of multivesicular bodies was increased. Occasionally, dense paracrystalline structures were seen, appearing rectangular or as a square (Fig. 10A) and surrounded by a membrane similar to that of the dense bodies. Papillary tumors of 6-mo-old C3H mice shown in Fig. 10, A and B, were subjected to the silver stain in the same fashion as the Clara cell specimen in Fig. 5D. The dense and multivesicular bodies as well as the paracrystalline structures were argentaffin which proved that they were osmiophilic. Most of the dense bodies in the papillary tumors did not possess a homogeneous matrix. Frequently, electron-lucent areas were present, shaped as crescents or vacuoles, and often located eccentrically (Fig. 10B). All older papillary tumors possessed cells with lipid vacuoles or droplets that were frequently surrounded by glycogen deposits (Figs. 9F and 10, A and C). The cell shape was usually tall columnar, and the nuclei were regularly cleaved and occasionally contained nuclear pseudoinclusions of mature la-

Fig. 6. Electron micrographs of solid lung tumor from C3H mouse. In A, cells are round or pyramidal with microvilli on their free surface and numerous large, cytoplasmic lamellar bodies (lb); m, mitochondria. Uranyl acetate/ammonium silver nitrate, × 5,250. B, formation of lamellar bodies (lb) from multivesicular bodies (mvb); large, dense incomplete lamellar body in upper-right corner, whorled round cytoplasmic sequestrations (sq) with glycogen granules. Uranyl acetate/ammonium silver nitrate, × 32,400.
HISTOGENESIS OF MOUSE LUNG TUMORS

In the periphery of all older papillary tumors, cells were present with cytoplasmic accumulations of osmiophilic, laminated material that was only preserved in epoxy resin-embedded material (Fig. 9G). Fig. 9F shows a semithin section of a papillary tumor with many cells possessing glycogen and lipid vacuoles as well as macrophages with crystal-like inclusions of unknown origin. Ultrastructurally, these configurations were membrane bound and electron lucent.

DISCUSSION

The two morphological variants of mouse lung tumors have commonly been considered benign (solid/alveolar adenoma) or malignant (papillary carcinoma) growths arising from alveolar type II cells (2). The papillary pattern of growth has been found both as a uniform feature (1) and in foci within solid tumors (4, 42). To our knowledge, no malignant solid neoplasms have been described in detail, although such tumors are transplantable (4).

The present study on solid mouse lung tumors induced by transplacental exposure to NEU confirms their origin from the alveolar type II cell. Tumor cells in tissue sections were consistently large, with round profiles and round nuclei, abundant pleomorphic lamellar bodies, and a strongly positive immunohistochemical reaction for intracytoplasmic surfactant apoproteins using two different antibodies.

No biochemical, immunocytochemical, or morphological evidence could be found for the proposed Clara cell origin (11) of the papillary lung tumor in either C3H or Swiss Webster mice. Therefore, suspected strain differences (43) do not seem to be justified. As previously shown by others (1, 5, 44), serial sections failed to reveal original growth from the bronchioles, the site of the Clara cell. According to recent understanding of lung differentiation (32, 33, 45–47), an early, strictly separate development of the alveolar and the bronchiolar systems is recognizable in the mouse from the 14th day of gestation onward. Therefore, it appears unlikely that Clara cells are present in alveolar walls (15) or that type II cells are transformed into Clara cells. Serial sections of larger, grossly visible, invading tumors can give no evidence as to their site of origin (8, 9). Few of the early tumors seen in serial sections in the present study were not separated by alveolar walls from bronchiolar lumina. These probably arose from the alveolar tubes (46) (formerly termed the distal part of the respiratory bronchiole). As the tumors increased in size and invasive growth with age, more neoplasms of both morphological types were found within bronchioles. This may account for different rates of airway invasion noted by others (48). However, 50% of both tumor types originated from alveolar walls adjacent to the pleura, where mouse lung tumors have always been well recognizable at necropsy (2, 49).

The present study of serial sections from mixed tumors containing both solid and papillary structures gave no evidence for invasion of one neoplasm by the other (11, 13). As suggested previously (4, 42, 50, 51), the mixed lung tumors probably represent a stage of tumor progression from a benign solid, alveolar type II cell adenoma to a less well-differentiated, malignant papillary tumor. In lung tumors arising naturally in mice not given a carcinogen, tumor appearance and dedifferentiation start relatively late and can extend over almost the entire life span of the mouse (50). Development is accelerated in chemically induced tumors and varies with the strain and age of the mouse at treatment (4), type of compound, and dosage (52). In transplacental carcinogenesis, the target tissue
is different from that of postnatal lung, and the induction period of lung tumors is reduced even further (24, 44). Therefore, direct comparisons of lung tumor latency periods according to tumor type (23) should only be done if the conditions of tumor induction are considered accordingly. It seems very unlikely that papillary mouse lung tumors should have a different origin depending on whether they arise de novo or from within solid neoplasms.

Dehydrogenase enzymes (mainly 3-hydroxybutyrate dehydrogenase) are present in large quantities in Clara cells of various species including the mouse and can be demonstrated histochemically by NBT formazan staining (25). Not a single lung tumor of C3H or Swiss mice studied for NBT reductase activity revealed a staining intensity exceeding that of the surrounding normal alveolar septa. In contrast, one conflicting report described 13 of 19 papillary tumors induced transplacentally in Swiss mice by NEU as possessing significant activity (17). The reason for this difference is not immediately clear, since mouse strain, NEU dose, and the day of treatment were the same as ours. However, it must be remembered that...
type II cells also contain the enzyme but in smaller quantities (25). It is thus not a specific marker for Clara cells and can be expected to be present in some tumors of alveolar type II cell origin as observed in the present study, especially if the inhibitive pretreatment with formalin is omitted (26).

Major arguments for the Clara cell origin of papillary tumors were based on the presence of dense secretory granules, membrane-bound crystalline structures, numerous microvilli, and interdigitating membranes between adjacent cells, as well as the cuboidal-to-columnar cell shape and deeply cleaved nuclei (11, 53). The shape of papillary tumor cells bears a resemblance to Clara cells, but also resembles epithelial cells in the fetal mouse
likewise, the reappearance of primary cilia and the high glycogen content in some cells of the papillary tumors signal a regressive development resembling a fetal stage. Although glycogen is also present in developing Clara cells, this occurs only relatively late in gestation, whereas glycogen is consistently present in alveolar type II precursor cells. Previous biochemical studies also noted an enzyme expression in mouse lung tumors comparable to the fetal lung that was absent in mature alveolar type II cells (2). Parallel reexpressions of fetal properties are known for other organs and their tumors (54).

Increasing quantities of neutral lipids, found with age in the papillary tumors in the present study, could result from a biochemical transformation of the glycogen. Frequently, both were seen together within the same cell. Lipid droplets were found in mouse alveolar type II cells during early postnatal development (55) and in tissue cultures of normal alveolar type II cells from rats (56).

The use of antigenic markers utilizing immunological techniques for cell identification has become widely acknowledged. Also as shown previously (16), not a single papillary neoplasm of more than 150 tumors in the present study was immunoreactive for Clara cell antigens. However, if tested for mouse surfactant apoproteins with two different antibodies using the ABC and PAP technique and indirect immunofluorescence, all solid tumors and the majority of papillary tumors reacted positively. Staining intensity was lower in the papillary tumors than in normal type II cells, probably as a result of dedifferentiation with partial loss of mature antigenic determinants. These results clearly confirm those obtained with an anti-rat surfactant antibody showing mouse lung toms to be of type II cell origin (16). SAALS is able to detect type II cell antigens at a very early stage of lung development in the mouse (33; Day 14 of gestation versus first day of life for anti-SAP-M) and in the rat (36; Day 16 of gestation). Therefore it appears that SAALS is specific for a different range of surfactant antigens. Variations in antibody specificity could in part account for different ultrastructural localizations of surfactant (57–60), since the antisera were prepared at different locations, at different times, or according to different methods.

To clarify discrepancies between previous electron microscopic (11) and immunocytochemical findings (16), an additional ultrastructural study was performed. Other ultrastructural investigations, presenting the alveolar type II cell as origin for mouse lung tumors, sometimes did not strictly separate solid and papillary tumor types. Nevertheless, it is obvious from descriptions and micrographs that the authors dealt with papillary tumors (6, 7, 61–63), and we can confirm most of their observations. As evidence for the Clara cell origin of papillary tumors, osmiophilic bodies and dense secretory granules were shown in specimens stained conventionally with toluidine blue or uranyl acetate (11, 15). Therefore, no distinction was possible between nonspecific electron-dense secretory granules of Clara cells (64–66) and other osmiophilic dense bodies. Crystals, quoted as Clara cell markers (11, 15), have never been described in mouse Clara cells (64–73) and cannot, therefore, be considered as morphological evidence that mouse papillary tumors originate from Clara cells. Membrane-bound crystalline-like formations have previously (7, 74), however, been noted in lung tumors of mice, and we found them to be osmiophilic. Multiple microvilli, characteristic of papillary tumors, contrasted with the relatively smooth surface of Clara cells, and the tumors usually possessed more rough endoplasmic reticulum (75) as opposed to the abundance of smooth endoplasmic reticulum in Clara cells (67, 72, 73). Cleaved nuclei, interdigitating membranes, and tight junctions represent nonspecific features also found in normal alveolar type II cells and solid tumors. Characteristic mouse Clara cell mitochondria (65, 73), however, were not present in the papillary tumors.

Significant structures found in papillary tumors were osmiophilic multivesicular bodies (Figs. 9C and 10B). Multivesicular bodies have been noted as a source of lamellar body formation (40) and a site of localization of surfactant antigens in alveolar type II cells (57–60). Clara cells may also possess multivesicular bodies (68). However, these are infrequent, lack a dense matrix, and show no lamellar formations. Some of the dense cell organelles seen in the tumors are probably lysosomes, but many of them are most likely involved in the production of surfactant, explaining the high incidence of tumors reacting positively for SAP-M and with SAALS. The formation of mature or immature lamellar bodies depends on surrounding growth conditions (76, 77), and cell organelles can even be lost (77), as in the normal alveolar type I cell derived from the alveolar type II cell (78). Earliest prenatal alveolar cells possess membrane-bound granules with a dense matrix which have been suggested to represent precursors of lamellar bodies (47, 79).

Some lamellar configurations are known to occur as artifacts (80). Such structures can be of lysosomal origin and are found in all cell types; they are inducible by drugs (81). Myelin figures similar to lamellar bodies have not been described to occur in normal mouse Clara cells (64–73). One could argue against the specificity of lamellar bodies found in papillary tumors as evidence for alveolar type II cell function if the identification is based on morphology alone. However, the same should then be applied to their presence in solid tumors. Immunohistochemical studies on the localization of surfactant apoproteins in papillary mouse lung tumors should help clarify these questions.

Tubular myelin constitutes the hypophase of the surface-active alveolar surfactant layer (82, 83) and is phagocytosed by macrophages (84, 85). Tubular myelin was observed in the lumina of papillary mouse lung tumors, and myelin figures were also present in some of 18 putative Clara cell tumors (11). Its origin within complex papillary tumors could stem from some remaining normal alveolar type II cells or from tumor cells. Osmiophilic lamellar accumulations observed in alveolar type II cells surrounding or within papillary and mixed tumors (17) probably represent a disturbed host response that can also be induced by radiation (86) or bleomycin (87). The significance of membranes in the cytoplasm of tumor cells (Figs. 6 and 10, A and C) is unknown (6, 88, 89). They might be comparable to the bar-like structures seen in rat alveolar type II cells (90) and have been suggested to be involved in surfactant production (47).

Human lung tumors with immunological markers of alveolar type II cells have been described to consist of cuboidal or high columnar cells and to grow in a papillary pattern (91–94). These tumors showed intranuclear staining for surfactant that was attributed to ultrastructural tubular configurations and may represent nuclear membranes with surfactant apoprotein (93). Only recently have the tubular intranuclear inclusions been suggested as a marker for human alveolar cell carcinoma (95). In the present study and in others on mouse lung tumors (6, 61, 89, 96), nuclear pseudoinclusions consisting of membrane invaginations with cytoplasmic contents, mainly lamellar structures, were a consistent finding in older papillary tumors and are probably responsible for the positive intranuclear surfactant staining (16).

In summary, we could not find specific enzymatic, antigenic, morphological features in the papillary tumors of the mouse.
lung that were unequivocally specific for Clara cells and that would clearly support an origin of these tumors from Clara cells. Substantial evidence is presented, however, for the alveolar type II cell as the origin of the papillary tumor. These features include origin from the pulmonary acinus, the high incidence of tumors immunoreactive to the different surfactant apoproteins, and the ultrastructural presence of cell organelles specific for alveolar type II cells.

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