Surfactant Protein C Expression in Urethane-Induced Murine Pulmonary Tumors

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Mice injected with urethane develop tumors with distinct histological patterns, which are classified as solid, papillary, or a mixture of these two patterns within the same tumor. Most investigators agree that solid tumors arise from alveolar type II cells, but the cellular origin of papillary tumors is less certain, being attributed to either type II cells or nonciliated bronchiolar epithelial (Clara) cells. To characterize the state of differentiation of these tumors more precisely and to provide additional information on gene expression, we used immunocytochemistry and/or *in situ* hybridization to determine the cellular localization of surfactant-associated proteins A (SP-A), SP-B, SP-C, and SP-D; Clara cell-associated protein CC-10; and thyroid transcription factor-1. In normal mouse lung, the messenger RNAs (mRNAs) for SP-A, SP-B, and SP-D were expressed in both type II cells and Clara cells. SP-C mRNA, however, was expressed only in type II cells, and CC-10 expression of mRNA was restricted to Clara cells. All tumors examined, both solid and papillary, expressed SP-A, SP-B, SP-C, SP-D, and thyroid transcription factor-1, but not CC-10. However, SP-C expression was slightly diminished in larger (older) papillary tumors. These results demonstrate that urethane-induced murine lung tumors express the type II cell phenotype. (*Am J Pathol* 2000, 156:175–182)

Murine pulmonary adenomas have been used extensively to model human lung adenocarcinomas and to provide insight into the molecular events in carcinogenesis.¹ One unresolved question is the determination of the state of differentiation of these tumors. Expression of cell type-specific genes is important in the diagnosis of and search for micrometastases. Genes expressed in these tumors may also provide insight into the cell type of origin. Murine adenomas have been divided into different

histological growth patterns, that is, solid or papillary tumors.^{2–5} The relative proportion of these tumors within a single lung depends on the inbred strain, the stage of tumor progression, the inducing carcinogen, and the age of the animal at the time of tumor induction. The predominant pattern is that of small solid tumors. Most investigators agree that these tumors, which have ill-defined borders and extend into the parenchyma, are derived from alveolar type II cells. Many of the cells have lamellar inclusion bodies that resemble the storage organelle for pulmonary surfactant found in alveolar type II cells.^{4–6} The cells in these tumors have also been reported to contain surfactant proteins A (SP-A) and B (SP-B) but do not contain detectable levels of the 10-kD Clara cell secretory protein CC-10^{6–9}; the Clara cell secretory protein is also referred to as CC-16, uteroglobin, or urinary protein 1.¹⁰

The other histological pattern is that of a papillary tumor, which can occur by itself or mixed with cells organized as solid tumors. In general, papillary tumors have more defined borders, arise later after the administration of carcinogens, develop into larger tumors, and can progress into adenocarcinomas.^{1,5,11} The cell of origin and differentiated characteristics of papillary tumors are in dispute.^{12,13} Most investigators believe that these tumors arise in the lung parenchyma and not within small airways.¹⁴ Some investigators have concluded that these tumors arise from Clara cells, based on the lack of lamellar inclusion bodies; the presence of high levels of nitroblue tetrazolium reductase, succinate dehydrogenase, and glycerol-3-phosphate dehydrogenase; and the expression of high-molecular-weight cytokeratins found in airway epithelial cells.^{2,15–20} Papillary tumors contain SP-A and -B^{6,8,9} but lack immunodetectable CC-10.⁸

The surfactant proteins—and especially SP-C—can serve as differentiation markers for pulmonary epithelial cells. The four surfactant-associated proteins, SP-A, SP-B, SP-C, and SP-D, fall into two separate groups. SP-A and SP-D are collagenous glycoproteins that are hydrophilic and can function as calcium-dependent lectins.^{21,22} SP-A is found in type II cells and in Clara cells,

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and it is thought to serve as a modulator of surfactant homeostasis as well as a lectin for host defense. SP-D is also found in alveolar type II cells and Clara cells, but SP-D binds surfactant lipids poorly and is thought to function primarily in host defense. SP-B and SP-C are very hydrophobic proteins that are found in lamellar bodies in type II cells as well as in isolated purified surfactant. SP-B is found in type II cells and Clara cells, whereas SP-C is restricted to type II cells.^{23–25} Hence, the only surfactant protein that is restricted to alveolar type II cells is SP-C, which, therefore, can be used to identify this cell lineage. In the normal lung, CC-10 is found only in non-ciliated bronchiolar epithelial cells or Clara cells and can be used to identify these cells.^{26–28} However, expression of CC-10 is markedly diminished during airway inflammation and epithelial injury.²⁹

Differentiated gene products expressed by tumors are important for the detection and precise diagnosis of individual tumors and their metastases. A large number of adenocarcinomas express SP-A, and this observation has led to the diagnostic capability of distinguishing primary lung cancers from metastatic adenocarcinomas and from mesotheliomas, which can appear similar to adenocarcinomas.^{30–32} SP-A measurement in malignant pleural effusions has also been used diagnostically.^{33,34} More recently, thyroid transcription factor-1 (TTF-1), which is a regulator of surfactant protein gene expression, has been shown to be expressed in human lung adenocarcinomas.³⁵ Bejarano et al suggested that TTF-1 might be a more sensitive marker than the surfactant proteins in the identification of lung adenocarcinomas.³⁵ TTF-1 expression has not been heretofore reported in urethane-induced murine tumors.

This study was undertaken to define more precisely the phenotype of the epithelial cells in urethane-induced mouse lung adenomas. Because concerns have been raised that cell-specific marker antigens may result from phagocytosis of secreted proteins and may not represent synthesis by the cell type in question,¹² we used both *in situ* hybridization and immunocytochemistry to characterize the cells. Our data show that both solid and papillary tumors express SP-A, SP-C, SP-D, and TTF-1, but neither type of tumor expresses CC-10. Our results represent the first immunologic localization of SP-C and SP-D within these tumors.

Materials and Methods

Tissue Preparation

Urethane-induced mouse tumors were produced by the procedure of Malkinson and Born.³⁶ Briefly, strain A/J mice, 6 to 10 weeks of age, were given a single intraperitoneal injection of urethane (Sigma Chemical Co., St. Louis, MO) dissolved in 0.9% NaCl at a dose of 1 mg/g of body weight. Mice were sacrificed at 8 to 50 weeks after the injection of urethane and processed for immunocytochemistry and *in situ* hybridization as described below.

Immunocytochemistry

Sections of normal mouse lung and tumors were immunostained with a panel of rabbit polyclonal antibodies. These included anti-rat SP-A and anti-rat SP-D (gifts from D. Voelker, Denver, CO), anti-human pro-SP-C (a gift from J. Whitsett, Cincinnati, OH), and anti-rat CC-10 (a gift from G. Singh, Pittsburgh, PA). Different fixation and processing were required for each antigen. For SP-C, tissues were fixed in freshly prepared 4% paraformaldehyde; initial fixation was for 2 hours at room temperature, and then the tissue was cut into pieces and fixed for an additional 20 hours at 4°C. The fixed tissue was washed in phosphate-buffered saline (PBS), dehydrated, and embedded in paraffin. After deparaffinization and rehydration, sections were treated with 6 N guanidine hydrochloride for 30 minutes at room temperature followed by 0.2 mg/ml trypsin for 30 minutes at 37°C.^{37–39} Immunostaining was done by the standard ABC method³⁷ with the primary rabbit anti-pro-SP-C at a concentration of 1:5000 (v/v), followed by goat anti-rabbit immunoglobulin G (IgG) (Vector BA 1000) and, finally, streptavidin-biotin-horseradish peroxidase complex (RPN 1051, Amersham, Arlington Heights, IL). Diaminobenzidine was used as the substrate to generate a brown reaction product, and then the sections were counterstained with hematoxylin. For CC-10, the deparaffinized and rehydrated sections were treated with PBS and hydrogen peroxide for 30 minutes to inhibit endogenous peroxidase; the guanidine hydrochloride and trypsin steps were omitted. The CC-10 antibody was applied at a dilution of 1:5000, and the same ABC method was used as with SP-C. For SP-A and SP-D, lung tissue was fixed with acid alcohol (96% ethanol, 1% acetic acid, 3% H₂O). The sections were treated with hydrogen peroxide in PBS to diminish endogenous peroxidase, but were not treated with guanidine hydrochloride and trypsin. The purified rabbit IgG anti-SP-A and anti-SP-D antibodies were used as described previously.^{37,40} The primary antibody was incubated overnight at 4°C and washed away, and then the sections were incubated with biotinylated goat anti-rabbit IgG (Vector Laboratory, Burlingame, CA), followed by streptavidin-biotin-horseradish peroxidase and then diaminobenzidine, as previously described.^{37,38}

In Situ Hybridization

In situ hybridization was performed on 4-μm sections as described in detail previously,^{41,42} with the exception that [³³P] UTP (2000–4000 Ci/mmol; NEN Life Science Products) was used in the transcription of RNA probes. Radiolabeled RNA probes were prepared from full-length rat complementary DNA (cDNA) for SP-A,⁴³ SP-B,⁴⁴ and SP-C⁴⁵; a 275-bp fragment of rat CC-10 (a gift from A. Rishi, Boston, MA); and a 414-bp fragment of rat TTF-1 (a gift from L. Thet, Madison, WI), using a commercially available kit (Promega Biotech, Madison, WI). Full-length probes were hydrolyzed to 300 bp before use. After high-stringency washing, the slides were dipped in NTB-2 nuclear track emulsion (Eastman Kodak Co.,

Figure 1. Immunocytochemistry for SP-A, -C, and -D and CC-10 in urethane-induced adenoma. **A:** Histology of representative solid tumors. **B:** Histology of representative papillary tumors. Tumor cells were stained with H&E. **C, E, G, and I:** Immunostaining for SP-A, -C, and -D and CC-10, respectively, in solid adenoma. **D, F, H, and J:** Immunostaining for SP-A, -C, and -D and CC-10, respectively, in representative papillary adenomas. The methods for immunostaining required different fixatives for different antigens as stated in Materials and Methods. Thus the micrographs are not all from the same tumors. All panels are at the same original magnification.

Rochester, NY) and then developed after an exposure period appropriate for each probe. Hybridization with radiolabeled sense probes was done as a control in all experiments.

Results

Histology and Immunocytochemistry

The histological appearance of the two types of mouse adenomas is shown in Figure 1. At 20 weeks post-urethane treatment, most tumors appeared solid (Figure 1A) or papillary (Figure 1B), as has been previously reported in A/J mice.^{5,46} We found no difference between the solid and papillary tumors in either their expression of SP-A, SP-C, and SP-D or their lack of expression of CC-10. All tumors contained immunologically detectable SP-A and SP-D, which are also expressed in alveolar epithelial cells and Clara cells in the conducting airways (Figure 1, E–H). All tumors contained pro-SP-C, which is a specific marker of alveolar type II cells, and there did not appear to be any difference between solid or papillary tumors (Figure 1, C and D). pro-SP-C was not detected in the epithelial cells of the conducting airways. Finally, CC-10 was detected in the Clara cells of the conducting airways but not in any tumors, whether they were solid or papillary (Figure 1, I and J). The 50-week-old tumors included solid and papillary tumors, as well as mixed tumors with both papillary and solid features. The immunostaining of the older papillary or mixed tumors was similar to that of the tumors in younger animals (Figure 2). There was immunostaining for SP-A, pro-SP-C, and SP-D but not CC-10 (Figure 2; data not shown). Immunostaining for all three surfactant proteins was retained even in areas that displayed mucinous metaplasia. However, the intensity of the staining of pro-SP-C appeared to be slightly less in the more glandular than the more solid areas of these tumors.

Hence, all of the tumors reacted immunologically with antibodies to SP-A, pro-SP-C, and SP-D, but not CC-10.

In Situ Hybridization

Expression of surfactant messenger RNAs (mRNAs) in individual cells of both solid and papillary tumors was also assessed by *in situ* hybridization (Figures 2 and 3). *In situ* hybridization complements the immunocytochemistry

Figure 2. Pro-SP-C immunocytochemistry and SP-C and CC-10 *in situ* hybridization in a large papillary tumor. This tumor was harvested from a mouse 50 weeks after intraperitoneal injection of urethane. **A:** Histology with staining with H&E; **B:** Immunostaining for pro-SP-C. **C and D:** *In situ* hybridization for CC-10. **E and F:** *In situ* hybridization for SP-C. All panels are at the same original magnification.

Figure 3. *In situ* hybridization for SP-C, CC-10, and TTF-1 in the same tumors. Sections of a solid and papillary tumor were hybridized with riboprobes for SP-C (A–D), CC-10 (E–H), and TTF-1 (I–L). The pairs of micrographs are visualized by bright-field or dark-field illumination. These are consecutive sections from the same tumor fixed in 4% paraformaldehyde and processed as stated in the methods section. All panels are at the same original magnification.

and addresses issues of cross-reacting epitopes of similar proteins and phagocytosis of secreted proteins. The mRNAs for SP-A, SP-B, and SP-D were expressed by both type II cells and Clara cells in normal mouse lung (data not shown), as has been reported by others.^{47–49} However, SP-C mRNA was found only in alveolar type II cells and not in Clara cells (23; Figure 3). SP-A, SP-B, SP-C, and SP-D mRNAs were expressed in all tumors evaluated, from 8 to 50 weeks post-urethane treatment (data not shown). Most cells in solid tumors appeared to express all four mRNAs. There was no observable variability in the expression of SP-A, SP-B, or SP-D in the tumors. As shown in Figure 3, both solid and papillary tumors expressed SP-C and TTF-1. There was no expression of CC-10 in any of the tumors but ample expression in the airway epithelial cells (Figure 3, E and F). In the older tumors there was also expression of SP-C (Figure 2, E and F) but not CC-10 (Figure 2, C and D). However, in these tumors the expression of SP-C appeared diminished compared with that in the tumors of younger animals and type II cells in the parenchyma. These results indicate that all of the tumors examined in this study expressed differentiation characteristics of alveolar type II cells, because they all expressed SP-C. All of the tumors also expressed TTF-1, and its expression did not vary with the histological appearance of the tumor. In summary, the observations made by *in situ* hybridization confirmed and extended those made by immunocytochemistry.

Discussion

Urethane has been used extensively to produce both solid and papillary pulmonary adenomas in many strains of mice, although the relative percentage of each type of

histological tumor varies with the strain.⁴⁶ These adenomas have been studied in a variety of biochemical, genetic, and mechanistic investigations, including specific studies of surfactant phospholipid metabolism, and they have also been the subject of several symposia and proceedings.^{1,50–52}

Most investigators have concluded that the small solid adenomas induced in mice by urethane are derived from alveolar type II cells. This is based on their ultrastructure, expression of SP-A and simple cytokeratins by immunocytochemistry, and lack of expression of CC-10.^{4,5,7,8} Early studies indicated that the tumors arose from type II cells and that the initial pathologic response appeared to involve type II cells. Kauffman reported that urethane produced type II cell hyperplasia.^{53,54} When urethane was added to the drinking water, initially there was an apparent selective injury to the alveolar epithelium. This was followed by type II cell hyperplasia, which peaked at 6 weeks.^{53,54} The type II cell hyperplasia was thought to be caused by alveolar epithelial injury, and the neoplasia was thought to be caused by incidental molecular alteration in the hyperplastic response. Thus, alveolar epithelial cells appeared to be the selective target of urethane, and there was little evidence for abnormalities in bronchial epithelial cells. Our data on the expression of SP-C support this concept. However, more recently Forkert et al have reported Clara cell hyperplasia in A/J mice treated with urethane.⁵⁵

The differentiated phenotype of the epithelial cells in the papillary tumors, however, has been much more controversial.¹³ Kauffman and others have concluded that the epithelial cells of the papillary tumors arise from Clara or nonciliated bronchiolar epithelial cells. This conclusion was based primarily on morphologic criteria such as the lack of lamellar bodies; cytoplasmic granules similar to

those seen in Clara cells and nuclear morphology; histochemical determination of NBT reductase, succinate dehydrogenase, and glycerol-3-phosphate dehydrogenase; and expression of high-molecular-weight cytokeratins.^{2,5,15-17,19,20} In contrast, Ward et al¹⁸ and Rehm et al^{6,9} concluded that papillary tumors induced by nitrosoethylurea were derived from alveolar type II cells because they failed to express CC-10, yet did express SP-A, which is found in both alveolar type II and Clara cells. Our data extend these observations; all of the papillary tumors examined expressed SP-A, SP-B, SP-C, and SP-D, but none expressed CC-10. However, in the older and larger papillary tumors, the more solid components of these tumors expressed higher levels of SP-C than the cells in the more glandular and mucinous portions of the tumor. We conclude that all urethane-induced tumors express the type II cell phenotype, but, in the older papillary tumors, the expression of SP-C is slightly diminished. The significance of this latter observation is unclear.

Murine adenomas can also be induced by other agents.⁵⁶ Rehm et al induced murine adenoma in C3H and Swiss Webster mice with transplacentally administered *N*-nitrosomethylurea.^{6,9} These tumors arose in the pulmonary acinus and invaded the bronchioles only at a later stage, as the tumors grew. Mixed solid and papillary patterns within a nodule were thought to be due to the progression from a solid to a more papillary pattern and not the merging of two different tumors. These tumors expressed antigens found in surfactant, presumably SP-A and lysozyme, but lacked both the expression of CC-10 and the NBT stain for dehydrogenase enzymes found in Clara cells. In a detailed ultrastructural examination, characteristic mouse Clara cell mitochondria were not found in the papillary tumors.⁹ The electron-dense organelles seen in the papillary tumors lacked the characteristic lamellar bodies of type II cells and were considered to be multivesicular bodies and lysosomes, but not Clara cell secretory granules. It is interesting that there was some glycogen, which is found in fetal pulmonary epithelial cells, seen in some of the papillary and mixed neoplasms. Ward et al also detected SP-A but not CC-10 in murine adenomas induced by *N*-nitrosodimethylamine or nitrosomethylurea.⁸ Hence, adenomas induced by transplacental nitrosourea are similar to tumors induced by urethane and are thought to arise from alveolar type II cells. However, Rehm et al applied *N*-nitrosomethyl-bis-chloroethylurea or *N*-nitroso-tris-chloroethylurea topically on the skin of Swiss mice and induced squamous cell carcinomas and adenosquamous carcinomas that expressed CC-10 but not type II cell markers.⁵⁷ Hence, murine tumors can express CC-10, but they have a different pathologic appearance from tumors induced by urethane.

Forkert et al evaluated the expression of another Clara cell marker, the cytochrome P450 isoenzyme CYP2E1, in urethane-induced adenomas.⁵⁸ CYP2E1 and a reaction product of this enzyme, 2-S-glutathionyl acetate produced from the substrate 1,1-dichloroethylene, are readily identified in Clara cells in the normal lung. There is weaker staining in type II cells, which also contain cytochrome P450. CYP2E1 was markedly diminished in the

urethane adenomas, although there was some staining at the edge of the papillary tumors. Other investigators have noticed a hyperplasia of type II cells at the edge of some tumors.¹⁸ Regardless, Forkert's observations indicate that another Clara cell gene product, CYP2E1, is not highly expressed in urethane adenomas. This observation may reflect the cell type of origin or be a consequence of the neoplastic response wherein certain differentiation markers are lost.

Although our evidence strongly suggests that these tumor cells arise from alveolar type II cells, there are alternative interpretations of these observations. It is possible that these cells arise from a more primitive epithelial cell in the alveolar wall or that the distal lung epithelial cells can express different differentiation markers depending on their local milieu. In other words, there may be plasticity in the epithelial phenotype and gene expression. Based on the observation that squamous cell carcinomas arise from the mucociliary epithelium of the bronchus, Nettesheim has cautioned against relying solely on the cellular biochemical phenotype to define the cell of tumor origin.¹² In the fetal lung, there are primitive epithelial cells that can give rise to cells of both the alveolar and the bronchiolar cell lineages. Initially, all primitive epithelial cells express SP-C, but expression is extinguished in the proximal epithelial cells that eventually form the bronchial epithelium. Although there has been speculation that stem cells reside in the adult lung that could give rise to epithelial cells of both the alveolar and bronchial lineages, there are no convincing data that such cells exist.⁵⁹ The plausibility of putative stem cells in the adult lung that could give rise to cells of both the alveolar and the bronchial epithelial lineage is based primarily on the analogy to oval cells in the liver and pancreas.^{60,61} The hypothesis that the urethane tumors arise from primitive epithelial cells in the adult lung could explain the histological features of the tumors and gene expression. However, until these primitive epithelial cells are identified in the adult lung, this hypothesis remains unlikely.

The concept of epithelial plasticity is difficult to evaluate in vivo. To date, there is no evidence that Clara cells can express SP-C mRNA or pro-SP-C. Nevertheless, it is difficult to refute the issue of epithelial plasticity and the implied limitation in attributing cell of origin based on differentiation markers in an established tumor. In addition, there is some experimental evidence to support this concept of plasticity. Most adenocarcinomas induced in transgenic mice by overexpressing the simian virus 40 (SV-40) T antigen under control of the human SP-C promoter express CC-10 but not SP-C.⁶² However, cell lines derived from the tumors and maintained in vitro fail to express CC-10, but some express SP-C. One cloned cell line (MLE-15) lacked CC-10 mRNA when grown in vitro, but expressed CC-10 when grown as tumors in nude mice; some of the tumors expressed SP-C in one area and CC-10 in another area. These authors concluded that the expression of SP-C and CC-10 is altered significantly by the cellular environment. However, confirmation that the cell line is derived from a single clone needs to be established. Kitamura et al have observed a potentially

similar transition in human adenomatous hyperplasia.⁶³ They observed a type II cell phenotype in atypical adenomatous hyperplasia by electron microscopy and by surfactant protein (presumably SP-A) immunocytochemistry. However, they found urinary protein 1 (CC-10) in some of the early bronchioloalveolar cell carcinomas and overt bronchioloalveolar lung carcinomas, which are later stages in the oncogenic transformation.

Another observation that indicates epithelial phenotypic plasticity or the presence of alveolar epithelial stem cells in large airways was reported by Ten Have-Opbroek et al, who took canine bronchial segments free of alveolar tissue, transplanted them subcutaneously into the back, and treated these transplanted bronchial segments with carcinogens.^{64,65} These grafts developed cells that expressed SP-A, whereas there were no cells containing detectable SP-A in the bronchi before transplantation. None of these tumors expressed CC-10. The implication of these observations is that there are pluripotent stem cells in the bronchial epithelium or that bronchial epithelial cells can differentiate to express SP-A, a process that the authors refer to as oncofetal differentiation. Ten Have-Opbroek et al have extended these observations to human lung cancer and precancerous lesions and found SP-A-positive cells in early progressive lesions in the conducting airways, eg dysplastic, *in situ*, or microinvasive lesions.⁶⁶ These authors conclude that SP-A-positive and CC-10-negative cells are the precursor cells of these tumors. These observations are supported by those of Broers et al, who found a few scattered epithelial cells that express SP-A along human conducting airways.⁶⁷

Most human lung adenocarcinomas are traditionally thought to arise from the epithelial cells of the peripheral airways. Nearly all of the epithelial cells in the pseudostratified respiratory epithelium of the conducting airways lack immunologically detectable SP-A. In humans there is a very short segment of alveolar duct and respiratory bronchioles that stains positively for SP-A, and this area is considered the location of the human equivalent of rodent Clara cells. However, CC-10 immunostaining is also observed further up the airway,⁷ and, by *in situ* hybridization, there is wider distribution of cells positive for SP-A and CC-10.⁶⁷ Nevertheless, it was unexpected that the majority of human adenocarcinomas would express the surfactant proteins.^{31,67} Nicholson et al found SP-A immunoreactivity with the PE-10 monoclonal antibody in 6 of 10 pulmonary adenocarcinomas and 23 of 23 nonmucinous bronchioloalveolar carcinomas.³⁰ However, SP-A was not detected in 34 mucinous bronchioloalveolar carcinomas, 40 nonpulmonary adenocarcinomas, and 26 adenocarcinomas metastatic to lung.³⁰ They concluded that immunostaining for PE-10 was highly specific for adenocarcinomas primary to lung and useful for differentiating primary lung adenocarcinomas from adenocarcinomas metastatic to lung.^{31,32} Nomori et al also reported that the PE-10 monoclonal antibody for SP-A stained 65% of primary lung adenocarcinomas.⁷ CC-10 was detected in 10% of adenocarcinomas, 20% of the squamous carcinomas, and 12% of large-cell carcinomas. CC-10 was identified in tall columnar epithelial cells in the human respiratory epithelium that were mor-

phologically quite different from rodent Clara cells. Broers et al used *in situ* hybridization to evaluate SP-A and CC-10 in human lung tumors and found that 5 of 6 of the adenocarcinomas expressed SP-A, but only 1 out of 19 lung tumors expressed CC-10. A squamous cell carcinoma was tumor positive for CC-10.⁶⁷ Thus, adenocarcinomas are much more likely to express SP-A than CC-10. Shijubo et al³³ and Honda et al⁶⁸ demonstrated that about 40% of metastatic pleural effusions due to primary pulmonary adenocarcinomas had SP-A levels in the pleural fluid of >500 ng/ml, a value which is about 10-fold that of typical serum concentrations. Malignant effusions due to other types of lung tumors, metastatic adenocarcinomas from other organs, and pleural effusions due to tuberculosis had SP-A concentrations of less than 500 ng/ml. Shijubo et al also reported that SP-A levels in pleural fluid could differentiate effusions caused by pulmonary adenocarcinoma from those caused by mesotheliomas.³⁴

Expression of surfactant proteins might be useful to screen regional lymph nodes for micrometastases. RT-PCR was used to screen for micrometastases in 41 patients with non-small-cell lung cancer.⁶⁹ RT-PCR for SP-A and SP-C identified micrometastases that were not apparent on routine pathologic examination. RT-PCR analyses for SP-B and SP-D were not found to be useful because of apparent expression outside of the lung. These observations are an important step forward in staging pulmonary adenocarcinomas.

TTF-1 may be an even more useful marker of pulmonary adenocarcinomas. Bejarano et al used immunocytochemistry for TTF-1 to evaluate primary lung and breast tumors and found it highly specific for pulmonary adenocarcinomas.³⁵ In this study the antibody for TTF-1 had a higher sensitivity and specificity than antibodies for SP-A and SP-B. TTF-1 was not detected in squamous carcinomas. Our data demonstrate expression of TTF-1 in the murine adenomas and indicate that the murine model might be useful for future studies on regulation of TTF-1 expression in these tumors.

Our data support the concept that both solid and papillary murine pulmonary adenomas induced with urethane are derived from alveolar type II cells. In the rat, most pulmonary adenomas are also thought to be derived from alveolar type II cells.⁷⁰ However, this may not be the case in other species. In the hamster, peripheral lung adenomas induced with *N*-nitrosodiethylamine arise from nonciliated bronchiolar (Clara) cells.⁷¹ This conclusion is based on immunostaining for a Clara cell secretory protein in 50% of the tumors, ultrastructure of the tumors, and areas of focal hyperplasia in the epithelium of bronchioles in treated animals.⁷¹ In addition, there were areas of focal squamous metaplasia along the conducting airways.⁷¹ As the tumors grew larger, the expression of CC-10 diminished, and the expression of keratin increased. However, these tumors were induced by *N*-nitroso-diethylamine or irradiation and not urethane.

In summary, the expression of SP-C and the lack of expression of CC-10 indicate that urethane adenomas in mice arise from alveolar type II cells. There was no difference in the expression of type II cell markers in the

solid or papillary tumors from young animals. These murine tumors also expressed TTF-1, which has been suggested to be a specific marker of pulmonary adenocarcinomas. A significant number of adenocarcinomas may arise from alveolar epithelial cells, and these cells deserve more intense study as precursor cells for adenocarcinomas, which historically are thought to arise primarily from epithelial cells of the peripheral airways.

Note Added in Proof

Since our study was completed, we found a report that confirmed the SP-C expression in murine lung tumors. These authors used small oligonucleotide probes and studied tumors of untreated B6C3F1 mice.⁷²

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