A Transgenic Mouse Model for Lung Adenocarcinoma

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Abstract
Lung cancer is a leading cause of tumor-related deaths in humans but its origin and development are poorly understood. To study the biology of these tumors, appropriate animal and cell culture models will be of eminent importance. Uteroglobin is a marker protein for the nonciliated epithelial Clara cells lining the respiratory and terminal bronchioi of the lung. We have used the promoter and 5'-flanking sequences of the rabbit uteroglobin gene to target expression of the SV40 T antigen to the lung of transgenic mice. All transgenic founders as well as the descendants from an established line, UT7.1, developed multifocal bronchioloalveolar adenocarcinomas originating from Clara cells. At least three different stages in tumor development with progressive loss of the differentiated phenotype can be distinguished by immunohistochemical data and in situ hybridization. Only in the initial stage did bronchiolar cells express both uteroglobin and SV40 T antigen, whereas at later stages, only SV40 T antigen was detected, and the most advanced tumors were negative for both proteins. From the lungs of UT7.1 mice, a bronchiolar cell line was established that maintains the features of differentiated Clara cells. This system provides a useful model for further studying the development and progression of lung adenocarcinomas in vivo and in vitro.

Introduction
It is currently estimated that 25% of the overall human lung tumors are adenocarcinomas deriving from the Clara cells of the bronchiolar epithelium, the type II pneumocytes, or the mucin-producing cells (1). Tumors derived from nonciliated Clara cells or type II pneumocytes are summarized as bronchioloalveolar adenocarcinomas (2). The bronchiolar epithelium, which contains up to 75% of Clara cells, is one of the most common sites of transformation (3, 4). Clara cells are biochemically characterized by the presence of the cytochrome P-450 monooxygenase system, which is involved in the metabolism of xenobiotics (5). This enzymatic activity may also result in an activation of toxins because they are present, for example, in cigarette smoke. Therefore, these cells are predisposed for malignant transformation (6).

To generate manipulable animal and cell culture models for investigating the genesis and growth of lung adenocarcinoma, we have used the rabbit uteroglobin gene 5'-flanking region for targeting an oncogene to the Clara cells of the lung. Uteroglobin is a small globular protein, which was originally described in the uterine fluid of pregnant rabbits (7). Later, the protein was identified as a major product of the bronchiolar Clara cells in rabbits, humans, mice, and rats (8–12). Binding of polychlorinated biphenyl metabolites has been discussed as a major function of uteroglobin in the lung (13). However, uteroglobin is also expressed at lower levels in other secretory epithelia including the genital tract, stomach, and salivary gland (12, 14).

Expression of the uteroglobin gene in Clara cells was first detected at about day 17 of gestation and is glucocorticoid regulated in adult mice (12). Its abundant cell type-specific expression during lung development, which is temporally paralleled by an increase in circulating glucocorticoids and expression of the glucocorticoid receptor (15, 16), offers an opportunity to investigate the mechanisms of differentiation and transformation in the lung. In transgenic mice bearing the rabbit uteroglobin gene encompassing 4 kb of 5'-flanking sequences, the transgene is expressed in the same cell types as the endogenous gene (12, 16). Based on these results, we have targeted expression of the SV40 T antigen to Clara cells in transgenic mice by using a rabbit uteroglobin/T antigen fusion construct. These transgenic mice not only develop lung adenocarcinomas suitable for studying the various stages of tumor development, but they can also be used to establish differentiated bronchiolar cell lines in culture.

Results
Characterization of UG/TAG Transgenic Mice. Five transgenic founder mice bearing 1 to 10 copies of an intact fusion construct were obtained by microinjection of the rabbit UG/TAG fusion gene (Fig. 1A). Autopsy of the founders revealed the presence of several lung tumors that, in each of these animals, turned out to be bronchioloalveolar adenocarcinomas and led to the death of most carriers before transgenic descendents were obtained. No attempt has been made to precisely quantitate the number of tumors, but they were always multifocal, and as many as 30 nodules could be identified in a single animal by macroscopic inspection. The tumors appeared as white and glassy nodules localized in the lower parts of the lobes. In advanced cases, solitary, firm, solid tumors several millimeters in diameter in the periphery of the lung were present. The color of the lungs interspersed with tumor nodules was deeply red, in contrast to lungs of normal littermates, suggesting a high degree of vascularization. Apart from the lung adenocarcinomas, the transgenic mice developed tumors in several other organs known to express uteroglobin, including the salivary gland, the stomach, the uterus, and the

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3 The abbreviations used are: UG/TAG, uteroglobin/T antigen; SP-A, surfactant protein A; kb, kilobase(s).
transgenic prostate (14). We have generated one transgenic mouse line from founder 7.1 (UT7.1) that expresses the transgene at high levels in the lung and in which male and female mice develop lung tumors. Southern blot analysis of BamHI-digested DNA from UT7.1 mice showed that about 10 copies of the transgene have been integrated in tandem at a single genetic locus (Fig. 1B). Most of the transgenes are integrated in head-to-tail orientation, as demonstrated by the presence of approximately eight copies of a 4.3-kb transgenic fragment. The 6.0-kb fragment originates from a single insertion in tail-to-tail orientation; the 8.0-kb fragment results from cleavage at the 3'-end of the clustered transgenes and in the mouse flanking sequences.

Histopathological Analysis of Lung Tumor Development. The histological analysis of lung tissue from UG/TAg transgenic mice showed that all cells within the bronchiolar epithelium were morphologically normal in 2-month-old transgenic animals (see below). Tumors were not detected in any of these young UT7.1 mice. Analysis of the lungs of 4-month-old transgenic animals, however, revealed dysplastic bronchiolar epithelia and few small tumor nodules. In 5-month-old animals, the morphologically altered epithelia and the number of tumor nodules were increased, shown representatively in lung sections of the 5-month-old founder 10.1 (Fig. 2A). At this age, all stages of tumor development were present within one lung. In all animals, single and coalescent tumor nodules as well as regions showing a relatively normal tissue architecture were detected, with Clara cells surrounding the terminal and respiratory bronchioli and a normal alveolar parenchyma (Fig. 2A). Some dysplastic bronchioli were lined by epithelial cells with cytologically transformed appearance; however, their basal membranes were intact (Fig. 2A, dy, and G). We interpret this as the initial state of tumor development. The second stage was characterized by small tumor nodules that formed dense structures and grew along the bronchiolar epithelium without disturbing its function initially (Fig. 2, A and B, br). The tumor cells exhibited little cytoplasm and undifferentiated anaplastic cell nuclei. In the course of further progression, tumor cells grew to a high density and displaced the normal pneumocytes, thereby disrupting the interstitial alveolar parenchyma, and tumor cells filled the alveoli (Fig. 2, A and B, stn). The third stage of tumor development was marked by solitary, large, de-differentiated tumor nodules, detected only in fatally ill animals. A magnification of one of these advanced tumors is shown in Fig. 3B.

Expression of the UG/TAg Transgene and Clara Cell-Specific Genes during Tumor Development. Transcripts from the UG/TAg transgene and the endogenous uteroglobin gene were detected by Northern blot hybridization with RNA isolated from lungs of all founder animals (Fig. 1C, Lanes 7.1, 10.1, and 18.0). Expression of uteroglobin and T antigen in individual cell types was studied by immunohistochemistry. In all lung sections examined, antibodies to uteroglobin reacted specifically with the Clara cells of intact bronchioli as well as with the bronchiolar epithelium adjacent to small tumor nodules (Fig. 2, C and D). In 2-month-old animals, uteroglobin expression was restricted to the Clara cells of morphologically normal bronchiolar epithelium, where most cells stained positively (Fig. 3, A and C). In contrast, T antigen was detected only in subsets of Clara cells, which were frequently clustered (Figs. 2F and 3B). Increasing amounts of cells expressing T antigen were found in the dysplastic proliferating bronchiolar epithelium (Fig. 2H). Subsets of the initial dysplastic cells represent the only stage of tumor development where uteroglobin and T antigen appear to be expressed in the same structure, although the majority of the uteroglobin-positive cells were negative for T antigen (Fig. 2, G and H). The more advanced small tumor nodules were virtually negative for uteroglobin (Fig. 2, C and D) but showed maximal accumulation of T antigen (Fig. 2, E and F).

In situ hybridization confirmed that, in contrast to the bronchiolar epithelium, tumors did not contain transcripts of the mouse uteroglobin gene (Fig. 4A). Transcripts of the transgene, however, were restricted to the small tumor nodules (Fig. 4B). Cells of progressed solid tumors expressed less T antigen than those from small tumor nodules (Fig. 3A), although they were actively proliferating, as demonstrated by high expression levels of the proliferating cell nuclear antigen (Fig. 3D), a marker protein for cell proliferation (17, 18). These findings suggest that cell proliferation in advanced tumors was dependent on the activity of genes other than the T antigen transgene. One possible candidate is p53, which forms complexes with the T antigen, and is often increased or altered in transformed cells (19, 20). However, expression of activated p53 was found to parallel expression of T antigen at various stages of lung tumors. An example is shown in Fig. 3H, which shows p53 staining with an antibody recognizing only the activated form of p53 (21), as compared to expression of T antigen in a similar area of the tumor (Fig. 3G). P53 antibodies stained mainly the nucleus but also the cytoplasm, as reported for other tumor cells (22). Otherwise, its expression correlated with...
staining for T antigen. In advanced tumors, which were negative for T-antigen expression, no staining with the PS3 antibody was detected.

A common progenitor cell has been proposed for the alveolar type II pneumocytes and the Clara cells of the respiratory bronchioles, suggesting that the type II cells might be further differentiated Clara cells (2, 4). To investigate the possible relationship between T-antigen expression and further differentiation of bronchiolar cells, we analyzed the expression of the SP-A. This protein has been shown to be mainly produced in alveolar type II pneumocytes, of which it is considered a typical marker, and is expressed to a much lesser extent in Clara cells (2, 3). However, like uteroglobin, SP-A immunoreactivity was detected in the intact bronchiolar epithelium of transgenic mice but not in the tumors at any stages (data not shown).

Establishment of a Clara Cell Line. The UT7.1 transgenic mouse line has been used for establishing cell lines in which the control of proliferation and differentiation of bronchiolar cells can be investigated. Two bronchiolar cell lines from lungs of 3-month-old UT7.1 mice, which had no obvious tumor nodules, were established essentially as described (23, 24) and maintained in serum-free medium for more than 1 year. The bronchiolar cell line...
UT7.1/1 shows an epithelial-like morphology and has been stable over 1 year in culture (Fig. 5, A and B). These cells grew slowly at the beginning, but after about 12 transfers, the doubling time stabilized at about 24 h. At early passage number, UT7.1/1 cells stained positive for T antigen, but after 3 months in culture, they no longer expressed T antigen. However, the cells stained positive with an antibody against activated P53 (20) that stained also the perinuclear cytoplasm (Fig. 5D). Permeabilized cells incubated with antisera against rat uteroglobin and rat SP-A, known to cross-react with the murine proteins, revealed specific cytoplasmic immunoreactivity for both uteroglobin and SP-A (Fig. 5C and data not shown). Thus, the cells have maintained the differentiated phenotype found in the intact bronchioli with respect to these two marker proteins.

Discussion

The results presented here demonstrate that the rabbit uteroglobin promoter selectively directs T-antigen expression to the nonciliated epithelial Clara cells of the lung, leading to the formation of bronchiolalveolar adenocarcinomas, which could serve as a useful animal model for the development of human lung adenocarcinoma. Although the integration site and copy number of the transgene varied considerably among different founders, all transgenic animals consistently developed multifocal tumors in the lung. Within a single lung, adenocarcinomas exhibiting different stages of development and differentiation were present at a given time. Thus, it was possible to analyze the ontogeny of Clara cell tumors in five independent founders and in descendants of one transgenic line. The formation of dysplasia within the bronchiolar epithelium represents the initial stage of tumor development. In some bronchioli, normal epithelial cells coexist with actively proliferating dysplastic cells that punctually penetrate into the parenchyma. At this stage, most normal Clara cells stained positive for uteroglobin but negative for T antigen. Only dysplastic Clara cells in
the initial stages of tumor development expressed both T antigen and endogenous mouse uteroglobin. The topological correlation of T antigen and uteroglobin expression was no longer observed in proliferating cells within the evolving small tumor nodules that represent the second stage of tumor development. These cells did not express uteroglobin but stained positive for T antigen. These findings suggest that, as a consequence of long-term T antigen expression, Clara cells loose their ability to express the endogenous uteroglobin gene and are transformed in a process that probably involves additional genetic changes.

The third stage of tumor development is represented by more progressive solid tumors consisting of actively proliferating cells that no longer express T antigen. From the latter observation, we conclude that additional events must have occurred in advanced tumors that rendered cell growth increasingly independent of T antigen. Since one of the mechanisms of action of SV40 T antigen on stimulating cell proliferation appears to result from its interaction with the product of the p53 tumor suppressor gene (19, 20, 25), initial studies on p53 gene expression during tumor development were performed. Coinciding with T antigen expression, high amounts of p53 protein were detected in small tumor nodules, whereas in the solid tumors, nuclear p53 reactivity decreased with concomitant decrease in T antigen levels. This suggests that tumor progression at early stages may be partly promoted through the failure of normal p53 function in the control of cell proliferation (26, 27). However, p53 seems not to account for the aggressive proliferation of the advanced solid tumors. Further work will be required to define the additional genetic events that render growth of tumor cells independent of continuous T-antigen expression, but we have no indication that the tumor cells have lost the transgene.

In the established Clara cell line derived from the lungs of UT7.1 mice, expression of uteroglobin was maintained for over 1 year in culture. The fact that these cells no longer express T antigen was puzzling, but a similar situation was found in a differentiated, originally T-antigen-positive hepatocyte line established from the transgenic mouse strain SV-202 (23), which after several passages no longer expressed T antigen but preserved its proliferative potential and its ability to express liver-specific genes. It is possible that the proliferative potential in the Clara cell line is maintained by the expression of activated p53, which was detected to localize both in the nucleus and in the cytoplasm.

Previous work has suggested that type II pneumocytes are precursors of Clara cells. Therefore, we analyzed a possible relationship between the expression of T antigen and that of specific differentiation markers of bronchiolar cells during tumor development. Both uteroglobin and SP-A were found to be expressed in Clara cells as well as in the dysplastic cells during the initial stages of tumor development but not in tumors of later stages (data not shown). Therefore, we conclude that cells in late-stage lung tumors have undergone partial dedifferentiation. They have lost the ability to express bronchiolar epithelial markers, such as uteroglobin, and have not acquired the capacity to express the alveolar epithelial marker SP-A.

Heterogeneous pulmonary epithelial tumors have been observed in transgenic mice expressing activated forms of ras oncogenes under the control of either the albumin promoter (28), the mouse mammary tumor virus promoter (29), or the immunoglobulin/SV40 early-region promoter sequences (30), as well as in mice expressing T antigen under the control of the SP-C promoter (31). A similar approach as illustrated here was reported by De Mayo et al. (32), but the only transgenic line established was mosaic for the transgene, expressed very low levels of T antigen in the lung, and developed lung tumors with such low incidence that a spontaneous origin could not be excluded. Therefore, this is the first report of a useful transgenic mouse line as a model for the development of lung adenocarcinoma. The transgenic mouse line UT7.1 will be useful for further studies of the molecular events accompanying the genesis and progression of lung adenocarcinoma. In addition, the established Clara cell line maintaining uteroglobin and SP-A expression in culture will be useful to investigate the cooperation of various oncogenes and growth factors in regulating the growth and differentiation of lung cells.

Materials and Methods

Construction of UG/Tag Fusion Gene and Generation of Transgenic Mice. A blunt-ended EcoRI/BamHI SV40 T antigen fragment (nucleotides 5171 to 2541 of the T antigen encoding sequence) derived from the plasmid plysTD550 (33) was cloned into the blunt-ended SalI restriction site of the vector pSPT18/UG Kpn-B3 3' to the rabbit uteroglobin 5'-flanking region reaching from −4.7 kb to position +11 bp relative to the transcription start site (34). A NheI/Apal restriction fragment containing the fusion gene was purified by low-melt agarose gel electrophoresis and was microinjected into fertilized oocytes (33) from hybrid CD2 mice (F1 generation obtained by cross-breeding of inbred strains DBA x BALBc).

Analysis of Genomic DNA. Transgene insertion site and copy number were determined by Southern blot analysis of DNA extracted from mouse tails (35). Restricted DNA was electrophoresed through 0.8% agarose, transferred to nylon membranes, and hybridized with the random-labeled

\[ \text{D. Bauer, D. Paul, and M. Strauss, manuscript in preparation.} \]
transgene, formerly used for injection. Hybridization was performed essentially as described (12).

Analysis of RNA. Total RNA from lungs, quick-frozen in liquid nitrogen, was isolated and purified as described (36). The RNA was separated electrophoretically on 1.3% formaldehyde-agarose gels, transferred to nylon membranes, and hybridized successively with a rat uteroglobin cDNA (11), a 0.53-kb HindIII-SV40 T antigen fragment, and a mouse β-actin cDNA. The probes were labeled with a multiprime labeling kit (Amersham) to a specific activity of 6 × 10^6 cpm/µg.

Immunohistological Analysis. Tissues from transgenic mice were fixed in Bouin’s solution, embedded in paraffin, and processed for immunostaining according to standard protocols. Sections were incubated with a rabbit antiserum against rat uteroglobin, with a mouse monoclonal antibody to SV40 large T antigen (Pab 101), or with a mouse monoclonal antibody to P53 (Pab 240) for 1 h at room temperature. Prior to the incubation with anti-P53, tissues were treated with 0.5% pepsin in 0.01 N HCl for 10 min at 37°C. Blocking of endogenous peroxidase activity was generally achieved by a 10-min incubation in 3% H₂O₂. Following incubation with peroxidase-conjugated secondary antibodies (Dakoppats), the sections were processed for photomicroscopy. For counterstaining, the periodic acid Schiff’s reaction was used. Slides were incubated in 0.5% sodium periodate for 10 min, washed in water, and subsequently incubated in Schiff’s solution (Merck) for 20 min and 3 times in 0.45% sodium bisulfite in 0.05 N HCl for 5 min. They were washed again and finally counterstained with hemealun.

In Situ Hybridization. In situ hybridization with [α-³⁵S]UTP-labeled (specific activity of 1 × 10⁶ dpm/µg) antisense and sense cRNA transcribed from a HindIII-EcoRI rat cDNA cloned into pGEM 3 and 4 transcription vectors, respectively, was performed essentially as described (12). For hybridization with T-antigen antisense and sense oligonucleotides complementary to bases 3569-3589 (3’-CCTTACAACATGTGGTACGT-5’) and bases 3432-3452 (5’-TCCTAGCTCAAAGTCAAGCC-3’) respectively, oligonucleotides were labeled with [α-³²P]dATP by terminal deoxynucleotidyl transferase to specific activities of 1 × 10⁹ dpm/µg according to the supplier (Boehringer). Sections were hybridized overnight at 32°C in 20% formamide, 2× SSC, 1× Denhard’s solution, 10 mM Tris-HCl (pH 7.5), 0.05 M EDTA, 10% dextran sulfate, 10 mM DTT, and 0.5 mg/ml tRNA with a final activity of 3 × 10⁹ dpm/ml. The slides were washed four times with 2× SSC for 2 h. Autoradiography was performed with NTB-2 liquid emulsion (Kodak), and slides were developed with Kodak D19 developer.

Cell Cultures. Lung tissues were excised from 3-month-old transgenic mice, and the primary cells derived thereof were cultured as described (23, 24). Immunofluorescence assays were performed as described (37). For detection of uteroglobin and SV40 T antigen, primary antibodies were used as mentioned above. Staining of SP-A was performed with a polyclonal rabbit anti-rat SP-A antibody.⁵

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References


⁵ B. Müller, personal communication.


