A Novel SV40 TAg Transgenic Model of Asbestos-Induced Mesothelioma: Malignant Transformation Is Dose Dependent

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Abstract

Although it has been clear for >40 years that mesothelioma can be caused by asbestos, not all patients with this disease have a history of asbestos exposure. Other factors, including non-asbestos fibers and ionizing radiation, are known to cause malignant transformation of mesothelial cells. In addition, it is likely that genetics will play some role in susceptibility. Recently, it has been suggested that SV40 viral oncogenes could contribute to the carcinogenicity of asbestos. To better understand the role of SV40, we used the mesothelin promoter to construct MexTAg mice that express SV40 large T antigen (TAg) in the mesothelial compartment. We generated four MexTAg lines that carry high, intermediate, and low copy numbers of the transgene. All of these mice show a relatively low level of spontaneous tumor development. High-copy, 299h mice rapidly developed mesotheliomas when exposed to asbestos, and these tumors were faster growing and more invasive than those developing in wild-type and single-copy (266s) mice. In addition, we found a direct relationship between transgene copy number and survival after exposure to asbestos. A single copy of TAg was sufficient to immortalize mesothelial cells in vitro, but these cells did not show evidence of malignant transformation. In contrast, cell lines developed from mesothelial cells of animals carrying multiple copies of TAg were growth factor independent and could be cloned at limiting dilution in soft agar. These data provide the first in vivo demonstration of co-carcinogenicity between SV40 and asbestos. (Cancer Res 2006; 66(22): 10786-94)

Introduction

Mesothelioma is an aggressive fatal tumor originating from the mesothelial cells that line the pleural, peritoneal, and pericardial cavities. Epidemiologic studies first established that exposure to airborne asbestos fibers is a major cause of the disease (1). However, whereas around 5% of people exposed to asbestos develop mesothelioma (2, 3), not all mesotheliomas are associated with asbestos exposure, and the identification of other causes of this aggressive cancer is central to understanding the pathology (4). Other recognized causes of mesothelioma include erionite fibers and genetics (5) and, very rarely, ionizing radiation (6). More recently, it has been suggested that SV40 could play a role in the induction of mesothelioma. SV40 DNA sequences have been found in some human cancers, including mesotheliomas (7), ependymomas, osteosarcomas, and, occasionally, lymphomas (8–10). SV40 oncoproteins and asbestos have been shown to be co-carcinogenic in vitro, with phosphatidylinositol 3-kinase/AKT signaling playing an important role in the transformative process (11).

SV40 can induce mesotheliomas in rodents (12), and in vitro studies have shown that human mesothelial cells are particularly susceptible to transformation by SV40 (13). In 1994, Carbone et al. found SV40 sequences in 60% of human mesotheliomas (7); however, differing opinions prompted the International Mesothelioma Interest Group to investigate the potential involvement of SV40 in the development of mesothelioma in a blinded multi-institutional study. This study found that up to 83% human mesotheliomas contained SV40 sequences (14, 15). However, controversy still remains, and the possibility that laboratory contamination accounts for the positivity of some mesothelioma samples was suggested in a study by Lopez-Rios et al. A reanalysis of their samples under more stringent conditions found SV40 in 6% of mesothelioma cases rather than the previous finding of 60% (16).

There is, however, an increasing body of evidence that the virus plays some role, and a recent epidemiologic study found a positive correlation between the number of cases of asbestos-induced mesothelioma and the presence of SV40 (17).

It is envisaged that large-scale exposure of the human population to SV40 could have occurred because the polio vaccines that were produced in monkey kidney cell cultures, between 1955 and 1961, were frequently contaminated with live SV40 (18). These vaccines were given to hundreds of millions of people throughout the United States, Europe, Canada, Asia, and Africa. Since 1960, guidelines have been in place to ensure that polio vaccines are free from infectious SV40 particles. However, inactivation techniques may have been inadequate, as SV40 was recently detected in polio vaccines made after this date (19). It is also possible that SV40 is transmitted between humans either horizontally or vertically because SV40 sequences have been detected in humans either too old or too young to have received the contaminated polio vaccines (20). Thus, the effects of SV40 may be more prevalent than first anticipated.

Transformation of cells by SV40 is mediated by two proteins: small t antigen (tAg) and large T antigen (TAg; ref. 21). TAg binds and inactivates a set of tumor suppressor genes, including Rb, p107, p130, and p53 (22). These proteins are the key regulators of cell cycle checkpoints, and loss of their normal function causes cells to undergo uncontrolled proliferation and DNA replication. SV40 TAg binds p53, Rb, and p107 in mesothelioma samples (23, 24). In addition to tumor suppressor proteins, TAg binds several other cellular factors, including the transcriptional coactivators p300 and CBP, which may contribute to its transforming properties (22). TAg functions independently of tAg. Although the precise role of tAg is
not well understood, it seems to enhance transformation by TAg (25). This may occur through its ability to inhibit the protein phosphatase complex (PP2A), resulting in the activation of growth promoting signal transduction pathways (11, 26, 27). Small TAg also enhances cell cycle progression by the activation of other kinases, including the mitogen-activated protein kinase family, causing transcriptional activation of growth promoting genes (28–30). It has also been shown to affect the activity of telomerase in mesothelial cells (31).

To investigate the relationship between asbestos and SV40 in vivo, we constructed transgenic mice in which the expression of SV40 TAg was targeted to mesothelial cells. We chose to investigate TAg independently of TAg because transgenic models incorporating the complete early region of SV40 develop tumors at a high rate, independent of additional carcinogens (32, 33); thus, the co-carcinogenic effect of TAg and asbestos could not have been tested. In a prostate cancer model, development of the cancer was more analogous to the human disease in transgenic mice that contained TAg alone (34).

Injection of asbestos fibers into the peritoneum of mice induces mesotheliomas (35, 36). Continued instillation of asbestos has been reported to lead to the induction of disease in 20% to 30% animals over the following 30 to 50 weeks (35). In this study, we show that a single copy of SV40 TAg does not alter the rate of asbestos-induced tumor formation. In contrast, multiple copies of TAg increase the incidence of disease and accelerate tumor development. This model will enable a study of the early molecular changes that occur during mesothelioma development. It is also a relevant and tractable model that can be used for testing potential therapies.

Materials and Methods

Establishment of MexTAg transgenic mice. A 2,148-bp fragment containing the TAg open reading frame (ORF; SV40 strain 776) was generated with BglII and XbaI restriction endonuclease sites by PCR. The fragment was cloned downstream of L380 bp of the mesothelin promoter in pGL3MP1850 (37) to generate pMextTag. A 4.3-kb ClaI fragment, encompassing the promoter and ORF, was inserted into C57Bl/6J embryonic stem cells (Ozgene, Perth, Australia). Founders were identified by Southern blot and bred and maintained as heterozygotes. A 2,148-bp fragment encompassing the promoter and ORF, was inserted into C57/Bl 6J embryonic stem cells (Ozgene, Perth, Australia). SV40 TAg PCR with primers forward (GGATTTAAAACTGGAACGGTGAAGG) and reverse primer SVTrev281 (CACTGCTCCCATTCATCAG). Cycles were 95°C for 5 minutes and 35 cycles 60°C, 45°C, and 72°C for 30 seconds at each temperature followed by a final extension of 5 minutes at 72°C. β-Actin PCR used the same conditions with the primers forward (GGATTCTAAGATGGCTGTTAG) and reverse (TTGGTCTACCACTGAGTCA). All RNA extractions, RT and PCR reactions were set up in a designated clean area to prevent contamination with environmental SV40 (see notes in Supplementary Data). Amplifications were repeated at least thrice, including a blinded experiment. RNA was prepared separately from three 299h, three wild-type mice, and two contaminated with environmental SV40 (seenotesin Supplementary Data). PCR reactions were set up in a designated clean area to prevent contamination with environmental SV40 (seenotesin Supplementary Data). Tissue (100 mg) was digested overnight with proteinase K at 55°C. Debris was removed by centrifugation, and genomic DNA was purified and scored. A minimum of 20 fields was counted; plates were set up in triplicate, and colonies containing 0.28 mm in diameter were scored. A minimum of 20 fields was counted; plates were set up in triplicate, and experiments repeated at least thrice, with two representative cell lines per mouse line. Data were compared with AE17 cells as a positive control. AE17 is a malignant mouse mesothelioma cell line generated by injecting asbestos into the peritoneum of a C57/Bl mouse (36).

Cell culture. Mesothelial cells were removed from sections of peritoneum by incubating in trypsin (0.25% in EDTA buffer; Life Technologies, Grand Island, NY) for 30 minutes at 37°C. The peritoneum was then discarded, and cells were pelleted and cultured in RPMI 1640 containing 10% FCS, supplemented with 100 units/ml penicillin, 100 µg/ml streptomycin, and 2 mmol/L l-glutamine (Life Technologies). These lines were denoted TGM for transgenic mesothelial; although they exhibit some transformed properties, they are not cancer cell lines and do not grow when transplanted into syngeneic mice. For low-serum experiments, 1 × 104 cells per well of six-well plates were plated in triplicate, in the RPMI with 0.5% FCS. Experiments were repeated at least thrice, with two to three representative TGM cell lines independently derived from each transgenic mouse line.

Asbestos-induced mesothelioma. Asbestos fibers (IUCF reference sample of Wittenoom Gorge crocidolite) were suspended in PBS (15 mg/ml) and then autoclaved and passed through a 23-gauge needle several times. Groups of 8 to 15 mice (C57Bl/6J wild type and MexTAg transgenic) were injected with 3 mg asbestos, either as a single dose or two doses, 1 month apart. All experiments were repeated. Mice were monitored for up to 24 months, and the animals were euthanized when ascites became evident or sickness, distress, or loss of condition was noticed. All mice had disease before the end of experiment, 24 months after asbestos injection. Mesothelioma cell lines were established from ascites fluid and cultured as above. These lines might be expected to originate from malignantly transformed cells and therefore do not take the TGM prefix.

Tumorigenicity assay. To determine if these ascites-derived cell lines were indeed malignantly transformed, they were injected s.c. into syngeneic mice. Aliquots of cultured cells (5 × 106 in 0.1 ml) were injected s.c. into transgenic or wild-type mice (three animals per group). Development of tumor was monitored for up to 90 days.

Western blotting. Primary mesothelial cells were removed from mouse peritoneum as described above. Tissue culture cells were retrieved from flasks using a cell scraper. All cell pellets were lysed in Laemmli buffer and fractionated on 10% SDS-PAGE. Protein was transferred to polyvinylidene difluoride membrane and immunoblotted under standard conditions. SV40 proteins were detected using the mouse monoclonal antibodies pAb 101 and pAb 419 (Santa Cruz Biotechnology, Santa Cruz, CA) at 1:1,000 dilution and an anti-mouse horseradish peroxidase (HRP) conjugate (Sigma, St. Louis, MO) and visualized using chemiluminescence.
Histology and immunohistochemistry. Tissue and tumor samples were fixed in 4% paraformaldehyde for 2 days and preserved in 70% ethanol until preparation into paraffin blocks by standard procedures. Five-micrometer sections were cut from paraffin blocks, dewaxed in xylene, and rehydrated in a graded series of alcohols. The SV40 pAb 101 antibody was applied for 1 hour at 1:100 dilution at room temperature and detected using an anti-mouse HRP conjugate using the SuperPicTure Polymer detection kit (Zymed Laboratories, Inc., San Francisco, CA) according to manufacturer’s instructions.

Statistical analyses. Quantitative expression data were analyzed using \( r^2 \) correlations. The significance of differences in survival was assessed by the log-rank test. Other data were compared using the \( \chi^2 \) test for independence in contingency tables and Fisher’s exact test.

Results

Construction of MexTAg transgenic mice. To investigate the role of SV40 TAg in the development of mesothelioma, TAg expression was directed to mesothelial cells by cloning its ORF downstream of the mesothelin promoter (Fig. 1A). The tissue-specific activity of this promoter region has been described previously (37). Four transgenic founder mouse lines were generated, a high-copy line (designated 299h), a single-copy line (266s), and two intermediate lines (304i and 270i). Quantitative PCR from genomic DNA revealed that the 299h mice have \( \sim 100 \) copies of the MexTAg transgene, whereas the 266s line has a single copy. The intermediate lines 304i and 270i have 32 and 15 copies, respectively (Fig. 1B). Southern blotting of genomic DNA showed a single EcoRI restriction fragment from line 266s DNA corresponding to a single copy of DNA (Fig. 1B). Multiple restriction fragments from line 299h DNA correspond to the sizes of the predicted concatamers of the transgene. Quantitative analysis of the Southern blot was highly correlated with the copy numbers derived from PCR (81, 21, 2, and 1 in lines 299h, 304i, 270i, and 266s, respectively; \( r^2 = 0.99 \)).

The cumulative lifetime incidence of tumors in untreated line 299h mice was 5%, commonly on the ear or abdomen; however, no experimental 266s or wild-type mice developed spontaneous tumors. The 299h tumors were all classified as sarcomas.

Expression of TAg in mesothelial cells is related to transgene copy number. Mesothelial cells were digested from the peritoneum of each of the four lines of MexTAg transgenic mice and were cultured in vitro. Electron microscopy showed these transgenic mesothelial (TGM) lines had the typical features of mesothelial cells, including long thin microvilli, microfilaments, and...
and gap junctions (data not shown). Steady-state SV40 TAg mRNA levels from these cell lines correlated with the transgene copy number from the four transgenic mouse lines (r² = 0.78; compare Fig. 1B with C). The 90-kDa SV40 large TAg protein was identified with SV40 TAg antibodies in lysates from TGM cell lines derived from 299h, 304i, and 270i mice (Fig. 1D). The protein was not detected in TGM266s cell lysates probably because it was expressed at levels below the limit of detection. Qualitatively the Western blot shows good concordance between the transgene copy number and the level of TAg protein expressed by the different cell lines. TAg was detected by Western blot from as few as 1 x 10⁴ TGM299h mesothelial cells grown in culture. In contrast, TAg protein was not detected in 1 x 10⁴ freshly isolated primary mesothelial cells from 299h mice. Thus, TAg protein is expressed by mesothelial cells in situ at levels <1% of the level expressed by cells proliferating in vitro.

SV40 TAg is expressed in the mesothelial cells of MexTAg mice. Mesothelin expression is limited to mesothelial cells that line the pleural, pericardial, and peritoneal cavities and the tissues within these cavities (39). To assess whether the mesothelin promoter similarly directs SV40 TAg expression to mesothelial cells, tissue samples were tested for SV40 TAg mRNA expression by PCR. Line 299h and 304i mice express SV40 TAg in all tissues, including muscle, brain, and skin, that do not have a mesothelial cell component (Fig. 2A). In contrast, line 266s and 270i mice expressed TAg only in tissues containing mesothelial cells (Fig. 2A). Despite the expression of mRNA, we were unable to detect TAg protein by immunohistochemistry in any of the transgenic mouse lines in any normal tissues using pAb 101 or pAb 419 TAg antibodies. Using in situ hybridization, SV40 TAg mRNA expression was found specifically localized to the mesothelial cells (Fig. 2B).

Mesothelial cells from MexTAg mice generate immortalized cell lines in vitro. Normal mesothelial cells expand logarithmically in culture for 8 to 10 population doublings. These cells then senesce and survive for many weeks without further division. In contrast, six independent TGM299h lines continued to divide for >100 population doublings (Fig. 3A). Single-copy TGM266s mesothelial cell lines expanded initially at a slower rate (Fig. 3B); nevertheless, three independent TGM266s lines were cultured in excess of 100 population doublings. Two independent TGM304i and TGM270i lines were also cultured for >100 population doublings, with growth rates similar to TGM299h lines. The growth rates of TGM266s cell lines were similar to TGM 299h lines after 60 population doublings (1.3 and 1.1 doublings per day, respectively).

MesTAg mesothelial cells exhibit the properties of transformed cell lines. Transformed cells have the capacity to divide in low-serum conditions and form anchorage-independent colonies in semisolid medium. TGM299h, TGM304i, and TGM270i cells grew in low serum conditions, whereas single-copy TGM266s and normal mesothelial cells did not (Fig. 3C). The TGM299h mesothelial cells formed colonies in soft agarose at a frequency 14 times higher than a fully transformed malignant mesothelioma cell line AE17 (Fig. 3D). Intermediate TAg copy TGM304i and TGM270i cell lines formed ~3-fold fewer large colonies (>0.28 mm diameter), but 2- to 3-fold more small colonies (<0.28 mm diameter) than TGM299h cells. However, single-copy TGM266s cells, like wild-type
mesothelial cells, were unable to grow in semisolid medium, suggesting that a differential threshold level of SV40 TAg expression is required for transformation and immortalization. None of the TGM lines grew when transplanted s.c. into syngeneic mice.

**SV40 acts synergistically with asbestos to induce mesotheliomas in vivo.** Asbestos fibers were injected i.p. into MexTAg transgenic and wild-type C57/Bl 6J mice. The high-copy line 299h mice developed ascites more rapidly than wild-type and single-copy 266s mice (median survival was 35, 63, and 55 weeks, respectively; Fig. 4A; Table 1). The survival of 299h mice after exposure to asbestos was significantly shorter than that for 266s and C57/Bl mice (log-rank test, χ² = 23.9, P < 0.0001). At 39 weeks after asbestos injection, all 299h mice had developed ascites, whereas 87% of wild-type and all 266s mice remained healthy. The period in which disease developed was significantly shorter for 299h mice compared with both 266s and wild-type mice (P < 0.0001; Table 1). When two doses of asbestos were given sequentially 1 month apart, the first case of mesothelioma in 299h mice occurred at 18 weeks, 8 weeks before a single dose (Table 1). The median survival after two doses was significantly shorter (24 weeks; χ² = 6.4, P = 0.011; Fig. 4B). Thus, increasing the dose of asbestos accelerates development of mesothelioma in these mice. For 304i mice, the median survival was intermediate (36 weeks) and significantly different from the other two transgenic lines (log-rank test, P = 0.005 between 299h and 304i and P = 0.01 between 266s and 304i). We found a direct relationship between copy number and survival after exposure to asbestos (r² = 0.89 when the four lines are compared). As found for a single exposure

![Figure 3](cancerres.aacrjournals.org)
to asbestos, median survival for 266s mice and wild-type mice was not significantly different after two doses of asbestos (55 and 56 weeks; \( P = 0.33 \)). Within the 24-month experimental time period, all asbestos treated mice developed disease as judged by abdominal distension. There were no deaths due to other cancers or other diseases, and untreated mice did not develop mesothelioma in this time period (10 mice per group). We wanted to differentiate between malignant mesotheliomas and benign effusions, both of which manifest as ascites. We have, therefore, used a combination of methodologies, including histology and a malignancy assay, to determine the number of cases of malignant mesothelioma.

Asbestos uniformly induces malignant mesothelioma in 299h MexTAg mice. We cultured samples from every ascites in vitro. Mesothelioma cells from the ascites of 24 of 24 (100%) line 299h mice exposed to asbestos were established as cell lines, whereas only 11 of 15 (73%) of 266s ascites and 15 of 27 (55%) of wild-type ascites could generate cell lines (Table 1). Ascites fluid from 299h mice was significantly more likely to generate an established cell line in vitro than fluid from either 266s or wild-type mice (\( \chi^2 = 13.7, P = 0.001 \)). Ascites lines were established and cultured for >100 population doublings from 299h (13 lines), 266s (7 lines), and wild-type mice (7 lines). In addition, transmission electron microscopy of 17 early-passage (3-6) transgenic or wild-type lines showed that the cells had multiple or irregular nuclei and abundant microvilli, microfilaments, and tight junctions, features consistent with mesothelial cell differentiation (data not shown). 299h mice had significantly more macroscopically visible nodules or masses at autopsy than both 266s and wild-type mice (\( \chi^2 = 6.9, P = 0.0318 \); Table 1).

Immuno histochemical markers for the identification of mouse malignant mesothelioma are not available; thus, malignancy was determined both by the examination of H&E sections of mouse tissues and s.c. injection of ascites fluid into syngeneic mice. The tissue samples taken for histology were diaphragm, liver, kidney, spleen, pancreas, and testis or ovary and, additionally, any nodules or masses seen at autopsy. Tissues were collected from 8 to 13 mouse cases for each line (Table 1). The cases that were selected for histologic examination were not biased for the presence of macroscopically visible tumor (Table 1). Of the thirteen 299h mice examined by histology, 7 (54%) had visible tumors; whereas, for the 266s mice, 2 of 8 (25%) and for wild-type mice, 4 of 11 (36%) had

Table 1. Comparison of MexTAg and wild-type mouse mesotheliomas

<table>
<thead>
<tr>
<th></th>
<th>MexTAg 299h</th>
<th>MexTAg 266s</th>
<th>C57/Bl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time to develop mesothelioma (median, wk)</td>
<td>35 (24)*</td>
<td>55 (55)*</td>
<td>63 (56)*</td>
</tr>
<tr>
<td>Time to develop mesothelioma (range, wk)</td>
<td>26-39 (18-38)*</td>
<td>41-97 (27-85)*</td>
<td>30-98 (32-78)*</td>
</tr>
<tr>
<td>Samples of ascites fluid that were established as cell lines</td>
<td>24/24 (100%)</td>
<td>11/15 (73%)</td>
<td>15/27 (55%)</td>
</tr>
<tr>
<td>Cell lines confirmed as mesothelioma by electron microscopy</td>
<td>11/11 (100%)</td>
<td>Not done</td>
<td>6/6 (100%)</td>
</tr>
<tr>
<td>Macroscopically visible nodules or masses*</td>
<td>13/24 (54%)</td>
<td>4/20 (20%)</td>
<td>7/26 (26%)</td>
</tr>
<tr>
<td>Histology</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Malignant</td>
<td>9/13 (69%)</td>
<td>1/8 (12.5%)</td>
<td>2/11 (18%)</td>
</tr>
<tr>
<td>Indeterminate</td>
<td>1/13 (8%)</td>
<td>1/8 (12.5%)</td>
<td>2/11 (18%)</td>
</tr>
<tr>
<td>Benign</td>
<td>3/13 (23%)</td>
<td>5/8 (62.5%)</td>
<td>5/11 (45%)</td>
</tr>
<tr>
<td>Normal</td>
<td>0/13</td>
<td>1/8 (12.5%)</td>
<td>2/11 (18%)</td>
</tr>
<tr>
<td>Tumorigenicity of established cell lines*</td>
<td>9/9 (100%)</td>
<td>5/6 (83%)</td>
<td>2/8 (25%)</td>
</tr>
</tbody>
</table>

*After two doses.
† Total from three experiments.
‡ The data refer to individual cases from each mouse line. A case was judged to be malignant if any one of the six tissues (diaphragm, liver, kidney, spleen, pancreas and testis or ovary) or the tumor itself, when present, was found to contain malignant cells.
§ Complete data are recorded in Supplementary Table 2.
visible tumors. These numbers are small, but to detect any bias, we used a generalized linear model with binomial error and logit link.

This confirmed that there was no bias in the selection of specimens for histology based on visible tumor at autopsy ($z$ test for indicator coefficient of $0.67, P = 0.50$).

Malignant mesotheliomas were identified by the presence of large, pleomorphic cells, often spindled, with abnormal mitoses (Fig. 5A) and were found to be sarcomatoid in all cases except one 299h tumor that showed both sarcomatous and epithelioid cells. Tumor tissue formed as a distinct nodular growth, extending out from the mesothelial layer on the surface of the tissue (Fig. 5F and G), or it infiltrated tissue (Fig. 5D). 299h mice had a significantly higher incidence of malignancy, determined by histology, than wild-type or 266s mice (9 of 13, 2 of 11, and 1 of 8, respectively; Table 1). The presence of macroscopically visible tissue nodules and masses correlated exactly with histologic findings of malignancy in the three mouse lines using both histology and tumorigenicity assay data are as follows: 13 of 13 cases (100%) of line 299h, 3 of 11 (27%) wild-type C57/Bl, and 6 of 8 (75%) line 266s (Supplementary Table S2). The incidence of malignancy was significantly different among the three lines ($\chi^2 = 14.86, P = 0.0006$). The number of cases of malignant disease in 299h mice was significantly higher than in wild-type mice (Fisher’s exact test, $P = 0.0002$). The incidence of malignancy in 266s mice was not significantly different compared with 299h mice ($P = 0.133$) or wild-type mice ($P = 0.070$).

These data also suggest that a negative diagnosis based on histology of tissue biopsies needs to be evaluated cautiously.

**Transgenic mouse mesotheliomas express SV40 TAg.** Sections of tissues from 299h asbestos exposed mice all showed strong expression of SV40 TAg in the nucleus using the pAb101 antibody (Fig. 5D-G). Reactive mesothelium and spindle cell/fibroblastic reactive tissue found at the surface of tissues was also TAg positive.

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**Figure 5.** Histology and immunohistochemistry of lesions in MexTAg mice following exposure to asbestos. H&E sections: (A) frank malignant mesothelioma, (B) benign reactive mesothelium (arrow) and reactive spindle cell/fibroblastic proliferation (circle) adjacent to normal spleen tissue (square), (C) indeterminate tissue on the surface of the pancreas. The triptych in (D) shows malignant tumor invading the muscle tissue of the abdominal wall: left, H&E; middle, no primary antibody; right, stained for TAg using pAb101. E, TAg expressed in a benign reactive section of mesothelium and a reactive spindle cell/fibroblastic proliferation in the spleen. F, tumor infiltrating the pancreas. G, tumor in a wild-type mouse at the surface of the liver.
SV40 TAg Transgenic Mice

Discussion

Murine models have provided an important tool to understand and study cancer (40, 41). Because of the long latency and unpredictable development of disease following inoculation with asbestos, many investigators have used models of mesothelioma in which cultured malignant cell lines are injected s.c. into syngeneic mice (36, 42). Although this cell transplantation model generates tumors that are clearly mesotheliomas, the disease is anatomically dislocated. Another clear difference between this type of model and the actual disease is that mesotheliomas are typically heterogeneous, whereas s.c. tumors are less so, developing from a clone of differentiated cells that were highly selected in vitro. We wanted to create a more authentic model without these negative features. In addition, we wanted to test how the key SV40 oncogene TAg might affect the development of malignant mesothelioma.

The majority of cases of human malignant pleural mesotheliomas fall into three distinct histologic subtypes: epithelial, sarcomatoid, and mixed. The most common diagnosis is epithelial (50%), and patients with this subtype are generally thought to experience a more indolent disease with longer survival than the other two subtypes (43, 44). We found that ~90% of SV40 TAg mouse tumors were sarcomatoid type, a much higher percentage than that found in humans. However, this accords with the finding that the presence of SV40 correlates with sarcomatoid mesotheliomas in humans (45). The significance of this is tempered by the observation that all of the two mesotheliomas we examined from asbestos exposed wild-type mice in this study were also of sarcomatoid type.

All of the high-copy animals develop mesothelioma after asbestos injection within a relatively short time period; thus, we present a model that will support the analysis of therapies in vivo. We found that tumor development was accelerated when two doses of asbestos were given. This not only makes the model more tractable, but it is also highly analogous to mesothelioma in humans where epidemiologic evidence shows that an increased exposure to asbestos is highly correlated with an increased risk of disease.

The onset of disease after asbestos treatment was the same in 266s and wild-type mice. However, the incidence of malignant mesothelioma in 266s mice was 75% compared with 27% in wild-type mice (100% in 299h mice). These numbers are small and approach significance \( P = 0.070, 266s \) versus wild type, and it is interesting to note that the 266s mice have an intermediate phenotype. The incidence of malignant mesothelioma in wild-type mice in these experiments is in agreement with previously published data (36, 42). Overall, we found a direct relationship between transgene copy number and the development of malignant disease after exposure to asbestos.

All four lines of transgenic mice expressed the linked TAg in the mesothelial compartment. Although it is clear that some inappropriate expression is found, particularly in the high-copy animals, taken collectively, the different mice show that the immediate 2-kb upstream fragment of the mesothelin promoter directs expression of a linked gene to mesothelial cells. We hypothesize that inappropriate expression is the result of the action of local transcriptional control mechanisms at the different sites of DNA integration. These anomalies have relatively mild effects on transcription in mesothelial cells because we saw a good correlation between copy number and expression.

The creation of transgenic mouse lines with a range of copies of TAg should provide us with the opportunity to investigate the clinical relevance of low levels of SV40 TAg, which in some mesotheliomas are estimated to be less than one copy of SV40 per cell (46–48). In our preliminary analysis, we show that a single copy of TAg does not affect the rate of asbestos induced tumor formation, although it has a clear biological effect on cells grown in vitro. A single copy was nevertheless sufficient to enable the immortalization of mesothelial cells so that these cell lines were able to grow indefinitely in vitro. Cells with multiple copies had additional properties associated with transformed cells; higher copy TGM cell lines were able to grow in low-serum conditions and in an anchorage-independent fashion. Although the highest copy number line 299h produced the highest frequency of large colonies in semisolid medium, the total number of colonies was similar to the total number formed by the two intermediate copy lines 304i and 270i. Taken together, these observations suggest that a different threshold concentration of TAg is required for immortalization and transformation.

SV40 TAg protein was not detected in normal mesothelial cells of any of the transgenic lines by immunohistochemistry. This is consistent with our finding of very few spontaneous tumors. No tumors occurred in 21 line 266s mice reaching an age of between 17 and 28 months, and in the high-copy line, the lifetime cumulative total was only 5.4%. TAg mRNA could be detected by PCR in normal transgenic mouse tissues that contain mesothelial cells, and using in situ hybridization, these transcripts were shown to be specifically located in the mesothelial cells. This suggests that quiescent mesothelial cells transcribe the gene without accumulating any protein. This might be for lack of synthesis because the message remains untranslated or it may represent a rapid turnover of the protein. TAg protein was detected in all TGM cells with the exception of the TGM266s, suggesting that in vitro culture conditions, perhaps associated with some component of the process of activation, cell cycle, and proliferation, leads to the expression of TAg protein in vivo. This is consistent with a two-step model for tumor induction with a single initiating stimulus. Thus, asbestos fibers cause irritation and damage, leading to repair processes, including mesothelial cell proliferation. Mesothelial cells that express TAg are more likely to become transformed, leading to malignancy. This hypothesis accounts for the low level of spontaneous tumors in the absence of asbestos in these transgenic animals. Furthermore, this model predicts that any damage that leads to mesothelial proliferation will be associated with a high level of transformation in these mice.

Interestingly, in TAg transgenic mice injected with asbestos, TAg was readily detected by immunohistochemistry in reactive mesothelial cells as well as in the tumor tissue. Clearly, the
distribution of the TAg gene is different in the transgenic animals (universal) to the infection situation (restricted), but because of the tissue specific promoter, there are many similarities to the pattern of expression as described in humans (7, 49). In human mesotheliomas, SV40 is detected in malignant cells, benign reactive mesothelium (as described here), but not in adjacent normal tissue (again as described for the model).

The profile of disease incidence described here could be useful for many other studies and may provide more insight into the potential role of SV40 in human disease. In particular, the finding that all 299 mice inoculated with asbestos develop mesothelioma in a predictable short time frame offers a unique opportunity to test novel therapies. These features make the model useful for early intervention studies and to investigate the most proximal biological changes associated with tumor development.

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