

Antisense to SV40 Early Gene Region Induces Growth Arrest and Apoptosis in T-Antigen-positive Human Pleural Mesothelioma Cells

Ishrat Waheed, Z. Sheng Guo, G. Aaron Chen, Todd S. Weiser, Dao M. Nguyen, and David S. Schrupp¹

Thoracic Oncology Section, Surgery Branch, National Cancer Institute, NIH, Bethesda, Maryland 20892

Abstract

Although SV40 oncoproteins have been detected in malignant pleural mesotheliomas (MPMs), their role in the pathogenesis and clinical behavior of these neoplasms remains controversial. In the present study, we sought to define the relevance of SV40 T/t antigen expression in established human mesothelioma cell lines deficient for p16^{INK4a} as well as ARF expression. SV40 early region sequences were readily detected in genomic DNA isolated from pleural mesothelioma lines; however, levels of SV40 T/t antigen expression were highly variable in these cells. An adenoviral vector expressing an antisense transcript to SV40 early region inhibited T antigen expression and mediated significant growth inhibition and apoptosis in T-antigen-positive mesothelioma cells and SV40-transformed COS-7 cells. Abrogation of T/t antigen expression coincided with enhanced p21/WAF-1 expression, suggesting that restoration of p53-mediated pathways may have contributed to the growth inhibition and apoptosis induced by the antisense construct. These effects were not observed after similar treatment of mesothelioma or lung cancer cells containing no SV40 DNA sequences. Collectively, these data suggest that SV40 oncoproteins contribute to the malignant phenotype of pleural mesotheliomas and indicate that interventions designed to abrogate their expression may be efficacious in the treatment of individuals with these neoplasms.

Introduction

MPM² are relatively rare, but highly lethal, neoplasms that have been associated with exposure to asbestos. These neoplasms are unique in that they rarely if ever exhibit Rb and p53 mutations, which frequently disrupt G₁ restriction point control in thoracic malignancies (1–5); these observations suggest that growth constraints mediated by these tumor suppressor genes are circumvented by mechanisms more subtle than those typically observed in solid tumors. Nearly 75% of mesothelioma cell lines exhibit allelic deletions involving 9p21–p22, which simultaneously inactivate p15 and p16^{INK4a} (6, 7). Although not as yet formally studied in mesotheliomas, allelic deletions involving p16^{INK4a} could conceivably affect the expression of p14/ARF, a tumor suppressor encoded via an alternative reading frame in the *p16* locus that modulates p53 expression and facilitates p53-mediated apoptosis in response to activated ras and viral oncoproteins (8, 9).

SV40 is a polyomavirus of rhesus macaque origin that is known to be tumorigenic in animals and has recently been associated with pleural mesotheliomas as well as several other relatively rare malignancies in humans (reviewed in Refs. 10 and 11). The SV40 early region encodes a *M_r* 90,000 large tumor (T) antigen that functions to sequester the Rb, p107, p130, and p53 tumor suppressor gene products

as well as p300 (12, 13). Rat pleural mesothelioma cells expressing SV40 T exhibit disrupted cell cycle progression, abnormal mitoses, and aneuploidy (14). A *M_r* 18,000–20,000 protein known as small tumor (t) antigen, encoded by alternative splicing in the SV40 early region, induces cyclin D₁ expression via extracellular signal-regulated kinase and mitogen-activated protein kinase pathways (15); both oncoproteins are essential for mesothelioma induction in rodents (16). SV40 early region DNA sequences have been detected in nearly 60% of primary human MPMs (10, 11, 17, 18), and SV40 T antigens isolated from these neoplasms bind Rb, p107, and p130 as well as p53 (19, 20), indicating that SV40 oncoproteins are expressed and functional in MPMs.

Faced with cytogenetic and molecular analyses consistently demonstrating a high frequency of mutational events targeting 9p21–22 that might simultaneously disrupt the Rb and p53 tumor suppressor pathways (8, 21), we sought to address the role (if any) of SV40 T/t antigen expression in pleural mesotheliomas. We reasoned that if SV40 oncoproteins were relevant for the transformed state in pleural mesothelioma cells, abrogation of their expression would inhibit proliferation and possibly induce apoptosis via restoration of p53 pathways. Herein we demonstrate that an adenoviral vector expressing an antisense to SV40 early gene region mediates profound growth inhibition and apoptosis in T-antigen-positive pleural mesothelioma cells, suggesting that SV40 oncoproteins contribute to their malignant phenotype.

Materials and Methods

Cell Lines and Plasmids. Human pleural mesothelioma cell lines H28, H2052, H2373, H290, and H2595 as well as the lung cancer lines H1299, H596, and H460 were established from primary tumor specimens at the National Cancer Institute (Bethesda, MD). REN mesothelioma cells were provided by S. Albelda (University of Pennsylvania, Philadelphia, PA). COS-7 cells were purchased from American Type Culture Collection (Rockville, MD). All cell lines were maintained in RPMI 1640 containing 10% fetal bovine serum and antibiotics. pRSV-Tag carrying the SV40 strain 776 early gene region was kindly provided by Dr. Curtis Harris (National Cancer Institute).

Genomic DNA, mRNA Extraction, PCR, and RT-PCR Analysis. Genomic DNA was isolated from the above cell lines according to the protocol provided by the Wizard genomic DNA purification kit (Promega Corp., Madison WI). Total RNA was likewise purified according to the instructions provided by the RNeasy Mini kit (Qiagen, Valencia, CA). Oligonucleotide primer pairs were custom prepared (Life Technologies, Inc., Gaithersburg, MD). SV40 primers included a 5' sense primer spanning the start site in the exon-1 of SV40 (strain 776) T antigen, AGT CCT CGA GTC TTT GCA GCT AAT GGA CCT, and a 3' antisense primer from exon-2, AGT CTC TAG ATC CTT TGT GGT GTA AAT AGC. Genomic DNA (1 μg) was amplified for 35 cycles at 94°C for 40 s, 58°C for 40 s, and 72°C for 40 s using *Taq* DNA polymerase (Perkin-Elmer, Branchburg, NJ). PCR products were resolved on 1% agarose gel, followed by ethidium bromide staining. For RT-PCR reaction, 1–2 μg of total mRNA was used according to the instructions provided by the manufacturer's manual (Promega). The above-described primer pair was used for PCR amplification of the cDNA products, using PCR conditions described

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¹ To whom requests for reprints should be addressed, at Head, Thoracic Oncology Section, Surgery Branch, National Cancer Institute, Building 10, Room 2B07, 10 Center Drive MSC 1502, Bethesda, MD 20892-1502. Phone: (301) 496-2127; Fax: (301) 402-1788.

² The abbreviations used are: MPM, malignant pleural mesothelioma; Rb, retinoblastoma; RT-PCR, reverse transcription-PCR; Ad, adenovirus; CMV, cytomegalovirus; pfu, plaque-forming unit(s); MOI, multiplicity of infection; wt, wild type.

above. Primers and conditions for PCR and RT-PCR analysis of p16 and ARF have been published previously (22).

Northern Blot Analysis. A PCR fragment corresponding to the first 550 bp of the SV40 early mRNA was amplified from pRSV-Tag using a 5' sense primer described above and a 3' antisense primer, CTG AGC AAA ACT GGT TTT CC. The resulting product was excised from the gel and eluted in distilled water. The DNA fragment was then radiolabeled with ³²P[dATP] by random priming and used as a probe. Total RNA isolated from mesothelioma cell lines was resolved on 1% agarose formamide gel. After denaturation in 0.05 N NaOH/0.15 M NaCl for 30 min, the gel was renatured in 0.5 M Tris (pH 7.5)/0.15 M NaCl for 1 h. RNA samples were then blotted overnight on to a nylon membrane, Duralon UV (Stratagene, La Jolla, CA), through capillary transfer in 10× SSC buffer. The RNA blot was hybridized to ³²P-labeled, denatured Tag sense or antisense riboprobe (2.5 × 10⁶ dpm/ml) in the presence of 2 μg/ml denatured salmon sperm DNA at 68°C for 1 h (Protocols in Mol. Biol., Section 10:16). After washing twice with 2× SSC/0.1% SDS at room temperature and once with 0.1× SSC/0.1% SDS at 60°C for 30 min, the blot was exposed to a X-ray film for 2–8 h before developing.

Immunoprecipitation and Western Blot Analysis. Cellular extracts were isolated and quantified by the BCA protein assay kit (Pierce, Rockford, IL). The immune-precipitation procedure was adopted from Protocols in Mol. Biol. (Section 4:9) after slight modification. Approximately 250–500 μg of the total protein were incubated with the primary monoclonal antibody Pab 108 (PharMingen, San Diego, CA) directed to the NH₂ terminus of the T/t antigens in IP buffer (25 mM Tris, 150 mM NaCl, 0.1% BSA, 0.02% NaN₃, and 0.1% Triton X-100) at 4°C for 1 h. Ten μl of protein-A agarose were then added to the mixture and further incubated at 4°C for 45 min. The antigen-antibody-protein A/agarose complexes were washed twice with the same buffer before pelleting by centrifugation at 5000 rpm for 10 min.

For Western blot analysis, cell lysates (50–100 μg) were resolved on 15% SDS-polyacrylamide gel. Proteins were then electroblotted onto an Immoblot polyvinylidene difluoride membrane. Membranes were incubated in 5% nonfat dry milk at room temperature for 45 min before incubating the specific antibodies to T antigen (Pab 107 and Pab 108; PharMingen), p21/WAF-1 (PharMingen), and β-actin for 2 h at room temperature. Blots were washed several times with PBS-Tween 20. Blots were then incubated with an appropriate secondary antibody linked to horseradish peroxidase for 45 min at room temperature. After several washings with PBS-T, blots were developed according to the instructions provided in the ECL kit (Amersham, Arlington Heights, IL)

Recombinant Adenoviruses. Replication-deficient, E1- and E3-deleted recombinant Ad serotype 5 (Ad5) was used as the viral backbone. To construct Ad.CMV/T-S (Ad/T-S) and Ad.CMV/T-AS (Ad/T-AS), a 550:bp DNA fragment of the SV40 strain 776 (nucleotides 5138–4588) was inserted into pAd.CMV vector (23) in the sense or antisense orientation, creating the shuttle vectors pAd.CMV/T-S and pAd.CMV/T-AS, respectively. The adenoviruses were made by cotransfection of the shuttle vectors with pBHG10 (24) into human 293 cells, and recombinant virus plaques were isolated. Ad.CMV-LacZ containing the *Escherichia coli* LacZ gene with a nuclear localization signal driven by the CMV enhancer/promoter was generously provided by F. Graham (MacMaster University, Ontario, Canada). All viruses were amplified in human 293 cells and purified as described previously (23). Viral titers were determined as pfu/ml using plaque assays in 293 cells.

Viral Infection and Proliferation Assay. Cells were infected with the recombinant adenoviruses at a MOI that resulted in 50% transduction efficiency as determined by the 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside staining of Ad.CMV/LacZ-infected cells (25). For proliferation assays, cells were exposed in triplicate to either PBS alone or an appropriate MOI of Ad/T-S or Ad/T-AS for 2 h, after which the virus was removed and medium was replaced with prewarmed RPMI 1640 supplemented with 10% FCS. Cells were harvested 1, 3, and 5 days after infection, and viable cells (as determined by trypan blue exclusion) were counted in each case.

Flow Cytometry Analysis. Mesothelioma and control cells were harvested at days 1, 3, and 5 after transduction with recombinant adenovirus or exposure to normal media. Cells were washed twice with cold PBS fixed in 1% paraformaldehyde-PBS solution on ice for 20 min and stored overnight in 70% ethanol at –20°C. Apoptosis was evaluated by Apo-BrdU techniques using a FACScan apparatus (Becton Dickinson) and protocols provided in the Apo-BrdU kit (PharMingen).

Results

Genomic DNA was isolated from six different mesothelioma cell lines, *i.e.*, H2052, H28, H290, H2595, H2373, and REN, as well as SV40 transformed COS-7 cells and H1299 and H460 lung cancer cells according to protocols described in “Materials and Methods.” In preliminary experiments, RT-PCR analyses were undertaken that revealed the absence of p16 as well as ARF expression in all of the mesothelioma lines except REN (Fig. 1). Results of the p16 expression analysis were consistent with data reported by Kratzke *et al.* (4), demonstrating that the majority of mesothelioma cell lines (including H28, H290, H2595, and H2373) express Rb and lack expression of p16; absence of ARF expression in mesothelioma cell lines has not been reported previously.

To determine the presence of SV40 in mesothelioma cell lines, PCR experiments were performed using primers designed to amplify a 738-bp segment (5138–4400) of SV40 early gene region; this analysis revealed that all of the mesothelioma lines, except REN, had PCR products corresponding to those obtained from control COS-7 genomic DNA (Fig. 2a). PCR products from three of mesothelioma lines (H2052, H28, and H290) as well as COS-7 were sequenced and confirmed to be derived from the SV40 early gene region (data not shown).

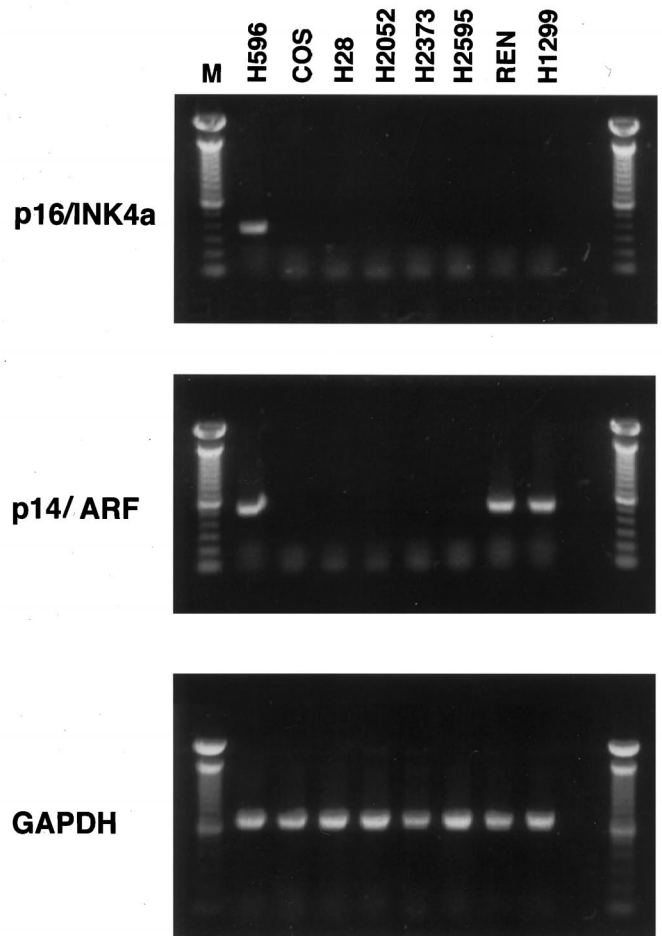


Fig. 1. RT-PCR analysis of p16 and ARF expression in cultured mesothelioma and lung cancer lines. PCR primers specific for p16^{ink4a} (top panel) and alternatively spliced p14/ARF (middle panel) were used to amplify corresponding 300- and 450-bp products from cultured mesothelioma cell lines (H28, H2052, H2373, H2595, and REN), H-596 and H1299 lung cancer cells, and COS-7 green African monkey kidney cells. Bottom panel, glyceraldehyde-3-phosphate dehydrogenase (GAPDH)-specific products confirming mRNA integrity.

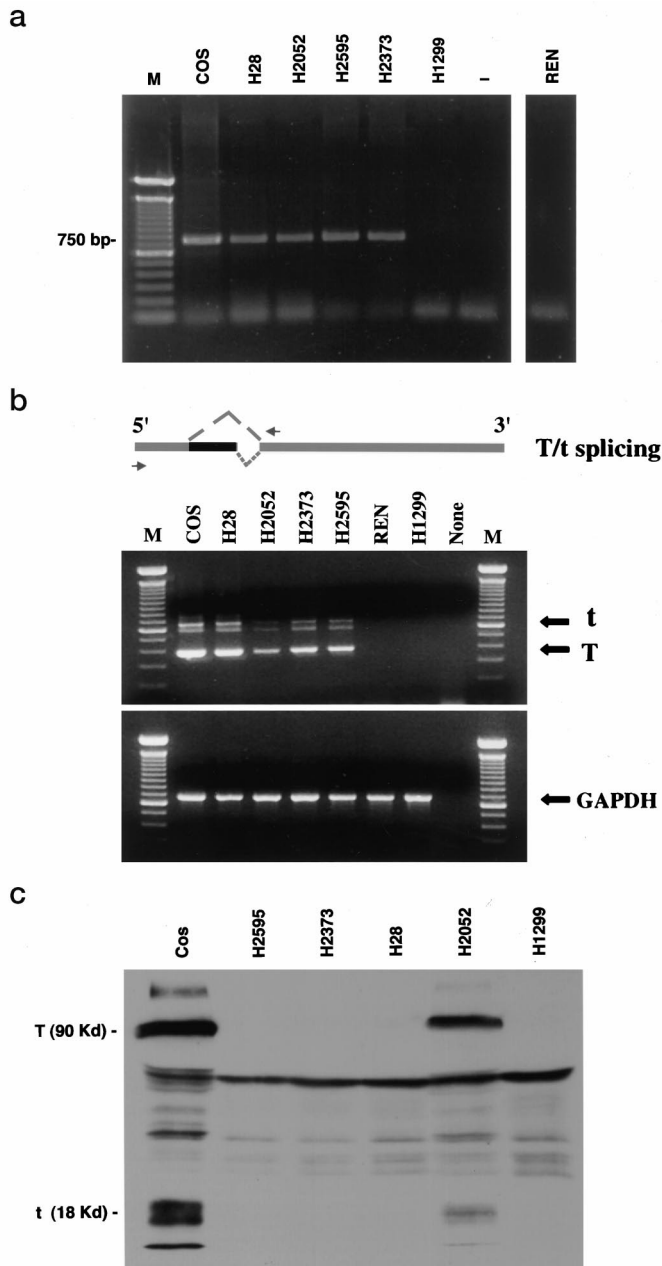


Fig. 2. *a*, genomic PCR analysis of integrated SV40 T antigen region in mesothelioma cell lines. Primers encompassing the first 750 bp of the T/t coding region were used for DNA amplification. *M*, 100-bp DNA ladder; *COS*, African green monkey kidney fibroblasts immortalized with multiple copies of SV40 sequences; *H28*, *H2052*, *H2595*, *H2373*, and *REN*, mesothelioma cell lines; *H1299*, a lung adenocarcinoma cell line; *-*, PCR control without any added template DNA. *b*, RT-PCR analysis of T/t expression in mesothelioma cells. As shown in the above schematic, RT-PCR primers (arrows) would amplify two distinct products as a result of RNA splicing. The larger bands (660 and 600 bp) correspond to the spliced forms of small t transcript, whereas the smaller (380-bp) band is the spliced first exon of large T mRNA. *Bottom panel*, a 650-bp PCR product of the glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) transcript that was used as an internal RNA loading control. The RT-PCR reactions for T/t RNA were performed under conditions described in the text. *c*, immune-precipitation and Western blot analysis of T/t gene products in mesothelioma cells. Total cell lysates were incubated with the NH₂-specific monoclonal antibody Pab 108, and the antibody-antigen immune complexes were precipitated with agarose-linked protein A. Western blot analysis of the complexes using the same monoclonal Pab 108 showed expression of large T (*M_r* 90,000) as well as small t (*M_r* 18,000) in H2052 and COS-7 cells.

To establish that SV40 early genes were expressed in mesothelioma cell lines that contained SV40 DNA sequences, a RT-PCR reaction using 1.0 μg of total cellular RNA was performed using oligo dT to generate the cDNAs. The cDNA products were then subjected to PCR

amplification (35 cycles) with T/t antigen-specific primer pairs that would simultaneously amplify two different sized products from the SV40 early gene region, *i.e.*, a 380-bp fragment corresponding to the spliced first exon of T transcript and a 650-bp spliced fragment corresponding to the t transcript. Under these conditions, all of the mesothelioma cell lines appeared negative for T-antigen-specific mRNA except H2052. However, when RT-PCR was performed using 2 μg of RNA and 40 amplification cycles, all of the mesothelioma lines that had SV40 T antigen sequences at the genomic level contained T/t antigen mRNA (Fig. 2*b*).

In additional experiments, T/t antigen protein expression was evaluated in MPM lines using Western blotting techniques and monoclonal antibodies recognizing the common NH₂ terminus of T/t antigens. Analysis of lysates from control COS-7 cells revealed two specific bands of molecular weight *M_r* 90,000 (T antigen) and *M_r* 18,000 (t antigen). None of the lysates from mesothelioma cells showed corresponding protein bands, indicating that the levels of T/t proteins in these cells were undetectable by conventional Western blotting techniques; subsequent analysis using immunoaffinity precipitation methods revealed T/t antigen protein bands in H2052 cells (Fig. 2*c*), results that were consistent with those obtained in RT-PCR experiments described previously. Collectively, these data indicate that SV40 T/t antigen expression levels are quite low in the majority of established mesothelioma cell lines; the mechanisms responsible for this phenomenon have not been established as yet.

Having verified low but detectable levels of SV40 oncoprotein expression in mesothelioma cell lines, we next sought to determine whether abrogation of T-antigen expression by antisense techniques would influence the malignant phenotype of these cells. Recombinant adenoviruses Ad/T-S and Ad/T-AS expressing, respectively, sense and antisense RNA to the first 550 bp of T/t antigen coding sequence spanning the 5' AUG and intron were constructed and characterized as described in "Materials and Methods." To evaluate whether the Ad/T-AS was functional in terms of inhibiting T/t antigen expression, a Northern blot analysis of cells transduced with this adenoviral construct was performed 48 h after infection at an MOI of 100 pfu/cell. The blots carrying total RNA from COS-7, H2052, and H1299 cells were hybridized to either sense or antisense ³²P-labeled probes spanning the first 300 bp of the first exon common to both large T and small t transcripts. This analysis confirmed expression of the antisense transcript in all three cell lines and a diminution of T/t mRNA levels in COS-7 and H2052 cells after transduction with the Ad/T-AS vector (Fig. 3*A*). Subsequent immunoprecipitation analysis revealed a reduction in T-antigen protein levels in H2052 and COS-7 cells after transduction with AD/T-AS but not the control vector (Fig. 3*B*).

Having established that Ad/T-AS could specifically inhibit SV40 oncoprotein expression, we next sought to investigate whether reduction of T/t antigen levels would inhibit the proliferation of mesothelioma cells *in vitro*. For these experiments, we used H2052 and REN mesothelioma cells that were clearly positive and negative, respectively, for SV40 oncoprotein expression, as well as COS-7 and H1299 cells (positive and negative controls, respectively). Preliminary experiments revealed that H2052 cells expressed Rb and wt p53 (data not shown). The mesothelioma and control cells were transduced with either Ad/T-S or Ad/T-AS at MOIs that resulted in comparable transduction efficiencies based on preliminary experiments (data not shown) using a replication-defective adenovirus expressing β-galactosidase. The antisense vector mediated a marked inhibition of proliferation in H2052 as well as COS-7 cells that expressed SV40 T/t oncoproteins; no effects were observed after viral treatment of REN or H1299 cells that lack SV40 viral gene sequences, suggesting that the antisense construct was specific for SV40 T antigen expression and

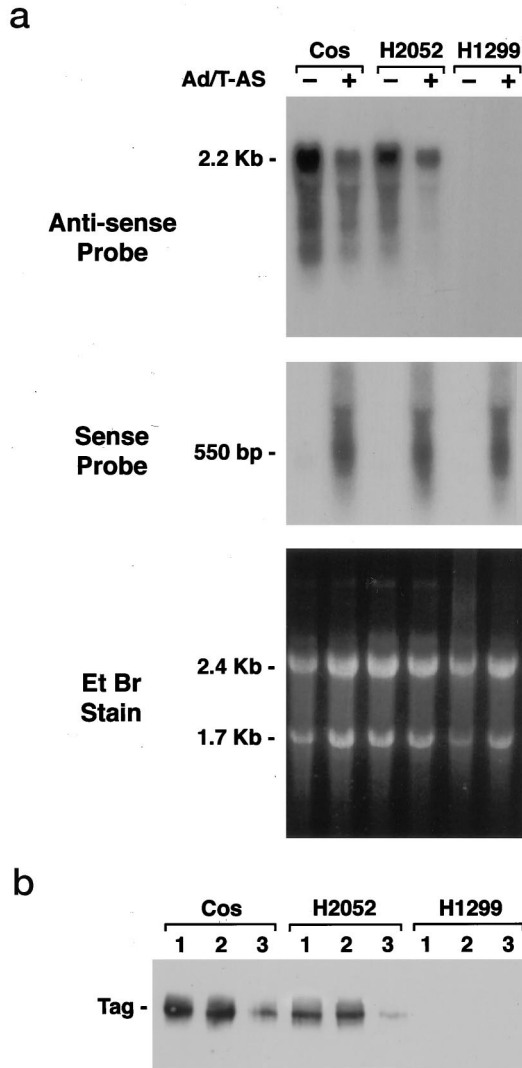


Fig. 3. Transduction of T antigen expressing COS-7 and H2052 mesothelioma cells by recombinant Ad/T-AS leads to a reduction in T/t gene products. A, Northern blot analysis of T/t transcripts in COS, H2052, and H1299 after exposure to normal medium (-) or Ad/T-AS (+). Top panel, a specific reduction in the full-length, 2.2-kb sense message in COS and H2052, as depicted by its hybridization to the ³²P-labeled antisense riboprobe. Middle panel, the relative expression levels of the 550-bp antisense transcript from the Ad/T-AS after hybridization to the sense riboprobe. Lower panel, ethidium bromide-stained gel of the total RNA prior to blotting. The 2.4- and the 1.7-kb bands correspond to the 28S and 18S rRNA, respectively. B, immune precipitation, followed by Western blot analysis of T antigen expression in COS, H2052, and H1299 cells after exposure to normal medium, vector control, or Ad/T-AS (Lanes 1, 2, and 3, respectively).

arguing against nonspecific toxicity related to the Ad/T-AS vector (Fig. 4a).

In subsequent experiments, APO-BrdU techniques were used to evaluate apoptosis in nonsynchronized cells 48 h after exposure to normal medium, Ad/T-S, or Ad/T-AS. This analysis revealed that the Ad/T-AS vector mediated pronounced apoptosis in H2052 mesothelioma and COS-7 cells; no effects were observed in H1299 or REN cells after similar treatment (representative experiment depicted in Fig. 4b). In four such independent experiments, apoptosis in COS-7 and H2052 cells ranged between 30 and 45% after antisense treatment, compared with 2–9% after exposure to the control vector. In additional experiments, p21/WAF-1 expression was evaluated using Western blot techniques in an effort to further define the mechanism of growth arrest and apoptosis in H2052 cells mediated by the adenoviral antisense construct. p21 levels appeared to be enhanced in

COS-7 as well as H2052 cells transduced with Ad/T-AS but not the control vector; p21 induction was not observed in H460 lung cancer cells containing wt p53 and lacking SV40 DNA sequences (Fig. 4c). These data suggest that restoration of p53 function contributed to the growth inhibition and apoptosis observed in H2052 cells after transduction with Ad/T-AS.

Discussion

SV40 was originally discovered in 1960 as a contaminant of human polio vaccines (11). Soon thereafter, Girardi *et al.* (26) reported that SV40 induced brain tumors and sarcomas in hamsters after i.v. inoculation. Subsequent experiments in Carbone's laboratory (16) revealed that virtually all hamsters developed mesotheliomas after receiving intrapleural instillation of SV40, and that the SV40 genome was present and expressed in 60% of human pleural mesotheliomas

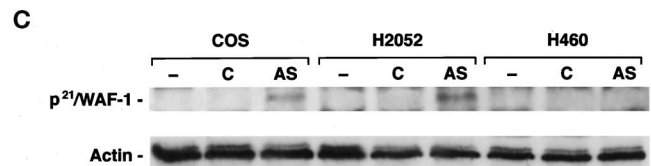
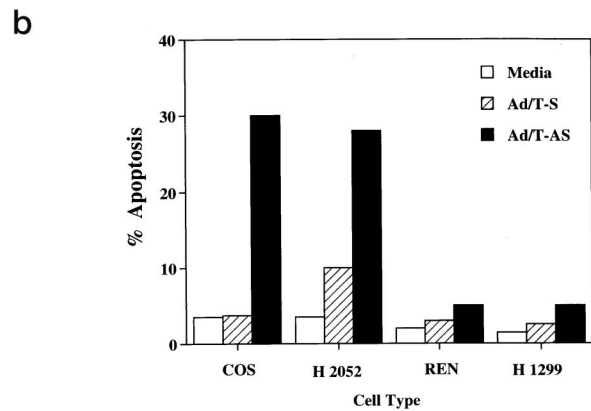
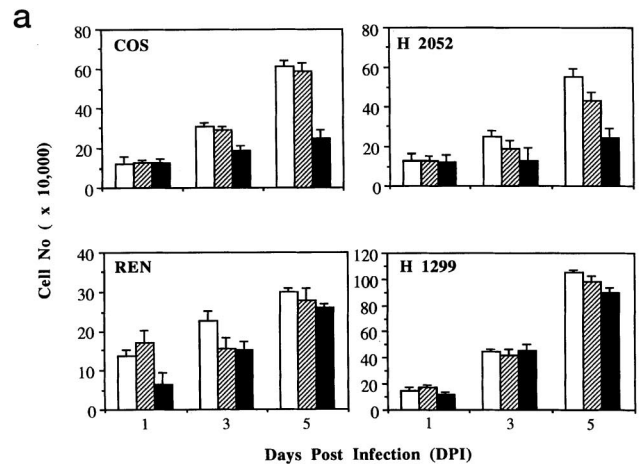


Fig. 4. a, proliferation of COS-7, H2052, REN, and H1299 cells after adenovirus transduction. Approximately 10⁵ cells in triplicate were treated with either medium alone (□), Ad/T-S (▨), or Ad/T-AS (■). The total numbers of viable cells were counted at days 1, 3, and 5 after infection; bars, SD. b, Apo-BrdU analysis of mesothelioma cells exposed to medium alone (□), Ad/T-S (▨), or Ad/T-AS (■). c, Western blot analysis of p21/WAF-1 expression in mesothelioma cells after infection with Ad/T-AS virus. Total protein lysates from COS-7, H2052, and H460 cells (all containing wt p53) were prepared 48 h after exposure to medium alone (-) vector control (C) or Ad/T-AS virus (AS). Western analysis with mouse monoclonal antibodies specific to p21/WAF-1 (top panel) and β-actin (lower panel) revealed a clear induction of p21 expression in COS-7 and H2052 cells but not H460 cells after infection with AdT-AS.

(17). Recent studies including a multi-institutional analysis have confirmed the presence of SV40 T/t antigen sequences in a significant percentage of primary human pleural mesotheliomas (10, 17, 18, 27). However, the origins of SV40 and its role in the pathogenesis of MPMs, as well as ependymomas and sarcomas that also have been shown to harbor SV40 sequences, remain unclear (10, 11, 28).

The oncogenic capacity of the SV40 virus relies almost entirely on its early gene region that gives rise to two major regulatory proteins, *i.e.*, large T and small t antigens. These proteins arise as the result of differential splicing events and share the first exon corresponding to 82 amino acid residues. This NH₂-terminal domain has been shown to be sufficient to enhance expression of a variety of proteins, including cdc-2 kinase, cyclin A, and cyclin D1 (15, 29, 30). However, more important immortalizing functions that are exclusive to T antigen rely on sequences encoded by its alternatively spliced second exon. This region of T antigen has been shown to bind Rb and its related proteins, p107 and p130, as well as p300/CBP and p53 (12, 13), all of which play crucial roles regarding cell cycle regulation. Disruption of their function by T antigen in the context of additional mutations involving proto-oncogenes such as *ras* facilitates malignant transformation (31).

Consistent with the possible role of SV40 oncoproteins in the pathogenesis of MPMs are observations that mutations involving Rb and p53 are exceedingly rare in these neoplasms (3, 5, 32, 33) and that SV40 T antigens isolated from MPMs can bind p53- and Rb-related proteins (19, 20). However, allelic deletions involving 9p21–22, which silence p16 (and presumably ARF) expression, are observed frequently in MPMs (6, 7); mutations involving the *Ink4a/ARF* locus may significantly disrupt Rb and p53 tumor suppressor pathways (8, 9), perhaps obviating any selective pressure for SV40 oncoprotein expression during malignant transformation in pleural mesothelia.

Given the apparent redundancy of 9p allelic deletions and SV40 oncoprotein expression in MPMs, we sought to determine whether abrogation of T/t antigen expression could reverse the malignant phenotype of pleural mesothelioma cells exhibiting characteristic 9p deletions. As demonstrated in this study, p16 as well as ARF expression was silenced in mesothelioma cell lines. To the best of our knowledge, this is the first demonstration of loss of ARF expression in MPM. Furthermore, although most of the mesothelioma cell lines in this study had an intact SV40 early gene region, levels of T/t mRNA and protein levels in these lines were quite low, with the exception of H2052 cells in which SV40 oncoprotein expression was readily detectable. Low-level SV40 oncoprotein expression could account in part for discrepancies concerning analysis of SV40 expression in mesothelioma cell lines and surgical specimens (particularly archival tissues) that frequently exhibit extensive stromal contaminants (reviewed in Ref. 34).

Our unpublished studies³ have indicated that adenoviral transduction of mesothelioma cells is relatively inefficient; typically, MOIs of 75–100 pfu/cell are required to achieve 50% transduction, as indicated by Ad/lacZ infection followed with 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside staining. Despite this inefficiency, a recombinant adenovirus expressing an antisense transcript targeted mainly at the initiation codon and the splice sites in the 5' region of SV40 early RNA mediated a clear reduction in T/t mRNA and protein levels in H2052 mesothelioma cells; inhibition of T/t antigen expression coincided with suppression of cell growth and induction of apoptosis in these cells. Interestingly, we have noted growth inhibition in several mesothelioma cell lines that contain SV40 T/t mRNA but lack readily detectable protein expression, suggesting that even very low levels of SV40 expression may be physiologically relevant; the fact that the

Ad-T-AS vector mediated no significant growth inhibitory effects in REN mesothelioma cells or H1299 and H460 lung cancer cells, which lack SV40 DNA sequences, suggests that the effects observed in T-antigen-positive mesothelioma cells are related to abrogation of SV40 T expression and not vector toxicity.

Data presented in this report pertaining to H2052 and COS-7 cells are consistent with those reported by Price *et al.* (35), who noted that proliferation rates of SV40-transformed human diploid fibroblasts correlated with SV40 T-antigen expression levels, and that even extremely low-level T antigen expression was sufficient to prevent senescence in transfected cells. Diminution of T-antigen expression coincided with restoration of normal morphology and contact inhibition in these transfectants. Our findings are also consistent with previous studies demonstrating senescence after abrogation of T-antigen expression in SV40 transformed cells (36). Antisense oligomers directed against the 5' splice sites of SV40 early transcripts act as potent inducers of RNase-H activity, leading to efficient degradation of the hybrid RNA species and reduced amounts of T/t antigens (37). Presumably, this mechanism contributes to inhibition of T/t antigen expression in mesothelioma cells after Ad/T-AS transduction, although this has not been formally evaluated.

Because the majority of mesotheliomas retain wt p53 (32, 33), it is tempting to speculate that this tumor suppressor protein is a primary target of T antigen in these neoplasms. p16 mutations appear to completely abrogate Rb-mediated growth constraints (38), and the resultant hyperphosphorylated Rb is relatively incapable of binding to SV40 T antigen (39). However, ARF mutations would not necessarily abrogate p53 function, particularly in response to genotoxic stress (9). Hence, in this context it is possible that very small amounts of T antigen are required to fully inactivate p53, given the fact that levels of this tumor suppressor protein might already be low because of concomitant loss of ARF expression (21, 40). Carbone *et al.* (19) observed the absence of p21 expression in mesothelioma specimens containing SV40 T antigen and wt p53. Our analysis indicated that abrogation of T-antigen expression in H-2052 cells coincided with enhanced expression of p21/WAF-1, which is known to be regulated by p53 (41). However, in all likelihood, the mechanisms of antisense T/t antigen-mediated growth arrest and apoptosis in mesothelioma cells are more complex than simple restoration of p53 function. Indeed, SV40 T and t antigens cooperate to induce cell entry in quiescent cells (42), and it is possible that some of the antisense-mediated inhibitory effects noted in this study are related to abrogation of t antigen expression. Additional studies using stable transfectants containing inducible constructs are in progress to further define the mechanisms of growth arrest and apoptosis in mesothelioma cells mediated by antisense SV40 sequences.

Data presented in this study suggest that SV40 oncoproteins contribute to the malignant phenotype of human pleural mesothelioma cells and imply that strategies designed to inhibit their expression may be efficacious in the treatment of individuals with MPMs (and possibly osteosarcomas or brain tumors) that harbor SV40. Conceivably, 9p allelic deletions and expression of SV40 oncoproteins are complementary, rather than redundant, events that simultaneously inactivate the Rb and p53 tumor suppressor pathways during malignant transformation in pleural mesothelia. The relevance of SV40 oncoproteins in the pathogenesis of malignant pleural mesotheliomas should not be underestimated, irrespective of their levels of expression.

Acknowledgments

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³ Unpublished data.

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