Evidence against a Role for SV40 in Human Mesothelioma

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Abstract

SV40 has been implicated in the etiology of 40% to 60% of human mesotheliomas. These studies could have important medical implications concerning possible sources of human infection and potential therapies if human tumors are induced by this agent. We did PCR-based analysis to detect SV40 large T antigen DNA in human mesotheliomas. None of 69 tumors in which a single copy gene was readily amplified contained detectable SV40 large T antigen sequences. Under these conditions, it was possible to detect one copy of integrated SV40 DNA per cell in a mixture containing a 5,000-fold excess of normal cells using formalin-fixed preparations. Kidney, a known reservoir of SV40 in monkeys, from some of these individuals were also negative for SV40 large T antigen sequences. A subset of mesotheliomas was analyzed for SV40 large T antigen expression by immunostaining with a highly specific SV40 antibody. These tumors as well as several human mesothelioma cell lines previously reported to contain SV40 large T antigen were negative for detection of the virally encoded oncoprotein. Moreover, mesothelioma cell lines with wild-type p53 showed normal p53 function in response to genotoxic stress, findings inconsistent with p53 inactivation by the putative presence of SV40 large T antigen. Taken together, these findings strongly argue against a role of SV40 by any known transformation mechanism in the etiology of the majority of human malignant mesotheliomas. (Cancer Res 2005; 65(7): 2602-9)

Introduction

SV40 has a well-characterized ability to trigger transformation of cells in culture. The early region of SV40 encodes two tumor antigen, large T and small t. Mutational analysis of SV40 large T antigen showed that regions of the protein that were necessary for full cellular transformation corresponded to sites of interaction of T antigen with several host proteins. These included p53 and the product of the retinoblastoma susceptibility gene, pRB. Thus, the transforming ability of SV40 is mediated, in part, by the large T antigen oncoprotein binding and inactivating cellular proteins with tumor suppressor activity (1–3). For example, a transformation-defective large T antigen mutant which cannot bind p53 is still capable of fully transforming cells which lack p53 (4). The small t antigen has also been implicated in the oncogenic activity of SV40. Similar to large T antigen, a role for interaction with cellular proteins has been shown. In this case, it is the binding of small t antigen to subunits of protein phosphatase 2A which contributes to the transformed phenotype.

In many cases, expression of SV40 large T antigen alone is necessary and sufficient for transformation of rodent cells. Use of a temperature-sensitive large T antigen supports the notion that this viral oncoprotein is not only important for the initiation of transformation but is also necessary for maintenance of the oncogenic state. In contrast to studies with rodent cells, additional events were shown to be needed for conversion of a variety of human cells to a fully tumorigenic phenotype in cell culture (1–3). The ability of SV40 virus to induce tumorigenesis in animals shows clear species specificity with newborn hamsters being the best characterized model. Depending upon the site of administration, tumors of a variety of different types can be observed. Thus, s.c. injection can lead to fibrosarcomas, whereas intrapleural injection results in mesotheliomas (5, 6).

SV40 became the focus of attention when it was discovered as a contaminant of poliovirus vaccines in the early 1960s. Although cell culture and animal studies argued against a role for SV40 in human disease, recently a number of reports have implicated SV40 in the etiology of 40% to 60% of human mesotheliomas based on detection of DNA sequences encoding the SV40 large T antigen and/or its protein expression in such tumors (7–11). These studies could have important medical implications concerning possible sources of human infection and potential therapies if human tumors are induced by this agent (12). To address this issue, we did PCR-based analysis to detect large T antigen DNA as well as protein expression in both primary human mesotheliomas and cell lines. Use of highly sensitive assays did not reveal any association of SV40 with human mesotheliomas.

Materials and Methods

Tissue specimens and cell lines. Formalin-fixed and paraffin-embedded tissue blocks of either malignant mesothelioma or normal kidney were obtained as follows. Forty-three mesothelioma cases were from the National Institute for Occupational Health, Johannesburg, Republic of South Africa. Sixty samples (30 mesothelioma cases with 30 matching normal kidney) were from Llandough Hospital, Wales, United Kingdom. Seventeen mesothelioma cases were obtained from the Department of Pathology, Mount Sinai Hospital, New York, NY and 23 mesothelioma cases were from Croatia. An experienced pathologist histologically evaluated the paraffin sections to verify the diagnosis and to measure tumor content. Tumor content of sections used for analysis typically was in the range of 80% to 100%. For the tissue specimens and DNA samples, all identifiers were removed and the samples were tested anonymously and blindly. All samples were obtained during the period of 1995 to 2002, with an age distribution of 37 to 82. Information concerning asbestos exposure was only available for

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the patients from Croatia and South Africa (26 of 27 had history and/or evidence of exposure). Cell lines were obtained from the American Type Culture Collection (Rockville, MD) and used within three to four passages.

**DNA extraction.** For each paraffin block, sets of 10-μm serial sections were deparaffinized and tissues containing 5,000 cells were collected. The cells were resuspended in 30 μl of lysis buffer containing 10 mmol/L Tris (pH 8.3), 1 mmol/L EDTA, 0.5% Tween 20, and 2 mg/ml proteinase K. Samples were incubated at 55°C for 24 hours followed by boiling for 10 minutes to inactivate the proteinase K as previously described (13). The Puregene Cell and Tissue Kit was used to extract DNA from blood samples according to the manufacturer's protocol (Gentra, Minneapolis, MN).

**PCR primers, amplification conditions, and detection of PCR products.** SV40 DNA was PCR amplified using four sets of forward and reverse primer (Fig. 1A). Cdc25C amplification used forward primer 5'-TGGTTGGGCAAAACTATCC-3' and reverse primer 5'-ATCGTTGAGGC-TCGCGATCACC-3' to generate a 100-bp product. PCR amplification was carried out with genomic DNA in a volume of 50 μl containing 20 mmol/L Tris·HCl (pH 8.4), 50 mmol/L KCl, 2.5 mmol/L MgCl2, 200 μmol/L deoxynucleotide triphosphate, 50 pmol of forward and reverse primers, 20 μg/ml bovine serum albumin, 5 units of Taq DNA polymerase (Invitrogen, Carlsbad, CA), and 6 μl crude DNA extracted from paraffin blocks or 100 ng purified DNA from blood specimens or cultured cells. The reaction mix was denatured at 94°C for 1 minute and subjected to 40 cycles (P/Q; N'/K') in a Mastercycler (Eppendorf, Hamburg, Germany). Each cycle consisted of 94°C for 1 minute, annealing for 30 seconds, and 72°C for 30 seconds. The annealing temperatures were 58°C for cdc25.for/cdc25.rev and SV.for3/SV.rev, or 80°C (P/Q, N'/K') in cycles 1-30. PCR was completed with a final cycle of 72°C for 10 minutes. Crude DNA extracts from paraffin-embedded COS-1 and CV-1 cells, as well as a water blank, served as positive and negative controls and were included with each amplification. The COS-1-positive control was always done after the tumor samples to avoid contamination. The CV-1 and no template negative controls were always the final samples in a series to verify that no contamination occurred.

**Figure 1.** Multiple primer sets detect DNA encoding the SV40 large T antigen. A, quality of the DNA extracted from paraffin-embedded samples was determined by PCR amplification of a fragment of the cellular gene cdc25C. Primers used are in bold. B, a schematic of the SV40 genomic DNA encoding the large T antigen from nucleotides 5163 to 2693. A single intronic sequence (4918-4571) is removed in the mature mRNA. DNA corresponding to four regions of the SV40 large T antigen that have been implicated in its ability to transform cells (shaded boxes). Each of the four primer sets used in this analysis is in bold. The indicated sequence corresponds to the sequence of strain 776 (NCBI entry AF316139). C, DNA extracted from formalin-fixed and paraffin-embedded COS-1 cells (containing a single copy of the SV40 genome) or CV-1 cells (parental cell line containing no SV40) was used as templates in PCR reactions with the indicated primer sets. Bracket and asterisk, primers or primer-dimers.
contamination had occurred during the analysis. Sterile microbiological techniques were employed at all times with PCR reactions being done in a HEPA-filtered hood with positive airflow. PCR products were resolved on a 3% agarose or a 15% polyacrylamide gel and visualized using ethidium bromide.

**Immunoblotting.** Cells were lysed in a buffer containing 0.5% sodium deoxycholate, 2% NP40, 50 mM/L NaCl, 25 mM/L Tris-HCl (pH 7.5), and the protease inhibitors, phenylmethylsulfonyl fluoride (1 mM/L), aprotinin (50 μg/mL), and leupeptin (50 μg/mL) for 10 minutes on ice. Lysates were spun at 15,000 rpm for 10 minutes; 100 μg of each lysate was electrophoresed on a 10% SDS polyacrylamide gel, transferred to nitrocellulose paper, and probed with PAb 101, which recognizes an epitope on the carboxyl end of SV40 large T antigen (14). D0-1 which recognizes human p53, or a commercially available antibody against actin. Second antibody was a horseradish peroxidase-conjugated goat anti-mouse IgG, and the signal was detected by the enhanced chemiluminescence method (Amersham, Piscataway, NJ).

**Immunohistochemical staining.** Five-micrometer sections were incubated with undiluted hybridoma supernatant containing PAb 101. Secondary antibody was a horseradish peroxidase-conjugated goat anti-mouse IgG.

**Flow cytometry.** Cells were fixed in 70% ethanol for at least 2 hours and treated with 1 mg/mL RNase A and 20 μg/mL propidium iodide. Analysis was done in a FACS Calibur cytometer using CellQuest software.

**p53 sequencing.** DNA was isolated with Ultracprep (Biotec, Houston, TX) and 1 μg was used immediately for cDNA synthesis with SuperScript reverse transcriptase (Life Technologies, Gaithersburg, MD) according to the manufacturer's instructions. Primers used for PCR amplification were F78 5′-TGCTCAAGCTGGCGCTAAA-3′ (sense) and R1786 5′-GGACAGC- TTCCCTGTTAGTACG-3′ (antisense).

PCR reactions were set up as already described. The reaction mix was denatured at 94°C for 1 minute and then subjected to 30 cycles, each cycle consisting of 94°C for 2 minutes, annealing at 55°C for 45 seconds, and then 72°C for 1 minute. PCR was completed with a final cycle of 72°C for 10 minutes. PCR products were purified using the QIAquick PCR purification kit (Qiagen, Chatsworth, CA). Primers used for sequencing were F263 5′-GCAGTCAGATCCTAGCGTCGAG-3′ (antisense), R1548 5′-TGCTTCTGACGCACACC-3′ (antisense), R906 5′-TGCTTCTGACGCACACC-3′ (antisense). Primers designations refer to the starting nucleotide in the cDNA using the numbering of the prototype Center for Biotechnology Information (NCBI) entry NM_000546 with the ATG start codon at nucleotide 252 and the TGA stop codon at nucleotide 1431.

**Results**

The majority of published studies using a PCR-based approach to detect SV40 DNA sequences in primary tumor samples relied on the use of a single set of primers (SV.for3 and SV.rev; ref. 15). Those samples capable of generating a detectable cdc25C product were then used with primers to detect SV40 DNA. None of the 69 informative cases showed detectable SV40 DNA in these assays (Table 1). Representative PCR amplifications for a subset of primary mesothelioma samples are shown (Fig. 3A-D). In this panel, 7 of 10 samples from the United Kingdom were informative for amplification of a region of the cdc25C gene (Fig. 3A). Of these seven samples, four were primary mesotheliomas and three were derived from normal kidney. None showed amplification using any of the three sets of SV40-specific primers (Fig. 3B-D).

Previous studies have suggested that the kidney serves as a reservoir for SV40 in infected individuals, although it remains unclear how long such infections persist (17). Thus, 30 normal kidney samples (derived from the matched U.K. mesothelioma cases) were analyzed. Of 20 informative for cdc25C, none showed detectable SV40 DNA (Table 1). Representative samples are included in Fig. 3A-D.
Published reports also have identified immunoreactive SV40 large T antigen in malignant mesothelioma samples (8). Subsets of samples were therefore analyzed by immunohistochemistry. PAb 101 is a monoclonal antibody that specifically reacts with the COOH-terminal region of the SV40 large T antigen (14). It does not cross-react with the closely related large T antigens of the human viruses, JC or BK (18, 19). The specificity of this antibody was revealed by immunoblotting. By immunoblot analysis, this antibody readily detected a single band with the appropriate mobility for T antigen in lysates of COS-1 cells, whereas T antigen–negative CV-1 cells showed no immunoreactive species (Fig. 5B). We confirmed the usefulness of this antibody for the specific detection of T antigen by immunostaining of formalin-fixed and paraffin-embedded COS-1 and CV-1 cells. PCR was performed with the indicated primer sets. Bracket and single asterisk, primers or primer-dimers. Bracket and double asterisk, a nonspecific PCR product. B, sensitivity of the PCR assay was also determined by adding a known amount of genomic DNA equivalents derived from COS-1 cells to a sample of DNA extracted from formalin-fixed, paraffin-embedded CV-1 cells (the parent cell line to COS-1, lacking any SV40 sequences). Each template contained the DNA equivalent of ~ 1,000 cells. The lanes labeled CV-1 and COS-1 are reaction using only DNA extracted from formalin-fixed and paraffin-embedded cells. PCR was performed with the indicated primer sets. Bracket and single asterisk, primers or primer-dimers.

<table>
<thead>
<tr>
<th>Source</th>
<th>Tissue type</th>
<th>cdc25C PCR</th>
<th>SV40 PCR</th>
<th>T antigen immunohistochemistry</th>
</tr>
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<td>Mesothelioma</td>
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<td>0/16</td>
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genomic DNA. No sequences corresponding to SV40 T antigen were detectable (data not shown). It should be noted that these results contrast to a previous study which found detectable genomic SV40 sequences in H28 and H2052 cells as well as T antigen expression by immunoblotting in H2052 cells (20).

SV40 large T antigen has a well-characterized role in binding and inhibiting the activity of p53. Sequence analysis of reverse

Figure 3. PCR analysis did not detect SV40 large T antigen in samples of mesothelioma or normal kidney. A-D, DNA derived from formalin-fixed, paraffin-embedded samples were subjected to PCR performed with the indicated primer sets. Bracket and single asterisk, primers or primer-dimers. These included mesotheliomas (Lanes 2, 3, 5, and 10) and normal kidney samples (Lanes 4, 6, 7, 8, 9, and 11).

Figure 4. Immunostaining did not detect SV40 large T antigen in samples of mesothelioma. Formalin-fixed, paraffin-embedded samples were stained using the monoclonal antibody Pab 101. CV-1 cells, COS-1 cells, and two examples of mesotheliomas obtained from the United Kingdom are shown.
transcription-PCR amplified p53 mRNA revealed that five of the mesothelioma lines contained wild-type p53 (data not shown). The sixth cell line (NCI-H2452) expressed a truncated p53 (Fig. 6A). Treatment with doxorubicin, a DNA-damaging agent, led to inducible expression of p53 in all five wild-type p53 expressing lines (Fig. 6A), and each showed a corresponding increase in the expression of the p53 target, p21 (Fig. 6A), and growth arrest as measured by flow cytometry (Fig. 6B). These findings exclude the possibility of an occult virally encoded protein inhibiting p53 function. As expected for a truncated p53 protein, no increase in p53 or p21 protein levels was observed for the NCI-H2452 cell line (Fig. 6A) nor did it undergo cell cycle arrest (Fig. 6B).

**Discussion**

A number of studies have reported detection of SV40 in human mesotheliomas and other tumors (8). Recently, an Institute of Medicine report has emphasized both the potentially serious public health implications and need for further investigation of these findings (12). With this in mind, our present studies used a combination of sensitive PCR-based molecular diagnostic and immunologic approaches to address this question in tumor samples and mesothelioma cell lines. We provide strong evidence against any role of SV40 in the etiology of this tumor. It has been argued that differences in the published frequency of SV40 detection in human malignant mesothelioma may be related to geographic variation in populations exposed to the virus. Only some lots of poliovaccine are known to have been contaminated with SV40 in the United States and the United Kingdom, and some countries did not receive SV40-containing vaccines at all. Using one set of the very same PCR primers used here, a published report detected SV40 DNA in over 60% (29 of 48) of the malignant mesothelioma samples tested, a frequency that is sufficient to expect that the samples examined by us should similarly have revealed SV40 positivity (7). Yet in results presented here, among 69 tumors analyzed including 16 from the United States, none were found to contain detectable SV40 T antigen DNA using three distinct primer sets under conditions shown to be capable of demonstrating the presence of T antigen sequences in less than one in one thousand cells. The continued application of properly designed, analytic approaches as described here should resolve any remaining controversy concerning the presence of SV40 in human tumors and the potentially important public health ramifications involved.

Epidemiologic studies have examined the possible increased incidence of disease among different populations known to be exposed to contaminated poliovirus vaccines. No clear association has yet been shown (12). Indeed, examination of specific populations known to have been exposed to contaminated vaccines failed to show any increased risk of malignancy (21, 22). Careful analysis of studies using serologic data suggests that reported presence of seropositivity for SV40 in many cases is likely due to cross-reactivity with the closely related JC and BK viruses (23). Taken together, population-based studies also do not support a role for SV40 in human disease (12, 23, 24).

The early region of the SV40 genome is differentially spliced to produce both small and large tumor antigens. The commonly used primers (SV.for3 and SV.rev) amplify a region of SV40 genomic DNA that is used as a coding region for the pRb binding site on large T antigen and also span a DNA sequence that represents an intron that is removed in the mature mRNA for small t antigen. Of note, the small t intron is commonly found in a variety of commercially available expression and reporter vectors (25). This raises the possibility that detection of the 105-bp DNA sequence between nucleotides 4476 to 4372 may be due to laboratory contamination. The negative findings with control samples in some studies (8) would presumably make this possibility less likely. However, consistent with our results are recently reported negative findings with respect to SV40 detection in malignant mesothelioma (26–29). A recent publication similarly did not confirm earlier reports of SV40 sequences in a large cohort of non-Hodgkin's lymphomas from the United Kingdom (30). The differences in findings among many of these published reports can not be readily reconciled.

Some studies that have detected SV40 DNA in tumors have indicated its presence in a small subset of tumor cells (8). Our studies indicate that if SV40 were present in these samples, it must occur in <2% of the cases analyzed by us and/or only in a very small subset of tumor cells. Studies with a temperature-sensitive SV40 large T antigen have shown that its expression is required for maintenance of the transformed phenotype (31, 32). Thus, the presence of less than one SV40 genome per tumor cell is
inconsistent with a role of large T antigen in tumor maintenance. A "hit and run" mechanism in mesothelioma has been proposed to account for the low frequency of T antigen–positive cells (33). For example, it has been suggested that infection of only a small subset of tumor cells may lead to full tumorigenesis due to the presence of an SV40 origin of replication contributing to genomic instability (34–36). There are occasional reports of the persistence of a transformed phenotype after loss of viral oncoprotein expression (37, 38). In some cases, this is accompanied by mutation of p53 (39, 40). However, these are isolated cases and clear, direct experimental support for the dispensability of T antigen in tumor maintenance remains lacking. The human papillomavirus (HPV), which is an established etiologic agent of human cancers (41), expresses oncoproteins with a similar mode of action as T antigen. That is, HPV E6 and E7 proteins bind and inactivate p53 and pRb, respectively (41). Both experimental and clinical studies have shown that in HPV-associated human cancers, these virally encoded proteins persist in the tumor cells. In fact, loss of expression of the E6 and E7 viral oncoproteins has been shown to inhibit the further proliferation of such tumor cells in culture (41). Thus, there is no published evidence consistent with a hit and run model of T antigen–dependent transformation.

It is formally possible that our tumor samples did not include those from patients who were exposed to contaminated vaccines. However, the age range and geographic distribution make this unlikely. Whereas these results may not reflect the situation for all mesotheliomas, the subset of tumors from various parts of the world examined here were negative for evidence of SV40 in these assays. Coupled with the lack of supporting epidemiologic evidence (12) and given the high sensitivity of the assays used here for both SV40 T antigen DNA and protein, our results strongly argue against an etiologic role for this monkey virus in human mesothelioma.

Acknowledgments

Received 7/9/2004; revised 12/29/2004; accepted 1/25/2005.

Grant support: International Environmental Research Foundation, New York, NY, and National Cancer Institute grant POICA080058 (S.A. Aaronson).

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We thank Drs. S. Patel and D. Burstein (Mount Sinai School of Medicine) for providing advice and technical support; Simun KriZanac (Faculty of Medicine, Zagreb University) for providing the Croatian mesothelioma cases; Jay Vallabh and the mortuary staff of the NIOH for their technical assistance; and the Panel of Scientific Advisors: E. Rubin, E. McConnell, and G. Gibbs for their many helpful suggestions and advice.

Figure 6. Mesothelioma cell lines have an intact p53-dependent response to DNA damage. Indicated cell lines were either untreated or treated with 0.1 μg/mL doxorubicin for 24 hours. A, protein extracts were prepared and then electrophoresed, transferred to nitrocellulose, and immunoblotted with a monoclonal antibody against p53 or its transcriptional target, p21. B, corresponding dishes of cells were subjected to flow cytometric analysis and the percent of cells in S phase is shown in the plot. Shaded columns, untreated cells; black columns, cells treated with doxorubicin. HCT116 is a colorectal tumor cell line expressing wild-type p53 included as a positive control.

References

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