Short Communication

Simian Virus 40 and Pleural Mesothelioma in Humans

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Abstract

It has been reported that DNA of SV40, a virus of Asian macaques that is tumorigenic for rodents and can transform human cells *in vitro*, is present in pleural mesotheliomas and in several other cancers. To verify these observations, we tested paraffin sections from mesothelioma tissues of 50 patients for SV40 DNA using PCR with two separate sets of primers. The analytic sensitivity for detection of SV40 DNA was 1–10 genome copies. We also tested the specimens for β-globin by PCR to assess the suitability of the specimen DNAs for amplification. β-Globin amplification was detected in 48 of the 50 specimens, but SV40 DNA was not detected in any tumors, with either of two SV40 primer sets. Furthermore, sera from 34 additional patients with mesothelioma, 33 patients with osteosarcoma (another cancer reported to be SV40-related) and 35 controls were tested for SV40 antibodies by a plaque neutralization assay. The serological data, like the DNA results, did not support an association of SV40 with mesothelioma or with osteosarcoma; antibodies to SV40 were detected in three mesothelioma patients, in one osteosarcoma patient, and in one control. These findings call into question the association of SV40 with mesothelioma.

Introduction

SV40 infects Asian macaques in nature. Experimentally, it can induce tumors in rodents (1, 2) and can immortalize or transform many different cell types *in vitro*, including cells of human origin (3, 4). The possibility that SV40 may be associated with human cancers has been investigated several times in the past, with equivocal results (5–10). Recently, DNA of SV40 or an "SV40-like" virus has been detected in human cancers using PCR (11, 12). Pleural mesotheliomas in adults (11) and ependymomas and choroid plexus tumors in children (12) were shown by PCR to contain SV40 DNA sequences in a large percentage of cases. In addition, a number of the tumor specimens tested were also shown by immunochemistry to contain SV40 large T antigen (11, 12). In a subsequent PCR study, SV40 DNA was reported to be present in osteosarcomas, osteoblastomas, chondrosarcomas, giant cell tumors, Ewing's sarcomas, liposarcomas, and Li-Fraumeni cell lines (13).

The public health implications of a relationship between SV40 and human cancers could be substantial. More than one hundred million people worldwide were exposed to SV40 during the late 1950s and early 1960s, because polio and adenovirus vaccines, which were grown in monkey-kidney cell cultures, were inadvertently contaminated with SV40 (14). Formalin-inactivated as well as live-attenuated vaccines contained infectious SV40 (14). Despite occasional reports of SV40 in human cancer (5, 6), epidemiological and virological studies have failed to demonstrate any increases in cancers associated with vaccine-related SV40 exposure (7–10, 14). Furthermore, vaccines manufactured after 1961 were required to be free of SV40 contamination (15). Therefore, the finding of SV40 in cancers of individuals who could not have received SV40-contaminated vaccines (*e.g.*, those born after 1961), implies not only that human infection with SV40 continues to occur, but that, in the case of children with ependymomas and choroid plexus papillomas, the infection is transmitted from infected mothers to their offspring.

We examined human pleural mesotheliomas for the presence of SV40 DNA sequences and analyzed sera from mesothelioma and osteosarcoma patients for antibody evidence of infection with SV40.

Materials and Methods

Tumor Specimens. Paraffin-embedded surgical biopsy specimens from 50 pleural mesothelioma patients were retrieved from the archives of the Department of Pulmonary and Mediastinal Pathology, Armed Forces Institute of Pathology (Washington, D.C.). These cases had been diagnosed between 1987 and 1992 in private hospitals and in hospitals serving military personnel and their families throughout the United States. Patients' ages about the time of diagnosis ranged between 43 and 88 years (median, 68 years). Paraffin sections from each tumor were examined microscopically to confirm the diagnosis and to verify that the sections contained tumor material. For PCR diagnosis, two sequential 5-μm sections were placed on separate slides, taking precautions to avoid section-to-section contamination.

Serum Specimens. The National Cancer Institute Immunodiagnosis Serum Bank, established by the National Cancer Institute through a contract with the Mayo Clinic, provided sera from 35 pleural mesothelioma patients, 35 osteosarcoma patients, and controls with non-tumor-related gastrointestinal ailments. The sera were collected by the serum bank between 1975 and 1991 from patients presenting to the Mayo Clinic with any of an assortment of 85 different malignancies, benign tumors, and non-tumor-related conditions (16). For this study, we selected all available cases of mesothelioma and an equal

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number of osteosarcomas. Controls were individually sex and age (±5 years) matched to the mesothelioma patients. The median age of mesothelioma patients was 60 years (range, 32–78 years), the same as for controls (range, 32–75). Osteosarcoma patients were younger with a median age of 20 years (range, 7–70 years). All sera were stored at −70°C until tested.

PCR Amplification and Diagnosis of SV40. Sections on glass slides were scraped off with sterile scalpel blades into 1.5-ml microfuge tubes and treated with 1 ml of octane for 5 min to remove paraffin. The octane was then removed, and the pellets were washed twice using 500 μl of ethanol and dried. One hundred μl of digestion buffer containing 200 μg/ml of proteinase K were added to the dry pellets, and the specimens were digested overnight at 55°C. Proteinase K was then inactivated by heating specimens to 95°C for 10 min.

Three separate amplification reactions were performed on each specimen. Two primer sets were targeted to the T antigen region of SV40: *SV.For3* and *SV.Rev*, which amplifies a 105-bp fragment, and *PYV.For* and *SV.Rev*, which amplifies a 203-bp fragment. These primers were among the reagents used in the recent studies of mesotheliomas and choroid plexus tumors (11, 12). The third primer set *GH20* and *PC04* was targeted to a 268-bp fragment of the β-globin gene. To 10 μl of the digested specimens, 40 μl of PCR buffer was added, yielding a final concentration of 10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM of each deoxynucleotide triphosphate, 0.5 μM of each primer, and 1.25 units of *Taq* polymerase. Forty cycles of amplification were conducted (1 min each at 94°C for denaturing, 52°C for annealing, and 72°C for extension) using a Perkin-Elmer Corp. 9600 thermocycler. The products of these amplifications were denatured, drawn onto a nylon membrane (IEC Biotrans), and immobilized by UV light on a transilluminator.

Filters were hybridized at 56°C in 5× saline-sodium phosphate-EDTA with 0.1% SDS and then washed in 2× saline-sodium phosphate-EDTA with 0.1% SDS also at 56°C. Hybridization was performed with biotinylated probes using the Amersham enhanced chemiluminescence system according to the manufacturer's instructions. The amplification products resulting from PCR with the two sets of SV40 primers were hybridized with *SV* probe, and the amplification products resulting from the β-globin primers were hybridized with *PC03* probe.

The following controls were included on each plate. To assess the analytic sensitivity of the assays, we used 10^3 COS-1 cells, which contain one copy of the SV40 early region in each cell, and titrated them in 10-fold steps with 10^4 SV40-negative BSC-1 cells. As additional positive controls, we used SV40-transformed human WI-26 cells and a pool of SV40 virus prepared in BSC-1 cells. As negative controls, we used SV40-negative BSC-1 cells placed in eight wells (10^3 cells in each well).

Serology. Sera were examined for antibodies that can inhibit SV40 plaque formation (17). Briefly, sera were diluted 1:10 in balanced salt solution, inactivated at 56°C for 30 min, and mixed with a dilution of SV40 that contained 20–30 plaque-forming units. The serum-virus mixtures were incubated at 37°C for 1 h and inoculated onto duplicate plates of confluent monolayers of BSC-1 cells. After adsorption at 37°C for 0.5 h, the plates were overlaid with 1% Oxoid agar. Plaques were counted on day 10 after addition of 0.01% neutral red to the plates.

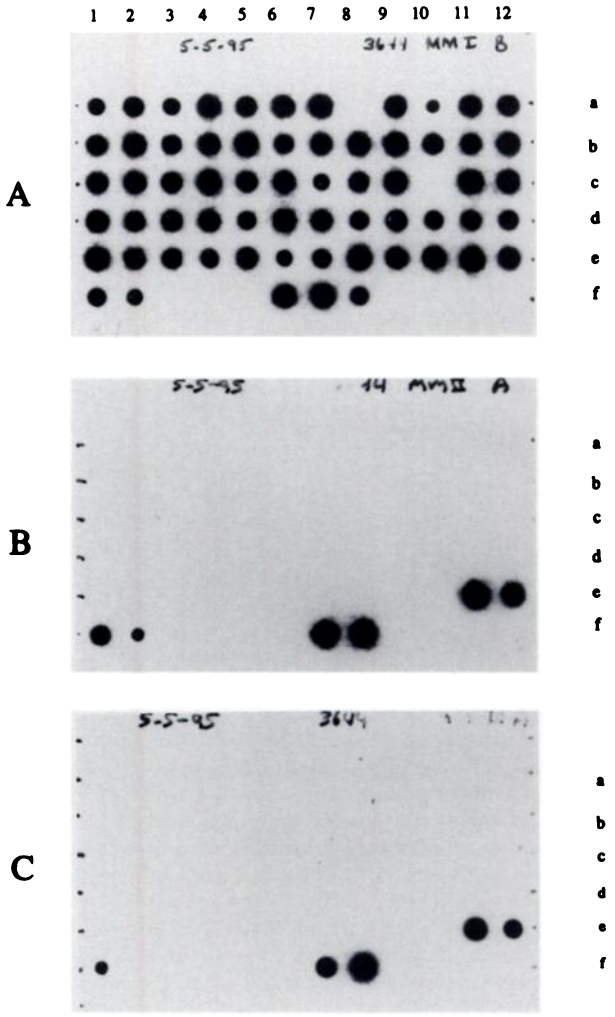

Fig. 1. Hybridization of PCR products with β-globin and SV40 probes. *A*, specimens amplified by β-globin primers and hybridized with β-globin *PC03* probe; *B*, specimens amplified with SV40 primers *SV.For3* and *SV.Rev* and hybridized with *SV* probe; *C*, Specimens amplified with SV40 primers *PYV.For* and *SV.Rev* and hybridized with *SV* probe. Fifty tumor specimens are shown in *rows a–e10*, along with 8 SV40-negative controls (10^3 BSC-1 cells) at locations *a7*, *b2*, *b9*, *c4*, *c11*, *d6*, *e1*, and *e8*. SV40-positive COS-1 cells (10^3) are titrated in 10-fold steps with 10^4 SV40-negative BSC-1 cells beginning at location *e11* (undiluted) and continuing through *f5* (1 COS-1 cell/10^3 BSC-1 cells). Normal BSC cells (10^3) are in location *f6*. SV40-transformed WI-26 cells (10^4) are in location *f7*, and a pool of SV40 virus prepared in BSC-1 cells is in location *f8*.

Results

Tumor Specimens. The PCR results using the three sets of primers are shown in Fig. 1. β-Globin was amplified satisfactorily from 48 of the 50 mesothelioma tumor specimens as well as from controls that contained human or simian cells (Fig. 1*A*). The analytic sensitivity of SV40 detection was 1 copy of SV40 genome for primer pair *SVFor3* and *SV.Rev* (Fig. 1*B*), and 10 copies of SV40 genome for primer pair *PYV.For* and *SV.Rev* (Fig. 1*C*). Both SV40 primer pairs also amplified SV40 DNA from SV40-transformed WI-26 cells (location F7), as well as from an SV40 virus pool prepared in BSC-1 cells (location F8). However, SV40 DNA was not detected in any of the mesothelioma tissues by either of the two SV40 primer pairs (Fig. 1, *B* and *C*).

Serum Specimens. Of the 105 serum specimens, 102 were tested satisfactorily. Results of three specimens (obtained from two osteosarcoma patients and a mesothelioma patient) could not be interpreted because of fungal contamination of the inoculated plates. Ninety-seven sera were completely negative for SV40 antibodies in the plaque neutralization assay. The five sera that reduced the number of SV40 plaques by more than 50% were scored as positive, but none of these blocked plaque formation completely. The donors of the five antibody-positive sera were: three mesothelioma patients (a 68-year-old male and two women, 55 and 58 years of age), one osteosarcoma patient (a 60-year-old male), and one control with benign gastrointestinal disease (a 56-year-old male). Thus, seroprevalence rates (and 95% confidence intervals) were 9% (0%, 20%) for patients with mesothelioma, 3% (0%, 10%) for patients with osteosarcoma, and 3% (0%, 10%) for controls. We note that the confidence intervals are broadly overlapping and, therefore, that the small differences between groups do not approach statistical significance.

Discussion

We investigated whether SV40 or an SV40-like virus was associated with pleural mesotheliomas in humans by testing tumor specimens for SV40 DNA and sera of patients for antibodies to SV40. The subjects studied were generally in age groups that made it possible that they had received SV40-contaminated polio vaccines in the late 1950s or early 1960s (7). However, we did not detect SV40 DNA in any of the 48 mesothelioma tissue specimens that were tested satisfactorily by PCR, and we did not find serological evidence to suggest that SV40 infection was associated with mesothelioma. In view of these negative results, it is important to point out that the PCR assay was highly sensitive and detected 1–10 copies of the SV40 genomes. Likewise, the plaque neutralization assay used to detect SV40 antibodies is a sensitive indicator of prior SV40 infection (17, 18). We did not test osteosarcoma tissues for SV40 DNA, but we did not find serological evidence linking this tumor with SV40 infection.

In keeping with our negative findings, natural SV40 infection of humans has never been documented. Furthermore, if, instead of SV40 itself, a previously unrecognized human SV40-like virus was prevalent in mesotheliomas, we should still have detected it. Our two PCR assays were targeted to the same DNA sequences and used primers and a probe that yielded positive results in earlier studies (11, 12).

The data are now in conflict regarding a relationship of SV40 or an SV40-like virus with pleural mesothelioma. By extension, other reported associations with human cancers may be considered less certain than before (13). Given the potential public health implications, it is essential that the role of SV40 in human cancers be confirmed or refuted by additional studies.

References

1. Eddy, B. E., Borman, G. S., Berkeley, W. H., and Young, R. D. Tumors induced in hamsters by injection of rhesus monkey kidney cell extracts. Proc. Soc. Exp. Biol. Med., *107:* 191–197, 1961.

2. Diamandopoulos, G. T. Induction of lymphocytic leukemia, lymphosarcoma, reticulum cell sarcoma, and osteogenic sarcoma in the Syrian golden hamster by oncogenic DNA simian virus 40. J. Natl. Cancer Inst., *50:* 1347–1365, 1973.

3. Koprowski, H., Ponten, J. A., Jensen, F., Ravdin, R. G., Moorhead, P., and Saksela, E. Transformation of cultures of human tissue infected with simian virus 40. J. Cell. Comp. Physiol., *59:* 281–292, 1962.

4. Lechner, M. S., and Laimins, L. A. Human epithelial cells immortalized by SV40 retain differentiation capabilities in an *in vitro* raft system and maintain viral DNA extrachromosomally. Virology, *185:* 563–571, 1991.

5. Soriano, F., Shelburne, C. E., and Gokcen, M. Simian virus 40 in a human cancer. Nature (Lond.), *249:* 421–424, 1974.

6. Krieg, P., Amtmann, E., Jonas, D., Fischer, H., Zang, K., and Sauer, G. Episomal simian virus 40 genomes in human brain tumors. Proc. Natl. Acad. Sci. USA, *78:* 6446–6450, 1981.

7. Shah, K., and Nathanson, N. Human exposure to SV40: review and comment. Am. J. Epidemiol., *103:* 1–12, 1976.

8. Geissler, E. SV40 and human brain tumors. Prog. Med. Virol., *37:* 211–222, 1990.

9. Mortimer, E. A., Jr., Lepow, M. L., Gold, E., Robbins, F. C., Burton, G. J., and Fraumeni, J. F., Jr. Long-term follow-up of persons inadvertently inoculated with SV40 as neonates. N. Engl. J. Med., *305:* 1517–1518, 1981.

10. Fraumeni, J. F., Jr., Stark, C. R., Gold, E., and Lepow, M. L. Simian virus 40 in polio vaccine: follow-up of newborn recipients. Science (Washington DC), *167:* 59–60, 1970.

11. Carbone, M., Pass, H. I., Rizzo, P., Marinetti, M., Di Muzio, M., Mew, D. J. Y., Levine, A. S., and Procopio, A. Simian virus 40-like DNA sequences in human pleural mesothelioma. Oncogene, *9:* 1781–1790, 1994.

12. Bergsagel, D. J., Finegold, M. J., Butel, J. S., Kupsky, W. J., and Garcea, R. L. DNA sequences similar to those of simian virus 40 in ependymomas and choroid plexus tumors of childhood. N. Engl. J. Med., *326:* 988–993, 1992.

13. Carbone, M., Rizzo, P., Giuliano, M. T., Procopio, A., Gephardt, M., Hansen, M., Malkin, D., Pompetti, F., Bushart, G., Levine, A. S., Pass, H. I., and Garcea, R. Incidence of SV40-like sequences in human tumors. Proc. Am. Assoc. Cancer Res., *36:* 201, 1995.

14. Fraumeni, J. F., Jr., Ederer, F., and Miller, R. W. An evaluation of the carcinogenicity of Simian virus 40 in man. JAMA, *185:* 713–718, 1963.

15. Shah, K. V. Evidence for an SV40-related papovavirus infection of man. Am. J. Epidemiol., *95:* 199–205, 1972.

16. DiMagno, E. P., Corle, D., O'Brien, J. F., Masnyk, I. J., Go, V. L., and Aamodt, R. Effect of longterm freezer storage, thawing, and refreezing on selected constituents of serum. Mayo Clin. Proc., *64:* 1226–1234, 1989.

17. Steinbaugh, S., and Melnick, J. L. Plaque formation by vacuolating virus, SV40. Virology, *16:* 348–349, 1962.

18. Shah, K. V., McCrumb, F. R., Jr., Daniel, R. W., and Ozer, H. L. Serologic evidence for a simian-virus-40-like infection in man. J Natl. Cancer Inst., *48:* 557–561, 1979.

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