A Possible Contributory Role of BK Virus Infection in Neuroblastoma Development

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ABSTRACT

The tumor suppressor protein p53 is aberrantly localized to the cytoplasm of neuroblastoma cells, compromising the suppressor function of this protein. Such tumors are experimentally induced in transgenic mice expressing the large tumor (T) antigen of polyomaviruses. The oncogenic mechanisms of T antigen include complex formation with, and inactivation of, the tumor suppressor protein p53.

Samples from 18 human neuroblastomas and five normal human adrenal glands were examined. BK virus DNA was detected in all neuroblastomas and none of five normal adrenal glands by PCR. Using DNA in situ hybridization, polyomaviral DNA was found in the tumor cells of 17 of 18 neuroblastomas, but in none of five adrenal medullas. Expression of the large T antigen was detected in the tumor cells of 16 of 18 neuroblastomas, but in none of the five adrenal medullas. By double immunostaining BK virus T antigen and p53 was colocalized to the cytoplasm of the tumor cells. Immunoprecipitation revealed binding between the two proteins. The presence and expression of BK virus in neuroblastomas, but not in normal adrenal medulla, and colocalization and binding to p53, suggest that this virus may play a contributory role in the development of this neoplasm.

INTRODUCTION

Neuroblastomas are the most common extracranial solid tumors of infancy and childhood. The cause of the tumor is unknown. The clinical course ranges from total spontaneous regression to malignant progression, with metastases and therapy-resistant tumors. Neuroblastomas are frequently incurable beyond the age of 1 year. Cytogenetic analyses have shown that some of the most aggressive neuroblastomas contain amplified copies of the N-myc oncogene (1). Furthermore, a striking absence of p53 gene mutations (2, 3) and an aberrant, cytoplasmic localization of the p53 tumor suppressor protein have been demonstrated (4–6). The latter translocation defect will compromise the suppressor function of p53 and may play a role in the tumorigenesis (5).

The human BKV and JCV infect children all over the world, seemingly without giving any serious symptoms (7). After primary infection, the viruses establish latent or persistent infections and may become reactivated by immunosuppression (8). In addition, it was recently demonstrated that SV40, originally a macaque monkey virus, has established itself within human populations and seems to represent an etiological cofactor for some forms of human cancer (9–11).

BKV and SV40 share a number of characteristics, which indicate that they may act as cofactors in the initiation, development, or progression of human tumors (9, 12). Both are able to induce tumors after experimental inoculation into animals and also to transform cells in culture. Furthermore, the large T antigens of these primate polyomaviruses may induce extensive chromosomal instability and rearrangements, bind and inactivate p53 and Rb-1, as well as other tumor suppressor proteins, and cooperate with cellular oncogenes to transform human cells (9, 12).

Neuroblastomas with T antigen expression and increased levels of p53 have been induced in transgenic mice carrying SV40 or JCV DNA (13). Large T antigen/p53 complexes are present in cell lines established from the neuroblastomas of animals expressing an SV40 large T antigen transgene (14). On the basis of this background, and given the opportunity to use new and more sensitive techniques, we initiated a search for polyomavirus DNA, as well as large T antigen and p53, in human neuroblastomas.

MATERIALS AND METHODS

Samples. Tumor specimens were obtained from 18 children with neuroblastomas of all different stages. The clinical material is part of an ongoing Swedish neuroblastoma study (1). Of those specimens, 13 were samples obtained from the primary tumors during operation before any treatment, four were obtained after cytotoxic treatment, whereas the last one was a liver metastasis obtained during autopsy.

Normal adrenal glands were obtained from two of the neuroblastoma patients and a child with Wilms’ tumor. From autopsies of two children, aged 2 and 15 years, two additional adrenal glands were obtained.

All tissue samples were quick-frozen in liquid nitrogen and stored at -70°C until used for DNA extraction and cryostat sectioning.

DNA Extraction. DNA was extracted from the samples by treatment overnight at 60°C with lysis buffer (500 μg/ml Proteinase K; Promega), 100 mM Tris- HCl (pH 8.0), 10 mM NaCl, 40 mM EDTA, and 1% SDS). The DNA was purified by the phenol-chloroform procedure and ethanol-precipitated. Negative control samples containing only reaction mixtures were processed in parallel with the tissue samples during the DNA extraction.

Oligonucleotide Primers and Probes. A set of primers amplifying 342 bp from the APC gene was used to assess the adequacy of the DNA extracts (15).

For the detection of polyomavirus early gene sequences, the primers PYV-for and PYV.rev were used (10). These primers amplify a conserved region of the large T antigen gene of BKV, JCV, and SV40 (Fig. 1.). The PCR products were run on an agarose gel and examined by Southern blot using a specific alkaline phosphatase-linked BK virus probe called NCCR-probe (sequence: ACAGGGAGGAGCTGCTTAC).

The BKTT1 and BKTT10 primers were used to amplify the viral NCCR of BKV (16, 17; Fig. 1). The PCR products were run on an agarose gel and examined by Southern blot using a specific alkaline phosphatase-linked BK virus probe called NCCR-probe (sequence: ACCGAGGAGGCTGCTTAC). The hybridization was performed at 55°C.

PCR Amplification. PCR amplifications were performed in a Perkin-Elmer GeneAmp PCR System 2400. Both PCR reactions and DNA extraction procedures were performed under strict conditions, involving separate pre- and post-PCR rooms, as well as designated equipment and reaction solutions for PCR and extraction, to avoid contamination (18).

All PCRs were conducted in parallel with positive and negative controls. The positive controls were viral or plasmid DNA from JCV, BKV, and SV40. The negative controls were water samples processed in between the clinical and positive samples.
In Situ DNA Hybridization. In situ hybridization was performed essentially as described (19), using DIG-end-labeled oligonucleotide probe mixtures of the oligonucleotides BKTT10 and PYV.for. These oligonucleotides will detect JCV, SV40, and BKV. A negative control, containing the hybridization mixture without end-labeled oligonucleotides, was prepared from each tissue. In addition, a persistently BKV-infected human osteoblastoma cell line (U2OS-BKV) was used as a positive control, whereas sections from a normal suprarenal gland were used as negative controls in each run.

Immunohistochemistry, Immunoprecipitation, and Immunoblotting. Immunoperoxidase staining for BKV large T antigen was performed on frozen sections using a combination of NK2- (aa 1–81) and COOH- (aa 644–695) terminal large T antigen-specific antibodies (20). Preimmune sera from the antibody-producing rabbits were used as negative controls. The streptavidin-coupled horseradish peroxidase/diaminobenzidine (DAKO) system was used for detection. Double immunofluorescence staining for large T antigen and p53 was performed using the same large T antigen antibodies as above, in addition to a human-specific monoclonal antibody for p53 (DO-1; Santa Cruz Biotechnology). Fluorolink Cy-2-labeled antirabbit IgG (Amersham Corp.) and TRITC-conjugated antimouse IgG (Sigma Chemical Co.) were used for detection. Nuclear integrity was verified by staining for proliferating cell nuclear antigen (Santa Cruz Biotechnology).

Immunoprecipitation was carried out on protein extracts (2 mg of total protein) from frozen neuroblastoma and normal adrenal tissue using a protein A-agarose-coupled polyclonal p53 antibody (FL-393; Santa Cruz Biotechnology). The protein concentration was determined by the Lowry method (Bio-Rad). The resulting immunoprecipitates were then immunoblotted with a monoclonal antibody raised against the COOH-terminal domain of large T antigen (MAB8505; Chemicon International Inc.) and alkaline phosphatase-labeled antiantimouse IgG (Sigma Chemical Co.). Detection was performed using the CDP-Star system (Boehringer Mannheim).

RESULTS

Detection and Characterization of Polyomavirus DNA Sequences in Tissue Extracts. PCR with the primer pair PYV.for and PYV.rev give amplicons corresponding to the viral T antigen coding region of polyomaviruses. Southern and dot-bLOTS with viral species-specific probes demonstrated that 18 of 18 neuroblastomas, but none of five normal adrenal medulla glands contained BKV-specific DNA sequences (Table 1). No samples tested positive using the SV40-specific probe. Sequencing the PCR products from nine of the neuroblastoma tumors revealed BKV-specific sequences (results not shown). No JCV sequences were found.

PCRs designed to amplify BKV NCCRs were carried out by the

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Fig. 1. BK viral genome, primers, and probes. Schematic diagram of the viral genome and the oligonucleotide primers and probes used for PCR amplification and detection of the BKV large T-antigen and the NCCR. The nucleotide positions are numbered according to the Dunlop strain (Ref. 24) for the following primers (BKTT1, 5106–5134; BKTT10, 413–441; PYV.rev, 4389–4411; PYV.for, 4547–4569) and probes (NCCR-probe, 144–173; PYV-probe, 4443–4463).

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Fig. 2. In situ hybridization and immunoperoxidase staining of neuroblastoma tissue sections demonstrating the presence of BKV DNA and expression of BKV large T antigen in tumor cells. a, negative control of in situ hybridization using the hybridization mixture without end-labeled oligonucleotides. b, neuroblastoma cells staining for BKV specific DNA sequences. c, negative control of immunohistochemistry using preimmune serum. d, immunoperoxidase staining using antibodies against BKV large T-antigen. Specific staining was localized both to the nucleus and cytoplasm.
Polyomavirus DNA Detection in Situ. To verify that the amplified polyomavirus DNA originated in tumor cells, in situ DNA hybridization was performed on sections from all neuroblastoma and normal adrenal gland samples. Seventeen of 18 neuroblastomas, but none of the five adrenal gland medullas, demonstrated specific polyomavirus hybridization, as illustrated in Fig. 2. The hybridization signals were localized to the nuclei of the tumor cells. The percentage of positive cells differed in each sample and between samples, ranging from 5–40% of the tumor cells.

Large T Antigen and p53 Expression. Using an immunoperoxidase staining method with antibodies raised against the BKV version of the protein, polyomavirus large T antigen was detected in situ for 16 of 18 neuroblastomas (Fig. 2). For the last two neuroblastoma samples, conclusive results were not achieved due to nonspecific staining with the preimmune sera from the rabbits immunized with large T antigen. Those two samples contained BKV DNA examined by PCR, and demonstrated specific polyomavirus hybridization. Large T antigen-specific staining was localized both to the nucleus and cytoplasm of the tumor cells. The percentage of positively stained cells was between 10 and 50%, but local variation within the same section was common. None of the normal adrenal glands gave staining for large T antigen in the medullae.

Using an immunofluorescence method for double staining of both T antigen and p53 revealed colocalization of these proteins in the cytoplasm of the cells (Fig. 3).

Western blot experiments from two different lysates of neuroblastoma specimens precipitated with antibody against p53 also revealed the presence of a 95-kDa T antigen-like protein (Fig. 4), suggesting that T antigen and p53 were physically associated. Normal adrenal medulla lysate gave no such bands.

**DISCUSSION**

Our findings indicate that the human BKV is present and its large T antigen is expressed in the tumor cells of neuroblastomas, but not in normal adrenal medulla cells. T antigen is colocalized with and bound to p53. SV40 DNA was not detected in the neuroblastomas.

In previous studies, SV40 sequences were not detected in extracts of neuroblastoma tissues, but a recent study reported the presence of SV40 DNA in a subset of neuroblastomas. This discrepancy may be due to differences in the methods used for detecting SV40 DNA.

The results of this study suggest that the BKV is involved in the development of neuroblastomas, and that its large T antigen may play a role in the tumorigenic process. Further studies are needed to determine the mechanisms by which the BKV interacts with other viral and cellular factors to induce tumor formation.

Fig. 3. Immunofluorescence double staining for BKV T-antigen (FITC) and p53 (TRITC) in neuroblastoma tissue. a, negative control immunofluorescence for T-antigen using preimmune serum. b, negative control immunofluorescence for p53 using negative serum. c, staining for BKV-Tag. d, staining for p53. Specific staining was localized mainly to the cytoplasm of both proteins.

Fig. 4. Immunoprecipitation. Western blot experiments from two different lysates of neuroblastoma specimens precipitated with antibody against p53 revealed the presence of a 95-kDa T-antigen-like protein (Lanes A and B, see arrow). Normal adrenal medulla lysates gave no such band (Lanes C–E). 2 mg of protein was immunoprecipitated in each sample.
from any of the 12 neuroblastomas studied, but they did not examine for BKV (10). BKV DNA sequences were detected in extracts from three of four human neuroblastoma cell lines, as well as neuroblas-
toma tissue from one patient (21). Our results are in accordance with those findings. No in situ or immunohistochemistry techniques were used during the studies referred to.

The PCR-products corresponding to the NH₂-terminal part of large T antigen were sequenced and definitely shown to originate in BKV, whereas Southern blots conferred the NCCRs to BKV. In other studies that have demonstrated polyomaviruses in human tumors, in situ techniques and immunohistochemistry have not been used, at least not to the same extent as in the present work. BKV was not detected in normal adrenal medullas, including two patients with neuroblastoma, indicating that the virus is not a common passenger in the cell type from which neuroblastomas originate.

The presence and expression of BKV sequences in tumor cells of neuroblastomas does not by itself establish a cause-and-effect relation-
ship with respect to the initiation or development of the tumors. Most individuals become infected with BKV by the age of 10 years (7), and viral DNA sequences could simply be an incidental finding in tissues of persons previously infected. Different lines of evidence do, however, support a contributory role of the presence of polyomavirus and the oncogenic T antigen and the development of neuroblastoma in humans. Adrenal neuroblastomas have been observed in JCV T antigen transgenic mice (22). Furthermore, neuroblastomas develop in transgenic mice expressing polyomavirus large T antigen (13, 14, 23).

In transgenic mice carrying the JCV regulatory region and SV40 T antigen region, adrenal neuroblastomas developed (13). The tumor tissues stained positive for large T antigen immunohistochemically and also for p53, indicating increased p53 levels. In neuroblastoma cell lines from transgenic mice, a physical association between wt p53 and SV40 T antigen has been demonstrated (14). It is believed that this interaction between T antigen and p53 blocks the antiproliferative function of wild-type p53, thereby promoting tumorigenesis.

The p53 in human neuroblastomas is usually wild type, but trans-
located to the cytoplasm, with a corresponding lack of growth-arresting function (22). Abnormal cytoplasmic sequestration of wild type p53 occurs in human neuroblastomas (4), and human neuroblastoma cell lines exhibit impaired p53-mediated cell cycle arrest after DNA damage (5). This naturally occurring translocation defect compromises the suppressor function of p53 and likely plays a role in the tumorigenesis of these tumors (4, 5).

We demonstrated T antigen in the cytoplasm of the tumor cells, colocalized with and bound to p53. These findings reflect large T antigen/p53 formation in vivo and retention of complexes in the cytoplasm of human neuroblastoma cells. Hence, BKV might inactivate the cell-cycle regulation and apoptotic effects of p53 and, thus, make adrenal cells more susceptible to malignant transformation.

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