Expression of Human Neurotropic Polyomavirus JCV Late Gene Product Agnoprotein in Human Medulloblastoma

Luis Del Valle, Jennifer Gordon, Sahnila Enam, Serena Delbue, Sidney Croul, Selvajothi Abraham, Sujatha Radhakrishnan, Martha Assimakopoulou, Christos D. Katsetos, Kamel Khalili

Background: The human neurotropic polyomavirus, JCV, contains an open reading frame within the late region of the viral genome that encodes a 71-amino-acid protein, agnoprotein. Because accumulating evidence supports an association between JCV infection and human brain tumors, including medulloblastomas, we assessed the presence of JCV Agno gene sequences and the expression of agnoprotein in a series of 20 well-characterized medulloblastomas. Methods: Formalin-fixed, paraffin-embedded tumor tissue samples were used for Agno gene amplification and for immunohistochemical analysis. Adjacent sections were stained with an antibody to agnoprotein and with antibodies to cellular structural and regulatory proteins, including the JCV early gene product, T antigen. Results: Analysis of amplified DNA from paraffin-embedded samples revealed the presence of the Agno gene in 11 (69%) of 16 samples. Immunohistochemical analysis showed cytoplasmic localization and widespread distribution of agnoprotein in the neoplastic cells in 11 (55%) of 20 samples. The JCV early gene product, T antigen, was present in the nucleus of some, but not all, of the neoplastic cells. Some medulloblastoma samples that expressed agnoprotein had no sign of T-antigen expression. p53 was detected in only six of the 11 tumors in which agnoprotein was expressed. None of the 20 samples showed expression of the viral late capsid proteins, ruling out productive infection of the tumor cells with JCV. Conclusions: Our data provide evidence that the JCV late gene encoding the auxiliary agnoprotein is expressed in tumor cells. The finding of agnoprotein expression in the absence of T-antigen expression suggests a potential role for agnoprotein in pathways involved in the development of JCV-associated medulloblastomas. [J Natl Cancer Inst 2002;94:267–73]

The human polyomavirus JCV has received special attention because of its established role in the fatal demyelinating disease, progressive multifocal leukoencephalopathy (PML), which is frequently seen in patients with acquired immunodeficiency syndrome (AIDS) (1,2). Similar to other polyomaviruses, the genome of JCV is composed of a double-stranded, circular, closed DNA, which contains coding sequences for the viral early protein T antigen and the late capsid proteins VP1, VP2, and VP3 as well as the auxiliary agnoprotein (3,4). Much attention has been paid to the multifunctional viral protein T antigen, whose expression is pivotal for initiation of the viral lytic cycle through stimulation of the DNA replication and late gene expression (5). Furthermore, in the absence of viral lytic infection, the production of the T antigen in an in vitro system results in transformation of neural cells (6–8); moreover, in several in vivo animal models, T antigen induces the formation of a broad range of neural-origin tumors [for review, see (9)]. For example, when JCV is inoculated intracerebrally in experimental animals, it induces a broad range of tumors, including medulloblastomas, astrocytomas, and glioblastomas (10–12). Finally, several transgenic mouse lines containing the sequences for the T antigen under the control of the JCV promoter have developed tumors of neuroectodermal origin (13–15). The oncogenic potential of the T antigen is due, at least in part, to the ability of this protein to interact with and functionally inactivate several cellular tumor suppressor proteins, including p53 and pRb, which are involved in the control of cell growth and proliferation (16,17).

The biologic role of the auxiliary agnoprotein in the viral lytic infection cycle and its effect on various cellular regulatory pathways remain unknown. The most recent studies from our laboratory (18), however, demonstrate that the interaction of the JCV T antigen with agnoprotein may have a functional consequence on the ability of the T antigen to enhance transcription and replication of the viral genome. We investigated the presence of the Agno gene and the expression of the agnoprotein in human medulloblastomas and in several PML samples.

Materials and Methods

Clinical Samples

A total of 20 formalin-fixed, paraffin-embedded biopsy and autopsy samples of medulloblastomas were collected from the pathology archives of St. Christopher’s Hospital for Children and from MCP-Hahnemann University, in Philadelphia, PA. Three samples of PML cases (two from human immunodeficiency virus-1 [HIV-1]-infected patients with AIDS and one from a patient without AIDS) were obtained from the Manhattan HIV-1 Brain Bank at Mount Sinai Medical Center in New York, NY. All of the samples were obtained in accordance with the guidelines from the institutional review boards of the participating universities.

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Development of Anti-agnoprotein Antibody

Three peptides, as shown below, that correspond to various regions of the agnoprotein were synthesized by Macromolecular Resources (Colorado State University, Fort Collins, CO). The sequences of the peptides are as follows: JAG-38—MLVRQLSRRASKVKSSTWGSTKKAQRLIFDFLLELDLC; JAG-36—LDDFCTGEDSVDGGKRRQHSGLTELQYYSLPEPDKC; and JAG-34—GTTKRAQRLIFDFLLELDFFCTGEDSVDGKRRQRC.

Approximately 2 mg of each peptide was pooled and used for injection of rabbits (Lampire Biological Laboratories, Pipersville, PA). The final sera were obtained 98 days after the initial injection and used in these studies.

Infection and Western Blot Analysis

SVG-A cells, which were subcloned from the parental line SVG that was derived from human primary fetal glial cells transformed with origin-defective simian virus 40 (SV40), were provided by Dr. Walter Atwood (Brown University, Providence, RI) for use in infection experiments. These cells express the SV40 T antigen but do not express the viral late genes. Approximately 5 × 10⁶ SVG-A cells were infected with approximately 500 hemagglutination units of the Mad-4 strain of JCV in Dulbecco’s modified Eagle medium containing fetal calf serum. After 4 hours, fetal calf serum was added to the culture medium (final concentration = 10%), and the cells were harvested 5, 10, and 15 days after infection. The protein extracts from the uninfected or infected cells (50 µg) were analyzed directly by western blot or were first reacted with preimmune or anti-agnoprotein antibody, and the immune complex was analyzed by western blot according to the standard procedures described previously (18).

Histologic and Immunohistochemical Analyses

Formalin-fixed, paraffin-embedded tissue was sectioned at 4-µm thickness and stained with hematoxylin–eosin for histologic studies. The classification of the tumors was based on the latest World Health Organization Classification of Tumors (19). Immunohistochemical analysis was performed with the use of an avidin–biotin–peroxidase complex system according to the manufacturer’s instructions (Vectastain Elite ABC Peroxidase Kit; Vector Laboratories Inc., Burlingame, CA). The protocol includes deparaffinization in xylenes and rehydration of the tissue through descending grades of alcohols up to water. Nonenzymatic antigen retrieval was performed by heating the sections to 95 °C in 0.01 M sodium citrate buffer (pH 6.0) for 40 minutes in a vacuum oven. After cooling for 30 minutes, the slides were rinsed in phosphate-buffered saline (PBS) and incubated in methanol–3% H₂O₂ for 20 minutes to quench the endogenous peroxidase. The sections were then rinsed with PBS and blocked with 5% normal horse or goat serum in 0.1% PBS–bovine serum albumin for 2 hours at room temperature. Primary antibodies, including those reactive against viral proteins and cellular markers, were incubated overnight at room temperature in a humidified chamber. Primary antibodies used in this study included rabbit polyclonal antibody against agnoprotein (1:500 dilution), rabbit polyclonal antibody against JCV capsid proteins (1:1000; provided by Dr. Walter Atwood), mouse monoclonal antibody for the detection of SV40 T antigen that cross-reacts with JCV T antigen (clone pAb416, 1:100 dilution; Oncogene Science, Boston, MA), mouse monoclonal antibody against p53 (clone DO-7, 1:100 dilution; Dako, Carpinteria, CA), mouse monoclonal antibody for GFAP (i.e., glial fibrillary acidic protein; clone 62F, 1:100 dilution; Dako), and mouse monoclonal antibody against the 38-rod form of synaptophysin (clone MAB332, 1:500 dilution; Chemicon International, Temecula, CA). After the sections were rinsed in PBS, biotinylated anti-mouse or anti-rabbit secondary antibodies were incubated for 1 hour at room temperature and rinsed in PBS. The tissue was subsequently incubated with avidin–biotin–peroxidase complexes for 1 hour at room temperature. Finally, the sections were developed with a diaminobenzidine substrate counterstained with hematoxylin and mounted under coverslips with Permount (Fisher, Pittsburgh, PA).

DNA Extraction and Polymerase Chain Reaction Amplification

DNA was prepared from 10 sections of 10-µm thickness from each of the tissue samples by use of the QIAamp DNA Tissue Kit, according to the manufacturer’s instructions (Qiagen, Valencia, CA).

For amplification of the Agno gene, a set of primers that specifically recognize JCV Agno gene was used, and the amplified DNA was hybridized with the use of JCV-specific DNA probe as depicted in Fig. 1. Southern blot analysis was performed according to a procedure that has been established in our laboratory and described previously (20,21). Amplification of the JCV early sequence was performed with the use of a set of primers that recognize the N-terminal region of the JCV T antigen, and hybridization was performed with the use of JCV-specific probes as described earlier (21).

RESULTS

The association of JCV with human brain tumors in general and with embryonal neuroepithelial tumors of the cerebellum in particular prompted us to investigate the expression of the viral late gene product, agnoprotein, in a series of well-characterized cerebellar medulloblastomas. For these experiments, we selected 20 surgically excised and autopsy tumor samples obtained from the pathology archives of St. Christopher’s Hospital for Children and MCP-Hahnemann University. These samples represented the different histologic varieties, i.e., classical (nine samples), desmoplastic medulloblastoma (seven samples), and medulloblastoma with neuroblastic differentiation (four samples). They were obtained from patients who were between the ages of 3 and 18 years. In addition, three archival autopsy samples from PML patients, either AIDS-related (two samples) or non-AIDS-related (one sample), were included in this study.

In the first series of experiments, a pair of primers that specifically recognize JCV DNA corresponding to the Agno gene was used for gene amplification, followed by Southern blot analysis. Fig. 1, A, illustrates the position of the Agno gene in the JCV genome (left panel), a representative gene amplification/Southern blot (top, right panel), and the DNA sequence highlighting the Agno gene sequence. Results from DNA analysis revealed that 11 (69%) of the 16 samples had sequences corresponding to the Agno gene (Table 1). It is interesting that some, but not all, of the tumors positive for the Agno gene also contained DNA sequences corresponding to the early gene of JCV, T antigen (data not shown). Examination of the T-antigen sequences was performed according to our earlier procedure.
using a set of primers that recognize the N-terminal region of the JCV T antigen followed by hybridization using a JCV-specific DNA probe (21). Seven (44%) of 16 samples that were examined contained DNA sequences corresponding to the T antigen and the Agno gene, whereas three were positive for the T antigen and negative for the Agno gene, and four contained the Agno gene but not the T antigen.

To investigate the expression of the auxiliary agnoprotein, we developed a specific polyclonal antibody that recognizes the JCV agnoprotein. Synthetic peptides derived from the various
regions of JCV agnoprotein (as described in the “Materials and Methods” section) were used for the development of a polyclonal antibody. To examine the ability of antisera to recognize JCV agnoprotein, we performed western blot analysis using protein extracts from SVG-A cells infected with JCV Mad-4 at various times after infection. As shown in Fig. 1, B, an approximately 8-kd protein, corresponding to the 71-amino-acid JCV agnoprotein, was detected 5 days after infection, and its levels increased 10 and 15 days after infection. We obtained no band corresponding to agnoprotein in protein extracts from uninfected cells (lane 1 in Fig. 1, B); in an immunoprecipitation experiment, the preimmune sera failed to precipitate any protein corresponding to the agnoprotein from the infected cell extract (lane 5 in Fig. 1, B). Furthermore, the anti-agnoprotein antibody showed no immunoreactivity with proteins from uninfected cells in the immunoprecipitation assay. These results confirmed the specificity of our antibody in recognizing agnoprotein.

In the next series of experiments, to examine the expression of agnoprotein in tumor cells and to determine its subcellular localization, we conducted an immunohistochemical analysis on the samples. Fig. 2 illustrates a desmoplastic medulloblastoma containing small, polar cells with neuritic processes (panel A), which exhibited a strong immunoreactivity for synaptophysin (panel B). Nine of the tumor samples showed nuclear immunoreactivity when they were tested with a monoclonal antibody against the T antigen, corroborating our earlier observations on the detection of the T antigen in human medulloblastomas. Immunoreactivity for the T antigen was observed in the nuclei of neoplastic cells in nine samples. By immunohistochemistry, p53 (a protein that, upon association with the T antigen, remains nonfunctional but in a stable form) was localized in the nuclei of the neoplastic cells, which corresponded to the same cellular compartment in which T antigen was detected (Fig. 2, C and D, respectively). Again, agnoprotein was found in cytoplasmic perinuclear regions of the neoplastic cells (Fig. 2, E). No evidence for the production of the viral late protein VP1 was observed in the neoplastic cells (Fig. 2, F). Examination of brain samples from patients with PML revealed an extremely weak immunoreactivity of the infected cells with an anti-T-antigen antibody, which is a typical feature of cells under lytic infection by polyomavirus due to the fact that viral early gene expression is reduced during later stages of infection when the capsid proteins are expressed (Fig. 2, G). In contrast, robust cytoplasmic perinuclear and nuclear localizations were detected, respectively, for agnoprotein and VP1 in JCV-infected, enlarged oligodendrocytes (Fig. 2, H and I, respectively). Table 1 summarizes the results from immunohistochemical and polymerase chain reaction analyses of medulloblastoma and PML samples. Immunohistochemical analysis showed cytoplasmic localization and widespread distribution of agnoprotein in the neoplastic cells in 11 (55%) of 20 samples. Double labeling of the tumor samples with antibodies to the T antigen and the agnoprotein showed differential localization of the T antigen in the nuclei and agnoprotein in the cytoplasmic perinuclear compartment within single neoplastic cells. Fig. 3, A and B, illustrates a representative field of a classical medullo-

### Table 1. Immunohistochemical and polymerase chain reaction (PCR)–Southern blot analyses of medulloblastoma and progressive multifocal leukoencephalopathy (PML) samples

<table>
<thead>
<tr>
<th>Case No.</th>
<th>Diagnosis</th>
<th>Sex—age, y</th>
<th>GFAP</th>
<th>SY-38</th>
<th>T-Ag</th>
<th>AGNO</th>
<th>p53</th>
<th>T-Ag</th>
<th>Southern</th>
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<td>−</td>
<td>−</td>
<td>+</td>
<td>−</td>
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<td>+</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
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<td>−</td>
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*Diagnosis of the tumors is based on the World Health Organization criteria for medulloblastomas, as detailed in the “Materials and Methods” section. The sex and the age of each patient at the time of surgical resection or autopsy are shown. n/d = not done (indicates that samples were not in appropriate condition for PCR–Southern blot analysis); − = negative (0% of cell labeling); + = 23% of cell labeling; ++ = 58% of cell labeling; +++ = 71% of cell labeling; GFAP = glial fibrillary acidic protein; SY-38 = synaptophysin; T-Ag = T antigen; AGNO = agnoprotein; AIDS = acquired immunodeficiency syndrome. 
blastoma after double-labeled immunohistochemistry for the agnoprotein and T antigen. In a PML case, co-immunostaining of the brain also showed differential localization of the agnoprotein in the cytoplasmic perinuclear region and VP1 in the nuclei of an infected oligodendrocyte (Fig. 3, C and D, respectively).

**DISCUSSION**

For the first time, to our knowledge, these data provide evidence that the polyomavirus late gene product agnoprotein is detected in pediatric brain tumors, more specifically in medulloblastomas. Medulloblastomas are the second most common primary brain tumor in children (after astrocytomas), accounting for 20% of all childhood brain tumors, and represent the most frequent malignant tumor of the brain in children (22–24). The majority of childhood medulloblastomas originate in the cerebellar vermis, presumably from the transformation of immature neuroepithelial cells of the external granular layer (25). In adolescents and young adults, the tumors have a predilection for the cerebellar hemispheres and may exhibit features of the desmoplastic variant (25,26). Histologically, medulloblastomas are composed of numerous sheaths of poorly differentiated, blue cells with scant cytoplasm. Features of early, albeit incomplete, neuronal differentiation are the neuroblastic rosettes and the areas of neoplastic neuritogenesis (“pale islands”) that typify the desmoplastic medulloblastomas. Divergent glial differentiation is rare (25).

The etiology and pathogenesis of medulloblastomas are not fully understood. Risk factors associated with the development of medulloblastomas are radiation exposure, certain genetic heritable syndromes, which include neurofibromatosis type I, Turcot’s syndrome (27,28), and the nevoid basal cell carcinoma syndrome or Gorlin’s syndrome (29,30). Although other genetic alterations, including p53 germline mutations (31,32), unbalanced translocations, deletions and duplications of chromosomes 1 and 10q, and isochromosome 17q, have been detected in approximately 50% of the cases (33,34), the majority of these malignant embryonal tumors are sporadic and their etiology remains unknown.
The development of medulloblastoma in transgenic mice containing the JCV early genome provided the rationale for earlier efforts by our group and other groups (35–37) to investigate the association of JCV with human medulloblastoma. In that setting, the major emphasis was on the viral early gene and on the expression of the T antigen in tumor cells. Detection of the JCV DNA sequence in surprisingly high numbers of cases prompted us to increase the scope of the study to examine the expression of viral proteins that are encoded by the late regions in human medulloblastoma. Although we found no evidence for the expression of the late capsid proteins VP1, VP2, and VP3 (data not shown) in these samples, results from immunostaining showed the detection of agnoprotein in the tumor cells. It is interesting that, in some tumor samples, DNA sequences corresponding to the Agno gene and the expression of agnoprotein were found in the absence of the JCV early gene sequence and its oncogenic product, T antigen. This observation may imply a role for agnoprotein in the development of medulloblastoma.

Infection with JCV occurs in early childhood, with 65% of the population infected by 14 years of age (38), most likely through spread from parent to child (39). Reviews (40) have discussed a role for B lymphocytes in the circulation of the virus throughout the body because virus has been detected in B cells from healthy individuals. Although the specific mechanism by which JCV may enter the brain is unknown, one can envision a scenario in which initial infection with JCV in children results in virus entry into the brain by the infected B cells during cerebellar development, resulting in infection of the cells that are thought to give rise to medulloblastoma.

The detection of agnoprotein in the perinuclear cytoplasmic region of both tumor cells as well as in infected oligodendrocytes from patients with PML indicates that subcellular localization of the protein remains unchanged during the course of these two distinct pathologic events. Although the function of agnoprotein remains to be investigated, one may envision a role for this protein in enhancing various cellular processes, such as transcription and replication, which are essential for the viral lytic cycle. In the absence of viral lytic infection, stimulation of cellular events by agnoprotein may lead to rapid and perhaps uncontrolled growth of the cells and to the development of neoplasm. Indeed, not mutually exclusive, one may also assume an anti-apoptotic role for agnoprotein, which is beneficial for virus replication in the infection pathway and the maintenance of tumor cells and growth in the neoplastic pathway.

The detection of JCV in human medulloblastoma may indicate a role for the virus in a subset of medulloblastoma. We have demonstrated viral DNA sequences in tumors that do not express T antigen or agnoprotein, as well as the presence of only one of the viral proteins. Chromosomal instability has been attributed to the T antigen leading to loss of viral genes during the course of tumorigenesis, which can explain why the whole genome of JCV may not be detected in every tumor sample (41). In addition, studies have indicated that the T antigen may not be required to maintain a transformed phenotype, leading to loss of its expression (42). Thus, the absence of the T antigen in some tumor cells with or without agnoprotein is not surprising. Cooperation between the agnoprotein and the T antigen in early stages of tumor development, however, remains to be investigated.

REFERENCES


NOTES

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