Effects of JC Virus Infection on Anti-Apoptotic Protein Survivin in Progressive Multifocal Leukoencephalopathy

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Progressive multifocal leukoencephalopathy (PML) is a fatal demyelinating disease of the central nervous system resulting from the productive infection of oligodendrocytes by the opportunistic polyomavirus JC virus (JCV). Apoptosis is a host defense mechanism to dispose of damaged cells; however, certain viruses have the ability to deregulate apoptotic pathways to complete their life cycles. One such pathway involves survivin, a member of the inhibitor of apoptosis family, which is abundantly expressed during development in proliferating tissues but should be absent in normal, terminally differentiated cells. Immunohistochemistry performed in 20 cases of PML revealed the presence of survivin in JCV-infected oligodendrocytes and bizarre astrocytes within demyelinated plaques. Survivin up-regulation was also found in oligodendroglial and astrocytic cultures infected with JCV. Cell cycle analysis and DNA laddering demonstrated a significantly lower number of cells undergoing apoptosis on JCV infection compared with noninfected cultures; small interfering RNA inhibition of survivin resulted in a dramatic increase in apoptotic cells in JCV-infected cultures. This is the first report describing the activation of survivin by JCV infection *in vitro* and in PML clinical cases. These observations provide new insights into the anti-apoptotic mechanisms used by JCV to complete its lytic cycle and may suggest new therapeutic targets for PML. (Am J Pathol 2007, 170:1291–1304; DOI: 10.2353/ajpath.2007.060689)

is the result of an opportunistic infection by the human neurotropic virus JC virus (JCV). Before the acquired immune deficiency syndrome (AIDS) pandemic, PML was considered a rare disorder associated with immunocompromising diseases such as leukemias and lymphomas or was seen in renal transplant and chemotherapy patients as a complication of immunosuppressive therapies. However, recent reports indicate that more than 4% of all human immunodeficiency virus (HIV)-1-infected patients will develop PML.¹ From the histopathological point of view, PML is characterized by extensive areas of demyelination in the subcortical white matter of the brain, attributable to the productive infection and cytolytic destruction of oligodendrocytes. Other histological hallmarks of PML include eosinophilic intranuclear inclusion bodies in oligodendrocytes, which represent the site of active viral replication, giant bizarre astrocytes with atypical nuclei, microglial nodules, perivascular cuffs of lymphocytes, and foamy activated macrophages.^{2,3}

JCV is a member of the *Polyomaviridae* family of DNA viruses and is widely spread among the human population, with ~85% of adults world-wide exhibiting JCV-specific seropositivity.^{4,5} Infection with the virus is thought to be subclinical and occurs in early childhood.⁶ The virus remains latent in healthy individuals until it reactivates under immunosuppressive conditions to cause PML. Based on the multifocal nature of the demyelinated lesions, it is likely that the virus reaches the brain by hematogenous spread, perhaps carried by lymphocytes in which the presence of JCV has been well documented.^{7,8} The clinical symptoms and signs depend on the location of the demyelinated lesions; because the most frequently affected location is the frontal lobe, symptoms include headaches and cognitive and motor

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Progressive multifocal leukoencephalopathy (PML), a subacute and fatal disease of the central nervous system,

impairments. PML is a fatal disease with a poor survival, which ranges from 4 to 6 months after the onset of symptoms.⁹

The viral genome consists of a closed, circular, double-stranded DNA within an icosahedral capsid of ~38 to 40 nm in diameter. The prototype strain of JCV, Mad-1, contains 5130 nucleotides¹⁰ and can be functionally divided into three regions: an early coding region, a late coding region, and a noncoding regulatory region. The regulatory region encodes the viral origin of DNA replication and contains a bidirectional promoter including two 98-bp repeats that controls transcription and is located between the early and late coding regions. The viral early genes encode the viral regulatory proteins, large and small T-antigens, and are transcribed before DNA replication, whereas the viral late genes encode the structural proteins of the capsid, VP-1, VP-2, and VP-3, as well as the accessory agnoprotein, and are transcribed after DNA replication.¹⁰

Programmed cell death is a mechanism necessary during embryonic development and in certain circumstances in adult tissues to maintain tissue homeostasis by eliminating senescent, damaged, or potentially harmful cells, including virus-infected cells.¹¹ The delicate balance between cell death activation and cell survival depends on the interactions between proapoptotic proteins and inhibitors of apoptosis.^{12,13} Among these anti-apoptotic factors is survivin, a member of the inhibitor of apoptosis protein family. The survivin gene is located in the long arm of chromosome 25, spans 15 kb, and contains an open reading frame of 426 nucleotides, which encodes a small protein of 142 amino acids and a molecular mass of 16.3 kd.¹⁴ Survivin is normally expressed at high levels during embryonic development, but its expression is completely silenced in adult and fully differentiated tissues, suggesting that the expression of survivin is regulated by an inducible promoter.¹⁵

The expression of survivin is highly regulated in a cell cycle-dependent manner. The protein can be detected selectively in the nucleus at G₂ phase, and at the beginning of mitosis survivin associates with microtubules of the mitotic spindle. Survivin also seems to be important in cell cycle progression, as shown by experiments performed with HeLa cells, in which disruption of survivin expression by anti-sense targeting resulted in spontaneous apoptosis.^{16,17} As expected, dysregulation of the normal suppression of survivin results in reduced cell death and abnormal cell viability, which may play an important role in the pathogenesis of cancer.¹⁸ The presence of survivin has been reported in a wide variety of human neoplasias. Expression of survivin has been shown using reverse transcriptase-polymerase chain reaction (RT-PCR) in colorectal carcinomas,¹⁹ esophageal carcinomas,²⁰ pancreatic cancer,²¹ and epithelial malignancies of the lung.²² Expression of survivin has been demonstrated by immunohistochemistry in colorectal cancer,^{23,24} gastric carcinomas,²⁵ breast malignancies,²⁶ bladder carcinomas,²⁷ thyroid carcinomas,²⁸ adrenal neuroblastomas,^{29,30} ovarian tumors,³¹ melanomas,³² and high-grade non-Hodgkin lymphomas.³³ As would be expected, expression of survivin is considered

an important prognostic factor that strongly correlates with tumor malignancy and poor prognosis.^{34–37}

The mechanism of apoptosis inhibition by survivin involves the binding and suppression of caspases, the well-known initiators and executioners of cell death, thus preventing maturation and proteolytic activity of initiator and effector caspases, including the direct suppression of caspases 3 and 7 activation.^{38–40} Survivin also seems to control the activation and activity of caspase 9 because a physical interaction between the two proteins has been demonstrated *in vivo* during mitosis.⁴¹ Overexpression of survivin has been associated with inhibition of cell death initiated via either the extrinsic or intrinsic pathways of caspase activation.

Although apoptosis has been demonstrated in several viral encephalitides and other diseases of the brain caused by viruses, there is little evidence supporting its occurrence in PML, suggesting that the machinery that controls programmed cell death may be disrupted by the presence of JCV. In the present study, we analyzed the status of survivin expression in glial cells infected by JCV in archival cases of PML and *in vitro*, and we studied the anti-apoptotic effects of survivin activation in oligodendrocytes, which could determine the fate of JCV-infected cells and play an important role in enhancing the life cycle of JCV.

Materials and Methods

Clinical Samples

A total of 20 formalin-fixed and paraffin-embedded autopsy brain samples of PML were collected from the archives of the Pathology Institute, University of Lausanne, Lausanne, Switzerland (10 cases) and from the Manhattan Brain Bank (R24MH59724) at the Mount Sinai Medical Center in New York, NY (10 cases). Sections of normal brain from three patients who died of nonneurological conditions were used as negative controls.

Histological and Immunohistochemical Analysis

The formalin-fixed, paraffin-embedded tissue was sectioned at 4- μ m thickness and stained with hematoxylin and eosin (H&E) for routine histological diagnosis and characterization. A special staining for myelin (Luxol fast blue) was performed to evaluate the extent of the demyelinated lesions. Immunohistochemistry was performed using the avidin-biotin-peroxidase complex system according to the manufacturer's instructions (Vectastain Elite ABC peroxidase kit; Vector Laboratories Inc., Burlingame, CA). Our protocol includes deparaffinization in xylenes, rehydration through descending grades of alcohol up to water, and nonenzymatic antigen retrieval in 0.01 mol/L sodium citrate buffer (pH 6.0) heated to 95°C for 40 minutes in a vacuum oven. After a cooling period of 30 minutes, the slides were rinsed in phosphate-buffered saline (PBS) and treated with 3% H₂O₂ in methanol for 25 minutes to quench endogenous peroxidase. Sections were then rinsed with PBS and blocked with 5% normal horse serum (for mouse monoclonal antibodies) or goat serum (for rabbit polyclonal antibodies).

ies) in 0.1% PBS/bovine serum albumin for 2 hours at room temperature. Primary antibodies were incubated overnight at room temperature in a humidifier chamber. Primary antibodies used in this study included: a rabbit polyclonal antibody against the JCV capsid protein VP-1 (1:1000 dilution; kindly provided by Dr. Walter Atwood, Brown University, Providence, RI) and a mouse monoclonal antibody specific for human survivin (clone D-8, 1:100 dilution; Santa Cruz Biotechnology, Santa Cruz, CA). Cellular markers included mouse monoclonal antibodies against glial fibrillary acidic protein (GFAP) (clone 6F2, 1:100 dilution; DAKO, Carpinteria, CA), myelin basic protein (clone SMI-94R, 1:500 dilution; Sternberger Monoclonals, Lutherville, MD), and galactocerebroside (clone MAB342, 1:250 dilution; Chemicon International, Temecula, CA). Biotinylated secondary anti-mouse or anti-rabbit antibodies were incubated for 1 hour at room temperature. Finally, sections were incubated with avidin-biotin complex (ABC kit; Vector Laboratories) for 1 hour at room temperature, rinsed with PBS, and developed with diaminobenzidine (Sigma, St. Louis, MO). Sections were counterstained with hematoxylin and mounted with Permount (Fisher Scientific, Fair Lawn, NJ).

Double-Labeling Immunofluorescence and Deconvolution

The first part of our protocol is similar to the methodology described above. After overnight incubation with a first primary antibody, sections were rinsed with PBS, and a rhodamine-conjugated secondary antibody was incubated for 2 hours at room temperature in the dark. After washing thoroughly with PBS, the sections were re-blocked and a second primary antibody was incubated overnight at room temperature. Finally, a second fluorescein isothiocyanate-tagged secondary antibody was incubated for 2 hours in the dark, and sections were coverslipped with an aqueous-based mounting media (Vectashield; Vector Laboratories), visualized in a Nikon UV inverted microscope (Nikon, Tokyo, Japan), and processed with a deconvolution software (Slidebook 4.0; Intelligent Imaging, Denver, CO).

Glial Cell Cultures and Infections

Human astrocytes and growth medium were purchased from Cambrex Bio Science Inc., Walkersville, MD (Clenetics astrocyte cell systems). Primary human oligodendrocytes were isolated as described previously.⁴² In brief, central nervous system tissue obtained from biopsies of patients undergoing lobectomies for epilepsy were trypsinized, filtered through mesh, and centrifuged on a 30% Percoll gradient. The initial mixture of dissociated glial cells was suspended in minimal essential medium (Life Technologies, Inc., Grand Island, NY) with 5% fetal bovine serum, 50 U/ml penicillin, and 50 μ g/ml streptomycin and then cultured for 48 hours in culture flasks. With this protocol, adherent cells such as astrocytes and microglia were separated from the non-adherent oligodendrocytes. The oligodendrocyte fraction was plated at 10^5 cells/well onto poly-L-lysine-coated

wells of 16-well chamber slides and cultured for 2 weeks in minimal essential medium and 5% fetal bovine serum. The phenotype of the cells and the purity of the cultures were corroborated by immunohistochemistry with specific markers such as galactocerebroside (Gal-C) and GFAP. Human oligodendrocyte and astrocytes cultures were infected with 100 hemagglutination units of the Mad1/SV Δ strain of JCV, equivalent to a multiplicity of infection of 1, in the absence of serum for 3 hours at 37°C. This hybrid JCV contains the sequences for all JCV coding regions and a modified noncoding region in which the distal portion of the second 98-bp repeat sequence has been replaced with an analogous portion of a 72-bp repeat sequence of SV-40, resulting in a more effective viral replication. After infection, cells were washed and refed with growth media supplemented with 15% fetal bovine serum. The efficiency of viral gene expression and viral replication in the infected cell cultures was evaluated by Western blot and immunocytochemistry using anti-T-antigen and anti-VP1 antibodies, respectively.

Northern and Western Blot Analyses

RNA and proteins were extracted from cells 0, 5, 10, and 15 days after infection and used for Northern and Western blot analyses. For the Northern blot analysis, RNA was isolated using guanidine isothiocyanate, separated using a 1.2% agarose gel containing formaldehyde, and transferred to a polyvinylidene difluoride membrane (Amersham Biosciences, Piscataway, NJ). Survivin cDNA used as a probe was labeled by a random-primed labeling reaction using Klenow enzyme and α [³²P]dCTP. For the Western blot analysis, cells were lysed in TNN buffer (50 mmol/L Tris, pH 7.4, 50 mmol/L NaCl, 5 mmol/L MgCl₂, and 0.5% Nonidet P-40). Proteins were separated using a 15% acrylamide gel and transferred to a supported nitrocellulose membrane. Fifty μ g of protein was loaded for each condition. A polyclonal rabbit anti-survivin antibody (Novus Biologicals, Littleton, CO) was used at a 1:500 dilution and a goat anti-rabbit secondary antibody (Pierce, Rockford, IL) was used at a 1:10,000 dilution.

Cell Cycle Analysis

Aliquots of cells (1×10^6 /ml) were fixed in 70% ethanol for 30 minutes at 4°C; cells were then centrifuged at 1600 rpm, and the resulting pellets were resuspended in 1 ml of freshly prepared propidium iodide/RNase solution. Cell cycle distribution was analyzed with the GuavaEasy Cyte mini system by using the Guava CytoSoft Cell Cycle Program according to the manufacturer's instructions (Guava Technologies, Hayward, CA). Based on the intensity of the propidium iodide fluorescence, the flow cytometry program will separate resting cells with one copy of each chromosome (G_0/G_1), cells that have replicated and contain double DNA content and thus double intensity of fluorescence (G_2/M), cells in S phase and cells with a low DNA content, indicative of apoptosis (sub- G_1).

Table 1. Clinical Information of PML Cases

Case number	Age (years)	Gender	Diagnosis	Associated	Region affected
1	73	Female	Non-AIDS-PML	Renal transplant	Frontal lobe
2	46	Male	Non-AIDS-PML	Lymphocytic leukemia	Frontal and parietal lobes
3	31	Female	AIDS-PML	AIDS	All lobes
4	36	Male	AIDS-PML	AIDS	Frontal and parietal lobes
5	37	Male	AIDS-PML	AIDS	Frontal, temporal, and occipital lobes
6	48	Female	AIDS-PML	AIDS	All lobes, cerebellum
7	44	Male	AIDS-PML	AIDS	Temporal lobe and internal capsule
8	38	Male	AIDS-PML	AIDS	Frontal, parietal, and temporal lobes
9	35	Female	AIDS-PML	AIDS	All lobes, cerebellum, and brainstem
10	44	Female	AIDS-PML	AIDS	All lobes
11	52	Male	AIDS-PML	AIDS	Frontal and occipital lobes
12	47	Male	AIDS-PML	AIDS	Frontal, temporal, and occipital lobes
13	35	Female	AIDS-PML	AIDS	Frontal and parietal lobes
14	44	Male	AIDS-PML	AIDS	All lobes
15	28	Male	AIDS-PML	AIDS	All lobes
16	36	Male	AIDS-PML	AIDS	Frontal, parietal, and temporal lobes
17	40	Female	AIDS-PML	AIDS	Parietal, temporal, and occipital lobes
18	37	Female	AIDS-PML	AIDS	All lobes
19	31	Male	AIDS-PML	AIDS	Frontal and parietal lobes
20	35	Female	AIDS-PML	AIDS	Frontal and occipital lobes
21	38	Male	Normal brain	Seizures	N/A
22	65	Female	Normal brain	Ovarian cancer	N/A
23	58	Male	Normal brain	Hypertension	N/A

N/A, not available.

In Situ Detection of Apoptosis

Apoptotic cells were identified by *in situ* terminal dUTP nick-end labeling (TUNEL) assay. In brief, primary human oligodendroglial cell cultures uninfected and infected with JCV were fixed using 4% buffered paraformaldehyde for 3 minutes, 10 days after infection, permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate, and labeled with TUNEL reaction mixture (Roche Applied Science, Indianapolis, IN). The rhodamine-labeled cultures were manually counted under direct visualization in an UV fluorescence microscope and were analyzed by flow cytometry using the Guava TUNEL kit and Guava TUNEL EasyCyte program (Guava Technologies). For manual counting, 1000 cells under phase contrast microscopy (50 cells per field, 20 fields) were counted, followed by the number of positive cells in the same fields, labeled with TUNEL assay tagged with rhodamine. Cell counts were performed in triplicate. Two samples that were labeled but without the terminal transferase enzyme were included in each experimental set as negative controls.

Staurosporine Induction of Apoptosis

For induction of apoptosis, oligodendroglial cultures non-infected and infected with JCV were treated with 10 nmol/L staurosporine (Upstate Cell Signaling, Lake Placid, NY) for 16 hours before fluorescence-activated cell sorting analysis. HL60 cells provided by the manufacturer, untreated and treated with 3 μ mol/L daunorubicin to induce apoptosis, were used as negative and positive controls as suggested by the manufacturer.

Small Interfering RNA (siRNA) Inhibition of Survivin

The siRNA technique functions by directing the sequence-specific degradation of messenger RNAs containing the siRNA sequence.⁴³ A specific small interfering RNA for targeting survivin, corresponding to the coding region 206 to 404 relative to the start codon, was designed and used to down-regulate the production of survivin (GenBank accession no. NM001168). All siRNA duplexes were obtained from Dharmacon (Lafayette, CO). The survivin siRNA sequence was 5'-AAGGACCACCG-CAUCUCUACA-3', and the nontarget (NT) control siRNA sequence was 5'-AAUGAAAAUUGUAUUGAA-3'. For all experiments, 100 nmol/L siRNAs were transfected using an oligofectamine delivery system according to the manufacturer's specifications (Invitrogen, Carlsbad, CA). For the infection experiments, cells were infected on day 0 in T162 flasks and split into six-well dishes on day 1. The infected cells were transfected with siRNAs on days 2 and 5, and all cells were collected on day 9 for analysis.

Results

Clinical Samples

The first set of experiments aimed to assess the expression of the anti-apoptotic protein survivin was performed in a collection of 20 archival cases of PML. Eighteen samples were from HIV-1-infected patients and two cases were non-AIDS-related (one case associated with chronic lymphocytic leukemia and the second with suppressive therapy after transplant). The clinical data of all

Figure 1. Histological characterization of PML. **A:** Low-magnification view of the subcortical white matter in a case of PML reveals a large circular plaque of myelin loss (H&E). **B:** Multiple foci of demyelination are more evident with a special staining for myelin (Luxol fast blue). **C:** An enlarged oligodendrocyte harboring an intranuclear eosinophilic inclusion body (H&E). **D:** A bizarre astrocyte with an atypical, hyperchromatic nucleus (H&E). **E** and **F:** Immunohistochemical detection of the JCV capsid protein VP-1 in the nuclei of enlarged oligodendrocytes and in the nucleus and cytoplasm of a bizarre astrocyte, respectively. Original magnifications: $\times 100$ (**A, B**); $\times 1000$ (**C–F**).

cases are shown in Table 1. Histologically, demyelinated lesions were present in different parts of the brain, including the frontal, parietal, occipital, and temporal lobes in the majority of the cases, two cases involving the cerebellum and one case affecting the brainstem. PML lesions were characterized by several foci of demyelination located in the subcortical white matter (Figure 1A), which were more evident with a special staining for myelin (Figure 1B, Luxol fast blue). Diagnostic cells of PML were found in abundance within the demyelinated plaques, including enlarged oligodendrocytes harboring intranuclear eosinophilic inclusion bodies (Figure 1C) and bizarre atypical astrocytes (Figure 1D). Immunohistochemistry for JC viral proteins demonstrated the presence of capsid protein VP-1 in the intranuclear inclusion bodies of oligodendrocytes (Figure 1E), and in the cytoplasm and nuclei of bizarre astrocytes (Figure 1F), indicating active viral replication in these glial cells, whereas glial cells in adjacent nonaffected areas of the brain showed no expression of JCV proteins.

Detection of Survivin in Oligodendrocytes and Astrocytes of PML Samples

Immunohistochemical experiments using a mouse monoclonal antibody specific for human survivin demonstrated robust expression of this anti-apoptotic protein in the intranuclear inclusion bodies of oligodendrocytes (Figure 2, A and B) in all PML cases, and in the cytoplasm of bizarre astrocytes within demyelinated plaques (Figure 3, A and B). Noninfected glial cells in adjacent normal areas of the brain were negative, as well as oligodendrocytes

and astrocytes in the white matter of normal control brains (insets). Survivin was found in every single cell affected by JCV within the demyelinated plaques (oligodendrocytes harboring inclusion bodies and bizarre astrocytes) in every case.

Although JCV-infected cells in PML are unmistakable, we performed double-labeling immunofluorescence to corroborate their glial nature. We found a prominent network of cellular processes labeled with myelin basic protein (Figure 2C), surrounding the survivin-expressing oligodendrocytes (Figure 2E). Double labeling of bizarre astrocytes demonstrated their phenotype, with cells expressing cytoskeletal GFAP (Figure 3C, Amca) and cytoplasmic survivin (Figure 3E). Finally, to prove that the expression of survivin is exclusive of cells infected by JCV, we performed double-labeling immunofluorescence with survivin and VP-1 antibodies. Enlarged oligodendrocytes, which show abundant survivin expression (Figure 2B, fluorescein), are strongly labeled with the JCV capsid protein (Figure 2D, rhodamine), as shown by double labeling (Figure 2F). In the same manner, bizarre astrocytes in which we find cytoplasmic expression of survivin (Figure 3B, rhodamine) show robust immunolabeling for VP-1 in both the nuclei and cytoplasm (Figure 3D). Double labeling demonstrates the presence of both proteins, survivin and VP-1, in the same cellular compartment (Figure 3F). Table 2 shows the immunohistochemical profile of astrocytes and oligodendrocytes in all cases of PML studied, which demonstrates the up-regulation of survivin in the nuclei of oligodendrocytes and the cytoplasm of astrocytes in all cases studied.

Figure 2. Detection of survivin in JCV-infected oligodendrocytes of PML. **A** and **B**: Immunohistochemistry for survivin in the nuclei of enlarged oligodendrocytes harboring intranuclear inclusion bodies (fluorescein). **Inset** shows an area of unaffected brain. **C** and **E**: Immunofluorescence for myelin basic protein shows an abundant network of oligodendroglial processes (**C**, rhodamine), surrounding an infected cell labeled for survivin (**E**, double labeling). **D** and **F**: Intense VP-1 expression (**D**, rhodamine) demonstrates the active viral infection of oligodendrocytes in which survivin expression is up-regulated (**F**, double labeling). Original magnifications, $\times 1000$.

Induction of Survivin Expression in Glial Cell Cultures on JCV Infection

In the next series of experiments, we infected glial cell cultures of oligodendroglial and astrocytic origin with

JCV. RNA as well as proteins were extracted from cells harvested after 5, 10, and 15 days after infection. Northern blot analysis of astrocytic cultures for the detection of specific survivin mRNA demonstrated a very weak signal in uninfected cells; however, a dramatic elevation was

Figure 3. Immunohistochemical detection of survivin in bizarre astrocytes of PML brain samples. **A** and **B**: Bizarre astrocytes within a demyelinated plaque in a case of PML, exhibit robust cytoplasmic immunoreactivity for survivin (rhodamine). **Inset** shows an area of normal white matter from the same case. **C** and **E**: GFAP is strongly expressed in the cytoplasm of these bizarre cells (**C**, Amca), corroborating their astrocytic phenotype (**E**, double labeling). **D** and **F**: The JCV capsid protein VP-1 was also detected in the nuclei and cytoplasm of bizarre astrocytes (**D**), in which expression of survivin is up-regulated (**F**, double labeling). Original magnifications, $\times 400$.

Table 2. Immunohistochemical Characterization of PML Cases

No.	Age (years)	Gender	Diagnosis	Associated	Immunohistochemistry					
					Oligodendrocytes			Astrocytes		
					VP-1	Agno	Survivin	VP-1	Agno	Survivin
1	73	Female	Non-AIDS-PML	Renal transplant	++ n	++ cy	+ n	++ n/+ cy	++ cy	++ cy
2	46	Male	Non-AIDS-PML	Lymphocytic leukemia	+++ n	+ cy	+ n/+ cy	++ n	+ cy	++ cy
3	31	Female	AIDS-PML	AIDS	+++ n/+ cy	+++ cy	++ n	++ n	++ cy	+++ cy
4	36	Male	AIDS-PML	AIDS	+++ n	++ cy	+++ n/+ cy	++ n/+ cy	++ cy	++ cy
5	37	Male	AIDS-PML	AIDS	+++ n	+++ cy	++ n/cy	+++ n	++ cy	++ cy
6	48	Female	AIDS-PML	AIDS	+++ n	++ cy	+ n	++ n/+ cy	+++ cy	++ cy
7	44	Male	AIDS-PML	AIDS	+++ n/+ cy	+ cy	++ n	++ n/+ cy	++ cy	+ cy
8	38	Male	AIDS-PML	AIDS	++ n	+ cy	+ n	++ n	+ cy	+ cy
9	35	Female	AIDS-PML	AIDS	++ n	++ cy	+++ n/+ cy	++ n/+ cy	+ cy	+ cy
10	44	Female	AIDS-PML	AIDS	++ n/+ cy	++ cy	+ n	++ n	++ cy	+++ cy
11	52	Male	AIDS-PML	AIDS	+++ n/+ cy	+++ cy	++ n	+++ n/+ cy	++ cy	++ cy
12	47	Male	AIDS-PML	AIDS	++ n	+ cy	+ n	++ n	+ cy	+ cy
13	35	Female	AIDS-PML	AIDS	+++ n	+++ cy	+++ n/+ cy	+++ n/+ cy	++ cy	++ cy
14	44	Male	AIDS-PML	AIDS	+++ n/+ cy	++ cy	++ n/+ cy	++ n	+ cy	+ cy
15	28	Male	AIDS-PML	AIDS	++ n/+ cy	++ cy	++ n/+ cy	++ n/+ cy	++ cy	+ cy
16	36	Male	AIDS-PML	AIDS	++ n	+ cy	++ n	++ n	+ cy	++ cy
17	40	Female	AIDS-PML	AIDS	+++ n	++ cy	+++ n/+ cy	+++ n	+++ cy	+++ cy
18	37	Female	AIDS-PML	AIDS	+++ n	+++ cy	++ n	++ n/+ cy	+ cy	++ cy
19	31	Male	AIDS-PML	AIDS	++ n/+ cy	+ cy	+ n/+ cy	++ n	++ cy	++ cy
20	35	Female	AIDS-PML	AIDS	+++ n	++ cy	++ n	++ n	++ cy	+ cy
21	38	Male	Normal brain	Seizures	—	—	—	—	—	—
22	65	Female	Normal brain	Ovarian cancer	—	—	—	—	—	—
23	58	Male	Normal brain	Hypertension	—	—	—	—	—	—

n, nuclear; cy, cytoplasmic; —, negative; +, weak immunoreactivity; ++, moderate immunoreactivity; +++, robust immunoreactivity.

observed at 5 days after infection, which decreased slightly by day 10 (Figure 4, A and B). Immunocytochemistry with survivin-specific antibodies in the astrocytic cultures showed high expression in the nuclei of infected cells (GFAP-positive) but no expression in the uninfected cultures (Figure 4C). Next, Western blot analysis performed with extracts from oligodendroglial (Figure 4D) and astrocytic cultures (Figure 4E) showed, as expected, no detectable expression of survivin in uninfected cells but high levels of expression by 5 days after infection. At the 10-day time point, the intensity of survivin expression decreased but recuperated after 15 days. Because expression of survivin is enhanced in neoplastic tissues, HeLa cells were used as positive control for the Western blot. Once the up-regulation of survivin had been demonstrated by Northern and Western blot, we determined its cellular location by immunocytochemistry. Uninfected oligodendroglial cultures, which were marked with Gal-C, showed no detectable expression of survivin, whereas JCV-infected cells, also expressing cytoplasmic Gal-C, showed a prominent nuclear labeling with the anti-survivin antibody (Figure 4F).

Cell Cycle Distribution of Oligodendroglial Cells after Infection with JCV

Flow cytometric analysis was performed in control, uninfected, and in JCV-infected oligodendroglial cultures at 10 days after infection. Cell cycle distribution demon-

strated that 7.58% of uninfected cells were in the sub-G₁ phase. Cells in sub-G₁ are characterized by a DNA content below the one observed in the G₁ phase of the cell cycle, which is usually associated with apoptosis (Figure 5A). After infection with JCV, the number of the sub-G₁ population decreased to 3.90%, suggesting that infection with the virus results in the inhibition of apoptosis (Figure 5B). Interestingly, other phases of the cell cycle were only minimally affected by infection with JCV. Similar results were obtained when apoptosis was induced by treatment with a low concentration of staurosporine. Cell cycle analysis showed 15.35% of uninfected cells undergoing apoptosis (Figure 5C); however, the number of apoptotic cells decreased nearly twofold in the JCV-infected culture (8.21%, Figure 5D), corroborating the protective effects of JCV infection against apoptosis.

Evaluation of Apoptosis in Uninfected and JCV-Infected Oligodendroglial Cultures

To verify that the results obtained by fluorescence-activated cell sorting analysis were attributable to apoptosis, we performed TUNEL assay in the same oligodendroglial cultures uninfected and infected with JCV, in which apoptosis was induced by a low concentration of staurosporine. Results from these experiments demonstrate a significantly higher number of cells undergoing apoptosis in the staurosporine-induced, uninfected culture (15.5%, Figure 6A) than in cultures infected with JCV (7.6%, Fig-

Figure 4. Up-regulation of survivin in JCV-infected glial cell cultures. **A** and **B**: Northern blot of mRNA from human astrocytes probed with a survivin cDNA. **C**: Double-labeling immunofluorescence for GFAP (fluorescein) and survivin (rhodamine) in noninfected and JCV-infected astrocytic cultures. **D** and **E**: Detection of survivin levels by Western blot in JCV-infected cell cultures at different time points after infection (**D**, astrocytes; and **E**, oligodendrocytes). **F**: Double-labeling immunofluorescence for Gal-C (fluorescein) and survivin (rhodamine) in noninfected and JCV-infected oligodendroglial cultures.

ure 6B). Flow cytometric analysis of the TUNEL-labeled oligodendroglial cells confirmed the results obtained by manual counting. After induction of apoptosis with a low

concentration of staurosporine, 24.38% of cells underwent apoptosis in the uninfected culture (Figure 6C), in comparison to only 6.46% of apoptotic cells in the JCV-

A. Control Uninfected Cells

DNA Histogram Results :

		G1	S	G2/M	Sub-G1
Total Cells	% Positive	52.62	16.02	23.78	7.58
4827	Count	2540	773	1148	366

B. JCV Infected Cells

DNA Histogram Results :

		G1	S	G2/M	Sub-G1
Total Cells	% Positive	54.52	16.29	25.29	3.90
4634	Count	2526	755	1172	181

C. Staurosporine. Uninfected Cells

DNA Histogram Results :

		G1	S	G2/M	Sub-G1
Total Cells	% Positive	50.71	13.51	20.43	15.35
4611	Count	2338	623	942	708

D. Staurosporine. JCV Infected Cells

DNA Histogram Results :

		G1	S	G2/M	Sub-G1
Total Cells	% Positive	55.04	14.82	21.93	8.21
4426	Count	2436	656	971	363

Figure 5. Cell cycle distribution of oligodendroglial cell cultures noninfected and infected with JCV. Analysis of flow cytometry distribution in control noninfected (A), JCV-infected (B), staurosporine-treated noninfected (C), and staurosporine-treated JCV-infected oligodendroglial cultures (D).

infected culture (Figure 6D). In an additional experiment, after induction of apoptosis in the oligodendroglial culture by serum starvation and withdraw from growth factors, flow cytometry of TUNEL-labeled cells showed very similar results, with 20.17% of cells detected in apoptosis in the noninfected culture (Figure 6E) and a significant decrease to 6.24% in the JCV-infected cells (Figure 6F).

siRNA Inhibition of Survivin; Effects of Cell Cycle and Apoptosis

We have demonstrated so far that infection with JCV leads to expression of survivin in glial cells and that these events are associated with protection from apoptosis. In the next series of experiments, we used a siRNA strategy targeting survivin mRNA to determine whether infection with JCV is still protective when survivin levels have been

attenuated. Protein extracts from uninfected and JCV-infected oligodendroglial cultures, in the presence and absence of siRNA treatment, were analyzed by Western blot and revealed the effective inhibition of survivin expression at 5, 10, and 15 days after infection. Nonspecific siRNA was used as a reference sample, in which survivin levels remain comparable with the levels detected in nontreated samples (Figure 7A). Then, we evaluated the cell-cycle distribution of JCV-infected cultures after siRNA inhibition of survivin. 4.79% of cells undergoing apoptosis were found in JCV-infected oligodendrocytes treated with a nonspecific siRNA (Figure 7B), a similar number to the JCV-infected cultures (Figure 5B). However, treatment with survivin siRNA resulted in a dramatic increase in the number of apoptotic cells to 17.93% (four-fold), strongly supporting the role of survivin in the protection of infected cells against apoptosis.

Figure 6. Effects of JCV-induced survivin activation in apoptosis. Evaluation of TUNEL-labeled cells in a noninfected (A) and a JCV-infected (B) oligodendroglial cell culture. C and D: Results from flow cytometric analysis of TUNEL-labeled cells in staurosporine-treated cultures, noninfected and JCV-infected. E and F: Results of apoptosis evaluation in cultures induced by withdraw from tropic support.

Finally, fluorescence-activated cell sorting analysis of TUNEL-labeled cells, after induction of apoptosis with staurosporine in JCV-infected cultures show the most dramatic effect of survivin inhibition in apoptosis. Al-

though the staurosporine-induced, JCV-infected culture treated with a nonspecific siRNA showed 5.64% of cells undergoing apoptosis (Figure 7D), similar to the 6.46% of cells in the staurosporine-treated, JCV-infected culture

A. siRNA Inhibition of Survivin

B. JCV Infection. Non-Specific siRNA

DNA Histogram Results :

		G1	S	G2/M	Sub-G1
Total Cells	% Positive	55.05	13.39	26.77	4.79
4547	Count	2503	609	1217	218

C. JCV Infection. Survivin siRNA

DNA Histogram Results :

		G1	S	G2/M	Sub-G1
Total Cells	% Positive	47.92	13.79	20.36	17.93
5174	Count	2479	714	1053	928

D. Staurosporine. JCV Infected Cells. Non-Specific siRNA

TUNEL Results: Dot Plot Gated

	Count	Cells /mL	% of Gated	x-Mean
Marker 1	1887	2.66e05	94.36 %	18.61
Marker 2	113	1.84e04	5.64 %	114.49
Total Cells	2000			

E. Staurosporine. JCV Infected Cells. Survivin siRNA

TUNEL Results: Dot Plot Gated

	Count	Cells /mL	% of Gated	x-Mean
Marker 1	570	4.68e04	28.51 %	12.84
Marker 2	1430	1.17e05	71.49 %	120.99
Total Cells	2000			

Figure 7. siRNA inhibition of survivin and its effects on cell cycle distribution. **A:** Protein extracts from oligodendroglial cell cultures infected with JCV and treated with a nonspecific siRNA and with a survivin-specific siRNA, were used for a Western blot analysis, which shows the effective inhibition of survivin expression at 5, 10, and 15 days after infection. Flow cytometric analysis of TUNEL-labeled cells demonstrates the effects of siRNA inhibition in JCV-infected cells (**B** and **C**), and JCV-infected cells treated with staurosporine to induce apoptosis (**D** and **E**).

presented before (Figure 6D), inhibition of survivin with a specific siRNA resulted in 71.49% of cells undergoing apoptosis (Figure 7E).

Discussion

Apoptotic cell death is a normal host defense mechanism necessary to maintain tissue homeostasis by disposing of senescent and damaged cells, including virus-infected cells. Apoptosis has been extensively demonstrated in a variety of viral infections outside the brain, including mononucleosis⁴⁴ and hepatitis B and C,^{45,46} and is also well documented in several viral infections of the brain such as herpes encephalitis,^{47,48} poliomyelitis,⁴⁹ rabies encephalitis,⁵⁰ West Nile encephalitis,⁵¹ subacute sclerosing panencephalitis,⁵² and particularly, AIDS encephalopathy.^{53–55} However, there is a noticeable lack of evidence to support the occurrence of apoptosis in PML.

An understanding of the lytic cycle of JCV is useful to explain some of the histopathological features seen in cases with PML. JCV has the ability to infect glial cells, especially astrocytes and oligodendrocytes and replicates within the nuclei of oligodendrocytes. This results in the presence of eosinophilic inclusion bodies, which eventually cause the lysis of the myelin-producing cells leading to the formation of demyelinated plaques. However, several questions regarding the physiopathology of PML remain unanswered, including the mechanism leading to the formation of bizarre astrocytes and the mechanism of oligodendrocyte destruction. To date, there is only one controversial report documenting apoptosis in oligodendrocytes in PML.⁵⁶ Other studies, however, have failed to show apoptotic markers in PML samples and have suggested nonapoptotic cell death and necrosis as the mechanisms of oligodendrocyte destruction.⁵⁷ Cells undergoing apoptosis display a characteristic pattern of structural and morphological changes in the nucleus and cytoplasm, including chromatin condensation, which eventually leads to nuclear pyknosis and fragmentation, and cytoplasmic shrinkage and disintegration.⁵⁸ However, none of these characteristic changes is present in JCV-infected oligodendrocytes or astrocytes in cases of PML. It could be argued that the presence of inclusion bodies may mask these alterations; however, electron microscope studies have demonstrated the absence of apoptosis-related changes in the nuclei of oligodendrocytes in areas adjacent to viral particles.^{57,59}

Although resistance to apoptosis is closely associated with tumorigenesis, certain viruses have the ability to deregulate apoptotic pathways to complete their life cycles. One such pathway involves a member of the inhibitors of apoptosis family, survivin, a protein that is necessary for control of cell proliferation during embryogenesis and expression of which is completely absent in adult tissues. In our first set of experiments, we demonstrate for the first time the expression of the normally dormant survivin in clinical samples of PML by immunohistochemistry.⁶⁰ Interestingly, survivin is located to the nuclei of JCV-infected oligodendrocytes where JCV replication takes place, whereas in bizarre astrocytes it is located in

the cytoplasm. As expected, no other cell in the samples expressed survivin, including normal astrocytes and oligodendrocytes, in adjacent areas of the brain. These observations lead us to hypothesize that JCV is capable of up-regulating the survivin gene in infected cells.

To investigate the effect of JCV infection on survivin expression, we used glial cell cultures of oligodendroglial and astrocytic origin for molecular experiments to detect survivin at the transcriptional and post-transcriptional level. We observed production of survivin mRNA and protein expression at different time points after infection. Interestingly, in both types of glial cells, we observed high levels of survivin 5 days after infection and these levels decline by day 10 only to recuperate by day 15. These *in vitro* experiments corroborate the findings of immunohistochemistry in the clinical samples and suggest that induction of the normally absent survivin is an early event in the JCV infection cycle, which may be an important event in preventing cells from undergoing apoptosis before viral DNA replication is initiated. Curiously, although expression of survivin was consistently nuclear in oligodendrocytes from tissue and cell cultures, the cellular location varied in astrocytes, with bizarre astrocytes in the brain expressing cytoplasmic survivin and infected astrocytes in culture showing a nuclear location. This difference may be attributable to a variation in the biological behavior of astrocytes in response to JCV infection under both conditions. Although astrocytes *in vitro* are capable of supporting active and productive infection, astrocytes in the brain become transformed and acquire a bizarre phenotype.

Once the JCV specificity in the activation of survivin was established, we investigated the consequences of JCV infection in the fate of infected cells. We consistently found a significantly decreased number of apoptotic cells in JCV-infected glial cultures either under basal conditions (twofold) or after induction of apoptosis (threefold to fourfold), and targeted siRNA inhibition of survivin resulted in a dramatic increase in apoptosis, corroborating the specificity and importance of survivin activation by JCV in the prevention of apoptotic death. The molecular mechanisms that regulate the reactivation of survivin expression are yet to be determined; however, preliminary results suggest that a likely candidate to mediate this event is the JCV regulatory protein T-antigen, which we have shown to be able to bind and activate the survivin promoter (data not shown).

The consequences of increased survival in both types of glial cells infected by JCV would be beneficial for viral replication and propagation but detrimental for the brain. In oligodendrocytes, avoiding apoptosis would constitute a key element for virus survival, allowing JCV the time necessary to complete its life cycle and increasing the risk for the disease to progress rapidly because the apoptosis impaired cells are fertile ground for viral replication and release through either necrosis or lysis of oligodendrocytes unable to contain such a large viral load. On the other hand, the fate of JCV-infected astrocytes, which do not undergo necrosis or lytic destruction, would allow them enough time to produce constant levels of T-antigen, which in turn

can bind, sequester, and inactivate important cell cycle regulator proteins such as p53 and pRb. In addition, it is also known that the presence of T-antigen in several cell types induces DNA damage and chromosomal aberrations, which in this case explains the bizarre appearance and the transformed phenotype and may contribute as an early event in the development of JCV-associated brain neoplasms.

In conclusion, results from these experiments show for the first time the activation of the normally dormant anti-apoptotic survivin in a viral disease of the brain and provide new insights in the pathophysiology of PML. This information invites new studies to determine the molecular mechanisms involved in the activation of survivin by JCV. Understanding of this pathway may lead to the development of more effective therapies targeting the survivin or caspase pathway against a thus far incurable and fatal disease.

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References

- Berger JR, Concha M: Progressive multifocal leukoencephalopathy: the evolution of a disease once considered rare. *J Neurovirol* 1995, 1:5–18
- Valle LD, Croul S, Morgello S, Amini S, Rappaport J, Khalili K: Detection of HIV-1 Tat and JCV capsid protein, VP1, in AIDS brain with progressive multifocal leukoencephalopathy. *J Neurovirol* 2000, 6:221–228
- Major EO, Amemiya K, Tornatore CS, Houff SA, Berger JR: Pathogenesis and molecular biology of progressive multifocal leukoencephalopathy, the JC virus induced demyelinating disease of the human brain. *Clin Micro Rev* 1992, 5:49–73
- Taguchi F, Kajioaka J, Miyamura T: Prevalence rate and age acquisition of antibodies against JC virus and BK virus in human sera. *Microbiol Immunol* 1982, 26:1057–1064
- Walker DL, Padgett BL: The epidemiology of human polyomaviruses. *Polyomaviruses and Human Neurological Disease*. Edited by JL Sever, D Madden. New York, Alan R. Liss Inc., 1985, pp 99–106
- Padgett BL, Walker DL: Prevalence of antibodies in human sera against JC virus, an isolate from a case of progressive multifocal leukoencephalopathy. *J Infect Dis* 1973, 127:467–470
- Houff SA, Major EO, Kats D, Kufta CV, Sever JL, Pittaluga S, Roberts JR, Gitt J, Saini N, Lux W: Involvement of JC virus infected mononuclear cells from the bone marrow and spleen in the pathogenesis of progressive multifocal leukoencephalopathy. *N Engl J Med* 1988, 318:301–305
- Tornatore C, Berger JR, Houff SA, Curfman B, Meyers K, Winfield D, Major EO: Detection of JC virus DNA in peripheral lymphocytes from patients with and without progressive multifocal leukoencephalopathy. *Ann Neurol* 1992, 31:454–462
- Aksamit AJ: Progressive multifocal leukoencephalopathy: a review of the pathology and pathogenesis. *Microsc Res Tech* 1995, 32:302–311
- Frisque RJ, White III FA: The molecular biology of JCV, causative agent of progressive multifocal leukoencephalopathy. *Molecular Neurovirology*. Edited by Roos RP. Totowa, Humana Press, 1992, pp 25–158
- Adams JM: Ways of dying: multiple pathways to apoptosis. *Genes Dev* 2003, 17:2481–2495
- Shibata S, Kyuwa S, Lee SK, Goto N: Apoptosis induced in mouse hepatitis virus-infected cells by a virus-specific CD8+ cytotoxic T-lymphocyte clone. *J Virol* 1994, 68:7540–7545
- White MK, McCubrey JA: Suppression of apoptosis: role in cell growth and neoplasia. *Leukemia* 2001, 15:1011–1021
- Ambrosini G, Adida C, Altieri DC: A novel anti-apoptosis gene, survivin, expressed in cancer and lymphoma. *Nat Med* 1997, 3:917–921
- Li F, Altieri C: Transcriptional analysis of human survivin gene expression. *Biochem J* 1999, 344:305–311
- Fortugno P, Wall NR, Giodini A, O'Connor DS, Plescia J, Padgett KM, Tognin S, Marchisio PC, Altieri DC: survivin exists in immunchemically distinct subcellular pools and is involved in spindle microtubule function. *J Cell Sci* 2002, 115:575–585
- Li F, Ambrosini G, Chu EY, Plescia J, Tognin S, Marchisio PC, Altieri DC: Control of apoptosis and mitotic spindle checkpoint by survivin. *Nature* 1998, 396:580–584
- Thompson CB: Apoptosis in the pathogenesis and treatment of disease. *Science* 1995, 267:1456–1462
- Sarela AI, Macadam RC, Farmery SM, Markham AF, Guillou PJ: Expression of the antiapoptosis gene, survivin, predicts death from recurrent colorectal carcinoma. *Gut* 2000, 46:645–650
- Kato J, Kuwabara Y, Mitani M, Shinoda N, Sato A, Toyama T, Mitsui A, Nishiwaki T, Moriyama S, Kudo J, Fujii Y: Expression of survivin in esophageal cancer: correlation with the prognosis and response to chemotherapy. *Int J Cancer* 2001, 95:92–95
- Satoh K, Kaneko K, Hirota M, Masamune A, Satoh A, Shimosegawa T: Expression of survivin is correlated with cancer cell apoptosis and is involved in the development of human pancreatic duct cell tumors. *Cancer* 2001, 92:271–278
- Monzó M, Rosell R, Felip E, Astudillo J, Sanchez JJ, Maestre J, Martin C, Font A, Barnadas A, Abad A: A novel anti-apoptosis gene: re-expression of survivin messenger RNA as a prognosis marker in non-small-cell lung cancers. *J Clin Oncol* 1999, 17:2100–2104
- Kawasaki H, Altieri DC, Lu CD, Toyoda M, Tenjo T, Tanigawa N: Inhibition of apoptosis by survivin predicts shorter survival rates in colorectal cancer. *Cancer Res* 1998, 58:5071–5074
- Kawasaki H, Toyoda M, Shinohara H, Okuda J, Watanabe I, Yamamoto T, Tanaka K, Tenjo T, Tanigawa N: Expression of survivin correlates with apoptosis, proliferation, and angiogenesis during human colorectal tumorigenesis. *Cancer* 2001, 91:2026–2032
- Lu CD, Altieri DC, Tanigawa N: Expression of a novel antiapoptosis gene, survivin, correlated with tumor cell apoptosis and p53 accumulation in gastric carcinomas. *Cancer Res* 1998, 58:1808–1812
- Tanaka K, Iwamoto S, Gon G, Nohara T, Iwamoto M, Tanigawa N: Expression of survivin and its relationship to loss of apoptosis in breast carcinomas. *Clin Cancer Res* 2000, 6:127–134
- Swana HS, Grossman D, Anthony JN, Weiss RM, Altieri DC: Tumor content of the antiapoptosis molecule survivin and recurrence of bladder cancer. *N Engl J Med* 1999, 341:452–453
- Ito Y, Yoshida H, Urano T, Nakano K, Miya A, Kobayashi K, Yokozawa T, Matsuzuka F, Matsuura N, Kakudo K, Kuma K, Miyauchi A: Survivin expression is significantly linked to the dedifferentiation of thyroid carcinoma. *Oncol Rep* 2003, 10:1337–1340
- Adida C, Berrebi D, Peuchmaur M, Reyes-Mugica M, Altieri DC: Anti-apoptosis gene, survivin, and prognosis of neuroblastoma. *Lancet* 1998, 351:882–883
- Azuahata T, Scott D, Takamizawa S, Wen J, Davidoff A, Fukuzawa M, Sandler A: The inhibitor of apoptosis protein survivin is associated with high-risk behaviour of neuroblastoma. *J Pediatr Surg* 2001, 36:1785–1791
- Yoshida H, Ishiko O, Sumi T, Matsumoto Y, Ogita S: Survivin, bcl-2 and matrix metalloproteinase-2 enhance progression of clear cell and serous-type ovarian carcinomas. *Int J Oncol* 2001, 19:537–542
- Grossman D, McNiff JM, Li F, Altieri DC: Expression and targeting of the apoptosis inhibitor, survivin, in human melanoma. *J Invest Dermatol* 1999, 113:1076–1081

33. Adida C, Haioun C, Gaulard P, Lepage E, Morel P, Briere J, Dombret H, Reyes F, Diebold J, Gisselbrecht C, Salles G, Altieri DC, Molina TJ: Prognostic significance of survivin expression in diffuse large B-cell lymphomas. *Blood* 2000, 96:1921–1925
34. Das A, Tan WL, Teo J, Smith DR: Expression of survivin in primary glioblastomas. *J Cancer Res Clin Oncol* 2002, 128:302–306
35. Kajiura Y, Yamasaki F, Hama S, Yahara K, Yoshioka H, Sugiyama K, Arita K, Kurisu K: Expression of survivin in astrocytic tumors: correlation with malignant grade and prognosis. *Cancer* 2003, 97:1077–1083
36. Sasaki T, Lopes MB, Hankins GR, Helm GA: Expression of survivin, an inhibitor of apoptosis protein, in tumors of the nervous system. *Acta Neuropathol* 2002, 104:105–109
37. Chakravarti A, Noll E, Black PM, Finkelstein DF, Finkelstein DM, Dyson NJ, Loeffler JS: Quantitatively determined survivin expression levels are of prognostic value in human gliomas. *J Clin Oncol* 2002, 20:1063–1068
38. LaCasse EC, Baird S, Korneluk RG, MacKenzie AE: The inhibitors of apoptosis (IAPs) and their emerging role in cancer. *Oncogene* 1998, 17:3247–3259
39. Deveraux QL, Reed JC: IAP family proteins—suppressors of apoptosis. *Genes Dev* 1999, 13:239–252
40. Tamm I, Wang Y, Sausville E, Scudiero DA, Vigna N, Oltsersdorf T, Reed JC: IAP-family protein survivin inhibits caspase activity and apoptosis induced by Fas (CD95), Bax, caspases, and anticancer drugs. *Cancer Res* 1998, 58:5315–5320
41. O'Connor DS, Grossman D, Plescia J, Li F, Zhang H, Villa A, Tognin S, Marchisio PC, Altieri DC: Regulation of apoptosis at cell division by p34^{cdc2} phosphorylation of survivin. *Proc Natl Acad Sci USA* 2000, 97:13103–13107
42. Yong V, Antel JP: Culture of glial cells from human brain biopsies. *Protocols for Neural Cell Culture*. Edited by S Federoff, A Richardson. New York, Humana Press, 1992, pp 81–96
43. Brummelkamp TR, Bernards R, Agami R: A system for stable expression of short interfering RNAs in mammalian cells. *Science* 2002, 296:550–553
44. Aulesa C, Prieto M, Pinadero M, Mainar I: Apoptosis in infectious mononucleosis: its detection with the Beckman Coulter GenS hematology analyzer. *Lab Hematol* 2005, 11:1–7
45. McPartland JL, Guzail MA, Kendall CH, Pringle JH: Apoptosis in chronic viral hepatitis parallels histological activity: an immunohistochemical investigation using anti-activated caspase-3 and M30 cytodeath antibody. *Int J Exp Pathol* 2005, 86:19–24
46. Kountouras J, Zavos C, Chatzopoulos D: Apoptosis in hepatitis C. *J Viral Hepatol* 2003, 10:335–342
47. Aurelian L: HSV induced apoptosis in herpes encephalitis. *Curr Top Microbiol Immunol* 2005, 289:79–111
48. Kong H, Baerbig Q, Duncan L, Shepel N, Mayne M: Human herpesvirus type 6 indirectly enhances oligodendrocyte cell death. *J Neurovirol* 2003, 9:539–550
49. Blondel B, Colbere-Garapin F, Couderc T, Wirocius A, Guivel-Benhassine F: Poliovirus, pathogenesis of poliomyelitis, and apoptosis. *Curr Top Microbiol Immunol* 2005, 289:25–56
50. Lafon M: Modulation of the immune response in the nervous system by rabies virus. *Curr Top Microbiol Immunol* 2005, 289:239–258
51. Shrestha B, Gottlieb D, Diamond MS: Infection and injury of neurons by West Nile encephalitis virus. *J Virol* 2003, 77:13203–13213
52. Hayashi M, Arai N, Satoh J, Suzuki H, Katayama K, Tamagawa K, Morimatsu Y: Neurodegenerative mechanisms in subacute sclerosing panencephalitis. *J Child Neurol* 2002, 17:725–730
53. Gray F, Adle-Biassette H, Brion F, Ereau T, le Maner I, Levy V, Corcket G: Neuronal apoptosis in human immunodeficiency virus infection. *J Neurovirol* 2000, 6(Suppl 1):S38–S43
54. Kolson DL, Sabnekar P, Baybis M, Crino PB: Gene expression in TUNEL-positive neurons in human immunodeficiency virus-infected brain. *J Neurovirol* 2004, 10(Suppl 1):102–107
55. Ryan LA, Peng H, Erichsen DA, Huang Y, Persidsky Y, Zhou Y, Gendelman HE, Zheng J: TNF-related apoptosis-inducing ligand mediates human neuronal apoptosis: links to HIV-1-associated dementia. *J Neuroimmunol* 2004, 148:127–139
56. Richardson-Burns SM, Kleinschmidt-DeMasters BK, DeBiasi RL, Tyler KL: Progressive multifocal leukoencephalopathy and apoptosis of infected oligodendrocytes in the central nervous system of patients with and without AIDS. *Arch Neurol* 2002, 59:1930–1936
57. Seth P, Diaz F, Tao-Cheng JH, Major E: JC virus induces nonapoptotic cell death of human central nervous system progenitor cell-derived astrocytes. *J Virol* 2004, 78:4884–4891
58. Rogalińska M: Alterations in cell nuclei during apoptosis. *Cell Mol Biol Lett* 2002, 7:995–1018
59. Boldorini R, Cristina S, Vago L, Tosoni A, Guzzetti S, Costanzi G: Ultrastructural studies in the lytic phase of progressive multifocal leukoencephalopathy in AIDS patients. *Ultrastruct Pathol* 1993, 17:599–609
60. Del Valle L, Piña-Oviedo S, Enam S, Khalili K: Expression of anti-apoptotic protein survivin in glial cells of progressive multifocal leukoencephalopathy. 7th International Symposium on Neurovirology, 2006 May 30–Jun 3; Philadelphia, PA. *J Neurovirol* 2004, 10(Suppl 3):68