Expression of Bax, Bcl-2, and P53 in Progressive Multifocal Leukoencephalopathy

Bin Yang, M.D., Ph.D, Richard A. Prayson, M.D.

Department of Anatomic Pathology, The Cleveland Clinic Foundation, Cleveland, Ohio

It has been shown *in vitro* that JC viral protein can form a complex with wild-type p53 protein, which is a key regulator of both cell proliferation and cell death. Cellular factors, Bax and Bcl-2, are two essential downstream elements involved in p53-dependent apoptosis. To determine whether association of JC virus with p53 protein affects the expression of Bax and Bcl-2 in viral-infected cells in progressive multifocal leukoencephalopathy (PML), we studied the expression of Bax, Bcl-2, and p53 in 14 cases from 13 PML patients by using paraffin immunohistochemistry. Seven of 13 patients were known to be HIV positive. Overexpression of p53 was found in viral-infected oligodendrocytes and some astrocytes in all 14 cases. Intense immunostaining of Bax was strongly expressed in viral-infected oligodendrocytes and astrocytes. Bax immunostaining was also found in macrophages in the demyelinating lesions. Bcl-2 was not detected in viral-infected glial cells. The expression pattern of Bax positive/Bcl-2 negative in viral-infected glial cells suggests that the oligodendrocyte may be undergoing apoptosis which may in turn contribute to the demyelinating process in PML. The coexpression of p53 and Bax in the infected glial cells suggests that p53 detected by immunohistochemistry may still maintain its wild-type function.

KEY WORDS: Apoptosis, Bax, Bcl-2, JC virus, p53, Progressive multifocal leukoencephalopathy.

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Progressive multifocal leukoencephalopathy (PML) is a demyelinating disease of the central nervous system that results from infection of oligodendrocytes with predominantly JC virus (JCV), a papovavirus (1). PML occurs predominantly in immunocompromised individuals. The incidence of PML has increased in the past decade, mainly due to increased numbers of AIDS patients (2). His-

topathologically, PML is characterized by demyelination of white matter, abnormal oligodendroglial nuclei, and atypical astrocytes (Alzheimer type I astrocytes) (1).

The mechanism of JCV-mediated demyelination is still not completely understood. Recent molecular biological studies have shed light on the interaction between JCV and the tumor suppressor p53 protein. p53 is an important regulator involved with the processes of cell proliferation and programmed cell death. It has been shown *in vitro* that JCV large T antigen, similar to SV40 large T antigen, can form a complex with wild-type p53 protein (3). *In vitro* studies have demonstrated that such an association between viral large T antigen and wild-type p53 protein abrogates the transcriptional function of the p53 protein (3, 4). p53 protein, stabilized by JCV, can be immunohistochemically detected in JCV-infected oligodendrocytes due to a prolonged half-life (5, 6). Further studies have provided evidence that such loss of p53 function is associated with the overexpression of proliferative cell nuclear antigen (PCNA) and DNA amplification in JCV-infected cells (5, 7). The significance of such an association is not clear, since it is well known that PML is a predominantly demyelinating process.

Recent studies examining other demyelinating diseases, such as multiple sclerosis and spinal cord injury, indicate that oligodendrocyte apoptosis contributes to the loss of oligodendrocytes and may be responsible for the demyelination of axons in the central nervous system (8, 9). Further evidence suggests that p53 may be directly involved in oligodendrocytic apoptosis, which is induced by certain cytokines (10, 11). Apoptosis is a complex cellular process involving multiple factors and steps biochemically. The balance of two cellular protein families, a pro-apoptotic protein Bax and an anti-apoptotic protein Bcl-2, influences a cell's fate through a p53-dependent apoptotic pathway (12). Wild-type p53, but not mutant p53, directly upregulates the transcriptional expression of Bax and indirectly downregulates the expression of Bcl-2 (13–15). Overexpression of Bax and downregulation of Bcl-2 represents an actively apoptotic process in

the cells (12), whereas overexpression of Bcl-2 and downregulation of Bax is frequently detected in neoplastic cells (16). To determine whether human PML is an apoptotic process induced by JCV, we immunohistochemically studied the expression of p53, Bcl-2, and Bax in viral-infected glial cells in 14 cases from 13 PML patients.

MATERIALS AND METHODS

Fourteen formalin-fixed and paraffin-embedded PML specimens, derived from 13 patients (10 males and three females), including 10 biopsies and four autopsies were used in this study. One case had both biopsy and autopsy specimens available. The patients' mean age was 48 years (range, 29 to 79 years). Seven patients were known to be HIV positive, three patients had non-Hodgkin's lymphoma and had completed chemotherapy, one patient had Hodgkin's lymphoma and had completed chemotherapy, one patient had systemic lupus erythematosus with long-term immunosuppressive therapy, and one patient had renal cell carcinoma requiring regular hemodialysis (Table 1).

Tissue was routinely fixed in 10% buffered formalin with about 12 hours fixation for biopsy specimens and about 2 weeks fixation for autopsy specimens. Paraffin-embedded tissue sections were stained with either hematoxylin-eosin (biopsy specimens) or with modified Kluver Barrera (autopsy specimens). In nine cases, an ultrastructural evaluation was performed at the time of initial diagnosis.

Immunohistochemical analysis of p53, Bcl-2, and Bax expression was performed with a labeled streptavidin-biotin system by means of an automated immunostainer (Ventana Medical Systems, Tucson, AZ). After deparaffinization and dehydration in xylene and graded alcohols, the slide was immersed in distilled water, and the section was transferred to an acetate buffer solution for antigen retrieval at pH 7.0 and microwaved for 15 min. The

sections were then incubated with a mouse monoclonal anti-p53 (DO7, 1:10, DAKO Corporation, Carpinteria, CA), a rabbit polyclonal anti-Bax (1:25, DAKO), and a mouse monoclonal anti-Bcl-2 (Ventana Medical Systems). Negative controls consisted of equivalent non-immune mouse immunoglobulin G. Sections were sequentially incubated with biotinylated anti-mouse immunoglobulin G, streptavidin-horseradish peroxidase, with a chromogenic substrate aminoethylcarbazole, and hematoxylin counterstaining, with intervening buffer washes. Reddish brown nuclear staining was interpreted as being positive for p53; perinuclear cytoplasmic staining was interpreted as being positive for Bcl-2; and cytoplasmic staining was interpreted as being positive for Bax. The positive control for p53 staining was a resection specimen of human colon adenocarcinoma. Specimens used as negative controls for p53 included one case of non-necrotizing granulomatous inflammation and one case of herpes simplex encephalitis. The positive control used for Bax was a normal human colon mucosa. Positive control for Bcl-2 was a tonsil with lymphoid hyperplasia.

RESULTS

Histopathology of PML Lesions

Gross examination of four autopsy brains revealed multiple tan-gray soft lesions situated in the cerebral hemispheric white matter. The cortex and deep gray matter were spared. Microscopically, at least one demyelinating focus was identified in each of the biopsy cases. Multiple foci of demyelination were seen in all four autopsy cases and some of the biopsy specimens. Typically, demyelinating lesions exhibited a relatively hypocellular center and was sometimes marked by the formation of microcysts. An abundance of oligodendrocytes with enlarged nuclei and Cowdry A inclusions were usually found

TABLE 1. Clinical Data of Patients with Progressive Multifocal Leukoencephalopathy

Case No.	Age/Sex	Specimen	Location	Clinical Association
1	52/M	Biopsy and autopsy	L frontal lobe, brain stem and spinal cord	CLL, s/p chemotherapy
2	64/M	Biopsy	L frontal lobe	lymphoma
3	46/M	Biopsy	R occipital lobe	HIV positive
4	32/M	Biopsy	L frontal lobe	HIV positive
5	79/M	Biopsy	L occipital lobe	RCC, essential thrombocytosis
6	71/F	Biopsy	R parietal lobe	renal transplantation, s/p hemodialysis
7	35/M	Biopsy	R occipital lobe	HIV positive
8	35/M	Autopsy	bil frontal lobes	HIV positive
9	71/F	Autopsy	frontal and occipital lobes, basal ganglion	SLE, s/p steroid treatment
10	68/F	Biopsy	R frontal lobe	CLL, s/p chemotherapy
11	44/M	Biopsy	L frontal lobe	HIV positive
12	39/M	Autopsy	cerebral peduncle	Hodgkin's disease
			brain stem	HIV positive
13	29/M	Biopsy	R frontal lobe	HIV positive

M, male; F, female; R, right; L, left; bil, bilateral; s/p, status post; SLE, systemic lupus erythematosus; CLL, chronic lymphocytic lymphoma; HIV, human immunodeficiency virus; RCC, renal cell carcinoma.

at the peripheral zone of the lesions (Fig. 1A). Atypical astrocytes (Alzheimer I astrocytes) with enlarged and hyperchromatic nuclei were often intermixed. Prominent numbers of infiltrating macrophages were observed in foci of demyelination. Perivascular lymphocytic infiltrates were often present. The surrounding uninvolved white matter and adjacent gray matter were microscopically unremarkable. There was no significant morphologic difference between HIV positive cases and HIV negative cases.

Expression of P53 Protein

Strong nuclear p53 immunostaining was seen in infected oligodendrocytes in all cases. In well-formed demyelinating lesions, p53-positive oligodendrocytes were usually distributed at the peripheral zone of the lesions (Fig. 1B). Atypical astrocytes adjacent to the PML lesions were positive for p53 in eight cases. Macrophages within the demyelinating lesion were negative for p53. Oligodendrocytes and reactive astrocytes from two control cases, including herpes simplex encephalitis and non-

necrotizing granulomatous inflammation, were negative for p53.

Expression of Bax

Bax immunostaining was not observed in uninfected oligodendrocytes and astrocytes in uninvolved white matter. In contrast, strong cytoplasmic immunostaining of Bax was observed in the majority of the enlarged infected oligodendrocytes in all the demyelinating lesions of the PML (Fig. 1C). The number of Bax-positive oligodendroglial cells correlated with the number of p53-positive cells. Bax immunostaining was also observed in macrophages infiltrating in foci of the demyelination (Fig. 1C).

Expression of Bcl-2

No immunostaining with Bcl-2 was observed in either infected or uninfected oligodendrocytes and astrocytes (Fig. 1D). However, Bcl-2 immunostaining was observed in the infiltrating lymphocytes, both infiltrating within the lesion and surrounding

FIGURE 1. Expression of p53, Bax, and Bcl-2 in PML. A PML lesion in the white matter shows enlarged oligodendroglial nuclei with intranuclear inclusions at the peripheral zone of the lesion in hematoxylin and eosin stained brain tissue (A) at 200 $\times$ magnification. Immunohistochemical staining of the PML lesion with antibodies to p53 (B), Bax (C), and Bcl-2 (D) at 200 $\times$ magnification. Strong expression of p53 was detected in JCV-infected oligodendrocytes in B. C shows the expression of Bax in infected oligodendrocyte (**solid black arrow**), reactive macrophage (**white arrow**), and some atypical astrocytes. Expression of Bcl-2 was only detected in lymphocytes surrounding the perivascular space shown in D.

the perivascular space (Fig. 1D). Morphologically, these Bcl-2-positive lymphocytes were small with a smooth nuclear contour. Macrophages within the demyelinating lesions did not stain for Bcl-2.

DISCUSSION

PML is a demyelinating disease resulted from infection of oligodendrocytes with predominantly JCV, although other viruses (such as HHV6) may also play a role in the pathogenesis of PML (17). Histopathologically, PML is characterized by multiple areas of demyelination, enlarged oligodendroglial nuclei with basophilic viral inclusions, and Alzheimer type I astrocytes (1). Oligodendrocytes are the main myelin producing cells in the central nervous system (CNS). The mechanism of JCV-mediated demyelination in PML is yet unknown. It has long been speculated that JCV targets oligodendroglial cells and the gradual lytic destruction of oligodendrocytes contributes to the demyelination that characterizes the pathology.

Recent studies in other CNS demyelinating diseases have indicated that demyelination is partly the result of oligodendroglial cell apoptosis derived from a variety of causes (8, 9, 18). *In vitro* studies have demonstrated that upon exposure to growth stimulation, mature oligodendrocytes overexpressed PCNA and *c-fos* genes, but failed to divide, and ultimately underwent cell death by apoptosis (19). P53 plays an important regulatory role in programmed cell death in the central nervous system (11). In particular, p53 has been directly implicated in the process of oligodendrocyte apoptosis (10). It is believed that the intracellular balance of two apoptosis-related proteins, Bcl-2 and Bax, regulates the cell proliferation and cell death (12). Evidence from animal models likewise demonstrates that the balance of Bcl-2 and Bax is also critical in determining cell death in the CNS (20–22). Bcl-2 is an anti-apoptosis protein whose overexpression contributes to the uncontrollable proliferation of gliomas and other neoplasms (21, 23). In contrast, Bax, an antagonist of Bcl-2, is an apoptosis promoter and its overexpression often results in cellular apoptosis (21, 23, 24). It has been shown that wild-type, but not mutant, p53 protein directly regulates the transcriptional expression of Bax (15), and indirectly regulates the expression of Bcl-2 (13, 14). In PML, it has been demonstrated *in vitro* that the large T antigen of JCV can form a protein-protein complex and result in an accumulation of p53 protein that can be detected by immunohistochemical staining (5–7). Whether p53 protein in viral-infected oligodendrocytes totally loses its wild-type function or PML results from oligodendrocyte apoptosis, through either p53-dependent or p53-independent pathways, has not been elucidated.

We studied the expression of p53, Bcl-2, and Bax in 13 patients with PML by immunohistochemistry. Overexpression of p53 was mainly found in viral-infected oligodendrocytes in all the cases and occasionally in atypical astrocytes. Interestingly, overexpression of Bax was also detected in the infected oligodendrocytes at the edge of demyelinating lesions, whereas no expression of Bcl-2 was detectable either in the infected oligodendrocytes or in atypical astrocytes. Such an overexpression of Bax and downexpression of Bcl-2 pattern may indicate that the viral-infected glial cells are in an active apoptotic state and may eventually undergo cell death through apoptosis. Our results are consistent with *in vitro* studies of Bax/Bcl-2 in other demyelinating diseases (25–28).

Interestingly, overexpression of both p53 and Bax were observed in the infected oligodendrocytes. This finding suggests that p53 protein, although complexed with the virus in PML, may still maintain its wild-type function and promote apoptosis through the upregulation of Bax. The functional status of the p53 in JCV-infected cells was further supported by the evidence that p53 is able transcriptionally to inhibit JC virus replication *in vitro* (4). It is possible that upon JCV infection, p53 overexpresses itself in response to the cellular stress and the high level of p53 expression, halting viral replication and promoting apoptosis through upregulating Bax and downregulating Bcl-2 in infected cells. The new balance of increasing Bax and decreasing Bcl-2 may lead oligodendrocyte apoptosis and demyelinating lesion formation. However, whether JCV-infected oligodendrocytes are truly apoptotic needs to be further confirmed by other apoptotic markers or analysis, such as an anti-caspase-3 immunostaining, TUNEL or ISEL methods.

The relationship between JCV and p53 may be more complex than our current understanding because p53 is also a crucial regulator of cell proliferation and malignant transformation (29). *In vitro* studies show that JCV large T antigen interacts with the same domain of p53 protein as SV40 T-antigen (4). SV40 can transform cultured cells *in vitro* by inactivating the function of the p53 (3). Whether JCV T-antigen functionally blocks the p53-mediated inhibitory effect on cell growth is still unknown. A recent study showed that the accumulation p53 protein in oligodendroglial cells was associated with the overexpression of a PCNA in some of the PML cases studied (5). This observation has led some to speculate that inactivation of p53 protein by JCV may allow cells to disorderly engage in DNA synthesis as a prelude to mitosis and proliferation (5, 7, 30). However, the interpretation of the overexpression of a so-called “cell proliferation marker” should be made with caution. It has been shown that not all the cells expressing Ki-67 antigen were

actively proliferating cells because many cell-cycle arrested cells, expressing both wild-type p53 and a cyclin-dependent kinase inhibitor p21, also showed Ki-67 positivity (31). In the presence of tumor suppressor gene *p53*, overexpression of oncogenes and viral oncoprotein may induce cell death rather than cell proliferation (32–34). Our finding of Bax +/Bcl-2 - expression pattern in infected oligodendrocytes further suggests that these cells are most likely in an active apoptotic state rather than a proliferative state.

Overexpression of Bax and undetectable Bcl-2 was also observed in macrophages involved in the demyelinating process in PML. Although little is yet known regarding the functional significance of Bax and Bcl-2 expression in the microglial/macrophage lineage, marked changes in the pattern of their expression are likely to alter the life span of these reactive macrophages. Evidence of the role of Bax/Bcl-2 families in the apoptotic pathway of macrophages/monocytes has been recently studied in human atherosclerotic plaques, giant cell granulomas and osteopetrosis (35–40). It is possible that these macrophages, after engulfing the degraded myelins, undergo apoptosis, and apoptotic macrophages may further enhance the development of demyelinating lesions by releasing cytokines. Recently, Krajewski *et al.* (21) reported that macrophages and microglial cells were more vulnerable to apoptosis in pediatric patients with HIV encephalitis than those without encephalitis. Because most PML patients are either HIV positive or had immunosuppressive conditions, it would be interesting to know whether immune status influences the life span of microglial cells in the CNS.

In summary, our findings of overexpression of Bax and downexpression of Bcl-2 pattern in viral-infected oligodendroglial cells suggest that oligodendrocyte may be undergoing apoptosis, which may contribute to the amount of demyelination in PML. The overexpression of both p53 and Bax in the infected oligodendroglial cells also suggests that p53 detected by immunohistochemistry may still maintain its wild-type function. Further studies with apoptosis labeling markers in PML lesions and studies of the functional status of p53 in PML will help us to understand the molecular mechanism of human PML.

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