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Immunohistochemical Localization of Bcl-2 in the Spinal Cords of Rats with Experimental Autoimmune Encephalomyelitis

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Abstract

We examined the localization of the anti-apoptotic molecule Bcl-2 in the spinal cords of Lewis rats with experimental autoimmune encephalomyelitis (EAE). Western blot analysis showed that Bcl-2 was constitutively expressed in normal spinal cords, and weakly increased in response to complete Freund's adjuvant(CFA) immunization. In EAE, with infiltration of inflammatory cells into spinal cords, Bcl-2 declined during the peak stage and further decreased during the recovery stage. Immunohistochemically, some neurons and glial cells constitutively expressed Bcl-2 in normal rat spinal cords. In the spinal cords of rats with EAE, Bcl-2 was also immunoreacted in some perivascular inflammatory cells while some brain cells, such as neurons and GFAP (+) astrocytes showed less Bcl-2 immunoreaction.

These findings suggest that in EAE, Bcl-2 expression in the CNS host cells decreases with CNS inflammation, possibly progressing to cell death in some cases, while the survival of host cells, including neurons, astrocytes, and some inflammatory cells, is associated with activation of the anti-apoptotic molecule Bcl-2. Taking all into considerations, its is postulated that Bcl-2 either beneficially or detrimentally functions in some host cells depending on the activation stage of each cell type.

Key words : apoptosis, autoimmune encephalomyelitis, Bcl-2

Introduction

Experimental autoimmune encephalomyelitis (EAE) is an autoimmune disease of the central nervous system (CNS) that is used to study human demyelinating diseases such as multiple sclerosis [2, 12]. The clinical course of EAE is characterized by weight loss, ascending progressive paralysis, and finally spontaneous recovery. These steps are matched by the inflammatory response in the CNS, which is characterized by the infiltration of T cells and macrophages, and the activation of microglia and astrocytes at the peak stage [13, 16]. Apoptosis is one possible mechanism for the recovery in EAE, because invading cells are eliminated through apoptosis during the peak stage [1, 5, 10,15]. Apoptotic cells are found mainly in the parenchyma, where many apoptosis-related molecules are found, including p53 and Bax, while they are rarely found in perivascular EAE lesions [8]. Brain cells can survive in the CNS of EAE rats, despite the increased infiltration of inflammatory cells and the resulting secretion of many cytokines and cyto-toxic molecules [6, 11].

Bcl-2 is an anti-apoptotic molecule that is normally expressed in neurons and cancer cells [14, 17]. Although Bcl-2 is expressed in multiple sclerosis lesions [19] and its animal model EAE [3, 18], little is known about the localization of Bcl-2 in rat EAE in relation to escape from apoptosis in host and some inflammatory cells.

In this study, we examined the distribution of the anti-apoptotic molecule Bcl-2 in EAE lesions of the spinal cord in Lewis rats, and studied the relationship between the distribution of this molecule and apoptosis.

Material and Methods

Animals

Lewis rats of both sexes (7-12 weeks old) were obtained from the Korea Research Institute of Bioscience and Biotechnology, KIST (Daejeon, Korea) and bred in our animal facility.

EAE induction

EAE was induced in Lewis rats with a slight modification of a previously described method [16]. Briefly, each rat was subcutaneously injected in the hind footpads bilaterally with an emulsion containing equal parts of fresh rat spinal cord homogenates in phosphate buffer (mg/ml) and complete Freund's adjuvant (CFA; *Mycobacterium tuberculosis* H37Ra,

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mg/ml)(Difco, Detroit, MI, U.S.A.). On the day of immunization, the rats were injected with 2 g of pertussis toxin intraperitoneally (Sigma, St. Louis, MO, U.S.A.). Control animals received either CFA or pertussis toxin only. Immunized rats were observed daily for clinical signs of EAE. Clinically, EAE was separated into five stages (grade 0, no signs; grade 1, floppy tail; grade 2, mild paraparesis; grade 3, severe paraparesis; grade 4, tetraparesis or moribund condition [10, 16].

Tissue sampling

Tissue samples were taken on days 10-14 and 21-25 post-immunization (PI), during the peak and recovery stages of EAE, respectively. Experimental rats (n=3) in each group were sacrificed under ether anesthesia, and the spinal cords were removed and frozen in a deep freezer (-70 C) for protein analysis. Pieces of the spinal cords were processed for paraffin embedding after fixation in 4% paraformaldehyde in phosphate-buffered saline (PBS), pH 7.4.

Western blot analysis

Frozen nervous tissue was thawed at room temperature, minced, lysed in a buffer consisting of 40 mM Tris-HCl, pH 7.4, 120 mM NaCl, and 0.1% Nonidet P-40 (polyoxyethylene (9) p-t-octyl phenol) containing the protease inhibitors leupeptin (0.5μg/ml), PMSF (1 mM), and aprotinin (5 μg/ml), and homogenized. Equal amounts of protein (200 g/20 μl) were loaded in each lane, and electrophoresed under denaturing conditions in sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE). After electrophoresis, the proteins were electrotransferred onto nitrocellulose transfer membranes (Schleicher and Schuell, Keene, NH). Blotting with rabbit anti-Bcl-2 (1:200 dilution, Santa Cruz, CA) was performed as described in a previous paper [8]. Visualization was achieved using Amersham ECL reagents (Amersham Life Science, Little Chalfont, Buckinghamshire, UK). The results were quantified with a densitometer (M GS-700 Imaging Densitometer, Bio-Rad Laboratories, Hercules, CA).

Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end-labeling (TUNEL)

DNA fragmentation was detected by *in situ* nick end-labeling, as described in the manufacturer's instructions (Intergen, Purchase, NY). In brief, the paraffin sections were deparaffinized, rehydrated, and washed in PBS. The sections were treated with 0.005% pronase (Dako, glostrup, Denmark) for 20 minutes at 37℃ and subsequently incubated with TdT buffer solution (140 mM sodium cacodylate, 1 mM CoCl, 30 mM Tris-HCl, pH 7.2) containing 0.15 U/μL TdT and 0.004 nmol/L digoxigenin-dUTP for 60 minutes at 37℃, and then in TB buffer (300 mM NaCl, 30 mM sodium citrate) for 15 minutes. They were then reacted with peroxidase-labeled anti-digoxigenine antibody for 60 minutes. Positive cells were visualized by using a diaminobenzidine substrate kit and counterstained with hematoxylin.

Immunohistochemistry

Staining followed the labeled-streptavidin-biotin (LAB-SA) method (Histostain®-*Plus* Kits, Zymed Laboratories Inc, San Francisco, CA) according to the manufacturer's instructions. In brief, 5-m-thick sections of paraffin-embedded spinal cords were deparaffinized and treated with 0.3% H_2O_2 in methyl alcohol for 20 minutes to block endogenous peroxidase. The sections were exposed to normal goat serum, and then incubated in optimally diluted primary antisera including rabbit anti-Bcl-2 (1:200 dilution, Santa Cruz Biotechnology, Santa Cruz, CA) for 1 hour at room temperature. To distinguish cell types in the CNS, either rabbit anti-GFAP serum (1:800, Dako) specific for astrocytes or ED1 for macrophages was applied to adjacent sections. The peroxidase was developed with diaminobenzidine- H_2O_2 solution (0.001% 3,3′-diaminobenzidine [Sigma] and 0.01% H_2O_2 in 0.05 M Tris-buffered saline [TBS, pH 7.4]). The sections were counterstained with hematoxylin before mounting.

Results

Western blot analysis of Bcl-2 in EAE

Bcl-2 was constitutively expressed in the normal rat spinal cord (Fig. 1, lane 1), and expression increased in response to immunization with CFA (Fig. 1, lane 2). The degree of Bcl-2 expression in the early stage of EAE (G1, day 9 PI) (Fig. 1, lanes 3 and 4) was the same as in the

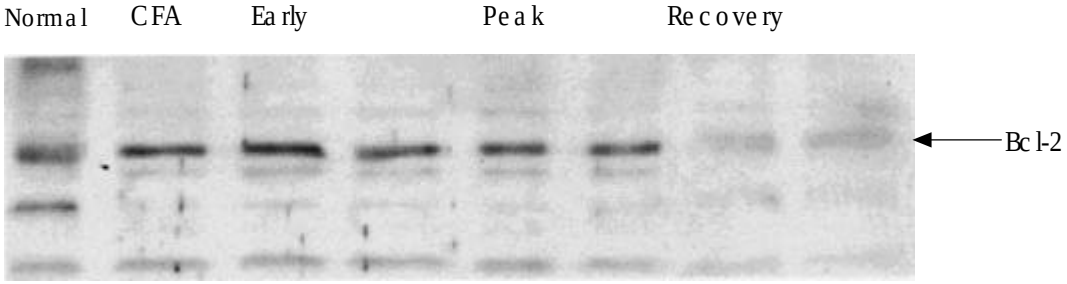

Fig. 1. A representative photograph of Western blot analysis of Bcl-2 in the spinal cord in normal, CFA-immunized, and EAE rats: lane 1; normal, lane 2; CFA immunized (day 9 PI), lanes 3 and 4; EAE (G1, day 9 PI), lanes 5 and 6; EAE (G3, day 12 PI), lanes 7 and 8; EAE (R0, day 21 PI).

Table 1. Bcl-2 immunoreactivity in cell phenotypes of the spinal cords of normal and EAE rats

	Normala	CFA	EAE(G3, day 12PI)a	EAE(R0, day 21 PI)a
Neurons	+++	+++	+	+
Astrocytes	++	++	+	+
Ependymal cells	+	+	+	+
Macrophages/activated microglia	−	−	++	+
T-cells	−	−	++	+

a Three to five animals were examined in each group.

b Normal and EAE spinal cord sections were analyzed using an apoptosis detection kit and immunohistochemistry was examined using antibodies to detect specific markers. Stained sections were scored according to the number of cells per field that were positive. The number of positive cells in an average of five randomly chosen 100 fields was scored as: −, no positive cells; +, <10 cells per field; ++, <30 cells; +++, 30 cells.

CFA-immunized group on the same day (day 9) (Fig. 1, lane 2). Surprisingly, Bcl-2 expression declined at the peak stage (day 12 PI, G3) (Fig. 1, lanes 5 and 6), and further decreased during the recovery stage (day 21 PI, R0) (Fig. 1, lanes 7 and 8). This suggests that Bcl-2 is constitutively expressed in normal adult CNS tissues, and its expression may increase in response to peripheral stimulation, such as immunization with CFA. However, relative amount of Bcl-2 expression decreases in the EAE affected spinal cords, suggesting that some cells exhibit less Bcl-2.

Distribution of Bcl-2 immunoreactivity and TUNEL reaction in EAE lesions

Bcl-2 was expressed in some neurons and glial cells in the normal rat spinal cord (Fig. 2A). In EAE lesions, Bcl-2 immunoreactivity was found in some inflammatory cells in the perivascular cuffing, rather than in those in the parenchyma, as well as in some neurons and glial cells (Fig. 2B). Table 1 summarizes the expression of Bcl-2 by cell phenotype. The intensity of Bcl-2 staining in neurons in EAE was weaker than in neurons in the normal and CFA-immunized groups. It suggests that the decreased expression of Bcl-2 in EAE by western blot might come from the less immunoreactivity of Bcl-2 in host cells, or the amount of Bcl-2 expression in normal spinal cords overwhelm those of both host cells and inflammatory cells in EAE lesions.

The localization of the TUNEL reaction (Fig. 3) was inversely related to Bcl-2 immunoreactivity (Fig. 2B) in EAE lesions. In EAE, TUNEL (+) apoptotic cells were scattered throughout the spinal cord parenchyma, but were rarely found in perivascular lesions (Fig. 3), as previously shown [8]. Moreover, the TUNEL reaction was barely seen in neurons and glial cells (Fig. 3), suggesting that host cells escape death in autoimmune CNS inflammation.

Discussion

Bcl-2 is a survival molecule that allows neuronal cells to survive *in vitro* [7], and is an anti-apoptotic factor in primary carcinoid tumors [20]. Consistent with previous findings, multiple sclerosis lesions were found to contain Bcl-2-expressing T lymphocytes, which may continuously injure brain tissues [19]. Previously, it was reported that in EAE, astrocytes and oligodendroglial cells expressing Bcl-2 do not undergo apoptosis [2]. Furthermore, the lack of apoptosis in perivascular cuffing in EAE is caused by the generation of superoxide in invading macrophages, at least in part [4].

Our results suggest that the apoptosis of inflammatory cells in EAE parenchyma cells lacking anti-apoptotic Bcl-2 requires additional molecules from the apoptosis cascade. In this study, in EAE spinal cords, the TUNEL reaction was barely seen in neurons and glial cells that showed Bcl-2 immunoreactivity, suggesting that the host cells escape death in autoimmune CNS inflammation. This is one possible reason why brain cells, including neurons and glial cells, survive autoimmune injury. Our finding that some inflammatory cells do not undergo apoptosis in perivascular lesions suggests that in these cells, during the peak stage of EAE, anti-apoptotic Bcl-2 predominates, rather than the cytotoxic effectors. Similar findings are consistently seen in cancer cells [17] and multiple sclerosis lesions [3]. Our findings are further supported by the observation that effector cells, such as oligodendroglial cells expressing many death signals, including Fas, do not undergo apoptosis in the murine EAE model, while homing inflammatory cells are selectively vulnerable to the death signals associated with apoptosis [2]. Moreover, Offen et al. [9] reported that, in MOG-induced EAE, Bcl-2 reduces axonal damage and attenuates the clinical severity.

In conclusion, we hypothesize that the anti-apoptotic molecule Bcl-2 allows the survival of host cells and perivascular inflammatory cells in autoimmune CNS inflammation, while inflammatory cells in the parenchyma undergo apoptosis because they lack survival genes.

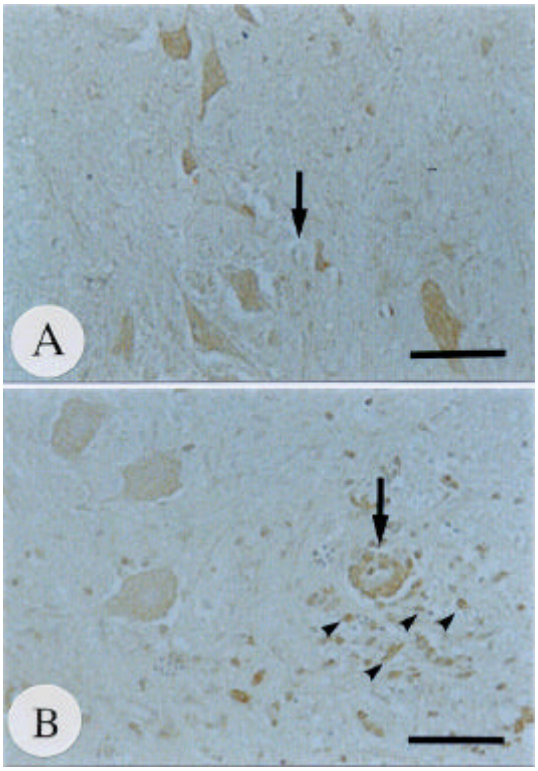

Fig. 2. Immunostaining for Bcl-2 (A, B) in EAE. Intense Bcl-2 immunoreactivity is found in neurons and glial cells in the spinal cords of normal rats (A). In EAE, intense Bcl-2 immunoreactivity is present in perivascular clusters (arrowhead) of inflammatory cells and weak immunoreactivity is seen in some neurons in the parenchyma during the peak stage (B). Arrows indicate blood vessels. Scale =30㎛. B; EAE (G3, day 12 PI).

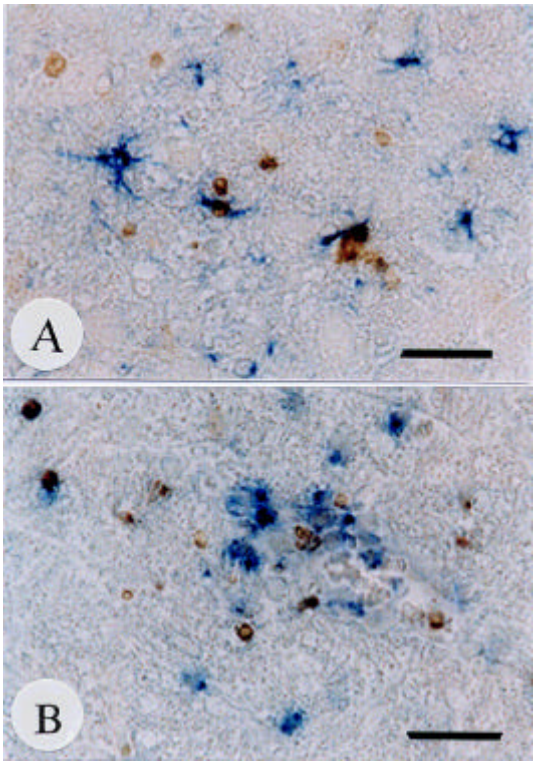

Fig. 3. TUNEL reaction in EAE lesions. TUNEL (+) Apoptotic cells were scattered throughout the parenchyma of the spinal cords of rats with EAE. The TUNEL reaction was commonly present around neurons and some GFAP (+) processes identical to astrocytes (A). Some apoptotic cells were colocalized with ED1 (+) cells (B). TUNEL and ABC-alkaline phosphatase reaction. A and B: TUNEL and either GFAP (A) or ED1 (B). Scale = 30㎛. B; EAE (G3, day 12 PI).

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