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자기면역성뇌척수염에서 apoptosis 유발인자 p53와 apoptosis 억제인자 Bcl-2의 발현

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Increased expression of p53 and Bax in the spinal cords of rats with experimental autoimmune encephalomyelitis

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Abstract

The expression of pro-apoptotic molecules, p53 and Bax, in the spinal cord of rats with experimental autoimmune encephalomyelitis (EAE) was examined. Apoptosis was confirmed by the terminal deoxynucleotidyl transferasemediated dUTP nick end-labeling (TUNEL) method. TUNEL (+) apoptotic cells were mainly either ED1 (+) macrophages or T-cells in the parenchyma of EAE. Western blot

analysis showed that both p53 and Bax expression significantly ($p < 0.01$) increased in the spinal cords of EAE rats at the peak stage, and thereafter declined. An immunohistochemical study showed that inflammatory cells (notably T cells) in the parenchyma express p53 and Bax, while brain cells, including neurons and glia, were devoid of these nuclear staining of these molecules. The nuclear expression of p53 largely matches apoptotic cells in the parenchyma of EAE. These findings suggest that pro-apoptotic molecules, p53 and Bax, may play an important role in eliminating T cells in the parenchyma in EAE.

Key words: apoptosis, experimental autoimmune encephalomyelitis, p53, Bax

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 [14]. The clinical course of EAE is characterized by weight loss, ascending progressive paralysis, and then spontaneous recovery. These steps are paralleled 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 [6, 16]. Subsequently, animals with EAE recover from the paralysis.

Programmed cell death, or apoptosis, is a fundamental biochemical process that plays an essential role in normal development and tissue homeostasis [3]. The host also utilizes apoptosis to defend against invading cells in EAE [6, 16]. There is a consensus that during this process Fas-related apoptosis plays an important role in the elimination of T cells in EAE [17]. Although Fas/Fas-L has been known as one of the important apoptosis-pathways in EAE, it is required to induce or activate other factors to reconstitute the response fully [13].

The nuclear phosphoprotein p53 has been characterized as a growth suppressor that is expressed in normal cells [4]. Therefore, the activation of p53 is commonly associated with apoptosis of macrophages [10] and T cells [18]. However, in EAE lesions, little is known on the expression of p53, one of the pro-apoptotic molecules. When DNA damage severely induced by several factors, i.e. nitric oxide, irradiation, serum deprivation, p53 causes apoptosis [8, 10], in part by promoting Bax up-regulation [11, 20].

In this study, we examined the changes of two apoptosis-associated molecules, p53 and Bax in EAE lesions. We found that p53 and Bax are increased in the spinal cords of rats with EAE.

Materials and Methods

Lewis rats of both sexes (7-12 weeks old) were obtained from the Korea Research Institute of Bioscience and Biotechnology, KIST (Taejeon, Korea) and bred in our animal facility. EAE was induced in Lewis rats using a slight modification of a previously described method [16]. Briefly, each rat was subcutaneously injected in the hind foot pads bilaterally with an emulsion containing equal parts of fresh rat spinal cord homogenate in phosphate buffer (mg/ml) and complete Freunds adjuvant (CFA; *Mycobacterium tuberculosis* H37Ra, 5 mg/ml; Difco, Detroit, Michigan). Control animals received CFA only.

Immunized rats were observed daily for clinical signs of EAE. Clinically, EAE is 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 [12].

Tissue samples were taken on days 14 and 21 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 at -70 ℃ prior to 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. The homogenate was electrophoresed under denaturing conditions in sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE) using a discontinuous procedure reported in a previous paper [2, 7]. Immunoblots of mouse anti-p53 and mouse anti-Bax (Santa Cruz Biotechnology, Santa Cruz, CA) were performed as previously described [20]. Immunoreactive bands visualized by developing the blot using Amersham ECL reagents (Amersham Life Science, Buckinghamshire, UK). The results were quantified with a densitometer (M GS-700 imaging Densitometer, Bio-Rad Laboratories, Hercules, CA). Western blots were further analyzed among groups (n = 3) using the post-hoc Student-Newman-Keuls procedure for multiple comparisons. Differences with a p-value < 0.05 were considered significant. Immunostainings of mouse anti-p53 and mouse anti-Bax (Santa Cruz Biotechnology, Santa Cruz, CA) were performed using a Histoplus

immunostaining kit (Vector Laboratories, Burlingame, CA) as outlined in the manufacturers instructions, with slight modifications as previously described [19]. To determine the cell type, either rabbit anti-GFAP (Dakopatte, Copenhagen, Denmark) for astrocytes or ED1 (Serotec, London, UK) for macrophages was applied to adjacent sections. DNA fragmentation was detected by *in situ* nick end-labeling, as described in the manufacturers instructions (Intergen, Purchase, NY).

Results

The clinical course of EAE is shown in Fig. 1. EAE rats immunized with spinal cord homogenates had floppy tails (G1) and severe paresis (G3) on day 14 PI (Fig. 1). All the rats subsequently recovered. Histological examination at the peak stage showed a large number of inflammatory cells infiltrating the perivascular lesions and parenchyma of the spinal cords of rats with EAE (Fig. 2A). No infiltrating cells were detected in the parenchyma of normal and CFA-immunized control spinal cords (data not shown). During the peak stage of EAE, apoptotic cells were scattered throughout the spinal cord parenchyma, but were rarely found in perivascular lesions (Fig. 2B). The TUNEL reaction was minimal in neurons and glial cells, suggesting that host cells escape death in autoimmune CNS inflammation. TUNEL (+) cells in parenchyma rats EAE lesion matched some ED1 (+) macrophages and many CD4 (+) T cells, as reported in a previous study [6]. Little p53 immunoreactivity was seen in normal and adjuvant-sensitized spinal cords(Fig. 3A). p53 immunoreactivity was significantly increased (about 8 fold) in the EAE rat spinal cords at the peak stage (day 14 PI, G3) (O.D. 51.16 12.31, $p < 0.01$) compared with adjuvant sensitized controls (O.D. 6.08 2.32), and declined thereafter (day 21 PI, R0) (Fig. 3A). Bax immunoreactivity was significantly increased (about 5 fold) in the spinal cords of rats with EAE at the peak stage (day 14 PI, G3) (O.D. 33.87 8.65, $p < 0.01$) compared with adjuvant sensitized control rats (O.D. 6.39 1.39), and declined thereafter (day 21 PI, R0) (Fig. 3B).

p53 was weakly expressed on some host cells (especially, neurons) in the normal and CFA-immunized spinal cords (Fig. 4A). It was present mainly in the cytoplasm, as reported in a previous *in vitro* study [5]. In EAE lesions, p53 was seen in some perivascular inflammatory cells, as well as in inflammatory cells in the parenchyma (Fig. 4B), while its expression was increased in neurons in

EAE lesions. In adjacent sections immunostained with ED1 or rabbit anti-GFAP, some p53 (+) cells in the perivascular and parenchymal EAE lesions were ED1 (+) macrophages, and not astrocytes (data not shown). Expression was seen mainly in the nuclei, suggesting that these cells were activated (Fig. 4B). In frozen sections immunostained with R73 (monoclonal anti-rat T cell receptor alpha and beta), small, p53-positive cells seen in the parenchyma were identical to T cells (data not shown). Bax was also seen diffusely in neuroglial cells in normal and CFA-immunized spinal cords (Fig. 4C), but few Bax (+) neurons were found. It was expressed on inflammatory cells in perivascular and parenchymal EAE lesions (Fig. 4D), but the intensity of Bax immunoreactivity was always weaker than that of p53. In an adjacent section stained with the TUNEL method, a few p53 (+) (Fig. 4E) and Bax (+) cells in parenchyma EAE lesions matched TUNEL (+) cells (Fig. 4F).

Discussion

This study is the first report that the intense increase of apoptotic molecules, p53 and Bax may regulate inflammatory cell apoptosis in EAE lesion. The tumor suppressor gene p53, which is able to induce growth arrest or apoptosis in cells with DNA damage, is one of the well-known apoptotic molecules found in many cultured cells. Although all cells expressing p53 do not undergo apoptosis, many apoptotic cells show nuclear localization of p53, suggesting that this molecule may be activated [5]. In this study, we found that some brain cells that were not apoptotic expressed p53 in the cytoplasm, while some inflammatory cells showed p53 nuclear staining and were TUNEL positive. This findings suggest that p53 is required for inflammatory cell death in EAE, but is not necessary for apoptosis of all infiltrating cells. Also, this may be further supported by the observation that target cells, such as oligodendroglia expressing several 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 [1]. p53 has been shown to activate the pro-apoptotic gene, including Bax [11]. Bax belongs to Bcl-2 family that the threshold for apoptosis is dictated by ratio of death agonists to antagonists [20]. Bax generally functions as a cell death agonist, and elevated levels of Bax have been shown to promote apoptosis in response to numerous cell

death-inducing stimuli [20]. The relationship between Bax and p53 expression appears to vary with cell type. In this study, we found that the pattern of Bax immunoreactivity was similar to that of p53, and these molecules co-localized in the inflammatory cells of the parenchyma in EAE. In conclusion, we speculate that p53 and Bax may involve apoptosis of the inflammatory cells in EAE as Fas does.

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Figure legends

Fig. 1. Clinical course of EAE in Lewis rats. Injecting 200mg of homogenized rat spinal cord on day 0 induced EAE. The symbol indicates the mean clinical score for a group (n = 3) on the day indicated.

Fig. 2. Histological finding and TUNEL reaction in the spinal cords of rats with EAE. The spinal cords of rats with EAE (G3) showed intercellular edema, perivascular cuffing, and inflammatory cells (A). TUNEL-positive cells were scattered throughout the spinal cord parenchyma, but were rarely found in perivascular lesions (B). A: H-E stain, Magnification: 33; B: Hematoxylin counterstain, Magnification: 132

Fig. 3. Western blot analysis of p53 (A) and Bax (B) in the spinal cords of adjuvant-immunized control rats and EAE rats. Minimal amounts of p53 and Bax were identified in the adjuvant-immunized spinal cords (A and B, lane 1). In the EAE spinal cords, both p53 and Bax were increased in rats with grade 3 paralysis (G3) (A and B, lane 2), but their expression decreased during the EAE recovery stage (R0) (A and B, lane 3). All tissues were collected on day 14 post-immunization with adjuvant or spinal cord tissue.

Fig. 4. Immunohistochemical detection of p53 (A, B, E) and Bax (C, D), and TUNEL reaction (F) in the spinal cords of adjuvant-immunized control rats (A, C), and those at the peak stage of EAE (B, D, E, F). Few p53 (+) glial cells were found in the spinal cords of the adjuvant-immunized control rats, but p53 was weakly expressed on the cytoplasm of some neurons (A). Some p53 (+) reactivity was seen on perivascular inflammatory cells, but it was mainly found in the parenchyma (B). In an adjacent section stained with the TUNEL method, a few p53 (+) cell (E, arrow) in parenchyma EAE lesions matched TUNEL (+) cells (F, arrow). Some Bax glial cells were found in the spinal cords of adjuvant-immunized control rats (C). In EAE, Bax was seen on perivascular and parenchymal inflammatory cells (D). A-F: Hematoxylin counterstain. A: Magnification: 33; B-F: Magnification: 132. All tissues were collected on day 14 post-immunization with adjuvant or spinal cord tissue.