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Bax-ablation attenuates experimental autoimmune encephalomyelitis in mice

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Abstract

Multiple sclerosis (MS) is an inflammatory disease of the central nervous system characterized by demyelination and axonal damage. Although the exact pathophysiology is unknown, apoptosis plays a crucial role. Here, we studied the role of the pro-apoptotic gene Bax in myelin oligodendrocyte glycoprotein (MOG)-induced experimental autoimmune encephalomyelitis (EAE), the animal model for MS. We demonstrate that the clinical signs were markedly reduced in the EAE Bax-deficient mice as compared to wild type (2.3 ± 0.5 vs. 1.02 ± 0.32, respectively, $P < 0.05$). Bax-deficient mice demonstrated less inflammatory infiltration and axonal damage, although they showed similar T-cell immune potency. In conclusion, ablation of the bax gene attenuates the severity of MOG-induced EAE and emphasizes the importance of apoptosis in the pathogenesis of EAE and MS.
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Multiple sclerosis (MS) is a degenerative disease of the central nervous system (CNS) characterized by destruction of myelin with accumulating axonal damage. Apoptosis is believed to be involved in regulating as well as promoting the disease process in MS and in experimental autoimmune encephalomyelitis (EAE) (for review see Ref. [16]). EAE is an inflammatory demyelinating disease of the CNS induced by myelin antigens, which is widely used as a model for MS. The apoptotic process is important in elimination of inflammatory cells such as T-cells and macrophages from the CNS, and is believed to be important in the recovery phase of EAE. Schmied et al. found that up to 49% of T-lymphocytes in EAE lesions showed signs of apoptosis in the recovery phase [18]. Kohji and Matsumoto found coexpression of Fas/FasL and Bax on infiltrating T-cells and microglia in the CNS, which is closely associated with apoptotic cell death during EAE [9]. A recent study found a significant reduction in the expression ratios of pro- to anti-apoptosis Bcl-2 members in peripheral lymphocytes from patients with active MS when compared to corresponding ratios in peripheral lymphocytes from patients with stable MS or other controls [19].

Moreover, CSF from MS patients with hypointense T1 lesions on magnetic resonance imaging caused neuronal apoptosis in culture which correlated with disability and reduced recovery from relapse [2,17]. Furthermore, caspase inhibitors, Ac-YVAD-cmk and Ac-DEVD-cmk, protect against neuronal apoptosis induced by CSF from MS patients [3].

It was also demonstrated that the pro-apoptotic regulators, bax and p53, are elevated and correlated with disease severity in EAE mice [14].

Recent studies have shown that apoptosis may contribute to the death of oligodendrocytes and neurons, a pathological process leading to accumulating neurological deficits. Meyer et al. demonstrate neuronal apoptosis in retinal ganglion cells in a rat model of MS [13]. In our previous study we have shown that transgenic mice over-expressing the anti-apoptotic gene bcl-2 in their neurons under the control of neuron-specific-enolase promoter were more resistant to myelin oligodendrocyte glycoprotein (MOG)-induced EAE than wild type (WT) mice [15]. Similarly, a strong association was found between the presence of bcl-2

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positive oligodendrocytes and the presence of remyelinating brain and spinal cord lesions. The highest proportion of bcl-2 positive oligodendrocytes was found in a subgroup of patients with a more benign, relapsing-remitting disease course [10]. In situ analysis of active MS lesions revealed increased p53 expression in oligodendrocytes in lesions featured by oligodendrocyte apoptosis and cell loss [22]. Moreover, expression of baculovirus p35 caspase inhibitor, an anti-apoptotic protein, protected mice from EAE [6]. Interestingly, transgenic mice that express anti-apoptotic proteins specifically in oligodendrocytes, and caspase-11-deficient mice are significantly more resistant to EAE induction [7].

The objective of this study was to examine the hypothesis that bax ablation in mice would provide protection from MOG-induced EAE. Bax is a member of the Bcl-2 family of cell death regulators, and it is a key promoter of apoptosis. Knock-out mutation of the bax gene in C57/bl mice by insertion of the neomycin resistance sequence into the bax gene sequence made the cells from these mice more resistant to apoptosis as shown in various experimental models [1,4,8]. Bax-deficient and WT mice genotype and phenotype were identified by PCR and Western blotting, respectively, as described previously [8].

EAE was induced by immunization with a peptide of rat MOG, encompassing amino acids 35–55. Synthesis was carried out by the Weizmann Institute Synthesis Unit, using a solid-phase technique on a peptide synthesizer (Applied Biosystems Inc., Foster City, CA). Eighty-six mice, 40 Bax-deficient and 46 WT mice, were injected subcutaneously in the flank with a 200 μl emulsion containing 300 μg MOG peptide in complete Freund adjuvant (CFA) and 500 μg Mycobacterium Tuberculosis (Sigma Israel). The other flank was similarly immunized 1 week later. MOG-treated mice were observed daily for 40 days and symptoms of EAE were scored as follows: 0, no clinical symptoms; 1, loss of tail tonicity; 2, partial hind limb paralysis; 3, complete hind limb paralysis; 4, partial frontal limb paralysis; 5, total paralysis; 6, death [12].

Spinal cords from bax-deficient and WT mice were removed 40 days after immunization with MOG. The tissues were dissected and fixed in 10% buffered formalin and embedded in paraffin. Five-micron thick sections were stained with hematoxylin and eosin (H&E). Bielshowesky's method was used to evaluate axonal integrity [11]. The presence of axonal pathology was supported by immunohistochemistry with anti-non-phosphorylated neurofilament H (SMI-32, 1:100, Sternberg Antibodies USA) [20]. CNPase antibodies (1:10, Sigma), which specifically stain oligodendrocytes and Schwann cells, were used to examine the damage to oligodendrocytes. Assessments of the differences in the histological staining were done using the Image Pro Plus analysis software (Media cybernetics, USA), demonstrated in arbitrary units representing optical density per area.

In order to asses the immune response of bax-deficient mice as compared to WT mice, splenocytes from bax-deficient and WT mice were exposed to several concentrations of MOG (0, 2, 5, 10, 25 μg/ml) and to concanavalin-A for 72 h followed by 24 h of incubation with [^{3}H]thymidine as previously described [5].

Statistical analysis was done using Student's *t*-test and the result was considered significant when $P < 0.05$ (two-tailed).

A statistically significant difference in the clinical severity of EAE was found between bax-deficient mice and WT mice ($P < 0.05$). While WT mice developed severe disease with complete hind limb paralysis (average score 2.3 ± 0.5), bax-deficient mice did not develop the disease, or showed only mild clinical signs (average score 1.02 ± 0.32) (Fig. 1). H&E staining of spinal cord sections from bax-deficient mice demonstrated a significant reduction in the inflammatory infiltrate compared to WT mice (0.051 ± 0.003 vs. 0.077 ± 0.008 arbitrary units representing the pixel area, $P = 0.0007$, Fig. 2). Moreover, axonal damage was reduced in bax-deficient mice compared to WT, as demonstrated by Bielshowesky's silver staining (0.742 ± 0.024 vs. 0.488 ± 0.017, $P = 0.0078$, Fig. 3). Immunohistochemical staining using antibodies against non-phosphorylated neurofilament H (SMI-32) demonstrated a marked reduction in the area stained by SMI-32 in bax-deficient mice compared to WT (0.595 ± 0.072 vs. 0.951 ± 0.107, $P = 0.0056$, Fig. 4). Immunohistochemical staining with anti-CNPase antibodies demonstrated an increased staining in bax-deficient mice but the difference did not reach statistical significance. The absence of the bax gene was irrelevant to the immune response of the induced mice since the immune potency of the strains was similar, as indicated by T-cell proliferative responses to MOG and to concanavalin-A. At MOG concentrations of 5, 10 and 25 μg/ml the [^{3}H]thymidine incorporation ratio was 238%,

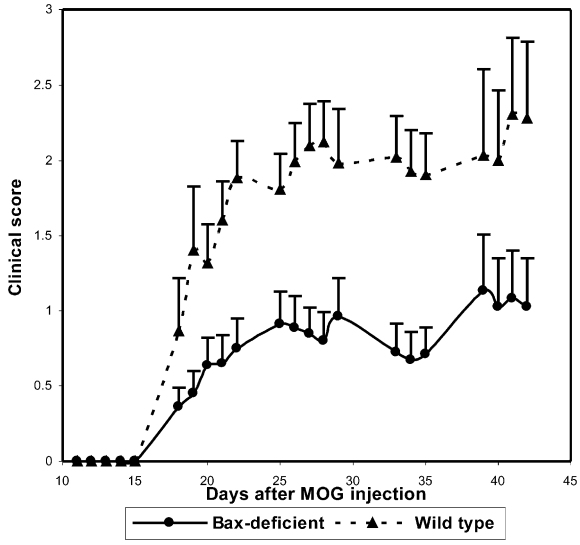

Fig. 1. EAE induction with pMOG 35–55 was attenuated in bax-deficient as compared to WT mice. The mean ± SD daily clinical score for each group ($n = 40$ and 46, respectively) is shown.

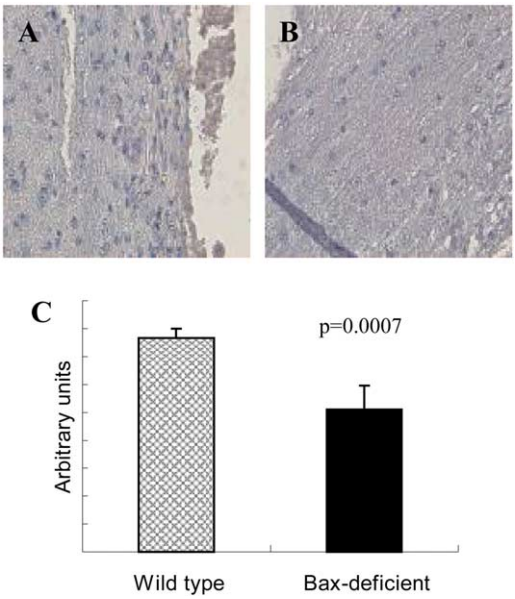

Fig. 2. H&E staining showed less inflammatory infiltration in spinal cord longitudinal sections from MOG-induced EAE bax-deficient mice (B) as compared to WT mice (A). Quantitative assessment of inflammatory infiltration was processed by Image Pro Plus analysis, and demonstrated in arbitrary units which represent optical density per area (C).

321% and 344% of baseline in bax-deficient mice vs. 278%, 273% and 373% of baseline in WT (the differences are not statistically significant).

Thus, this study demonstrates that the clinical signs were markedly reduced in the EAE Bax-deficient mice as compared to WT mice (2.3 ± 0.5 vs. 1.02 ± 0.32, respectively, $P < 0.05$). Bax-deficient mice demonstrated less

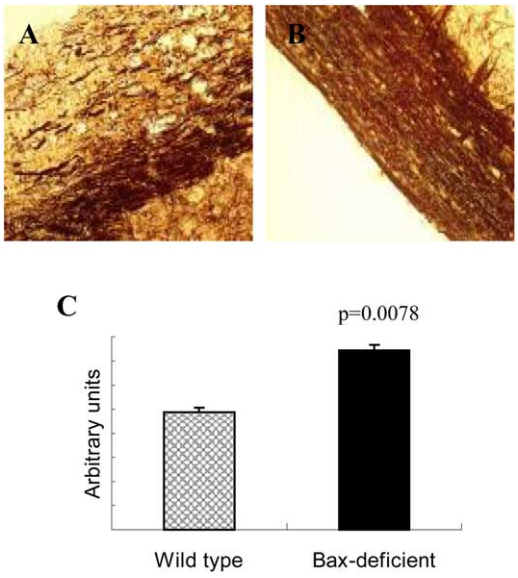

Fig. 3. Bielshowesky's silver axonal staining of MOG-induced EAE demonstrated lower axonal damage in bax-deficient mice (B) as compared to WT mice (A). Quantitative assessment of the stained axons was processed by Image Pro Plus analysis, and demonstrated in arbitrary units which represent optical density per area (C).

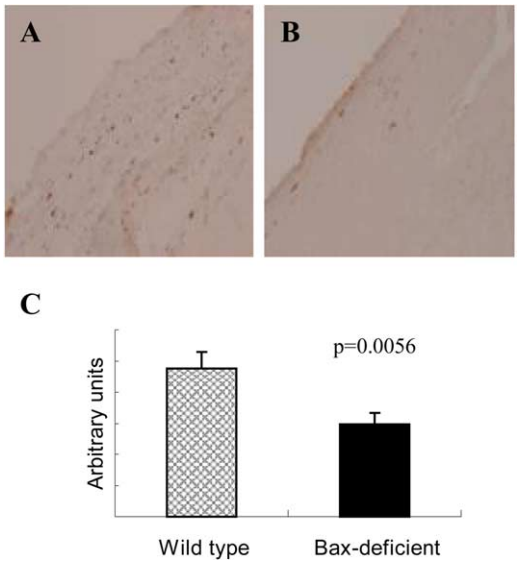

Fig. 4. Dephosphorylated neurofilament H immunostaining of MOG-induced EAE, using SMI-32 antibodies, demonstrated lower axonal damage in bax-deficient mice (B) as compared to WT mice (A). Quantitative assessment of the stained axons was processed by Image Pro Plus analysis, and demonstrated in arbitrary units which represent optical density per area (C).

inflammatory infiltration and axonal damage, although they showed similar T-cell immune potency.

The role of apoptosis, as well as bax, in EAE and MS is not yet clear. However, a protective effect of bax deficiency in EAE induction was clearly demonstrated in this study. The apoptotic process has a dual role in EAE and in MS. On one hand apoptosis eliminates the inflammatory cells and terminates the immune attack, and on the other hand it causes apoptotic death of oligodendrocytes and probably neurons, leading to accumulation of damage and disability. Although the mechanism by which bax deficiency protects from EAE is not yet understood, it might exert its action by attenuating the apoptotic cell death of oligodendrocytes and neurons which results in reduction of the inflammatory process and associated axonal damage.

Dong et al. found that oligodendrocytes cultured from bax-deficient mice are resistant to apoptosis, induced by cyclosporin-A or staurosporine, but not to cytotoxic necrosis induced by exposure to kainate. They also found that oligodendrocytes, but not neurons, better survive spinal cord hemisection in bax-deficient mice [4]. In a previous study using the same bax-deficient mice in a 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) model for Parkinson's disease, the neurons were much more resistant to apoptosis in comparison to WT [21].

In our model of bax-deficient mice, as well as in two other EAE models, p35-expressing oligodendrocyte mice [6] and caspase-11-deficient mice [7], the number of infiltrating cells was markedly reduced in the spinal cord. These findings suggest that the inhibition of oligodendrocyte apoptosis may result in inhibition of the inflammatory process in EAE, and support the idea that decreasing the

antigenic myelin fraction by inhibiting oligodendrocyte death may decrease immune activation [7]. Since each oligodendrocyte normally myelinates segments of ten to 20 different axons, the reduced apoptosis of oligodendrocytes may protect large areas of axons against secondary damage, as demonstrated by Bielshowesky's silver staining and by SMI-32 immunostaining.

In conclusion, our study suggests that inhibition of apoptotic cell death may reduce the severity of induced chronic EAE, and probably in MS. Our findings support the idea that more selective reagents that will target and protect oligodendrocytes from apoptosis might be a promising strategy for treating MS.

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