Differential expression of Bax and Bcl-2 in the brains of hamsters infected with 263K scrapie agent

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Received 28 February 2000; accepted 13 March 2000

INTRODUCTION

Scrapie, the archetype of a group of diseases known as transmissible spongiform encephalopathies (TSE) or prion diseases, occurs naturally in sheep and goats [1–3]. Its clinical symptoms are characterized by a long latent period, progressive ataxia, tremor, wasting and death [4]. It has been experimentally transmitted to laboratory rodents, and these species have become experimental models of TSE [5,6]. Neuronal cell death is a prominent feature of prion diseases; however, the pathogenesis and the molecular basis of neuronal cell death in these diseases have not been determined.

Cell death may occur by one of two general pathways, apoptosis or necrosis. Since many common neurodegenerative diseases such as Alzheimer’s disease, Parkinson’s disease and amyotrophic lateral sclerosis are characterized by a gradual loss of neurons without obvious inflammatory response, it has been hypothesized that cell death in these disorders is due to apoptosis [7]. However, controversy remains over whether cell death in these diseases is mediated by apoptosis or necrosis. Several results suggest that neuronal cell death in TSE is due to apoptosis [8,9]. Apoptosis was also observed when residues 106–126 of the human prion protein were added to primary neuronal cell cultures [10]. Other studies have suggested that neuronal cell death in scrapie-infected brain could be related to mitochondrial dysfunction induced by oxidative stress, or that the increase of Bax:Bax homodimers by the binding of PrP to Bcl-2 promotes apoptosis [11,12]. In order to ascertain potential therapeutic targets, it is important to clarify the mechanisms leading to neuronal cell death in TSE.

In the current study, we analyzed the levels of expression of Bcl-2 and Bax by RT-PCR, Western blot, and immunohistochemistry of the brains of hamsters infected with the 263K scrapie agent.

MATERIAL AND METHODS

Animal injection and assessment: Six-week-old female golden Syrian hamsters were divided into control and infected groups. Hamsters were inoculated intracerebrally by stereotaxic apparatus (Stoelting Co., USA) with 20 µl of 1.0% brain homogenate in 0.01 M phosphate-buffered saline (PBS). The brain homogenates were prepared from 263K scrapie-infected hamster brains. Control inoculum was prepared from brains of normal hamsters (NHB). After inoculation with the 263K scrapie agent, hamsters developed clinical symptoms (tremor, head-bobbing, ataxia) at 60 ± 5 days. Animals were sacrificed 5–7 days after the

To study the mechanism(s) of neuronal cell death during scrapie infection, we investigated the expression of Bax and Bcl-2 in brains of hamsters infected with 263K scrapie agent. The expression of Bcl-2 mRNA was significantly decreased in the brains of 263K scrapie-infected hamsters compared with controls, whereas the expression levels of Bax mRNA were significantly increased in scrapie-infected brain. The levels of Bax and Bcl-2 proteins in brains of scrapie and control animals reflected the difference in mRNA levels. Immunoreactivity for Bax and Bcl-2 were found predominantly within neurons. In scrapie-infected brains, the number of neuronal cells positive for Bcl-2 was significantly lower in the hippocampal CA3 region and was decreased in the cerebral cortex, whereas the number of neuronal cells positive for Bax was significantly increased in both regions. The possibility that differential regulation of Bax and Bcl-2 expression may play an important role in neuronal cell death induced by scrapie infection is discussed. NeuroReport 11:1–6 © 2000 Lippincott Williams & Wilkins.

Key words: Bax; Bcl-2; Neurodegeneration; Neuronal cell death; Scrapie
appearance of clinical symptoms. Hamsters injected with NHB were harvested at the same time.

**Preparation of brain extracts and Western blot analysis:** Hamsters were sacrificed and brains were removed rapidly. Whole brains were homogenized with a Teflon–glass homogenizer in 9 vol extraction buffer (pH 7.0) containing 0.32 M sucrose, 10 mM Tris–HCl, 1 mM EDTA, 5 mM 2-mercaptoethanol. The homogenates were centrifuged at 1000 x g for 10 min at 4°C, and the supernatants were collected. After centrifugation of the supernatant at 105,000 x g for 60 min at 4°C, the pellet was washed and resuspended with extraction buffer. Protein concentration of this fraction was measured by the method of Lowry [13,14]. SDS-PAGE was performed by the method of Laemmli [15]. After SDS-PAGE, immunoblots were performed by the method of Towbin et al. [16]. Protein samples (50 μg) were subjected to 15% SDS-PAGE. After electrophoresis and transfer, the blotted membranes were incubated for 1 h at room temperature with primary antibody, Bax (Calbiochem, USA) and Bcl-2 (Pharmingen, USA) diluted (1:100) in TBST (Tris-buffered saline; 0.1% Tween-20) containing 5% skim milk. The development of membranes used the chemiluminescence Western blotting system (ECL; Amersham, USA). Results were analyzed by scanning densitometry.

**RT-PCR analysis:** RT-PCR was performed from total RNA of control and 263K scrapie-infected hamster brains. Single strand cDNA synthesis from total RNA (2 μg) was performed using oligo (dT) with AMV reverse transcriptase (Promega, USA). The PCR was conducted using Taq DNA polymerase (Promega, USA). The nucleotide sequences of the primers were: for amplification of Bax, 5'-ACCAAGAAGCTGAGCGAGTGT-C-3' (sense), 5'-ACAA AGATGGTCACGGTGCTGACC-3' (antisense), generating a 374 bp fragment; Bcl-2: 5’-TGCACCTGACGCCTCCTAC-3’ (sense), 5’-AGACAGCCAGGAGAAATCAAAACG-3’ (antisense), generating a 260bp fragment; β-actin: 5’-ACC TTCAACACCCCAGCCATG-3’ (sense), 5’-GGCCATCTCTTGCTCGAAGTC-3’ (antisense) generating a 309 bp fragment. The specificity of each PCR product was confirmed by nucleotide DNA sequencing. Programmable temperature cycling (Techne, UK) was performed according to the following: after an initial time of 1 min for predenaturation at 95°C, PCR was performed using 30 cycles for Bax, Bcl-2 and β-actin with denaturation for 60 s at 94°C, annealing for 45 s at 60°C, and extension for 60 s at 72°C.

**Agarose gel electrophoresis:** Total PCR mixtures were subjected to electrophoresis in 1.8% agarose gels containing ethidium bromide. Separated PCR products were visualized under u.v. illumination.

**Immunohistochemistry:** Immunohistochemical procedures were carried out using the ABC kit (Vector, USA) by a modification of the avidin-biotin-peroxidase method. Briefly, sections (6 μm) of the brain were dewaxed with xylene and hydrated with graded ethanol, and then treated with 0.3% hydrogen peroxide in methyl alcohol for 20 min to block endogenous peroxidase. After washing three times with phosphate buffer, the sections were exposed to normal goat serum, and then incubated overnight at 4°C with primary antibody used at a 1:100 dilution for Bax (Calbiochem, USA) and at a 1:200 dilution for Bcl-2 (Calbiochem, USA). After washing three times with phosphate buffer, the sections were sequentially treated with biotinylated anti-rabbit immunoglobulin and avidin-biotin peroxidase complex, and developed with diaminobenzidine-hydrogen peroxidase solution (0.003% 3,3’-diaminobenzidine and 0.03% hydrogen peroxidase in 0.05 M Tris buffer), and finally counterstained with hematoxylin. The number of Bax and Bcl-2 immunolabeled neurons was counted in the hippocampal CA3 region and the cerebral cortex in five fields of 1 mm² each [17].

**Statistical analysis:** The statistical differences between control and infected groups were calculated by Student’s t-test; data are shown as mean ± s.d. Statistically significant values are shown as *p < 0.05 and **p < 0.01.

**RESULTS**

**Gene expression of Bax and Bcl-2:** We analyzed levels of gene expression of Bax and Bcl-2 in the brains of scrapie-infected and control hamsters by RT-PCR. Amplification using specific primers yielded bands of expected sizes (Bcl-2: 250 bp, Bax: 265 bp, β-actin: 215 bp). The expression of β-actin mRNA was used as a reference.

![Figure 1](image1.png)

**Fig. 1.** The expression of Bax and Bcl-2 mRNA in the brains of control and scrapie-infected hamsters. Each band was shown the amplified products of the expressed mRNAs of Bax, Bcl-2 and β-actin in the brains of 263K scrapie-infected and control hamsters. The expression of β-actin mRNA was used as a reference.

![Figure 2](image2.png)

**Fig. 2.** Western blot analysis of Bax and Bcl-2 protein levels in the brains of control and scrapie-infected hamsters. (a) Bcl-2 immunoreactivity in control (lane 1) and 263K scrapie-infected brains (lane 2). The band is at the predicted molecular size (26 kDa) for Bcl-2. (b) Bax immunoreactivity of control (lane 1) and 263K scrapie-infected brains (lane 2). The band is at the estimated molecular size (21 kDa) for Bax. Graphs at bottomconfined brains (lane 2). The band is at the estimated molecular size (21 kDa) for Bax. Graphs at bottom portion of the figure depict quantitave analysis of density for Bax (n = 5) and Bcl-2 immunoblot bands. Data are expressed as mean ± s.d. *p < 0.05, **p < 0.01.
Fig. 3. Representative photomicrographs showing immunohistochemical analysis of Bax. The Bax-positive neuronal cells in cerebral cortex (a,b) and CA3 region of hippocampus (c,d) in control (a,c) and 263K scrapie-infected brain (b,d), ×400. (e) Graphs at bottom of figure depict quantitative analysis of positive neurons for Bax immunostaining in the cerebral cortex and the CA3 region of hippocampus in 263K scrapie-infected group (n = 5) and control group (n = 5). Data are expressed as mean ± s.d., * p < 0.05, ** p < 0.01.
Fig. 4. Representative photomicrographs showing immunohistochemical analysis of Bcl-2. Bcl-2 positive neuronal cells in cerebral cortex (a,b) and CA3 region of hippocampus (c,d) in control (a,c) and 263K scrapie-infected brain (b,d), ×400. (e) Graphs at bottom of figure depict quantitative analysis of positive neurons for Bcl-2 immunostaining in the cerebral cortex and the CA3 region of hippocampus in 263K scrapie-infected group (n = 5) and control group (n = 5). Data are expressed as mean ± s.d. * p < 0.05, ** p < 0.01.
2, 260 bp; Bax, 374 bp; β-actin, 309 bp). Our results showed that Bcl-2 was expressed at low levels in the brains of scrapie-infected hamsters, whereas there was an increase of Bax mRNA expression in the brains of the scrapie-infected group compared to the control group (Fig. 1). Levels of mRNA of constitutively expressed β-actin were virtually identical in scrapie-infected and control groups (Fig. 1).

**Bax and Bcl-2 protein levels:** The levels of Bax and Bcl-2 protein were measured by Western blots. As shown in Fig. 2, Bcl-2 antibody detected a band at 26 kDa, the predicted mol. wt for the bcl-2 gene product. In the scrapie-infected brains, Bcl-2 protein was present at reduced levels, whereas protein levels of Bax were higher than in controls. The molecular weight for Bax was 21 kDa, which is the expected size. The lower portion of the figure shows densitometric analysis of the Western blots.

**Immunohistochemistry of Bax and Bcl-2:** Immunohistochemical methods were used to examine the distribution of Bax and Bcl-2 in brain tissue of control and scrapie-infected hamsters. Immunoreactivities for Bax and Bcl-2 were found mainly within neurons in both control and scrapie-infected brains (Fig. 3, Fig. 4). Immunoreactivity for Bax increased (Fig. 3), whereas that for Bcl-2 decreased (Fig. 4) within neurons of the hippocampal CA3 region and the cerebral cortex of the scrapie-infected group compared with the staining seen in control animals. The data in Fig. 3 and Fig. 4 were quantitated by analyzing the number of cells that stained positively in randomly chosen areas of 1 mm². The results are shown in the lower portions of Fig. 3 and Fig. 4. The number of stained cells revealed in scrapie-infected brain, Bcl-2 immunoreactivity in neuronal populations was reduced by 20% in the cerebral cortex and 50% in the CA3 region of the hippocampus ($p < 0.05$), while the number of Bax-immunoreactive cells was increased in the cerebral cortex by 2.7-fold and in the CA3 region of the hippocampus by 4-fold. These changes in Bax-immunoreactive cells were significant at $p < 0.05$ and $p < 0.01$, respectively.

**DISCUSSION**

Two modes of cell death can be distinguished: apoptosis and necrosis. In order to assess potential therapeutic targets in TSE diseases, it is important to clarify the mechanisms leading to neuronal cell death. The fact that apoptotic cells are rapidly removed via phagocytosis combined with the fact that the process often concludes in secondary necrosis means that it is difficult to ascertain the cause of initial damage to cells [18]. Nevertheless, apoptosis has been implicated as an important mechanism of cell death in many neurodegenerative diseases [19]. With regard to prion diseases, studies of primary neuronal cultures and neuronal cell lines have shown that PrP peptides can cause apoptosis [10], DNA cleavage, one of the key features of apoptosis, has been reported in scrapie-infected sheep brains [20]. However, the sequence of pathological events that lead to neuronal cell death in scrapie-infected animals is not clear.

In the current study, the major findings are as follows: the products of the proapoptotic gene Bax were detectable in normal hamster brain, but following 263K scrapie infection the expression of Bax was significantly increased. In contrast, the expression of Bcl-2 measured by mRNA and protein was reduced in hamsters clinically affected by scrapie compared to control hamsters. The results of the in vitro studies were confirmed by immunohistological analyses that showed increased staining for Bax in neurons of the cerebral cortex and the hippocampal CA3 region, whereas Bcl-2 staining was reduced in these neuron populations in scrapie-infected hamsters. These findings suggest that the mode of neuronal cell death observed in TSE is apoptosis resulting from differential expression of Bax and Bcl-2.

The cause of differential expression of Bax and Bcl-2 in the brains of scrapie-infected animals is unknown. Since p53 protein is a direct transcriptional regulator that upregulates Bax expression and down-regulates Bcl-2 expression, we suggest that p53 protein is involved in the differential regulation of Bax and Bcl-2 expression [21]. Although an increase of Bax and a decrease of Bcl-2 expression have been detected in scrapie-infected brains, the finding does not prove that apoptosis is the mode of cell death. There are several factors that contribute to this uncertainty. First, in addition to inhibition of apoptosis, Bcl-2 can also inhibit several forms of necrosis [22]. Second, in a previous study we showed that neuronal cell death in the brains of hamsters infected with the 263K scrapie agent is related to structural abnormalities (swelling) of mitochondria, which is a major feature of necrosis [11].

As noted above, the current results do not distinguish whether cells die because of apoptosis or necrosis in TSE. We suggest that neuronal cell death in these diseases may employ both mechanisms in a scenario in which cells initially undergo apoptosis and then are subject to secondary necrosis.

In future studies, we plan to examine signaling cascades activated before DNA fragmentation begins through analysis of expression of p53 and Fas/APO-1, transmembrane receptors, and the relationship to differential expression of Bax and Bcl-2. We will also assess the correlation between these values and the pathological changes in brains of scrapie-infected animals.

**CONCLUSION**

Apoptosis is implicated in neuronal cell death in prion diseases. In this study, we showed that there was an increased expression of Bax and a reduced expression of Bcl-2 in the brains of 263K scrapie-infected hamsters; the findings indicate that the differential regulation of Bax and Bcl-2 expression might be a decisive factor in the process of neuronal cell death in this scrapie strain-host model.

**REFERENCES**

Acknowledgements: We thank Hyoung-Gon Lee for his helpful comments and critical reading of the manuscript. This study was supported by The Hallym Academy of Sciences (1999), Hallym University, Korea.