Original Article

Temporal Correlation of Bax Expression and Axotomy-Induced Motoneuronal Apoptosis in Adult Rats: A Morphometric, Ultrastructural and Immunohistochemical Study

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ABSTRACT

Background and Objective: **As apoptotic cell death is extremely involved in physiological development and many pathological situations such as cancer and neurodegenerative diseases, the understanding of its molecular machinery can be useful in designing new therapeutic strategies. The present study investigated the temporal expression of the proapoptotic protein Bax in adult spinal motoneurons.**

Materials and Methods: **Following unilateral mid-thigh sciatic transection in adult rats, the incidence and nature of spinal motoneuron loss were evaluated by means of light microscopic cell count and electron microscopy 1 day, 1 week, 1 month and 3 months post-operatively. In all groups the temporal expression of Bax was immunohistochemically determined and the findings were compared with the results of the cell count.**

Results: **Following axotomy the related motoneurons underwent chromatolytic changes which increased up to one month and diminished in the 3-month group. One day following axotomy the number of motoneurons did not show any significant reduction, but thereafter a progressive cell loss occurred, which was most prominent after three months. Electron microscopic study confirmed the ultrastructural apoptotic nature of cell death. Bax immunohistochemistry indicated an increasing immunoreactivity up to one month post-axotomy, but in 3-month group it was clearly diminished.**

Conclusion: **Following transection of a peripheral nerve in adult animals, related motoneurons undergo chromatolytic changes which in some neurons may proceed to apoptotic cell death. Although the proapoptotic protein Bax has long been believed as the main apoptotic factor, other Bax-independent pathways may also participate in the axotomy-induced neuronal apoptosis which must not be ignored.**

Key words: Bax protein, Apoptosis, Motor Neurons, Axotomy

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Introduction

Apoptosis is a cell death program which is central to cellular and tissue homeostasis and is involved in many physiological and pathological processes (1). The morphological features of apoptosis are chromatin condensation, DNA and nuclear fragmentation, cytoplasmic shrinkage, and finally formation of apoptotic bodies which are subsequently phagocytosed by surrounding cells (2,3). One of the most studied extracellular signals of apoptosis is the axotomy-induced withdrawal of target-derived trophic support of neurons (3).

The proteins of Bcl-2 family are key regulators of apoptosis and their main function is to control mitochondrial permeability and particularly the release of apoptogenic proteins from this organelle (4). Based on their structure and role in apoptosis, the Bcl-2 family proteins can be divided into three groups: the antiapoptotic proteins such as Bcl-2 and Bcl-xL which are characterized by the presence of four Bcl-2 homology domain (BH1, BH2, BH3 and BH4); The proapoptotic proteins such as Bax and Bak which contain three homology domains (BH1, BH2 and BH3); and the BH3-only proteins such as Bid and Bad which contain only BH3 domain and induce apoptosis by activating proapoptotic proteins like Bax or by inhibiting anti-apoptotic proteins like Bcl-2 (1). Bcl-2 is placed on the outer mitochondrial membrane (OMM), while Bax might be on the same membrane or in the cytosol. Bax (Bcl-2 associated-x) protein is a major proapoptotic protein in neuronal cells which following an apoptogenic stimulus translocates from the cytosol to the mitochondria and causes the release of mitochondrial apoptogenic proteins and apoptosome formation which in turn activates caspase-dependent and -independent pathways (5,6). Mitochondrial form of Bax is a 21 Kda monomer weakly associated with the OMM or soluble in the cytosol. Upon the induction of apoptosis this monomer evolves into a high molecular complex of 96-260 KDa homo-oligomers, each consisting of 6 to 8 molecules of Bax, and inserts into the OMM (1).

Apoptosis is considered as an ideal therapeutic target for premature cell death despite the complexity of the various pathways involved, because it allows an opportunity for treatment of cell degeneration. It may not be so simple to develop therapeutic strategies without a thorough understanding of pathways regulating cell survival and cell death in specific cell types. In the present study, we investigated the temporal relationship between Bax expression and apoptotic cell death of spinal motoneurons up to three months following sciatic nerve transection in adult rats by means of morphometric, ultrastructural and immunohistochemical methods.

Materials and Methods

The animal care and all experimental procedures were carried out according to ethical guidelines established by the Shahed University. Forty young adult Sprague-Dawley rats (100-150 g) obtained from Razi Institute (Karaj, Iran) were housed under a 12 hour light/dark cycle with free access to food and water. Under general anesthesia with 35 mg/kg intraperitoneal injection of Nesdonal, the left sciatic nerve was transected at the mid-thigh level under the long head of biceps femoris, and to hinder innervation a 5 mm piece of the nerve was removed. The animals were randomly divided into four groups and sacrificed 1 day, 1 week, 1 month and 3 months post-operatively by an overdose of pentobarbital and transcardially perfused with heparin containing normal saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). After laminectomy the L4-L6 spinal cord segments were removed and in all samples the intact side of the spinal cord was considered as control. In each group the samples of 5 animals were used for morphometry and cell count, 2 for electron microscopy and 3 for immunohistochemistry.

Morphometry and Cell Count

For morphometry and cell count, after fixation of samples in Bouin's fixative and embedding in

paraffin, 8 μm transverse sections were serially cut and stained with Cresyl Fast Violet. Motoneuron counts were blindly made on every fifth section and cells with a large nucleus (>10 μm) and a large distinctly stained cytoplasm were counted. Using these criteria, less than 1% of the cells appear on two successive sections and therefore only an insignificant number of cells would be counted twice and no correction factor is needed (7). In each group the total number of motoneurons and the mean percentage of motoneuron reduction compared to the intact side were calculated and the results were analyzed for statistical significance by ANOVA and Tukey's tests.

Electron Microscopy

For electron microscopic studies, in every group nearly 1mm^3 pieces from both ventral horns of mid-L5 segment w ere dissected and fixed in 2.5% glutaraldehyde in phosphate buffer (0.1 M, pH 7.4) for 2 hours and postfixed in 1% osmium tetroxide in phosphate buffer (0.1 M) at 37 ºC for 1 hour, dehydrated in acetone and embedded in resin. After trimming the blocks and finding the proper points by tolouidine blue stained semithin sections, 50-70 nm ultrathin sections were collected on copper grids and stained with uranyl acetate and lead citrate and examined in a ZEISS EM-900 electron microscope. In each group, samples obtained from right ventral horn were considered as controls.

Immunohistochemistry

For immunohistochemical studies after fixation of samples in buffered formalin and preparing paraffin blocks, 8 μm transverse sections were mounted on glass slides and processed for Bax immunolabelling. In every group the ventral horn of unaxotomized side was considered as controls. For heat-mediated antigen retrieval, the de-waxed sections were placed for 30 minutes in boiling citrate buffer (0.01 M, pH 6). Then, the sections were preincubated for 30 minutes in 3% H2O2 in 0.01 M phosphate- buffered saline (PBS) to block endogenous peroxidase activity followed by incubation in 5% normal goat serum for 10 minutes. Then, the sections were incubated overnight with monoclonal rabbit anti-Bax antibody (1:2000, Serotec) as primary antibody followed by peroxidase-conjugated goat anti-rabbit immunoglobulins (1:200, Dako) for 30 minutes at room temperature as secondary antibody in a moist chamber. After a 10 minute incubation in a solution containing 0.05% diaminobenzidine + 0.003% H2O2, the sections were dehydrated, cleared and covered with coverslips. Omission of primary antibody was used as negative control. The percentage of Bax-positive neurons to the total neurons was expressed as mean ± standard deviation.

Results

Cell count

In each group the mean of motoneuron number and the percentage of reduction of neurons compared to the control groups were calculated and the results were statistically analyzed by ANOVA and Tukey's tests (Table 1). One day following axotomy the mean of motoneurons showed no significant difference as compared to the controls, but in 1 week and 1 month groups the difference was significant ($p<0.05$), and after 3 months the significance distinctly increased ($p<0.001$). These results indicate a late cell loss in axotomized spinal motoneurons in adult rats. Light microscopic morphological studies indicated chromatolytic changes in some of axotomized motoneurons, which were most abundant in the 1-week and 1-month groups and decreased 3 months following axotomy; but apoptotic features with the characteristic shrinkage and condensation of the cell body or karyopyknosis (Figure.1) showed a progressive time-matched increase up to 3 months following axotomy.

Table 1: The number of motoneurons in ventral horn of axotomized and intact side of spinal cord in experimental groups

Group	Mean of motoneurons (intact side)	Mean of motoneurons (axotomized side)	Reduction percentage
1 Day	1682 ± 186.47	1637 ± 154.09 in	2.68%
1 Week	1654 ± 193.98	1528 ± 104.26 *	7.62%
1 month	1606 ± 199.07	1431 ± 101.76 *	10.90%
3 months	1594 ± 129.54	1385 ± 83.82 **	13.11%

Data are expressed as means ± standard deviation and the reduction percentage of motoneurons in each group (in = insignificant, * = $p<0.05$, ** = $p<0.001$).

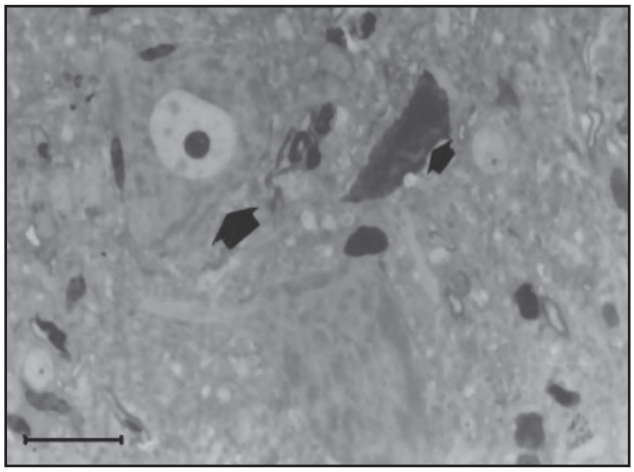

Figure 1. Photomicrograph of a tolouidine blue-stained semithin section of the axotomized ventral horn 1 week post-operatively. The great arrow denotes a normal motoneuron with a large round nucleus and a prominent nucleolus and the small arrow indicates a pyknotic apoptotic feature (scale bar =20 µm).

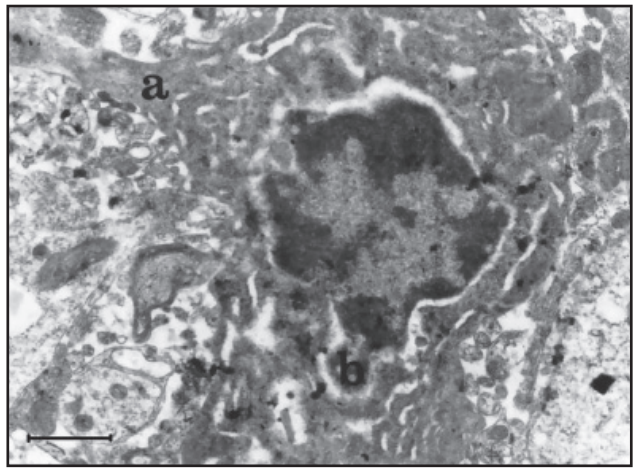

Figure 2. Electron micrograph of the axotomized ventral horn 1 month post-operatively, indicating an apoptotic motoneuron with characteristic condensation and margination of chromatin and some blebbing features (b). The axon hillock (a) is evident at the left of the figure (scale bar = 0.95 µm).

Electron Microscopy

Electron microscopic studies indicated clear ultrastructural changes in some of axotomized spinal motoneurons including chromatin condensation and clumping, nuclear fragmentation, cytoplasmic shrinkage and condensation, and finally dilatation and vacuolation of cytoplasmic organelles such as mitochondria (Figure 2). These ultrastructural features can be considered as reliable evidences of apoptotic cell death.

Immunohistochemistry

In our immunohistochemical study, the motoneurons with a dark brown-stained cytoplasm and light nucleus were considered as Bax-positive neurons, whereas the intact Bax-negative neurons which did not react with Bax antibody showed a light cytoplasm (Figure 3). In every experimental group, 10 sections were randomly chosen for Bax-immunolabelling. In every section the Bax-positive and negative neurons were counted and the mean percentage of Bax-positive neurons to the total number was calculated (Figure 4). Bax-positive neurons could be seen in all experimental groups, increasing up to one month following axotomy and decreasing in 3-month group.

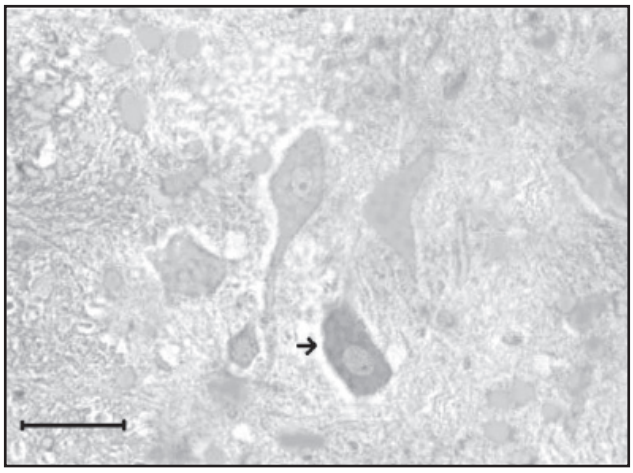

Figure 3. Photomicrograph of Bax-immunoperoxidase labelling of axotomized ventral horn 3 months post-operatively. The arrow denotes a Bax-positive apoptotic motoneuron (scale bar = 20 µm).

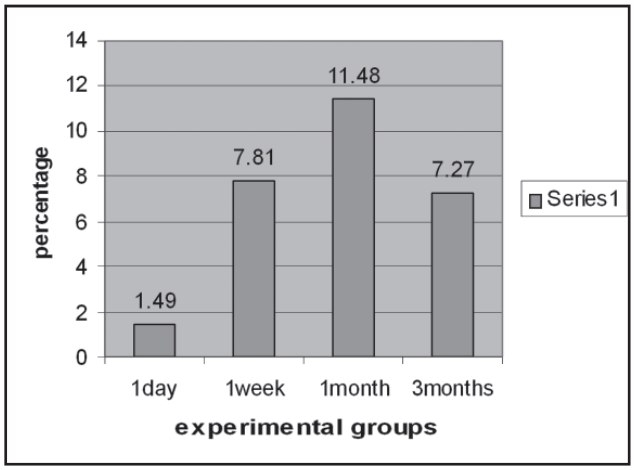

Figure 4. The mean percentage of Bax-positive motoneurons in the axotomized ventral horn in different experimental groups. The values increased up to 1 month post-axotomy, but in 3-month group it is diminished.

Discussion

The main purpose of this study was to evaluate the temporal relationship between Bax expression and apoptotic cell death. We used axotomy to induce apoptosis in spinal motoneurons in adult rats and applied cell count and electron microscopic studies to determine the extent and nature of cell degeneration. Peripheral nerve transection results in a disconnection of neuron from its target and a series of metabolic changes occur in the cell body that may cause apoptotic neuronal cell death. Although neonatal neurons are the most susceptible, peripheral lesion-induced cell death also occurs in adults (8). Transection of the adult sciatic nerve at mid-thigh level is reported to cause apoptotic cell death in 15-35% of ipsilateral L4 and L5 dorsal root ganglion neurons and related ventral horn motoneurons with a peak incidence at 2 weeks up to 2 months following injury (9-12). Our cell count findings indicated similar results with a cell loss in motoneurons of L4-L6 ventral column reaching to 13.11% three months following axotomy. Our light microscopic study of semithin sections showed chromatolytic features in 1-week and 1-month groups which decreased after 3 months. These results are consistent with the results of other researchers who reported axotomy-induced chromatolysis in adult rats (13,14). Electron microscopic study indicated the apoptotic characteristics of neuronal cell degeneration including chromatin condensation and clumping, nuclear fragmentation and blebbing, cytoplasmic shrinkage and condensation, and in some cells swelling of cytoplasmic organelles such as mitochondria which can be a feature of late apoptosis, though some studies have shown that mitochondrial swelling is not an absolute prerequisite during the in vivo apoptosis (15). Our cell count and electron microscopic results confirm the apoptotic nature of axotomy-induced degeneration of spinal motoneurons following transection of sciatic nerve in adult rats. Briefly, in adult animals following axotomy and interruption of target-derived trophic support, the injured motoneurons undergo chromatolytic changes which are usually reversible and the neuron survives the insult, but in some of the affected cells, chromatolysis progresses and results to the apoptotic cell death.

To assess the temporal relationship between apoptotic process and Bax expression, we carried out Bax-immunolabelling in all experimental groups. The results indicated an increasing Bax-immunoreavtity up to 1 month following axotomy, whereas samples obtained after 3 months showed decreased Bax-immunoreactivity which is contrary to the concurrent increased cell loss in the same group. The target-derived trophic factors, upon reaching the cell body, bind to tyrosine kinase receptors and activate a series of secondary intracellular signals,

which eventually inhibit the proapoptotic Bcl-2 family proteins (16). Most of these proteins contain a potential transmembrane domain involved in their localization to the membranes of organelles such as mitochondria and endoplasmic reticulum. These proteins can form homodimers and/or heterodimers, essentially through the interaction of their BH3-domain (1). Intracellular organelles, particularly mitochondria, are key participants in apoptosis. The mitochondrial involvement in apoptosis includes two crucial events: the release of intermembranous mitochondrial proteins such as cytochrome C and apoptosis inducing factor (AIF), and the onset of multiple parameters of mitochondrial dysfunction. Cytochrome C triggers a post-mitochondrial pathway forming an oligomeric apoptosome consisting of cytochrome C/Apaf-1/procaspase-9. Apoptosome by activating the initiator caspase-9 and subsequent cleaving of effector caspase-3 and -9 has an important role in apoptosis progress (17). Following a cell death signal the pro-apoptotic members of Bcl-2 family, such as Bax and Bak accumulate at the OMM, and form oligomers which disrupt the function of mitochondrial membrane and cause the release of the cytochrome C and other signaling molecules, which activate downstream executioners of death such as the caspases. Anti-apoptotic members of Bcl-2 family may act primarily by preventing the translocation or oligomerization of Bax/Bak (18,19). Although initial reports used to affirm the necessity of bax activity for apoptotic progress in the nervous system generally (20-23), recent reports have thrown doubt upon its veracity (5, 24-26). Miller et al reported that Bax deficiency can hinder axotomy-induced apoptosis (20), White et al found that Bax deficiency results in the elimination of developmental cell death in many neuronal populations in the nervous system (21), Lindsten et al indicated that the deletion of Bax may lead to a generalized increase in cell number in multiple regions of the brain (22), and Bar-Peled et al found that Bax deficiency suppresses motoneuronal apoptosis in dissociated spinal cord cultures (23). However some recent authors claim quite different opinions, which are in striking contrast with the central role of Bax in apoptosis. Some studies on the neurodegenerative diseases reported unchanged Bax expression compared to age-matched normal brains (24). Coulpier et al reported that Bax is not necessary for Bovine Spongiform encephalopathy-induced neuronal apoptosis and suggested the existence of a Bax-independent pathway in this pathological situation (25). In mice a 50-75% cell loss in the spinal cord between the 11th and 14th embryonic day has been reported. This may imply that if Bax deficiency could completely suppress the naturally occurring cell death, at the end of the developmental stage, the number of spinal motoneurons in the Bax -/- mice would be two to four times more than the normal Bax +/+ mice, but Kinugasa et al have only reported a 1.5 times increase, suggesting that Bax deficiency can suppress the naturally occurring cell death to some extent, but not completely (26). Also, Rogerio et al reported that following axotomy in neonatal rats the Bax-immunoreactivity remained virtually unaltered and they suggested that enhanced expression of Bax is not an essential step of the death process (5). The above mentioned studies are only a small number of a plenty of reports which have debated the issue of absolute indispensability of Bax in neuronal apoptosis. Although seemed to be the strongest factor promoting spinal motoneuron apoptosis following deprivation of peripheral trophic support, Bax is only one of the apoptosis-promoting factors. As mentioned previously, Bax operates in the mitochondrial path-way, but neuronal apoptosis may also be regulated through other pathways such as activation of plasma membrane receptors and endoplasmic reticulum (26). Thus, it can be suggested that at least a proportion of the cell loss occurring in the nervous system could be regulated by Bax-independent pathways.

Conclusion

Following transection of a peripheral nerve, related motoneurons undergo chromatolytic changes, which in most cases are reversible and the neuron survives the insult, but in some neurons chromatolysis proceeds to a late and long-lasting apoptotic cell death, with the highest incidence three months following axotomy.

Although the proapoptotic protein Bax is believed as the main apoptotic factor, other Bax-independent pathways may also be involved in the neuronal apoptosis, which must not be ignored.

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