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Caspase-dependent apoptosis in motor neurons of adult mouse spinal cord slices

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Abstract

In this study organotypic adult spinal cord slices were used to investigate whether caspases could participate in the apoptosis of motor neurons. The thoracic region of spinal cord was sliced using a tissue chopper and cultured in a medium for 6h. Morphological and biochemical features of apoptosis were assessed by fluorescent staining and terminal deoxynucleotidyl nick end labeling (TUNEL) method respectively. To investigate the role of caspases, general caspase inhibitor, Z-VAD.fmk, and immunohistochemistry for activated caspase-3 were used. After 6h in culture, many motor neurons displayed morphological features of apoptosis. In addition, the neurons appeared TUNEL positive. Z-VAD.fmk not only prevented apoptosis in the motor neurons but also increased motor neurons viability after 6h. At this time point, immunolocalization to activated caspase-3 was also detected in the cytoplasm and the nuclei of apoptotic motor neurons. Results of the present study suggest a caspase-dependent apoptosis in motor neurons of adult spinal cord slices.

Keywords: Apoptosis; caspase; motor neuron; spinal cord

1. Introduction

In the spinal cord, cell death occurs in motor neurons during development as a physiological process (Sendtner, 2000). These neurons may also die under pathological conditions such as spinal cord injury (Liu, 1997) and amyotrophic lateral sclerosis (Martin, 2000), a progressive neurodegenerative disorder leading to motor neuron loss, axonal denervation, muscular atrophy and death (Bigini, 2007). It has been shown that apoptosis contributes to cellular damage after traumatic spinal cord injury in human (Emery, 1998) and rat (Yong, 1998). Apoptosis might also be responsible for neuronal cell death during spinal cord injury in vitro. For instance, Casha and co-workers (Casha, 2005) in a model of spinal cord injury demonstrated neuronal apoptosis in spinal cord slices exposed to weight drop injury. We have recently shown apoptosis in adult spinal cord motor neurons at early time point of slice culture (Momeni, 2007).

Apoptosis is a form of programmed cell death which is characterized by morphological and biochemical changes (Ziegler and Groscurth, 2004). Apoptosis can be induced in neurons by a caspase-independent manner (Bigini, 2007, Momeni, 2008). Caspase-dependent apoptosis is also a known pathway in a wide variety of cells including neurons (Momeni, 2013, Raghavamenon, 2011; Weber, 2013). Following the induction of this form of apoptosis, a family of cysteine proteases, caspases, are activated either via death receptors or mitochondria (Wang, 2005). In most models in which caspases are involved, caspase inhibitors are shown to attenuate apoptosis (Thornberry and Lazebnik, 1998). Among all caspases, caspase-3 is considered to be responsible for the cleavage of key cellular proteins such as cytoskeletal (Chang and Yang, 2000) and nuclear (Robertson, 2000) proteins. In this context, caspase-3 has been reported to play a critical role in neurodegenerative diseases (Hartmann, 2000, Louneva, 2008) as well as in spinal white and gray matter after spinal cord injury in rat (Keane, 2001).

Several hypothesis such as glutamate excitotoxcicity (Pizzi, 2000) and calpain (Momeni, 2007), calcium dependent proteases, have been proposed to explain the mechanism(s) responsible for the apoptosis of motor neurons in adult spinal cord slices. However, it seems likely that such pathways are not be the only mechanisms by which apoptosis is induced in these neurons. Therefore organotypic adult spinal cord slices in culture were used to examine whether caspase could be involved in motor neurons apoptosis.

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2. Materials and Methods

a) Animals and preparation of organotypic spinal cord slices

Adult female Balb/c mice (23-25 g) were purchased from Pasture Institute, Tehran, Iran. The animals were housed in plastic cages at 20°C, a 12-h light/ dark cycle, and fed with standard commercial laboratory chew and water. The experiments were approved by the local ethical committee on research animal care at Arak University. The animals were deeply anesthetized by an intraperitoneal injection of sodium pentobarbital (60 mg/kg) and subsequently killed by heart puncture. The spinal cord was dissected and placed in ice cold phosphate-buffered saline (PBS), pH 7.4. The thoracic region of the spinal cord was then sliced transversally into 400 µm-thick sections using a McIlwain tissue chopper (Stoelting, USA). The slices were then placed in a four well sterile plastic plate where each well contained 450 µl medium composed of a mixture of 50% minimum essential medium, 25% Hanks balanced salt solution, 25% horse serum, 25 mM *N-2-hydroxyethyl piperazine-N'-2-ethanesulfonic acid* (HEPES), 6g/L glucose and 1% penicillin-streptomycin, pH 7.3-7.4). The cultures were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air for 6h in the absence (control) or presence of general caspase inhibitor, Z-VAD.fmk (N-Benzyloxycarbonyl-Val-Ala-Asp (O-Me) fluoromethyl ketone, Sigma, USA). The inhibitor was dissolved in dimethylsulfoxide (DMSO) as stock solution and stored at -20°C. The stock solution was directly added to the medium. Controls also received a corresponding amount of DMSO.

b) Fixation and sectioning

Freshly prepared (0h) and cultured slices were fixed in Stefanini's fixative (2% paraformaldehyde, 0.2% picric acid in 0.1 M phosphate buffer, pH 7.2) for at least 2h. The fixed slices were washed in PBS (3×5 min) and incubated overnight in 20% sucrose in PBS at 4°C. The slices were cut into 10 µm-thick sections in a cryostat. The sections were collected and mounted on Poly-L-lysine coated glass slides.

c) Assessment of apoptosis

Apoptosis was assessed by fluorescent staining, *terminal* deoxynucteotidyl nick end labeling (TUNEL) method and agarose gel electrophoresis. To study morphological features of apoptosis, the combination of propidium iodide (PI, Sigma, USA, 10 µg/ml in PBS, 15 min at room temperature) and Hoechst 33342 (Sigma, USA, 10 µg/ml in PBS, 1 min at room temperature) was used. The cryostat sections were washed in PBS (3×5 min), mounted in glycerol/PBS (1:1) and coverslipped. The percentage of motor neurons (n=20) viability was estimated by counting 12 randomly selected ventral horns from each experiment. Photographs were taken with an Olympus camera attached to an Olympus fluorescence microscope (Olympus Optical Co. Ltd, Japan) using the appropriate excitation and emission filters.

To evaluate biochemical analysis of apoptosis, TUNEL assay was used to detect apoptotic motor neurons using ApopTag plus Peroxides in Situ kit (Chemicon, USA) according to the manufacture's protocol. The motor neurons counterstained with methyl green showed normal nuclei. In contrast, the nuclei that contain DNA fragments were positively stained dark brown. The motor neurons were then photographed under a light microscope.

d) Immunohistochemistry

For immunohistochemistry, the cryostat sections were washed in PBS (3×5 min) and incubated with a 1:200 dilution of a rabbit antibody against the active form of caspase-3 (Cell Signaling and Technology, USA) in a moist chamber at 4°C overnight. The sections were washed in PBS (3×5 min) and incubated with goat anti rabbit Alexa 488 (Molecular Probes, USA) labeled secondary antibody at room temperature for 1h. For the assessment of non-specific immunostaining, alternative sections were incubated without the primary antibody. The sections were then washed in PBS (3×5 min), mounted in glycerol/PBS solution (1:1) and coverslipped. Photographs were taken with the fluorescence microscope.

e) Statistical analysis

Results were expressed as mean ±SD. The statistical significances were analyzed by analysis of variance (ANOVA). In all cases, $P<0.05$ was considered significant.

3. Results

a) Morphological and biochemical features of apoptosis in the motor neurons

Fluorescent staining was used to determine apoptotic cell death based on morphological changes (Fig. 1). In freshly prepared slices (0h) the motor neurons showed large cell bodies, large nuclei and the expected distribution of nuclear material and no apoptotic signs could be observed

within the motor neurons (Fig. 1A). After 6h, many motor neurons displayed characteristic morphological features of apoptosis such as cell shrinkage as well as nuclear and chromatin condensation (Fig. 1B). Results of the fluorescent staining were further supported by TUNEL assay (Fig. 2). Motor neurons from freshly excised slices revealed no TUNEL positive (Fig. 2A). In contrast, considerable TUNEL positive nuclei were detected in the motor neurons from slices cultured for 6h (Fig. 2B).

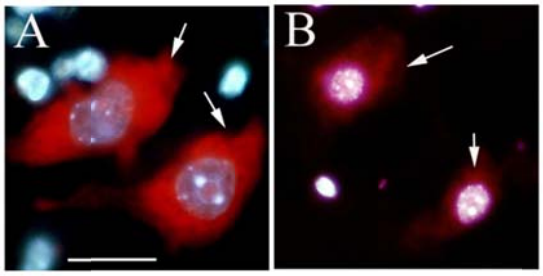

Fig. 1. Apoptosis in motor neurons. Sections from freshly prepared and cultured slices were stained with the combination of propidium iodide (red) and Hoechst 33342 (blue). A) Normal motor neuron at 0h with no apoptotic signs. B) Motor neurons from slices cultured for 6h displayed apoptotic features. Arrows: motor neurons. Scale bar A and B: 25µm

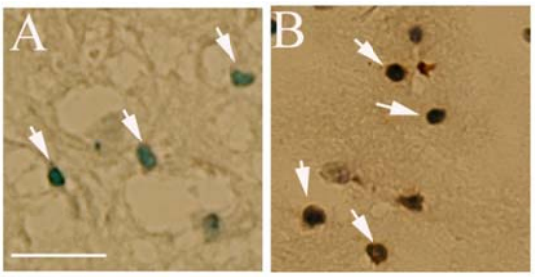

Fig. 2. TUNEL assay for the labeling of DNA fragmentation in spinal cord motor neurons. A) Motor neurons from freshly prepared slices (0h) showed no TUNEL positive. Motor neurons from slices cultured for 6h (B) displayed TUNEL positive nuclei. Arrows: motor neurons. Scale bar A and B: 25µm

b) Effect of caspase inhibitor on apoptosis and the viability of motor neurons

The finding that cell death occurred through apoptosis motivated us to investigate whether caspases participated in this event. The application of pan caspase inhibitor, Z-VAD.fmk (100 µM), effectively prevented the appearance of nuclear apoptotic changes in the motor neurons after 6h (Fig. 3A) compared to the control (Fig. 1B). Furthermore, the caspase inhibitor significantly increased the percentage of motor neurons viability in the ventral horns after 6h compared to the control (Fig. 3B).

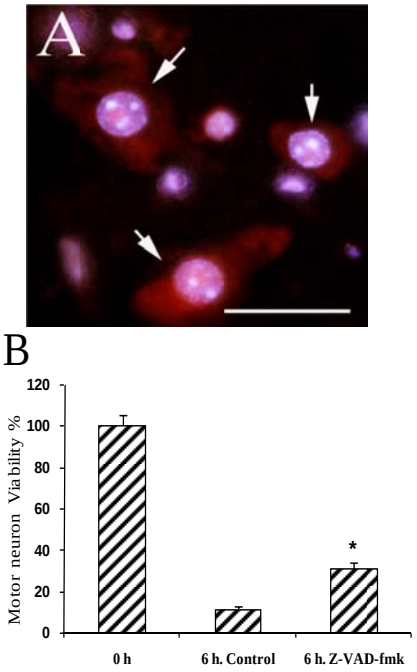

Fig. 3. The inhibition of apoptosis in motor neurons. Motor neurons from sections stained with the combination of propidium iodide (red) and Hoechst 33342 (blue). A) General caspase inhibitor, Z-VAD. fmk (100 µM) prevented apoptosis in the motor neurons compared to the control (Fig. 1B). Arrows: motor neurons. Scale bar: 25µm. B) Z-VAD.fmk (100 µM) significantly increased the percentage of motor neurons viability compared to the control. Mean ±SD, n=12. *p<0.01 versus control

c) Activated caspase-3 immunoreactivity in motor neurons

Since apoptosis could not be confirmed by just analyzing the morphological changes in the nucleus and chromatin, we decided to check the activation of caspase-3 in the motor neurons, using activated caspase-3 antibody. Motor neurons from freshly prepared slices, showed weak immunoreactivity for activated caspase-3 in the cytoplasm with no nuclear apoptotic changes (Fig. 4A, B and C). After 6h in culture, intense activated caspase-3 immunoreactivity was found both in the cytoplasm and the nuclei of the motor neurons where these neurons displayed nuclear and chromatin condensation (Fig. 4D, E and F).

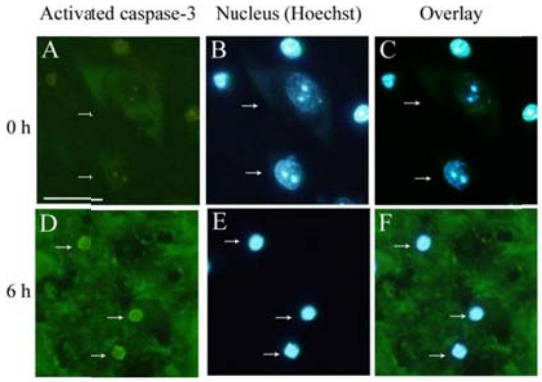

Fig. 4. Immunolocalization of activated caspase-3 in spinal cord motor neurons. Motor neurons were immunostained for activated caspase-3 (green) and counterstained with Hoechst 33342. (A-C) Motor neurons showed weak staining for activated caspase-3 in the cytoplasm at 0h with no nuclear apoptotic changes. (D-F) Intense activated caspase-3 immunoreactivity both in the cytoplasm and nuclei of motor neurons at 6h where they display nuclear and chromatin condensation. Arrows: motor neurons. Scale bar A-F: 25µm

4. Discussion

In the present study, organotypic spinal cord slices were used to investigate whether caspases could participate in the apoptosis of motor neurons. We demonstrated the involvement of caspase-3 and the neuroprotection effect of Z-VAD. fmk in adult motor neurons from cultured spinal cord slices. Results showed that the motor neurons displayed morphological and biochemical features of apoptosis after 6h. These features have been documented to be the hallmark of apoptosis (Ziegler and Groscurth, 2004). The binding of the fluorescent dye such as Hoechst 33342 to DNA allows the visualization of nuclear and chromatin condensation (Sgonc and Gruber, 1998), whereas TUNEL method is widely used to visualize DNA fragmentation at early stage of apoptosis (Das, 2005; Scholz, 2005; Zacharaki, 2010).

Molecular mechanisms behind the apoptosis of motor neurons in the cultured spinal cord slices have not been fully elucidated. One possibility might be the activation of caspases which play a critical role in the execution of apoptosis (Earnshaw, 1999). The role of these proteases have been reported in neuronal apoptosis following spinal cord injury (Keane, 2001) and neurodegenerative diseases (Eldadah and Faden, 2000). If caspases could be a possible candidate for apoptosis of the motor neurons, the inhibition of caspases should prevent apoptosis in such neurons. Interestingly, in the present study the general caspases inhibitor, Z-VAD.fmk, was able to provide effective motor neuron protection in the cultured slices. In this context, several reports suggest that Z-VAD.fmk attenuates caspase-dependent apoptosis in neurons (Scholz, 2005; Sharifi, 2009). Since caspase-3, one of the most important effector caspases, has been expressed in several kinds of apoptotic neurons (Scholz, 2005; Sharifi, 2009; Zacharaki; 2010) as well as in the white and gray matter of spinal cord following spinal cord injury (Keane, 2001), we hypothesize that caspase-3 might be activated in the apoptotic motor neurons. We found intense immunoreactivity for activation of caspase-3 in the cytoplasm and the nuclei of the apoptotic motor neurons when using an antibody specific to activated caspase-3. Taken together, our results provide a possibility to suggest that caspase-3 could be an important executor of apoptosis in the apoptotic motor neurons following slice culture. The finding, that weak activated caspase-3 immunoreactivity was observed within the cytoplasm of motor neurons in freshly prepared slices may suggest the presence of activated caspase-3 under physiological conditions. After 6h, the localization of activated caspase-3 in the nuclei of motor neurons was interesting and could be due to the translocation of this protease from the cytoplasm to the nucleus. Such translocation has been demonstrated by Scholz et al (Scholz, 2005) in the neurons of spinal cord dorsal horn after peripheral nerve injury.

When apoptosis is triggered by death signals, caspases, which exist as an inactive form (pro-caspases) are activated by proteolysis to form an active protease, thereby degrading multiple protein substrates in the cytoplasm and the nucleus (Kaufmann and Hengartner, 2001; Wang, 2005). Cytoskeletal proteins e.g. actin and fodrin (Chang and Yang, 2000), nucleoskeletal proteins, e.g. lamins, nuclear mitotic apparatus protein (NuMA) and poly (ADP-ribose) polymerase (PARP) which maintain cellular integrity are cleaved during apoptosis (Martelli, 1997). The appearance of activated caspase-3 in the motor neurons may explain cytoplasmic and nuclear apoptotic changes in these neurons. The apoptotic nuclear changes are conceivably due to both proteolysis of key nuclear polypeptides and DNA fragmentation (Martelli, 2001). Rapid proteolysis of the nucleoskeletal proteins has been suggested to be responsible for nuclear changes during apoptosis (Hendzel, 1998) and activated caspase-3 is considered for cleaving nuclear substrates to induce nuclear and chromatin condensation (Martelli, 1997). Endonucleases such as caspase activated DNase (CAD) could be another possibility for nuclear apoptotic changes in the motor neurons. Activated caspase-3 cleaves inhibitory of CAD (ICAD) to release CAD, leading to the active form of this protease. Once activated, it translocates into the nucleus to induce chromatin

condensation and DNA fragmentation (Jeong and Seol, 2008; Robertson, 2000). Therefore, it is reasonable to assume that the cytoplasmic and nuclear apoptotic changes in the motor neurons are due to the activity of protease(s) and nuclease(s).

In our experiments, the exact upstream death signals responsible for activation of caspase-3 are not yet defined. Two well-studied caspase activation pathways include the death receptor-mediated pathway and the mitochondria-mediated pathway (Wang, 2005). Both pathways participate in the downstream process, resulting in caspase-3 activation (Wong, 2006). Which upstream signals are responsible for apoptosis of motor neurons remain to be studied.

5. Conclusion

The immunolocalization of activated caspase-3 in apoptotic motor neurons as well as the possibility that the caspase inhibitor could delay apoptosis in these neurons and significantly increase motor neurons viability suggest that caspases, in particular caspase-3, might be involved in the apoptosis of adult spinal cord motor neurons.

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