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Expression of apoptosis-related proteins in model of anoxia *in vitro*

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Abstract

There has been growing evidence that different modes of cell death exist, among them the apoptosis is thought to be an important mechanism of nerve cell loss implicated in various pathological states. A number of proteins mediated with apoptotic process have been identified, including p53, BAX, BCL-2 and BCL-X.

We examined the expression of proteins related to programmed cell death in hippocampal neurons in vitro, exposed to pure anoxia or pretreated with apoptosis modulating agents: zinc and zinc chelator – TPEN. The results evidenced the noticeable differences in the expression of pro- and anti-apoptotic proteins in particular experiments. In the cultures exposed to pure anoxia, a significant increase of p53 and BAX immunoreactivity, associated with the decreased level of BCL-2 and BCL-X immunopositive cells was observed, related to the activation of apoptotic process. Hippocampal cultures pretreated with $ZnCl_2$ before anoxia showed decreased immunoreactivity for p53 and BAX, connected with BCL-2 overexpression, whereas the cultures exposed to zinc chelating agent – TPEN or TPEN connected with anoxia showed significant increase of immunorectivity for p53 and BAX.

This strong immunoreactivity of proapototic proteins (p53 and BAX) in hippocampal cultures exposed to anoxia or/and TPEN correlated with previous ultrastructural evidences of anoxia- and TPEN-induced apoptosis, while the overexpression of anti-apoptotic protein (BCL-2 and BCL-X) in zinc-pretreated cultures evidenced the protective ability of this metal against apoptosis in model of anoxia in vitro.

Key words: *apoptosis-related proteins, anoxia in vitro, zinc, TPEN, immunoreactivity*

Introduction

Apoptosis is an active process during which the genetic programme is realized. That is why one of biochemical marks of transcription and translation in the course of apoptosis is the RNA and proteins synthesis *de novo* [27,28]. Mechanisms present in the process of apoptosis are connected with an expression of several genes. Interactions between proapoptotic and antyapoptotic genes play a key role among factors connected with apoptotic proteins synthesis [20]. Some proteins /p53, BAX/ are activators of apoptosis, while other /BCL-2/ actively work as inhibitors of apoptosis.

A number of studies have been performed over the regulating influence of expression of proto-oncogenes

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and antineoplastic genes, especially families of the gene bcl-2 and the gene p-53. The best recognized group of restrictive genes of the apoptotic process is the family of bcl genes. Its expression has been observed in considerable intensity in cells stimulated to proliferation.

The response of neurons to oxygen deprivation is highly complex and is not yet fully understood [7]. It has been lately emphasized that the effects of hypoxic-ischemic injury may vary according to the severity of stress or brain maturation and that both necrotic and apoptotic types of neuronal death can coexist [1,4]. The activation of the certain mode of cell death is regulated by many different intracellular and extracellular factors [9]. Apoptotic death is initiated by the activation of endogenous proteases, which results in cytoskeletal disruption, cell shrinkage and membrane blebbing. The nucleus undergoes condensation due to endonuclease activation, resulting in nuclear DNA degradation.

Several enzymes have been qualified for early apoptotic markers, for instance endonuclease, a specific early protease poliADP-ribose and transglutaminases changing the structure of the proteins of the marginal part of the cytoplasm. Caspases make another group of enzymes which are activated at the beginning of apoptosis [24]. Active caspases activate subsequent procaspases, leading to cascade of reactions.

Our previous ultrastructural studies based on a model of anoxia *in vitro* revealed a characteristic pattern of nuclear chromatin clumping and formation of numerous apoptotic profiles, which were consistent with morphological criteria of apoptosis [21]. Moreover, it has been evidenced that some injured neurons share several features of both necrosis and apoptosis, which supports the hypothesis of the so-called, necrotic-apoptotic continuum.

In each organism apoptosis takes place as a consequence of many physiological and pathological factors. Zinc is thought to be one of essential elements for maintaining the cellular homeostasis but its precise role in necrotic and apoptotic cell death is largely unclear. We evidenced the protective effect of $ZnCl_2$ on development of late postanoxic changes connected with apoptosis, whereas the necrotic injury was not significantly reduced [22].

This study was performed to evaluate the process of apoptosis by characterization of the expression of different proteins implicated in this mode of cell death in a model of anoxia *in vitro* subjected to apoptosis modulating agents: zinc and zinc chelator – TPEN.

Material and methods

The experiments were performed on organotypic cultures of hippocampal formation prepared from 2-3-day-old Wistar rats. The hippocampus was dissected out under sterile conditions from both cerebral hemispheres, placed in dishes containing Eagle Minimal Essential Medium (MEM) and cut coronally into thin slices. The explants were placed on collagen-coated cover glasses with two drops of nutrient medium and sealed into Maximow chambers. The cultures were kept at 36.6°C in a medium consisting of 20% inactivated fetal bovine serum and 80% MEM supplemented with glucose to a final concentration of 600 mg% and antibiotics. The medium was renewed twice a week. On the 14-18th day *in vitro* the well differentiated cultures, sensitive to anoxic injury, were subjected to 20-minutes anoxia in pure nitrogen atmosphere in a flask adapted for permanent gas flow. Some cultures were pretreated with $ZnCl_2$ in concentration of 100 μM or with N, N, N'N'-tetrakis-(2-pyridylmethyl)ethylene-diamine (TPEN) in concentration of 15 μM added to the medium for 30 minutes prior to anoxia. Control cultures were maintained in standard conditions.

Immunohistochemical studies: After 30 minutes, 2 and 24 hours, 3 and 5 days post exposure the cultures from experimental and control groups were selected for immunohistochemical studies to determine the expression of proteins connected with apoptosis: p53, BAX, BCL-2 and BCL-X.

The expression of antigens was examined with complex biotin-streptavidine method (B-SA method). Cell cultures were fixed in 4% paraformaldehyde in PBS for 2 hours. The following monoclonal primary antibodies were used: p53 (Pharmingen International, 1:100), BAX (Sigma, 1:100), BCL-2 (Dako, 1:50), BCL-X (Dako, 1:100). The product of the reaction was showed by diaminobenzidine (DAB). The specimens were stained with hematoxylin.

Results

Cell immunoreactivity was measured in the marginal zone of the culture, within recognizable migrating glial cells and separated nerve cells.

In the control cultures grown in standard conditions weak immunoreactivity or the lack of clear reaction to p53 (Fig. 1a), BAX (Fig. 1b) and BCL-X (Fig. 1c) accompanied by the dispersed, weak reactivity for BCL-2 proteins were observed.

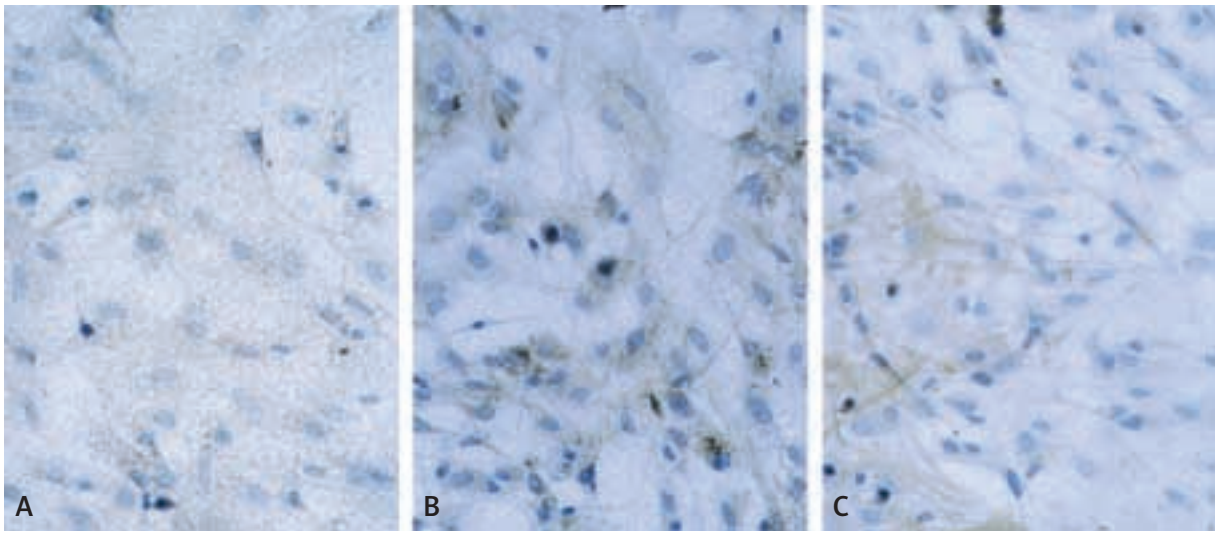

Fig. 1. Control cultures. **A.** p53 immunoreactivity, **B.** BAX immunoreactivity, **C.** BCL-X immunoreactivity. Magnification x 400

In the cultures exposed to 20-minutes anoxia, the distinct increase of immunoreactivity for p53 protein in comparison with the control cultures was noted. At 24 hours or 3 days post 20-minutes anoxia, numerous p53-immunonegative or weakly positive neurons with vacuolated cytoplasm occurred (Fig. 2a). After 3 days post anoxia the distinct increase in expression of p53 protein both, in the explants and in the outgrowth zone was detected within about 40% identified large pyramidal neurons (Fig. 2b). In the period of 3-5 days after anoxia, the more intense p53 immunoreactivity was ascertained within the cell nucleus and nerve cell bodies. In the marginal zone of growth, numerous dark aggregates composing of nuclear chromatin were visible, liable to reflect apoptotic bodies (Fig. 2c). They appeared both, in the neuronal processes and in the swollen cytoplasm of immunonegative glial cells.

In the investigated model of anoxia *in vitro*, the pattern of BAX immunoreactivity was similar to p53 expression in about 50% pyramidal cells. Strongly expressed reaction with the antibody against BAX was observed especially in the perinuclear region of strongly shrunk nerve cells, containing a dark nucleus with the narrow edge of the cytoplasm.

As opposed to strongly expressed immunoreactivity for p53 and BAX after 20-minutes anoxia, only the weak expression of BCL-2 was seen, similar to the expression of these proteins in control cultures at the same moment of growth. The expression of BCL-2 was evidenced in the population of proliferating glial cells and their processes, while large pyramidal neurons showed only slight reactivity or they were completely immunonegative (Fig. 2d). A few small apoptotic bodies could be seen on the background of dispersed BCL-2 immunoreactivity. In the majority of cultures exposed to anoxia, the weak expression of the BCL-X was observed in both, the nerve and glial cells, similar to control cultures.

Cultures exposed to 100 μM $ZnCl_2$ revealed only slight immunoreactivity for p53, BAX and BCL-2, regardless the time of observation.

In cultures exposed to anoxia pretreated with $ZnCl_2$, the p53 immunoreactivity was distinctly less expressed in comparison to the cultures exposed exclusively to anoxia. After 5 days of experiment, the expression of p53 was limited to a few (3-5%) scattered nerve and glial cells in the zone of growth. The quite well-preserved, weakly immunopositive pyramidal neurons were often seen (Fig. 3a). Immunoreactivity for BAX was similarly slightly expressed.

In the cultures exposed to anoxia pretreated with $ZnCl_2$, an overexpression of BCL-2 in nerve cells was seen, especially after 3 and 5 days. Numerous, well-preserved pyramidal neurons revealed strong immunoreactivity for BCL-2 within cytoplasm and their processes (Fig. 3b). Distinct immunoreactivity for BCL-2 was shown in about 60%-70% large pyramidal neurons. The immunoreactivity for BCL-X was strongly within cytoplasm of about 40% preserved pyramidal neurons.

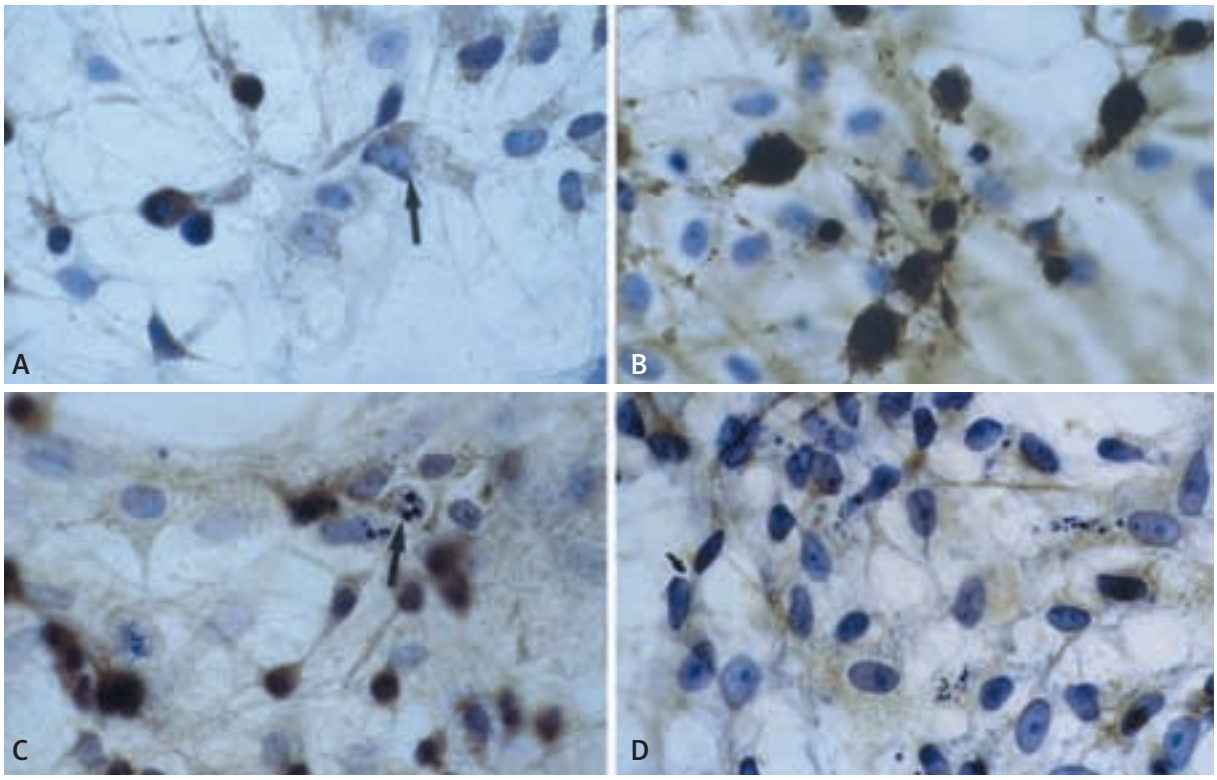

Fig. 2. Anoxia 20 minutes/3-5 days. **A.** Strong p53 immunoreactivity in shrunken neurons and weak positivity in large neurons with vacuolated cytoplasm (arrow). **B.** Significant increase of p53 expression. **C.** Numerous p53 immunoreactive neurons and dark apoptotic bodies (arrow). **D.** Slight, diffuse BCL-2 immunoreactivity and numerous apoptotic bodies. Magnification x 1000

The cultures exposed to 15 µM TPEN and cultures exposed to anoxia pretreated with TPEN showed intense immunoreactivity for p53. There could be noticed numerous p53-positive nerve cells (about 70% of large pyramidal neurons) in which the immunoreactivity appeared both in cell nuclei, in the cytoplasm and in neuronal processes (Fig. 4a). The cultures submitted to anoxia and TPEN revealed significant increase of p53 immunoreactivity (Fig. 4b). Neurons with characteristic features of apoptosis and numerous, dark apoptotic bodies, situated in the cytoplasm or processes of phagocytic cells were seen. More intense expression for BAX could be also seen in about 60% neurons, especially after longer period of observation. The immunoreactivity of BCL-2 was significantly decreased (Fig. 4c).

Discussion

In our study performed in a model of anoxia *in vitro* we noticed differences in the expression of pro- and anti-apoptotic proteins depending on apoptotic modulating agents added. Cultures exposed to pure anoxia showed high immunoreactivity for p53 and BAX related to activation of apoptotic process. The positive reaction appeared both, in the cell nuclei, perikaryons and neuronal processes. This could have been related to redistribution of the proteins in the cell cytosol and its location in membranes of cellular organelles, including mitochondrial, Golgi apparatus, endoplasmic reticulum and nuclear membranes [23].

Anoxia *in vitro* was related with the small number of immunopositive cells for BCL-2 and BCL-X, being the inhibitors of apoptosis. In turn, cultures pretreated with $ZnCl_2$ before anoxia, showed diminished immunoreactivity for p53 and BAX, accompanied by overexpression for BCL-2. These proteins appeared mainly in the cytoplasm of well preserved neuronal cells, which could be connected with its membranous location within cellular organelles and with the participation on the leackage of particles across these membranes. It seems that overexpression of BCL-2 can protect cells against different noxious factors.

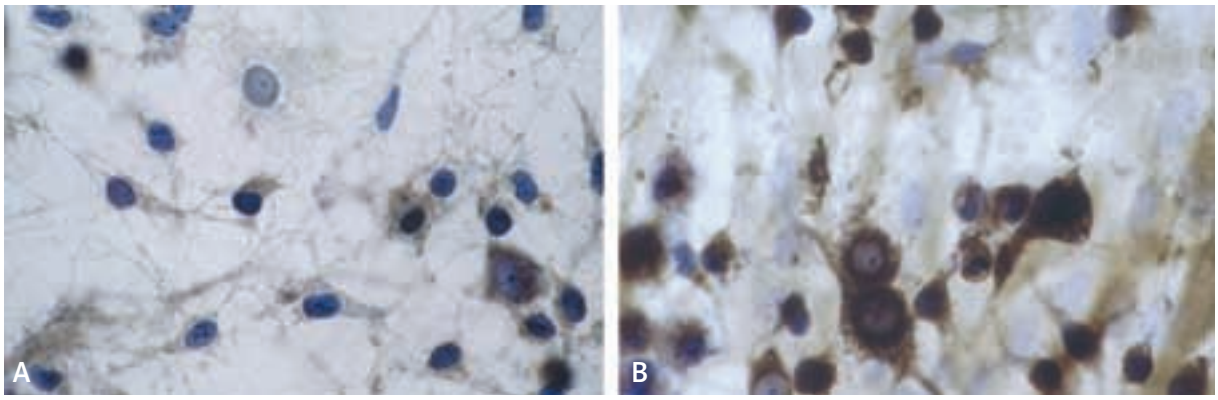

Fig. 3. Anoxia 20 minutes + 100 μM $ZnCl_2$/5 days. **A.** Slight p53 immunoreactivity. **B.** Well-preserved large pyramidal neurons with strong immunoreactivity for BCL-2. Magnification x 1000

In the cultures exposed to TPEN or to anoxia pretreated with TPEN, the marked apoptosis was connected with the expression of p53 and BAX, which are the promotors of apoptosis. The elevated immunoreactivity for p53 and BAX was noted especially after 3 and 5 days of observation.

The p53 protein is a product of the antineoplastic gene and appears in the wild form, being the regulator of the cellular cycle. The gene p53 is a suppressor gene whose damage or inactivation makes impossible the induction of programmed cell death. The distinct expression of the gene p53 was shown in both, proliferating and apoptotic cells [25]. Lack of the expression of this gene makes the induction of apoptosis in many cells impossible. This is observed in the course of different neoplastic diseases, particularly in leukemia [11,18] and can be used as a marker of the inefficiency of the antineoplastic therapy [18]. Disturbances of the expression of the p53 gene have been pointed out as a consequence of exposition to different toxic factors, to underline the role of this gene in the pathogenesis of cellular death [10,13,26]. However, the real part of p53 is not fully understood. It is the regulation of the apoptotic death process rather than activation of the cell reaction to damaging factors [1].

In turn, the bcl-2 gene is a restrictive gene of apoptosis. Its role is related to cell survival in reply to different noxious factors rather than to stimulating their proliferation [2,19]. The BCL-2 is widespread in CNS during the embryonic development, while the mature neurons revealed only weak expression of this protein [19]. Reports exist indicating that expression of BCL-2 in the mature brain is limited primarily to granular cells of dentate gyrus and cells of the olfactory bulb, which preserve ability to mitotic activity [3]. The expression of BCL-2 was noted also in neurons within the border zone between necrosis and healthy tissue, to underline the role of this protein in the determination of neuronal survival after anoxic-ischemic damages [5] and in reactive astrocytes [16].

Recently, 6 forms of genes from the bcl group were identified and the most important is probably the bcl-2 subgroup. Studies performed on distribution of the BCL-2 proteins showed that they were found primarily in cells already differentiated and less susceptible to apoptosis [12] as well as in numerous proliferating cells [24]. With reference to clinical conditions, more intensive expression of bcl-2 can be responsible for the chemotherapy resistance [8]. The BCL-2 proteins most likely work as elements of multiple complexes. The genes closely connected with bcl-2 consisted of two forms of bcl-x: bcl-xL (it appears only in mature tissue structures, mostly in the neuronal cells) and bcl-xS, as well as bax and bak. Products of these genes can unite with themselves, and their complex relations regulate the process of apoptosis. The likely mechanism of BCL-2 proteins activity was mediated by inhibition of particles from the group of proapoptotic factors, regulation of ions flow through mitochondrial membranes, stabilization of the membranous potential, regulation the cytochrome C release and inhibition of free radicals. The inhibition of apoptosis by BCL-2 is probably related with the inhibition of caspase 3 activation by blocking of cytochrome C release from mitochondria [14,17]. It has been evidenced that the BCL-2 and BAX proteins work

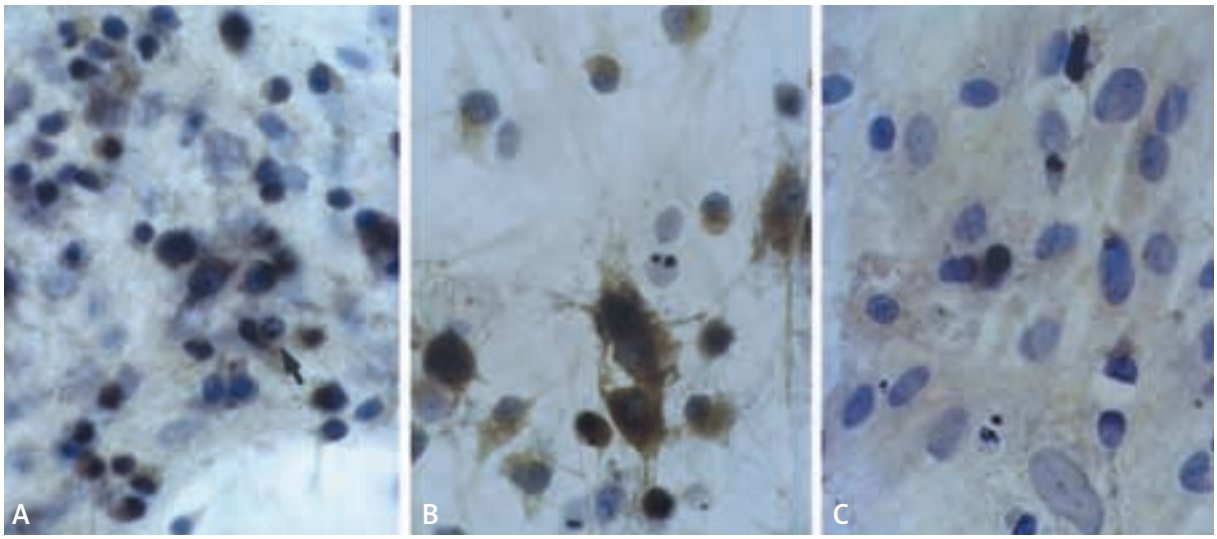

Fig. 4. 15 µM TPEN/5 days (A) or anoxia+TPEN/5 days (B, C). **A.** Marked p53 immunreactivity in numerous neurons with evidence of apoptotic features (arrow). **B.** Strong p53 immunoreactivity and dark apoptotic bodies. **C.** Weak, diffuse expression of BCL-2 accompanied by numerous small apoptotic bodies. Magnification x 1000

together and their quantitative relations modulate the cell conditions [6,15].

The strong immunoreactivity of proapoptotic proteins (p53 and BAX) documented in hippocampal cultures exposed to anoxia or/and TPEN supports the previous ultrastructural evidences of anoxia- and TPEN-induced apoptosis, while the overexpression of anti-apoptotic protein (BCL-2 and BCL-X) in zinc-pretreated cultures evidences the protective ability of this metal against apoptosis in model of anoxia *in vitro*.

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