ULTRASTRUCTURAL AND IMMUNOHISTOCHEMICAL EVALUATION OF APOPTOSIS IN FOETAL RAT LIVER AFTER ADRIAMYCIN ADMINISTRATION

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Abstract

The purpose of this study was to show apoptosis in foetal hepatocytes as a late effect of adriamycin given to female rats in a single dose (5 mg/kg of b.w., intraperitoneally) a long time before planned pregnancy. Rats were decapitated on 20th d of pregnancy and for further investigation the foetal liver was taken. Apoptosis was evaluated on the basis of the analysis of electron microscope picture of hepatocytes and the immunohistochemical identification of BCL-2 and BAX proteins. The BAX expression was significantly increased in the experimental group compared to the control group, whereas BCL-2 was not expressed (the same as in the control group). The high level of apoptotic index in the experimental group was caused by both the lack of BCL-2 protein expression and the intensive expression of the BAX protein. In the group of control foetuses apoptosis was not intensive, but was present as physiological natural cell death during embryogenesis process. In hepatocytes of foetuses whose mothers were treated with adriamycin in single dose a long time before pregnancy, there was observed increased apoptosis.

Key words: rat foetus, adriamycin, liver, apoptosis, electron microscopy, immunohistochemistry.

It is known that adriamycin (ADR), antineoplastic antibiotic cause apoptotic death in hepatocytes of adult rats (10, 16). Apoptosis is actually, without a doubt, one of the most often described phenomenon. Natural, physiological cell death - programmed death - happens as a result of organism aging, but not only because of this. Altruist cells commit suicide for the sake of the whole organism. Apoptotic physiological cell death is often determined as a result of the organizational comfort of cell structure disintegration, that is mitochondria, endoplasmic reticulum with a lack of typical necrotic signs. Physiological apoptosis appears, among others, in embryogenesis. During foetal life, part of new-arised cells decrease to leave the best cells in new organism. The present study evaluated foetal liver cells of rats whose mothers 4 weeks before the planned pregnancy were treated with adriamycin.

A dose of 5 mg/kg of body weight used in the present study is known as one which in experimental animals, including rats, allows for full-term pregnancy (11). It is also known that adriamycin administered to pregnant female rats causes tracheo-oesophageal fistulas with oesophageus atresia in foetuses, which could be used in a foetal rat model of oesophageal atresia (3, 12, 13). Osteotic changes in foetal rats exposed to adriamycin are also relatively more frequent (5).

Because of numerous metabolic pathways, the liver is especially exposed to the harmful effect of exogenous substances, including drugs. This study shows a typical physiological death of hepatic cells of healthy rat foetuses and apoptosis of rat foetal hepatocytes after the damaging effect of exogenous factors. In apoptosis study, the evaluation of a picture from electron microscope was used and BCL-2 and BAX proteins were identified using immunohistochemistry method. BAX protein is one of the main factors of apoptosis. BCL-2 is anti-apoptotic protein. Immunohistochemical detection of places of BAX protein expression could assess the localization and intensity of physiological and pathological apoptosis, which could help in distinguishing these two types of cell death.

Material and Methods

Sixteen foetuses were used in the experimental group, chosen randomly two from each of 8 female rats which were treated with adriamycin administered intraperitoneally 4 weeks before fertilization in a single
dose of 5 mg/kg of body weight. The control group consisted of 16 foetuses chosen randomly two from each of 8 female rats which were treated with 0.5 ml of 0.9% NaCl administered intraperitoneally in a single dose 4 weeks before fertilization.

After the decapitation of the pregnant females (on 20th d of pregnancy) the chosen foetuses were also decapitated and liver samples were taken from them. The samples taken for electron microscopy were fixed in fluid containing 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M Sørensen phosphate buffer. Then the preparations were treated with osmium tetraoxide, stained in uranyl acetate, dehydrated in increasing concentrations of ethanol and embedded in Aralchit ACM Fliska resins. The preparations were cut into 0.5-0.7 µm and 60 nm thick sections with Reichert Ultracut S ultramicrotom. The ultra-thin sections were stained with 8% solution of uranyl acetate in 0.5% acetic acid and lead citrate. The photographic documentation was performed with Tesla BS-500 electron microscope.

Samples taken for immunohistochemical studies were fixed in 10% formalin, and then after dehydratation and embedding in paraffin cut into 7 µm sections. To identify BAX and BCL-2 proteins, preparations from both groups were used. For each preparation a negative control was performed (a slide without primary antibody). The proteins expression level was evaluated with a standard three-step immunohistochemical procedure. Rabbit BCL-2 and BAX antibodies were used as a primary antibody. Then biotinylated secondary antibody was added, and then horse-radish peroxidase conjugated with streptavidin. Since streptavidin has a great affinity to biotin, it binds to the place where primary antibody coated the background, and after adding a chromatogen (DAB or AED) a reddish colour appears.

Results

Foetal hepatocytes from the control group showed some difference as compared to the hepatocytes from adult individuals known from manual descriptions, which was due to the developmental stadium, intensity of proliferation, and liver embryogenesis. Foetal hepatocytes were much smaller with a smaller amount of the cytoplasm in relation to the nucleus as compared to mature hepatocytes (Fig. 1). The lack of specialization was manifested with a smaller amount of cell organelles in the cytoplasm (Fig. 1).

Haematopoetic cell focuses in different stages of the development, as well as erythroblasts in blood vessel lumen were visible in the foetal liver (Fig. 2). The observed divisions of hepatocytes and blood cells were the evidence of intensive proliferation (Fig. 1). There were no signs of cell damage. (Fig. 2)

Some of the foetal hepatocytes from the experimental group were characterized by steatosis and necrosis. But the most frequent changes were apoptotic features.

Hepatocyte nuclei showed different shapes and size. In some cells pyknotic nuclei with chromatin condensed circumpherentially were found (Fig. 4). In some cases an evident vacuolization of the nuclei was observed. In some cells “rinsed” cytoplasm, without organelles - cells “empty inside” was observed (Fig. 3), while in other cells, the destruction of cell organelles was found. Mitochondria were often swollen, with bright matrix and cristal destruction, some of them were damaged, with ruptured mitochondrial membrane. Rough endoplasmic reticulum was quite often defragmented (Fig. 3). An increased amount of lysosomes was also found in hepatocyte cytoplasm. Numerous autophagosomes were observed close to cholangial tubules (Fig. 3). Perisinusal spaces were often dilated and swollen with the content of damaged hepatocyte cytoplasm. Connective tissue proliferation was observed, which was the evidence of cell membrane damage, which was also shown by the presence of erythrocytes in hepatocyte cytoplasm (Figs 3 and 4).

Changes were also observed in blood stem cells - deformed cells, “naked nuclei”. These changes as a result led to atrophia of haematopoetic focuses (Figs 3 and 4). Numerous cell divisions were the evidence of attempts at liver regeneration.

The expression of BAX protein was observed in the experimental (Fig. 8) and control groups (Fig. 6), but in the experimental group BAX was expressed more strongly (Fig. 8). In the control group apoptotic changed cells were disposed rather steadily. The most intensive expression in the experimental group was observed in perportal regions of hepatic lobes.

BCL-2 protein was not expressed in rat foetal liver preparations either in the experimental or in the control groups (Figs 5, 6, 7 and 8).

Discussion

The digestive system, together with the liver and pancreas, derives from the primary gut. The gut develops during the formation of the head and caudal and lateral folds of embryo. After the fold formation is finished, the gut can be divided into anterior, medium, and posterior gut. The anterior gut could be divided into the head part from the oral-throat membrane to the place of forming lung bud and the caudal part - from lung bud to the place where a liver bud is formed. The liver bud appears, as a epithelium thickening in the most caudal part of the primary anterior gut.

Adriamycin toxicity is known and proved. The histological ultrastructural changes present in this study (cell lysis, effacement of cell structures, swelling of mitochondria with brightening of the matrix and partial crest destruction, some mitochondria damage, defragmentation of rough endoplasmatic reticulum) fully confirm earlier reports, describing damage of adult rat liver after adriamycin treatment (10). These changes are typical for apoptosis. A few stages of apoptotic cell degeneration could be distinguished.
Fig. 1. Electronogram of part foetal liver of rat from control group. Visible cell in division (arrow) and nuclei (n). 3000x.

Fig. 2. Electronogram of part foetal liver of rat from control group. Hepatocytes and blood cells present. Erythroblast in blood vessel (arrows). 3000x.

Fig. 3. Electronogram of foetal hepatocytes of rat from experimental group. Significant cell damage, formation of autophagosomes (AF). 3000x.

Fig. 4. Electronogram of foetal hepatocytes of rat from experimental group. Visible pyknotic nuclei in hepatocytes. 3000x.
The early morphologic changes can include the damage of plasmatic membranes, decrease of cell volume, and nuclear pyknosis. More advanced changes are complete destruction and fragmentation of nuclei with forming of apoptotic bodies. The final stage is phagocytosis of apoptotic bodies and the remains of the destroyed cell by other cell with phagocytic capacities.

Asakura et al. (2) proved that adriamycin conjugated with glutathione via glutaraldehyde inhibited the activity of glutathione S-transferase, playing an important part in apoptosis induction, in rat hepatoma cells.

It was proved that therapeutical doses of adriamycin increase lipid peroxidation in microsomes and in mitochondria of the liver, especially in the presence of Fe^{3+} ions (8, 14).

Ganey et al. (7) showed that the toxicity of that drug is oxygen-dependent. In periportal regions of hepatic lobes, which are rich in oxygen, an increase in oxygen intake was proportionally dependent on adriamycin dose, whereas the compound had no influence on oxygen intake in hepatocytes in region of central vein, which is poor in oxygen (7). Perhaps that is why in the present experiment BAX protein expression present in experimental liver sections was located mainly in periportal regions of hepatic lobes in contrast to control group where BAX protein expression were disposed rather steadily in all preparations.

BCL-2 oncoprotein evaluated in the present experiments regulates programmed cell death (15) by providing a survival advantage to rapidly proliferating cells, while BAX protein promotes apoptosis by enhancing cell susceptibility to apoptotic stimuli. The multidominian proapoptotic molecule BAX is required to initiate the mitochondrial pathway of apoptosis (4). Increased expression of BAX protein could be the evidence that the mitochondrial pathway of apoptosis started. The presence of performed apoptotic process was confirmed by microscopical view.

Apoptosis is an important mechanism regulating cell number and their development in different organs and tissues, which is essential in embryogenesis and aging processes, as well as in removing harmful and useless cells from the body (6). Apoptosis of cells in the healthy human embryo was observed by Lichnowsky et al. (9). They noticed an increase in BCL-2 and BAX protein expression using the immunohistochemical method. They assessed BAX/BCL-2 apoptotic index. The low value of apoptotic index was mostly accompanied by high
expression of BCL-2 – antiapoptotic protein. It suggests the presence of apoptosis in embryogenesis, which is regulated by high level of antiapoptotic protein.

In the present study, increased expression of BCL-2 protein in the investigated rat foetal liver was not observed, which suggests the lack of proliferation and regeneration processes. BAX protein found in the control rat foetal liver shows the presence of the apoptosis process. Apoptosis in the control group dominated the proliferation processes; however, it was significantly smaller than in the experimental group. The high level of apoptotic index in the experimental group was caused both by lack of the BCL-2 protein expression and intensive expression of the BAX protein.

The increase in BCL-2 expression and decrease in BAX level in the liver was observed by Akcali et al. (1) during hepatocyte regeneration after hepatectomy. Increase in the BAX (1) during hepatocyte regeneration after hepatectomy. BAX protein found in the control rat foetal liver shows the presence of the apoptosis process. Apoptosis in the control group dominated the proliferation processes; however, it was significantly smaller than in the experimental group. The high level of apoptotic index in the experimental group was caused both by lack of the BCL-2 protein expression and intensive expression of the BAX protein.

In mammal cells two main ways of apoptotic signal transmission were described: external and internal (mitochondrial) way. The internal way is a response to factors causing DNA damage, and the process takes place mostly in mitochondria, most often in connection with proapoptotic proteins from BCL-2 group, including BAX protein.

The increase in the BAX protein activity observed in the present study and visible mainly thanks to immunohistochemical methods could suggest that the apoptotic signal was transmitted in mitochondrial way. A possibility is not ruled out that apoptosis could be initiated also in the ways not investigated in the present study.

References