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P-53 PROTEIN AND PROTEINS FROM BCL-2 FAMILY IN THE HEART OF RAT AFTER ADRIAMYCIN ADMINISTRATION. IMMUNOHISTOCHEMICAL EVALUATION

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ABSTRACT

Adriamycin (ADR) – the antineoplastic antibiotics has confirmed proapoptotic activity, mainly on neoplastic cells and young quick dividing cells. Cardiotoxicity of Adriamycin is limitating in antineoplastic therapy. The purpose of study was an evaluation of internal pathway of induction of signal to the apoptosis in myocardial cells of rat, which had administered Adriamycin. The sign of late cardiotoxicity after Adriamycin is coagulative necrosis. In present study was noticed also increased apoptosis of cells in rat heart, which was induced via mitochondrial pathway, with activation of p-53 protein and with BAX/Bcl-2 ratio > 1 – with prevalence of proapoptotic BAX protein.

Key words: apoptosis, heart, adriamycin, Bcl-2, BAX, P-53, immunohistochemistry.

INTRODUCTION

Adriamycin (ADR), the antineoplastic drug has already known cardiotoxical activity [4, 6, 8, 13, 14, 15, 22, 24]. Just that activity is limitating during of that drug therapy. The spectrum of Adriamycin activity is quite wide. It is used in treatment of leucemias, lung cancer, breast cancer, gastric cancer, cancer of urinary bladder and many others [2, 10, 11].

The mechanism of antineoplastic therapy of Adriamycin rely on incorporation of adriamycin between two purine/pyrimidine bases with following inhibition of DNA and RNA synthesis [2, 6, 21].

For cytotoxical including cardiotoxical activities of Adriamycin are responsible free radicals, which arised in biodegradation process of that drug [19]. The cardiotoxicity is assessed mainly after clinical features. The signs of unfavorable adriamycin action on heart are divided into early and late cardiotoxicity. Early cardiotoxicity includes arythmias and conduction disorders. As a base of late cardiotoxicity development were considered primary follicular

myofibrillar disintegration with following coagulative necrosis and also disorder in translation process [4]. Autors try to answer the question if coagulative necrosis is the only form of myocardial cells death, which takes place after treatment with Adriamycin. It was proved that apoptotic cells significantly vary from necrotic cells, although the end of inflammation process arising as a result of necrosis is the apoptosis of inflammation cells, in that way the inflammation process is shortened and health cells stay intact [25, 26]. The apoptosis after Adriamycin were observed in many organs of human and experimental animals [1, 16]. In present study to check if increased apoptosis after Adriamycin has place also in the rat heart, were investigated immunohistochemically proteins, which takes part in internal pathway of apoptosis.

MATERIALS AND METHODS

In experiment were used 16 female Wistar rats. Rats were divided into two equal groups: experimental and control. Female rats from experimental group were administered intraperitoneally ADR (Adriblastin; Farmitalia, Carlo Erba, Milan, Italy; D; 10mg was dissolved first in 5 ml water) in dose 5mg/kg of body weight. Female rats from control group were given 0.5 ml 0.9% NaCl also intraperitoneally.

After 4 weeks female rats were decapitated and two heart sections from each animal were collected from them for immunohistochemical investigations.

Sections taken to immunohistochemical studies were fixed in 10% formalin, and then after dehydratation and embedding in paraffin cut into 5µm slides.

Sections were placed on siliconised glasses. Preparations were deparaffinated in xylene and decreasing concentrations of ethanol. Then preparations were subjected with heat in acidic environment (10mM in citrate buffer pH 6.0) or in basic environment for Bcl-2 (1 mMEDTA buffer pH 8.0) – 7.5 minutes in microwaves with power 750W and after 5 minutes break 7.5 minutes with power 375W (antigen pretreatment). After cooling off during 20 minutes preparations were rinsed with distilled water, and then placed in TBS (TRIS Buffer Saline pH 7.6).

Then endogenous peroxidase was blocked by incubation during 20 minutes in 0.3% solution of H2O2 (99ml TBS, 1 ml 30% H_2O_2). After washing during about 10 minutes in TBS, preparations were incubated during 60 min. with mouse primary antibody p53 (Lab Vision) or BAX (Santa Cruz) in dilution 1/50 in TBS/BSA(Bovine Serum Albumine)1% buffer at room temperature (Bcl-2 in dilution 1/25). Next to obtain immunohistochemical reaction were used ready-to-use reagent kit – DakoCytomation corporation including: biotinylated secondary antibody against mouse antibodies (15 minutes of incubation), streptavidin conjugated with horse radish peroxidase (15 minutes of incubation), AEC (3-amino-9-ethylcarbazole) – substrate-chromogen reaction with horse radish peroxidase (10 minutes of incubation). After use of each reagent preparations were rinsed in TBS at least 10 minutes. After staining sections were counterstained with hematoxyline about 30 seconds, and then rinsed large amount of distilled water. Preparations were covered with coverslip using Aquatex liquid.

The analysis of microscopic picture in magnitude 125x, assessing the expression of the protein was performed using computer program Analysis-Pro 3rd version (Soft Imaging System Gmbh, Germany). From each slide was assessed 3 randomly selected places with field 781 193.35µm^2. Field of the sectioned surface of cells with positive reactions was measured. The range of colours assessed by the computer as a positive was set on intensive red, so red-pink or pink colour were not assumed as a positive.

Results were statistically analyzed using a ANOVA test. Averages, standard deviations and % of positive reaction in examined tissue field were determined. Differences were considered statistically significant when $P<0.05$.

Photographical documentation was performed using Jenaval Contrast Carl Zeiss Camera.

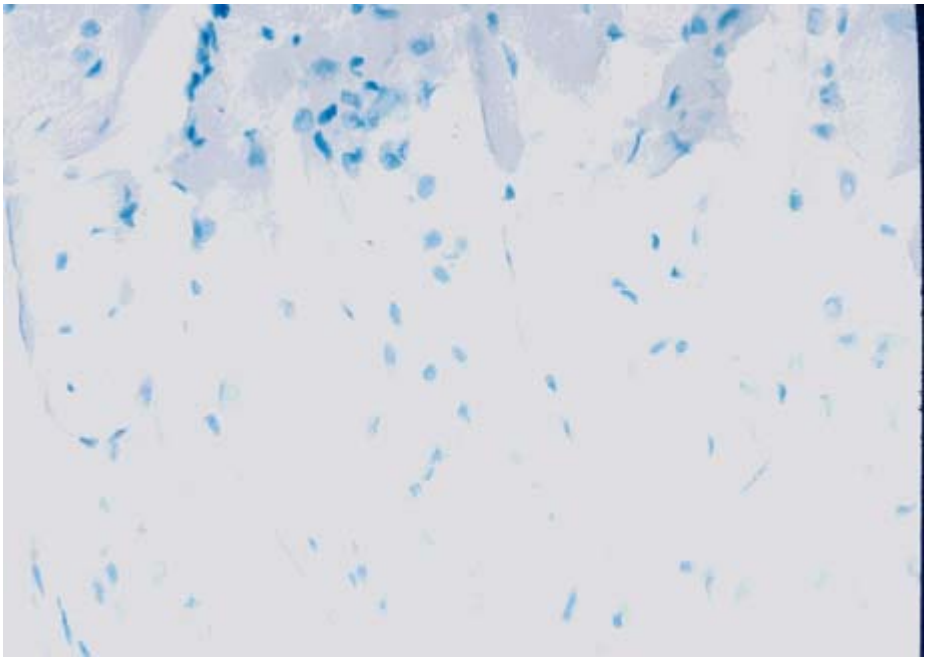
RESULTS

In immunohistochemical investigation in heart's cells with positive p-53, BAX, reaction were observed mainly follicular-diffused cytoplasmatic reaction. (Fig.1, 2)

The cytoplasm staining was from bright pink to red. Intensity of p-53, BAX, (+) positive cytoplasm staining of cells from experimental group (Fig.1) was much bigger than staining of p-53, BAX (+)positive cytoplasm cells from control group (Fig.2).

Fig. 1. Section of rat heart from experimental group. Pink BAX (+) positive reaction. AEC and Hematoxylin staining. Magn. 350x

Fig. 2. Section of rat heart from control group. BAX reaction. AEC and Hematoxylin staining. Magn. 350x



Positive p-53, BAX(+) reaction was visible mainly in the cytoplasm of heart cells (Fig.1, 2).

Bcl-2 positive reaction was not observed in each preparation as well control and as well experimental group (Fig.3). From 16 preparations from control group 2 (12.5%) have BAX **(+)** positive reaction, and 1 (6.25%) has p-53 (+) positive reaction. However in experimental group 16 from 16 preparations p-53, BAX **(+)** positive reaction was visible.

Fig. 3. Section of rat heart from experimental group. Bcl-2(-) negative reaction. AEC and Hematoxylin staining. Magn. 350x

The mean field with BAX (+) positive reaction in experimental group cover 23116.9 μm^2, which was statistically significantly higher than the mean field with BAX (+) positive reaction in control group – 73.3 μm^2 (P=0.0003) (Tab.1).

The mean field with p-53(+) positive reaction in experimental group cover 1630.5 μm^2, which was statistically significantly higher than the mean field with p-53 (+) positive reaction in control group – 48 μm^2 (P=0.0001) (Tab. 2).

The mean field with BAX (+) positive reaction in heart in control group was about 0.009%of examined area, and in experimental group 3% of examined area.

The mean field with p-53 (+) positive reaction in heart in control group was about 0.006% of examined area, and in experimental group 0.2% of examined area.

Table 1. The mean field with BAX(+) positive reaction in heart

	Control group (μm^2)	Experimental group (μm^2)
Mean	73.3	23116.9
Standard deviation	+/–136.5	+/–10046.3
Statistical significance	P=0.0003	

Table 2. The mean field with BAX(+) positive reaction in heart

	Control group (μm^2)	Experimental group (μm^2)
Mean	48.0	1630.5
Standard deviation	+/–144.1	+/–738.5
Statistical significance	P=0.0001	

DISCUSSION

To early signs of apoptosis are included: decreasing of cells volume and chromatin condensation in nucleus. Such features was also observed in present experiment. Similar changes was noticed by Lampides et al. in cardyomyocytal nuclei in cell culture exposed on Adriamycin activity [12] and Taylor et al. in isolated rabbit heart [4, 20]. That changes were determined as a first signs of early cardiotoxicity after Adriamycin. Free radicals arising as a result of biotransformation of Adriamycin alkylate and damage nucleic acids, proteins and structural lipids [17]. Peroxidation of biological membranes including mitochondrial membranes lead to its destruction an increase of permeability. In the cytosol appears Cytochrome C, which initiates mitochondrial pathway of apoptosis.

Other, but also mitochondrial way of apoptotic signal induction is stimulated by damaged DNA. Crucial role in that pathway play p-53protein. It is phosphoprotein activated by damaged DNA. It inhibits cell cycle in G1 phase and makes cell repairing possible. Proper cell produces small amount of p-53 protein. In damaged cells, p-53 protein stimulate production of p-21 protein [9], which inhibits kinases necessary in cell cycle, what inhibits cell cycle for more than 10 hours [18]. When repairing is not possible p-53 initiates the program of apoptosis [5] – increases Fas expression, increases the amount of BAX protein, blocks Bcl-2 gene. Proteins from Bcl-2 group regulate the apoptosis via its influence on essential cellular processes. They mostly regulate the outflow of proapoptotic agents from mitochondria (cytochrom C, AIF (apoptosis inducing factor) [23] through interaction with so called megachannels (permeability transition pore-PTpore). They influence also on mitochondrial intramembraneal potential [3]and activate caspases and DNA-ses. Proteins from Bcl-2 groups acts antiapoptotically (proteins: Bcl-xL, Bcl-w, Mcl-1, Brag) and also proapoptotically (proteins: Bax, Bcl-xS, Bak, Bik, Bad, Bim). Relationship antiapoptotic to proapoptotic proteins decide about death or survival of cell [7].

In present study was noticed evident increase of positive immunohistochemical reaction for p-53 and BAX proteins, but for Bcl-2 protein was not noticed increased positive reaction in experimental group as compare to control one. Results points the presence of the enlarged apoptosis in rat heart after Adriamycin administration.

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