IMMUNOHISTOCHEMICAL ASSESSMENT OF RENAL TUBULAR EPITHELIAL CELL DEATH AFTER ANTHRACYCLINES THERAPY

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Abstract

In the present study the quantitative and qualitative immunohistochemical examinations of rats’ renal tubular epithelial cells were conducted to determine the presence of proteins involved in adriamycin-induced apoptosis, in which death signalling was produced through the mitochondrial pathway involving proteins: Bcl-2, BAX, Apaf-1, and caspases 9 and 3; the extrinsic pathway - proteins of caspase 8 and 3; and the endoplasmic reticulum pathway - caspases 12 and 3. Moreover, caspase 1 – one of the post-necrosis inflammation markers, was evaluated immunohistochemically. Adriamycin-induced apoptosis in the renal tubular cells occurred after 4 weeks and intensified after 7 weeks (an increase in reaction to the executing caspase 3). Irrespective of the time factor, it developed through the mitochondrial pathway (statistically-significant increase in reaction for, BAX, Apaf-1, caspase 9) and reticular pathway (statistically-significant increase in reaction for caspase 12). No increased reaction was observed for caspase 1.

Key words: rat, kidneys, cell death, apoptosis, anthracyclines, immunohistochemistry.

Until the 70-ties of the XX Century, cell death was classified as apoptosis (programmed cell death) – genetically-planned cell death and necrosis – an accidental type of cell death (9). In 1973, Schweichel et al. (16) suggested a wider classification of cell death based on the observations of rat and mouse embryos and foetuses. They noticed that at least three different forms of cell death occur: apoptosis, in which phagocytic lysosomes are involved in the apoptotic cell degradation (9); autophagol programmed cell death based on the lysosomes of the dying cell (6); and non-lysosomal death - rarely observed. In 1990, this classification was changed and extended by Clarke (4). He distinguished four types of cell death: type I apoptosis – cell shrinkage, chromatin condensation, nucleosomal DNA fragmentation, formation of apoptotic bodies, activation of caspases and removal of cell residues by lysosomes of the adjacent cells or phagocytes; type II autophagol cell death – formation of autophagol vesicles containing fragments of the cytoplasm or cell organelles surrounded by a double or multiple membrane (5) and destruction of vesicles by lysosomes of the same cell (1, 2, 13); type IIIA non-lysosomal granular degradation; and type IIIB cytoplasmic degeneration. Since that time many classifications of cell death have been created, which divide it, among other things, into apoptosis, autophagy, and proteasomal degradation (during which the proteins previously bound with ubiquitine are broken down) (12, 14). At present, the most common-distinguished types of cell death (13) include type I apoptosis; type II autophagy; and type III necrosis.

Anthracyclines, which include adriamycin (ADR), induce apoptosis via free radicals and oxidative stress caused in the cell.

The present study quantitative and qualitative immunohistochemical examinations were conducted to determine the presence of proteins involved in adriamycin-induced apoptosis, in which death signalling was produced through: the mitochondrial pathway involving proteins: Bcl-2, BAX, Apaf-1, and caspases 9 and 3; extrinsic pathway - involving caspase 8 and 3; and endoplasmic reticulum pathway – involving caspases 12 and 3. Moreover, caspase 1 – one of the post necrosis inflammation markers, was also evaluated immunohistochemically.
Material and Methods

The study material consisted of 32 Wistar female rats of baseline body weight 200-250 g and aged 2.5-3 months. The animals received standard feed and water ad libitum. The rats were divided into four equal groups: group I – rats treated with a single intraperitoneal dose of adriamycin - 5 mg/kg b.w. - and decapitated after 4 weeks; group II - control rats treated with a single intraperitoneal dose of 0.9% NaCl - 0.5 ml and - and decapitated after 4 weeks; group III - rats treated with a single intraperitoneal dose of adriamycin - 5 mg/kg b.w. - and decapitated after 7 weeks; and group IV - control rats treated with a single intraperitoneal dose of 0.9% NaCl - 0.5ml - and decapitated after 7 weeks. The study was approved by the Local Ethics Committee.

The renal specimens collected for immunohistochemical examinations were fixed in 10% formalin, dehydrated in the alcohol series, cleared in xylene, and embedded in paraffin blocks. The blocks were cut into 5 µm sections, which were placed on the silanised glasses. Two kidney specimens collected from each animal were used. After removing paraffin and hydration, the specimens were rinsed in Tris Buffered Saline (TBS). Next endogenous peroxidase was blocked by incubation in 0.3% H2O2 solution. The specimens were rinsed in TBS against protein Bcl-2 (1mM EDTA). After cooling for 20 min, the preparations were rinsed with distilled water and placed in the Tris Buffered Saline (TBS). Next the DakoCytomation kit was used for immunohistochemical reactions, which included: biotinylated secondary antibody against mice and rabbit antibodies (Biotinylated Link Universal) (15 min incubation); streptavidin conjugated with horse-radish peroxidase (Streptavidin-HRP) (15 min incubation); and AEC substrate - HRP reaction dye (AEC Substrate chromogen) (15 min incubation). After the use of each reagent, the sections were rinsed in TBS for 10 min. After chromogen staining, the sections were stained with haematoxylin for about 1 min and thoroughly rinsed with distilled water. The sections were covered with coverslip using the Aquatex fluid. The photographic documentation was prepared using the computer-guided Colour Video Camera CCD-IRIS (Sony).

The renal specimens collected for immunohistochemical examinations were fixed in 10% formalin, dehydrated in the alcohol series, cleared in xylene, and embedded in paraffin blocks. The blocks were cut into 5 µm sections, which were placed on the silanised glasses. Two kidney specimens collected from each animal were used. After removing paraffin and hydration, the specimens were rinsed in Tris Buffered Saline (TBS). Next endogenous peroxidase was blocked by incubation in 0.3% H2O2 solution. The specimens were rinsed in TBS against protein Bcl-2 (1mM EDTA). After cooling for 20 min, the preparations were rinsed with distilled water and placed in the Tris Buffered Saline (TBS). Next endogenous peroxidase was blocked by incubation in 0.3% H2O2 solution. The specimens were rinsed in TBS for about 10 min and incubated with mice monoclonal primary antibody at room temperature for 60 min: BAX (Santa Cruz:sc-7480) in 1% TBS/BSA, dilution 1/25; Bcl-2 (Santa Cruz:sc-7382) in 1% TBS/BSA, dilution 1/25; and with rabbit primary antibody: caspase 1(Lab Vision RB-9259-PO) in 1% TBS/BSA, dilution 1/100; caspase 3 (Lab Vision Ab-4; RB-1197-PO) in 1% TBS/BSA, dilution 1/100; caspase 8 (Lab Vision Ab-4, RB-1200-PO) in 1% TBS/BSA, dilution 1/100; caspase 9 (Lab Vision RB-1205-PO) in 1% TBS/BSA, dilution 1/100; caspase 12 (Alexis/ProScience PSC-2327) in 1% TBS/BSA, dilution 1/100; and Apaf-1 (Lab Vision RB-9263-P0) in 1% TBS/BSA, dilution 1/100.

The positive BAX reaction was focally observed in all groups. Less intense reaction was found in the control group where the cytoplasm of tubular epithelial cells showed slightly intensified granular red reaction (Fig. 2). In experimental group III, the cytoplasmic reaction was more intense. Dark red granules were located in the cytoplasm of renal tubular epithelial cells around the nucleous.

The Bcl-2 reaction in the renal tubular cells was focal and slightly intensified in all groups. The cytoplasm of the cells with positive immunohistochemical reaction was slightly pink, mainly in the basilar part of the cells. The intensity of reaction was similar in all control and experimental groups (Fig. 3).

The results of immunohistochemical examinations were subjected to quantitative evaluation using the Analysis-pro software, version 3 (Soft Imaging System GmbH, Germany). The microscopic images, at magnification 125x were analysed assessing the protein expression in three randomly-chosen areas, 781,193.35 µm², each. The surface area of cells with positive reaction (+) was calculated. The statistical analysis using the one-way ANOVA test involved the differences in mean area with positive immunohistochemical reaction for all antibodies examined in the individual groups.

Results

A positive caspase 3 reaction was markedly more intense in the experimental groups. It was focal and concerned the cytoplasm of the tubular epithelial cells. In the control groups, the whole cytoplasm was pink (Fig. 1). In the remaining experimental groups, the cytoplasmic reaction was mostly intense red and concerned the apical parts of the tubular epithelial cells directed towards the lumen. Characteristic location the immunohistochemical reaction concerned those places in the tubular cells where the cytoplasm indended to the tubular lumen during the formation of apoptotic cells (cells “boiled”).Quantitative evaluation showed increased caspase 3 reaction in all experimental groups compared to control (group I - P=0.0002; group III - P<0.0001). The strongest reaction was seen 7 weeks after the drug administration (Table 1) Differences between both experimental groups were statistical significant (P<0.001).

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The Bcl-2 reaction in the renal tubular cells was focal and slightly intensified in all groups. The cytoplasm of the cells with positive immunohistochemical reaction was slightly pink, mainly in the basilar part of the cells. The intensity of reaction was similar in all control and experimental groups (Fig. 3).

Quantitative evaluation showed statistically significantly increased BAX reaction in all groups compared to controls (group I - P=0.002; group III - P<0.001). Its intensity was the highest 7 weeks after adriamycin administration. The lowest BAX reaction increase was observed in rats examined 4 weeks after adriamycin administration. The Bcl-2 reaction in all groups was comparable with the control (Table 1).
Fig. 1. Control group. The rat kidney section showing caspase 3 reaction of low intensity. AEC+H staining. 140x.

Fig. 2. Control group. Positive BAX reaction of low intensity in the rat kidney. AEC+H staining. About 280x.

Fig. 3. Experimental group III. Very weak Bcl-2 reaction in the kidney 7 weeks after adriamycin administration. AEC+H staining. About 140x.

Fig. 4. Control group. Apaf-1 reaction of low intensity in the kidney. AEC+H staining. About 280x.

Fig. 5. Experimental group III. The kidney of rats treated with adriamycin and examined 7 weeks later. Highly intense Apaf-1 reaction. AEC+H staining. About 280x.
The Apaf-1 reaction was visible focally in all groups (Figs 4 and 5). The most intense reaction was observed 7 weeks after Adriamycin administration. The bright pink, diffused reaction filled the whole cytoplasm of the renal tubular epithelial cells; however, it was much more intense in the apical part directed to the renal tubule lumen. In these places, the reaction was more granular, and dark pink and dark red in colour. Increased red reaction was also found in the lumen of renal tubules (in apoptotic bodies?). In experimental groups where the tubular immunohistochemical reaction was more intense, it was also observed in renal glomeruli, although its intensity was low (Fig. 5).

The quantitative evaluation using the image computer analyser showed statistically significantly increased Apaf-1 reaction in all groups compared to the control (group I - \( P<0.001 \); group III - \( P=0.0016 \)). The Apaf-1 reaction in rats examined 7 weeks after Adriamycin administration was the highest one (Table 1). There was no statistical significance between experimental groups (\( P=0.013 \)).

The caspase 9 reaction was diffused and focal in all experimental and control groups. The most intense
reaction was observed in rats 7 weeks after adriamycin administration. The staining was pink and evenly distributed in the whole cytoplasm of renal tubular cells. Increased pink reaction was visible in the lumen of renal tubules (in apoptotic bodies?).

Quantitative evaluation showed relatively strong caspase 9 reaction already in the control group; however, it increased further (statistically significantly) in the adriamycin group (group I - P=0.0042; group III - P=0.0003). Differences between both experimental groups were not statistically significant (P=0.009). (Table 1)

The immunohistochemical caspase 12 reaction was slightly intense in the control group. In both adriamycin groups the reaction was intensely increased, focal, and mainly in the apical part of the cells directed to the tubular lumen. The colour intensity ranged from bright to dark red (Fig. 6).

Quantitative computer-evaluation of caspase 12 reaction showed a statistically-significant increase in renal tubular cells 4 weeks after adriamycin administration (P=0.0002). The area occupied by positive cytoplasmic reaction in the rats examined 7 weeks after adriamycin administration increased even more (P<0.0001). Differences between both experimental groups were statistically significant (P=0.002).(Table 1)

The caspase 8 reaction was visible in all groups (Fig. 7). Quantitative evaluation showed that caspase 8 reaction was similar in control and experimental adriamycin groups (group I - P=0.58; group III - P=0.24) Differences between both experimental groups were not statistically significant (P=0.44) (Table 1)

In all groups, the caspase 1 reaction was focal, weak, bright pink, evenly filling the cytoplasm of renal tubular cells. In the adriamycin groups the reaction was also visible in the lumen of renal tubules. Quantitatively, there was no increase in the caspase 1 reaction in any group compared to controls (group I - P=0.23; group III - P=0.10). The intergroup differences were statistically insignificant (Table 1).

Discussion

Many literature reports describe the two main pathways leading to cell apoptosis (3):“EXTRINSIC” - this pathway is induced by an external signal and is connected with the membranous death receptors; and the “INTRINSIC” pathway in which mitochondria are involved. Sometimes both pathways overlap. This phenomenon is called the amplification of proapoptotic signalling. Numerous recent studies on apoptosis have widened the knowledge about the pathways of apoptotic signalling. It has been demonstrated that the EXTRINSIC pathway leads not only through the activation of membranous death receptors, which have an intracellular death domain (DD) (10), but also through the attack of cytotoxic T lymphocytes. These lymphocytes recognise damaged cells or those infected with viruses and initiate apoptosis to prevent neoplastic transformation or viral infection. T lymphocytes produce perforine and granzyme B. Perforine causes perforation of the cellular membrane incorporating into it and forming a large membranous channel, which enables the penetration of granzymes into the cell. Granzyme B - the proteolytic enzyme, activates the caspase cascade. Simultaneously, the cell is penetrated by Ca\(^{2+}\) ions from the extracellular fluid, so increased Ca\(^{2+}\) levels are thought to be death signals. Some cell-initiating apoptosis have both Fas and FasL membranous proteins. Their combination activates the caspase cascade.

The INTRINSIC pathway is activated by cellular stress (7, 8), which may occur during the cell exposure to radiation, chemical substances, or viral infection. Moreover, it may result from growth factor damage or oxidative stress initiating apoptosis via the involvement of mitochondria. Mitochondria can be involved indirectly. In this case DNA damage leads to the accumulation of p-53 protein in the cell and inhibition of the cell cycle. p-53 stimulates the production of BAX protein, which is capable of opening the mitochondrial channels from which cytochrome C is released. Cytochrome C, together with Apaf-1 protein, forms a complex activating procaspase 9 thus activating the caspase cascade. Direct damage to mitochondria also releases cytochrome C, which together with Apaf-1 and procaspase 9 forms an apoptosome-activating caspase 9 and hence initiating the caspase cascade. The INTRINSIC pathway can also initiate apoptosis due to damage to the endoplasmic reticulum, which leads to the activation of caspase 12 located inside the reticulum. The activation of caspase 12 triggers the caspase cascade (15).

In this study adriamycin-induced apoptosis in the renal tubular cells of rats occurred after 4 weeks and intensified after 7 weeks (an increase in reaction to the executing caspase 3). Irrespective of the time factor, it developed through mitochondrial pathway (statistically-significant increase in reaction for BAX, Apaf-1, caspase 9) and reticular pathway (statistically-significant increase in reaction to caspase 12). No increased reaction was observed for caspase 1- one of the biomarkers of inflammation.

References