IMMUNOHISTOCHEMICAL ASSESSMENT OF CELL DEATH IN HEPATOCYTES AFTER ANTHRACYCLINE THERAPY

AGNIESZKA PEDRYCZ, ZBIGNIEW BORATYŃSKI¹, IRENEUSZ OLESIŃSKI², AND MALGORZATA DUDZIŃSKA³

Department of Histology and Embryology with Laboratory of Experimental Cytology, Medical University of Lublin, 20-080 Lublin, Poland
¹Department of Animal Anatomy and Histology, Faculty of Veterinary Medicine, University of Life Sciences in Lublin, 20-950 Lublin, Poland
²Department of Neurology I, Neuropsychiatric Hospital, 20-442 Lublin, Poland
³Department of Lungs Diseases and Tuberculosis, Jan Bozy Hospital Lublin, 20-089 Lublin, Poland
apw4@wp.pl

Received for publication August 4, 2009

Abstract

The study material consisted of 32 Wistar female rats. The rats were divided into four equal groups. The rats from the experimental groups received adriamycin - 5 mg/kg b.w. to induce apoptosis and were decapitated after 4 and 7 weeks. After decapitation, specimens from the liver were collected, fixed in 10% formalin, and then embedded in paraffin blocks. Caspases 1, 3, 8, 9, and 12, Bcl-2, BAX, and Apaf-1 were detected using the standard three-step immunohistochemical method. Adriamycin-induced apoptosis in the hepatocytes occurred after 4 weeks and increased after 7 weeks (an increase for executing caspase). Irrespective of the time factor, it was induced mainly via the mitochondrial pathway (statistically-significant increase in reaction for BAX, Apaf-1, and caspase 9) and reticular pathway (statistically-significant increase in reaction for caspase 12). Apoptosis via the extrinsic pathway through caspase 8 activation was comparable to that in control (insignificant caspase 8 reaction). There was no increased reaction observed for caspase 1, one of the markers of inflammation.

Key words: rat, liver, cell death, anthracyclines, caspases, immunohistochemistry.

The present paper is a continuation of the series of authors’ studies concerning adriamycin-induced apoptosis. Furthermore, it is known that each cell is capable of destroying its own proteins and itself. Short-lived regulatory cytoplasmic proteins are mainly destroyed by cell proteasomes (22). Long-lived proteins are destroyed by lysosomes, to which they are transported in five different ways: simple transport, vacuolar degradation and transport, endocytosis, microautophagy, and macroautophagy (4).

Macroautophagy, also called autophagy, is a well-known mechanism of suicidal cell death (19). It was described in yeasts (9, 14), insects, and during embryonic development in humans (5). It plays a vital role in the pathogenesis of neoplastic tumours (15) and neurodegenerative diseases (17, 20).

Before the research on apoptosis is started, it is essential to differentiate between various types of cell death. The distinction between apoptosis and necrosis is relatively simple; however it is extremely difficult to determine the boundary between apoptosis and autophagial cell death. This is partly related to the fact that the apoptotic process may end with autophagy. The blockage of caspase activity is likely to result in the transition of apoptosis into type II cell death (16). And, the other way round, initiated autophagy may end in apoptosis. The apoptotic cells are markedly different from the necrotic ones, although the inflammatory process caused by necrosis ends with apoptosis of the inflammatory cells, thanks to which the inflammatory process gets shortened and healthy normal cells are not damaged. Necrosis is accidental death caused by an exogenous harmful factor, which blocks the life processes of the cell (e.g. respiration or production of high-energy compounds). It is a pathological, passive, catabolic, and degenerative process, which results in the substantial loss of many cells in the tissue and in an inflammatory reaction. Apoptosis is a programmed controlled death. It is the physiological mechanism used to eliminate single damaged and old cells without disturbing the integrity of tissues or eliciting an inflammatory response. The apoptotic cell shrinks, its organelles preserve full integrity, and DNA divided by endonuclease forms a characteristic apoptotic ladder visible during DNA electrophoresis on agarose gel. The size of DNA fragments corresponds to the segments...
between the adjacent nucleosomes. The forming apoptotic bodies are phagocytosed by the neighbouring cells.

Necrosis is different. The cell swells because its membrane becomes more permeable to water. The smooth and rough endoplasmic reticulum space widens, and mitochondria become oedematous. The plasmatic membranes undergo perforation. The cell ruptures, pouring its content into the intercellular space. The necrotic focus develops and is filled with lymphocytes, neutrophils, and macrophages involved in the inflammatory process. DNA is split into fragments of various length.

The process of apoptosis was divided into three phases: decisive, executive, and degradation ones (7). The decisive phase of apoptosis is reversible and lasts from the apoptotic signal generation to caspase cascade initiation. The executive phase is irreversible. Its essential elements are cysteine proteases from the interleukin-1-β-converting enzyme (ICE) family - called the caspase family (12). The caspases-digest proteins behind asparaginan residue use one of their cysteine residues (21), hence their name – cysteine-dependent asparaginan-specific proteases. The degradation phase of apoptosis is the phase in which apoptosis is accomplished. It begins with the destruction of the nuclear DNA.

In this study, proteins, which are activated during apoptosis, caspases 1, 3, 8, 9, and 12, Bcl-2, BAX, and Apaf-1 were detected in rats’ hepatocytes using the standard three step immunohistochemical method. We wanted to answer a question: which pathway involves adriamycin-induced apoptosis?

Material and Methods

The study material consisted of 32 white Wistar female rats weighing 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 in which the effects of adriamycin were evaluated (Table 1). Apoptosis was induced with a single intraperitoneal administration of adriamycin (Adriblastin; Farmitalia, D; 10 mg were dissolved in 5 ml of injection water) in the dose of 5 mg/kg b.w. (groups I and III). In control groups (II, IV) the rats received a single intraperitoneal dose of 0.5 ml of 0.9% NaCl. The study was approved by the Local Ethics Committee.

The animals were decapitated after 4 weeks (groups I and II) and 7 weeks (remaining groups). After decapitation, the specimens from the right hepatic lobe were collected for further examinations. The specimens were fixed in 10% formalin, dehydrated in the alcohol series, subjected to xylene, and embedded in paraffin blocks. The blocks were cut into 5µm-sections, which were placed on the silanised glasses.

To detect proteins in the liver cells, the three-step immunohistochemical method was used. In the first step, the removal of endogenous peroxidases from the sections (peroxidases are oxidised by hydrogen peroxide) and antigen-antibody reaction (detecting antigens: BAX, Bcl-2, caspases 1, 3, 8, 9, and 12, react with the primary antibodies) were performed. In the second step, the reaction of primary antibodies with biotinylated secondary antibodies took place. In the third step, the reaction of biotin with avidin (ABC-avidin-biotin complex) or streptavidin (BSA-biotin-streptavidin) conjugated with horse-radish peroxidise was carried out. The addition of the dye (AEC), which is oxidised by horse-radish peroxidase at the site of antigen-antibody reaction gives a red (AEC(3-amino-9-ethylcarbazole)) stain at the site of the occurrence of the antigen.

The results of immunohistochemical examinations were subjected to qualitative evaluation, taking into account the intensity of colour reaction at the antigen-antibody site in rat organs examined in individual groups.

The quantitative evaluation was performed using the Analysis-pro software, version 3 (Soft Imaging System GmbH, Germany).

The microscopic images, magnification 125x, were analysed assessing the protein expression in three randomly-chosen areas, 781,193.35 µm², each. The surface area of cells with a positive reaction (+) was calculated. Firstly, the area to be masked was determined. Secondly, the range of colours was selected, which corresponded to the colour of the antigen-antibody reaction over which the mask would be placed. The mask was placed (transition to black and white image - binarisation). The computer detected the masked areas (the areas, which became white due to the mask). At the end, summing up the masked areas took place. The statistical analysis was presented as means and standard deviation of the mean using the ONE WAY ANOVA test. Five percent error risk and statistical significance at P<0.05 were accepted.

The analysis involved the differences in mean area with positive immunohistochemical reaction for all antibodies examined in the individual groups.

Results

The most intense caspase 3 reaction was observed focally in hepatocytes in the rats 7 weeks after the administration of adriamycin. The reaction was bright, intensely pink and filled evenly the whole cytoplasm. Quantitative evaluation showed the area occupied by positive caspase 3 reaction in the liver in the adriamycin group examined 4 weeks after drug administration compared to the control group (P<0.006). However, the highest increase was found in the rats examined 7 weeks after the administration of adriamycin. (P<0.001) (Table 2). Differences between both experimental groups were statistically significant (P=0.0001). The BAX reaction observed in the liver was focal in all groups (Fig.1). The most intense reaction was observed in the rats 7 weeks after the administration of adriamycin (P=0.0004). The red, granular cytoplasmic reaction was mainly found around the hepatocyte nuclei.
Differences between both experimental groups were statistically significant (P=0.0001).

The Bcl-2 reaction was of low intensity in all groups examined. Quantitative evaluation showed a statistically-significant increase in BAX reaction in both adriamycin groups compared to the control group; a statistically-significantly higher increase was observed 7 weeks after the administration of adriamycin. The Bcl-2 reaction in all groups was comparable with the control (Table 2).

The Apaf-1 reaction was observed focally in the liver in all groups examined (Fig. 2). The most intense reaction was found in the group examined 7 weeks after the administration of adriamycin (P<0.0001). The dark red colour reaction filled the whole cytoplasm of hepatocytes. More intense reaction was noted around the nuclei. Quantitative evaluation showed statistically-significant increase in Apaf-1 reaction in both adriamycin groups compared to control; a statistically-significantly higher increase was observed 7 weeks after the administration of adriamycin. (Table 2). Differences between both experimental groups were statistical significant (P<0.0001).

The most intense caspase 9 reaction was observed in the hepatocytes of rats examined 7 weeks after the administration of adriamycin. The colour reaction filling completely and evenly the cytoplasm ranged from pink-violet to brown-red (Fig. 3). In all groups the reaction was visible focally. Quantitative evaluation showed statistically-significant increase in caspase 9 reaction in both adriamycin group compared to control; the highest increase was observed 7 weeks after the administration of adriamycin (P=0.0001 to control). (Table 2). Differences between both experimental groups were statistically significant (P<0.0001).

### Table 1
Animal groups and experimental design

<table>
<thead>
<tr>
<th>Group</th>
<th>Onset of experiment</th>
<th>4 weeks</th>
<th>7 weeks</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Experimental I</strong></td>
<td>Adriamycin</td>
<td>decapitation</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Control II</strong></td>
<td>0.9% NaCl</td>
<td>decapitation</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Experimental III</strong></td>
<td>Adriamycin</td>
<td>decapitation</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Control IV</strong></td>
<td>0.9% NaCl</td>
<td>decapitation</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### Table 2
Mean area (µm²) occupied by positive reaction for all proteins in the examined area of 781,193.35 µm² in the rat liver in all groups

<table>
<thead>
<tr>
<th>Control</th>
<th>Experimental</th>
<th>ONE WAY ANOVA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>After 4 weeks</td>
<td>After 7 weeks</td>
</tr>
<tr>
<td><strong>Caspase 3</strong></td>
<td>212.16 µm²±116.59</td>
<td>901.15 µm²±316.98</td>
</tr>
<tr>
<td><strong>BAX</strong></td>
<td>242.94 µm²±95.94</td>
<td>999.05 µm²±302.81</td>
</tr>
<tr>
<td><strong>Bcl-2</strong></td>
<td>152.87 µm²±100.43</td>
<td>400.17 µm²±137.52</td>
</tr>
<tr>
<td><strong>Apaf-1</strong></td>
<td>2,277.06 µm²±431.13</td>
<td>31,183.32 µm²±1,890.93</td>
</tr>
<tr>
<td><strong>Caspase 9</strong></td>
<td>1,863.61 µm²±118.69</td>
<td>5,362.15 µm²±522.88</td>
</tr>
<tr>
<td><strong>Caspase 12</strong></td>
<td>302.8 µm²±218.89</td>
<td>3,099.25 µm²±741.25</td>
</tr>
<tr>
<td><strong>Caspase 8</strong></td>
<td>243.59 µm²±262.43</td>
<td>287.44 µm²±243.93</td>
</tr>
<tr>
<td><strong>Caspase 1</strong></td>
<td>221.35 µm²±203.14</td>
<td>132.45 µm²±183.34</td>
</tr>
</tbody>
</table>
Fig. 1. Experimental group III. The liver section of the Adriamycin-treated rat examined 7 weeks later. Intense BAX reaction. AEC+H staining. About 280x.

Fig. 2. Experimental group III. The liver section of the Adriamycin-treated rat examined 7 weeks later. Intense Apaf-1 reaction. AEC+H staining. 140x.

Fig. 3. Experimental group I. The liver section of the Adriamycin-treated rat examined 4 weeks later. Intense caspase 9 reaction. AEC+H staining. About 140x.

Fig. 4. Experimental group III. The liver section of the Adriamycin-treated rat examined 7 weeks later. Intense caspase 12 reaction. AEC+H staining. 280x.

Fig. 5. Control group IV. Very weak caspase 1 reaction in the liver. AEC+H staining. About 140x.
The caspase 12 reaction was most intense in the group of rats examined 7 weeks after adriamycin administration (Fig. 4). The dark pink to intense red reaction was focal and concerned the whole cytoplasm, which it filled evenly. The neighbouring hepatocytes were characterised by various intensity of positive caspase 12 reaction. Quantitative evaluation showed statistically-significantly increased caspase 12 reaction in both adriamycin groups compared to control (group I: P=0.002; group III: P=0.0001); a statistically-significantly higher increase was observed 7 weeks after the administration of adriamycin (Table 2). Differences between both experimental groups were statistically significant (P<0.0001).

The caspase 1 and 8 reaction was focal in all examined groups. Qualitative and quantitative evaluation revealed weak reaction of both caspases in the rat liver in all experimental groups, which was comparable with the control (Fig. 5, Table 2).

Discussion

There are a number of reports describing adriamycin–induced apoptosis in various human and animal organs (1, 8, 13). However, only a few researchers have tried to define the signalling pathways of apoptosis. Asakura et al. (2) observed adriamycin-activated caspase 3, which indicated that the cells examined were in the course of apoptosis.

In the present study, adriamycin-induced apoptosis in the hepatocytes via the mitochondrial pathway (extrinsic) through the release of cytochrome C from mitochondria, formation of apoptosisome with Apaf 1, activation of caspase 9 and executing caspase 3. Similar observations were reported by Chen et al. (6), as well as Ascensao et al. (3). In the present study, the level of BAX also increased, while the level of apoptotic protein Bcl-2 was comparable to that in control. The second pathway of apoptotic signal transmission in the present study was through the activation of caspase 12. Apoptosis induced by adriamycin via this pathway was also described by Jang et al. (11). They administered adriamycin intraperitoneally, as in the present experiment, in the twice-higher dose - 10 mg/kg b.w. - and demonstrated that in the apoptotic pathway, through the destruction of endoplasmic reticulum and activation of caspase 12, the two remaining ones dominated - mitochondrial and receptor (extrinsic). Mehmed (18) showed that if during apoptosis the caspases were activated near the cell membrane (extrinsic pathway) or the mitochondrial membrane (mitochondrial pathway), caspase 12 was not activated. The third pathway of apoptotic signal induction – extrinsic (activation of caspase 8) in the present study was significantly less Intense and comparable with that in controls.

At all stages of the present study, the level of caspase 1, involved in the process of inflammation resulting from necrosis, was examined immunohistochemically. Caspase 1 was always at the level comparable with that in control, which is likely to indicate the lack of inflammation and marked predominance of apoptosis over potential necrosis. Moreover, necrosis was not confirmed by renal and hepatic images observed by authors under light and electron microscope.

In order to induce apoptosis in various cells, adriamycin was administered in the doses ranging from 3 mg/kg (10) to 10 mg/kg (11). According to Asakura (2) the apoptotic signal-activating caspase 3 was already induced 6 h after the administration of adriamycin. Jang et al. (11) observed apoptosis after 4 d.

Adriamycin induced apoptosis in the renal tubular epithelial cells and hepatocytes 4 weeks after its administration. Apoptosis developed via the mitochondrial and reticular pathway and increased during the observation period. Other types of cell death were not observed (necrosis, autophagy).

References


