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Over-expression of biomarkers of environmental stress in renal epithelial cells resulting from proapoptotic activity of adriamycin: an immunohistochemical assessment

Nadekspresja biomarkerów stresu środowiskowego w komórkach nabłonka kanalików nerkowych jako wynik proapoptotycznego działania adriamycyny: ocena immunohistochemiczna

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Summary

Introduction: In previous studies authors examined renal cells in rats after adriamycin administration and showed over-expression of proapoptotic effector caspase 3 and also histological changes typical for apoptosis.The mechanisms of adriamycin cytotoxic action involve DNA damage, action of free radicals formed in the process of degradation of this antibiotic, and interactions of both mechanisms.

The aim of the current study was to investigate the early answer of renal cells on proapoptotic action of adriamycin. Using the same model of renal epithelial cells from rats treated with adriamycin author determined the expression of proteins (HSP70, p-53) which are known as a biomarkers of cellular stress, using the immunohistochemical three-step method.

Results and discussion: The results showed that examined in this experimental study cells in response to DNA damage, increased synthesis of p-53 protein and in response to oxidative stress, started overproduction of heat shock protein 70 as a repairing factor. The areas of immunolocalization of these proteins were quantified using an image analysis program. Overproduction of HSP70 is extremely favorable for cells exposed to stress, however it blocks the repair action of p-53.

Conclusion: It is also likely that HSP70 inhibitors administered with adriamycin might result in more effective anti-neoplastic therapy for this drug.

Streszczenie

Wprowadzenie:W poprzednich pracach autorzy oceniając komórki nabłonka kanalików nerkowych po terapii adriamycyną (antybiotykiem przeciwnowotworowym) wykazali wzmożoną ekspresję proapoptotycznej, efektorowej kaspazy 3 oraz histologiczne zmiany w komórkach typowe dla apoptozy. Adriamycyna wywołuje cytotoksyczność uszkadzając DNA komórkowe oraz działając przez wolne rodniki powstające podczas jej biodegradacji.

Celem obecnej pracy była ocena wczesnej odpowiedzi komórki na uszkadzające działanie adriamycyny. Zbadano nerki szczurów poddanych uprzednio terapii adriamycyną, w których oceniono przy użyciu standardowej trójstopniowej metody immunohistochemicznej ekspresję białek HSP70 i p-53. Białka te znane są jako czułe biomarkery stresu komórkowego.

Wyniki i dyskusja: Wyniki badań wykazały, że komórki badane w niniejszym doświadczeniu odpowiedziały na działanie adriamycyny uszkadzające DNA zwiększoną syntezą białka-53, zaś na stres tlenowy wywołany przez wolne rodniki – nadekspresją HSP70. Wzmożona produkcja HSP70 w komórce narażonej na stres jest niezwykle pożądanym mechanizmem ochronnym. Z drugiej jednak strony HSP70 blokuje przeciwnowotworowe działanie p-53.

Wnioski: Może okazać się, że inhibitory HSP70 stosowane razem z adriamycyną przyczynią się do skuteczniejszej terapii tym lekiem.

Key words: biomarkers of environmental stress, HSP70, p-53, Kidney, Apoptosis, Immunohistochemistry
Słowa kluczowe: biomarkery stresu komórkowego, HSP70, p-53, nerki, apoptoza, immunohistochemia

Introduction

In previous reports author noticed that renal tubular epithelial cells in rat treated with Adriamycin (an antibiotic from anthracyclines group), were in a condition of oxygen shock [1]. In these cells author noticed that Adriamycin administered to rats led to typical apoptotic changes in renal tubular epithelial cells [2]. These changes were significantly greater in the Adriamycin-treated group than in the control one [2] with a statistically greater apoptotic index and increased expression of executive caspase 3.

In other studies author noticed that adriamycin-induced apoptosis in the renal tubular cells of

rats occurred after 4 weeks and intensified after 7 weeks (an increase in reaction for 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 for caspase 12). No increased reaction was observed for caspase 1- one of the biomarkers of inflammation.

The aim of the current study was to investigate the early answer of renal cells on proapoptotic action of adriamycin. Using the same model of renal epithelial cells from rats treated with adriamycin author determined the expression of proteins (HSP70, p-53) which are known as a biomarkers of cellular stress preventing the cells against adriamycin-induced apoptosis. The immunohistochemical three-step method was used.

Material and methods

The study material consisted of 32 albino Wistar female rats with baseline body weight – 200-250g, aged 2.5-3 months. They got standard food and water *ad libitum* and were housed 4 rats per cage with a natural light/dark cycle. The rats were divided into four groups: two an experimental (n=8) and a control groups (n=8). The rats from the experimental groups were injected with Adriamycin (Adriblastin - Farmitalia, Milan, Italy) intraperitoneally in a dose of 5mg/kg of body weight. The vehicle (solvent) for the Adriamycin was 0.9% NaCl. The rats from the control groups were given 0.5 ml 0.9% NaCl also intraperitoneally.

The rats were killed by decapitation after 4 weeks (group exp.-I, contr.-II) and after 7 weeks (group exp.-III, contr.-IV).

The experimental protocols received permission from the Ethics committee for animal experimentation of the Medical University of Lublin (No 32/2000, 551/2005).

The rat's kidneys were collected for immunohistochemical investigations. The kidneys for immunohistochemical studies were fixed in 10% formalin buffered in phosphate buffer pH 7.0, and after dehydration in ascending ethanols, cleared in xylene and embedded in paraffin. Microtome sections were cut at 5μm and then adhered to the siliconised slides.

To identify p-53, HSP70 proteins preparations from experimental and control groups were used. For each preparation, a negative control (a slide without primary antibody) was used.

The protein expression level was evaluated with a standard three-step immunohistochemical procedure LSAB using DakoCytomation kits according to the manufacturer's instructions. Mouse p53 (diluted 1:50) (Lab Vision Corporation, Fremont, CA, USA), and rabbit HSP70 (diluted 1:100 (Lab Vision)) antibodies were used as a primary antibody. Then a biotinylated secondary antibody was added, followed by horseradish peroxidase conjugated with streptavidin (DakoCytomation; Glostrup, Denmark). At the sites of immunolocalization of the primary antibodies, a reddish color appeared after adding a chromogen – AEC (DakoCytomation; Glostrup, Denmark). The colored reaction occured because streptavidin has a strong affinity to biotin.

The expression of all proteins was evaluated in preparations from 16 rats from the control group and 16 rats from the experimental group (two preparations from every individual for each from the antibodies; in total, for each antibody there were 32 control slides and 32 experimental slides). The slides were analyzed using a light microscope. The photographic documentation was performed with a CCD-IRIS Colour Video Camera (Sony) connected with a computer.

The analysis of the microscopic images at a magnification of 125x, to assess the expression of the protein was performed using the computer program analySIS®Pro 3.0 (Soft Imaging System, Munster, Germany). From each slide 3 randomly selected standard microscope fields of 781193,35μm^2 were assessed. The field of the sectioned surface of the kidney specimens with positive reactions was measured. The range of colors assessed by the computer as positive was set as intensive red; red-pink or pink were not assumed as a positive result.

The results were analyzed statistically using an ANOVA test and a student's t-test. The averages, standard deviations and the percentage of positive reactions in the examined tissue field were determined. The differences were considered statistically significant when $p<0.05$.

Results

Qualitative evaluation in the kidneys showed focal p-53 reaction in all groups (Fig 2a,b,c). The colour reaction was observed in the cytoplasm of renal tubular epithelial cells. The positive cytoplasm staining was weak pink in control groups (Fig 2a). In the experimental groups the reaction was markedly more intense - stained bright red (Fig.2b,c). The cytoplasm of the cells was stained at their bases in the vicinity of basal membranes of renal tubular epithelial cells. In the renal glomeruli, p-53 (+) reaction was not observed (Fig. 2a,b,c).

Quantitative evaluation revealed statistically significantly increased p-53 reaction in experimental groups ($p<0,001$). The highest increase was observed in the group 7 weeks after adriamycin administration ($p=0,006$, Table I).

The HSP70 reaction was visible focally in rat kidneys in all experimental and control groups (Fig 1a,b,c). The least intense staining reaction was observed in control groups (Fig 1a). Its colour ranged from bright to dark pink. The reaction filled the apical part of the cytoplasm of renal tubular epithelial cells. The most intense reaction was found in the cytoplasm of tubular epithelial cells in the 7 weeks after ADR group. It was dark pink in colour, finely granular, diffused, and filled the whole cells and focally the lumen of renal tubules (Fig. 1b,c).

Figures:

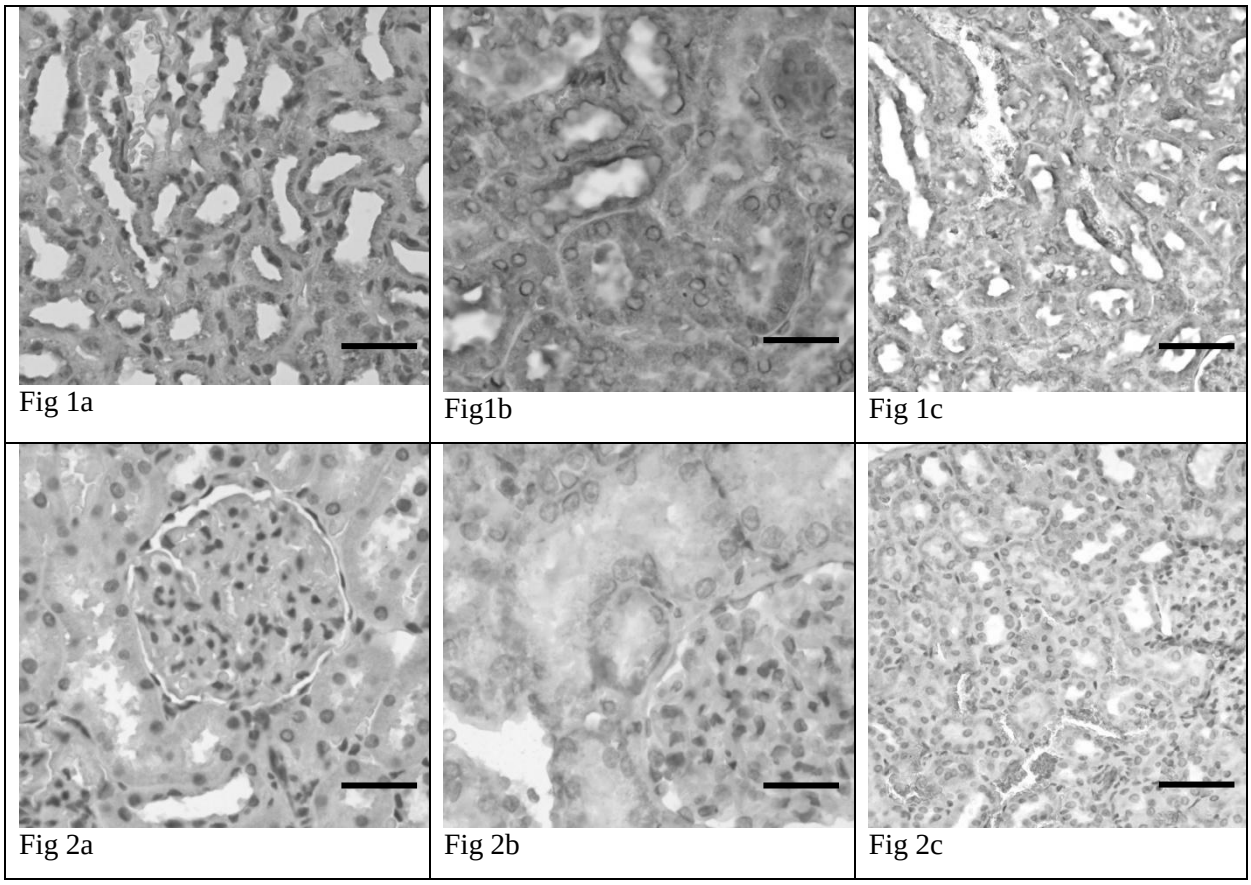

Figure 1. Immunohistochemical localization of HSP70 protein in the kidney from (a) control rats (quite intensive cytoplasmic HSP70 reaction) scale bar=36μm, and from (b) experimental rats, decapitated 4 weeks after Adriamycin administration (HSP70 reaction of medium intensity) scale bar=36μm, and from (c) experimental rats decapitated 7weeks after Adriamycin administration (HSP 70 showing strong staining) scale bar=18 μm. All preparations stained with AEC (AEC Substrate chromogen) and nuclei counterstained with hematoxylin.

Figure 2. Immunohistochemical localization of p-53 protein in the kidney from (a) control rats (weak cytoplasmic p-53 reaction), scale bar=54μm and from (b) experimental rats, decapitated 4 weeks after Adriamycin administration (p-53 reaction of medium intensity), scale bar=72μm, and from (c) experimental rats decapitated 7 weeks after Adriamycin administration (p-53 showing strong staining), scale bar=18 μm.

All preparations stained with AEC (AEC Substrate chromogen) and nuclei counterstained with hematoxylin.

Table 1. The mean area in μm^2 occupied by a positive reaction for p-53, HSP70 proteins in standard fields of 781193μm^2 in rat kidneys in the control and the experimental groups

Protein	**Control group** Mean±SD	**Experimental group I** Mean±SD	**Experimental group III** Mean±SD	**P value***
p-53	1882μm² +/-466	7116μm² +/-771	9716 μm² +/-981	p<0.001
HSP70	11388μm² +/-1455	50092μm² +/-221	172432μm² +/-12187	p<0.001

*One way ANOVA test, statistical significance

The HSP70 immunostaining reaction in the kidneys evaluated quantitatively using the image analysis computer program was quite strong in controls. A higher increase was observed 4 weeks after adriamycin administration ($p<0,001$). The highest HSP70 reaction was found in the adriamycin group 7 weeks after its administration ($p<0,001$, Table I). Differences between groups was statistical significant ($p<0,001$).

Discussion

There are several reports describing adriamycin-induced apoptosis in various human and animal organs [2,3]. About 5-15% of the administered dose of ADR is excreted in the urine within 5-7 days after administration [4]. This is why the renal cells, are therefore most exposed to the effects of free radicals formed in the process of biodegradation of adriamycin and responsible for the development of oxidative shock in the cell, which leads to apoptosis.

In the decisive phase of apoptosis, the key role is played by p-53 protein, which is a phosphoprotein activated by damaged DNA. It inhibits the cell cycle in phase G1 enabling the cell to repair itself. The normal cell produces small amounts of p-53. It was observing in this experiment too. The mean area occupied by a positive reaction for p-53 protein in rat kidneys in the control groups was almost 4 times less than in experimental group examined 4 weeks after ADR therapy and almost 5 times less than in experimental group examined 7 weeks after ADR therapy. In damaged cells, p-53 stimulates the production of p-21 protein [5,6], which inhibits kinases required in the cell cycle, inhibiting the cell cycle for several hours [7]. When repair is not possible, p-53 starts the apoptosis process[8]. It increases the expression of Fas, the amount of BAX protein, and blocks Bcl-2 gene.

The expression of genes encoding HSPs is induced by so-called environmental stress. This stress is caused by high temperature (hence the name: heat shock proteins), but also by other internal and external factors (molecular stressors) which include: analogs of amino acids, heavy metals, alcohols, free radicals, metabolic poisons, bacterial and viral infections, glucose deficiency, cytokines, UV radiation etc. [9]. It was observed in this experiment: the mean area occupied by a positive reaction for HSP70 protein in rat kidneys was almost 4,5 times bigger in the experimental group examined 4 weeks after ADR therapy and almost 15 times bigger in experimental group examined 7 weeks after ADR therapy than in control groups. The role of molecular chaperones, including HSP70, is to prevent the damage to proteins, which could lead to their degradation. Schmitz et al. [10] described HSP70 as a protein protecting the cell against the death signal.

Deminenko et al. [3] demonstrated that pharmacological induction of HSP70 production prevented apoptosis in the cells usually undergoing adriamycin–induced apoptosis.

After exposure of the renal cells to adriamycin in this experiment, two mechanisms preventing the cell against apoptosis were initiated: an increase in HSP70 expression as a cell response to the action of free radicals formed after ADR biotransformation and an increase in p-53 expression as a cell response to DNA–damaging effects of ADR.

In the present study increased HSP70 reaction was a response of cells to damaging oxidative stress (action of free radicals). An increase in the amount of HSP70 in the cells induced by adriamycin was too small to protect the cells against apoptosis.

However, it was sufficient to show that the cells got under the influence of destroying (proapoptotic) factors and made an attempt to repair the damage.

In the present study, adriamycin exerted strong proapoptotic effects on the cells examined, which were not inhibited even by HSP70, an endogenous factor preventing against apoptosis [11].

One of the theories concerning abnormal, neoplastic divisions of a normal cell indicates that this phenomenon occurs when p-53 is damaged or chaperones (including HSP70) fail. However, it appears that even when p-53 is undamaged, the cell can undergo neoplastic division.

According to Żylicz et al. [12], overproduction of HSP70 is extremely favorable for the cells exposed to stress, however it blocks the repair action of p-53, which leads to neoplastic proliferation of the cell as HSP70 produced in abundant amounts and also combines with p-53 resulting in the dysfunction of the blocked protein. The combination of HSP70 and p-53 leads to the inhibition of p-53 in the cytoplasm. P-53 cannot enter the nucleus and activate the genes encoding repairing proteins. It was observing in this experiment (especially in experimental groups): the presence of p-53 in the cytoplasm of cells and not their nucleus where they are most commonly detected.

The present study showed that adriamycin caused an increase in HSP70 as well as p-53 in the renal cells. In the tumor therapy, p-53 with its antineoplastic action is most desirable. According to Żylicz et al. [12], an increase in HSP70 concentration leads to the repair dysfunction of p-53 (although its amount in the cell in the present study increased in comparison to control). On the one hand, this is likely to be caused by too small an amount of p-53, on the other hand, by its binding by HSP70.

It is also likely that HSP70 inhibitors administered with adriamycin might result in more effective antineoplastic therapy with this drug as reduced amounts of the intracellular regulator (HSP70) decreasing or sometimes preventing the effect of apoptosis could contribute to an increase in the amount of p-53, which by counteracting neoplasia would increase the antineoplastic and proapoptotic effects of adriamycin.

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