POSSIBILITIES OF PROTEIN EXPRESSION MEASUREMENT IN IMMUNOHISTOCHEMICAL APOPTOSIS DETECTION IN THE IRRADIATED LUNG TISSUE EXAMPLE

[1]Michal KRÁLIK, [1]Jan ÖSTERREICHER, [2]Leoš NAVRÁTIL, [1]Jiří KNÍŽEK, [1]Jiřina VÁVROVÁ, [2]Jiří ŠKOPEK[2], [1]Aleš MACELA,
[1]Department of Radiobiology and Immunology, Purkyně Military Medical Academy, Hradec Králové
[2]Group of the Clinical Radiobiology, the [1st] Medical Faculty of Charles University, Prague

Summary

This study observes the development of apoptotic changes in type II pneumocytes in a time interval of 1, 2, 3, 4, 5, 8 and 12 weeks after local irradiation of the lungs with 15 Gy. We also measured the number of neutrophils in the lung tissue. Elevation of the apoptotic cells was observed 2 weeks and 4 weeks after irradiation. An increase in neutrophil infiltration was observed from 5 weeks after irradiation.

Key words: Radiobiology; Apoptosis; Immunohistochemistry; Detection.

Introduction

It is a well known fact, that after irradiation of the lungs radiation pneumonitis (RP) and radiation fibrosis (RF) appear. RP occurs as a result of ionizing radiation by X-ray or by gamma radiation (10). The clinical manifestation of RP was noted from the 16[th] to the 30[th] weeks after irradiation of the lungs with a single dose of 7 Gy and a higher dose. This illness is classified as Adult Respiratory Distress Syndrome (ARDS), sometimes known as diffuse alveolar damage (DAD) This nosologic unit is usually manifested in whole-body irradiated individuals after the recovery from the bone marrow syndrome or as a result of local irradiation of the lungs in oncological patients with a 10% probability of RP in the common therapeutic procedure (6).

Histopathologic manifestations of RP have already been described. Interstitial edema of the alveolar septa and infiltration of the alveolar septa by neutrophils are the most important of them. However the mechanism of inception of RP has not been described up to the present time. It is supposed that pneumocytes represent the decisive role in the occurrence of RP. They are maternal cells of alveolar epithelium. Their production of surfactant keeps low surface tension of alveoli. Type II pneumocytes are changed to type I pneumocytes, which maintain the integrity of the alveolar-capillary membrane. Hurley (5) states that type I pneumocytes with their tight junctions, rather than endotheial cells, check the penetration of fluid through the alveolar septa.

Apoptosis is a programmed physiological process playing an important role in homeostasis. During embryonal development apoptosis is used to eliminate of unnecessary cells and to form organs. Apoptosis maintains the balance of the cell populations of individual tissues as a contradiction of cell proliferation (2). Apoptosis is also induced by various harmful factors for example ionizing radiation (3, 7) and cytostatic agents (4, 1).

For the identification of epithelial apoptotic cells, the present method of Cytokeratine 18 fragments detection is used. The enzyme Caspase 3 plays an important role in Cytokeratine 18 fragmentation. Cytokeratine 18 ranks among the family of cytoskeletar proteins presented in epithelial cells. Caspase 3 is a part of the enzymatic cascade and together with additional Caspases is exprimed by such Ced genes as zymogenes, which are activated due to the influence of various apoptotic impulses. After the running of the enzymatic cascade, changes, which end with cell death, arise (12).

In recent years, the computer image analysis was established as a tool for quantitative measurement of various markers in evaluated histological samples.

We have decided to observe the influence of ionizing radiation on the development of apoptosis in the lung tissue, especially on type II pneumocytes because this has not been described in a satisfactory may up to now. For comparison of the occurrence of apoptotic processes in individual periods two markers were selected: the number of M30 positive cells in the viewing field (preparations were stained using the M30 Cytodeath kit (Boehringer Mannheim, Germany) and the average intensity of positive staining in the field which expresses the quantity of a new protein production. In connection with apoptosis the change in neutrophil infiltration of the lung tissue in individual time

periods after irradiation was evaluated. As a marker we monitored the number of neutrophils (preparations were stained using chloracetate esterase stain).

Material and Methods

Male rats (Wistar) aged 8-12 weeks and with a weight of 150-200 g were used for the experiment. The laboratory animals were irradiated locally in the area of the thorax using a ^{60}Co unit (Chisotron Chirana) at a dose rate of 1,0 Gy/min (the target distance was 1 m). The animals were slightly anaesthetised before irradiation by means of a solution composed of one portion of Rometa R (the Spofa Company, Prague) and 3 portions of Narkamon (The Léčiva Company, Prague) and 12 portions of physiological saline. This solution was applied intramuscularly in a 10 ml/kg dose. Local irradiation of the rat thoraxes was performed in a fixating box with a 10 cm thick layer of lead plates to prevent the irradiation of other parts of the body with a dose higher than 2-3% (WARD).

The irradiated laboratory animals were divided into 7 groups with a total of 42 individuals irradiated with 15 Gy and histologically examined 1, 2, 3, 4, 5, 8 and 12 weeks after irradiation. The non-irradiated animals were killed 12 weeks after the beginning of experiment. The values of the observed markers of 6 rats from the non-irradiated group were used as reference values.

Histologic Examination

The animals were killed and histologically examined 1,2,3,4,5,8 and 12 weeks after irradiation with 15 Gy. The lungs of these animals were histologically examined. When the given time intervals had passed, the rats were killed using cervical dislocation. During the dissection, the removed lungs were carefully fixed per trachea in a 10% neutral buffered formalin, embedded into paraffin then 4 μm tissue sections were cut, and were stained using Chloracetate esterase (to determine neutrophil granulocytes) and Gramm's stains (to evaluate bacterial infection).

Immunohistochemical examinations for direct detection of the apoptotic cells were performed using a standard peroxidase technique. For immunohistochemical detection of apoptotic cells the M30 Cytodeath kit (Boehringer Mannheim, Germany) was used. After blocking for 20 minutes, tissue sections were incubated with individual monoclonal antibodies, the M30 Cytodeath kit (Boehringer Mannheim, Germany) for 24 hours at 4 °C, and washed three times in a phosphate-buffered saline (PBS, pH 7,2). Then they were incubated with a horseradish peroxidase-coupled anti-mouse antibody (Boehringer Mannheim, Germany) for 30 minutes at room temperature. Excess antibodies were washed off with PBS. Finally, a 0,05% 3,3-diaminobenzidine tetrahydrochloride chromogen solution (Sigma, Prague, the Czech Republic) in PBS containing 0,02% hydrogen peroxide was added for 10 minutes at room temperature to visualise the formed antigen-antibody complex.

The Measurement of Apoptotic Cells

Immunohistochemical samples were evaluated using the IMT-2 light microscope (Olympus Company, Prague) and a computer image analysis - Image Pro (Media Cybernetics, MD, USA). Ten randomly selected viewing fields without bronchi and large vessels with a size of 10744,32 μm^2 were evaluated in each preparation at a 600-fold original magnification. In each viewing field, positive staining cells have been manually counted and the intensity of the staining has been automatically measured. It was established that positive staining pixels are in the range of Red: 117-170, Green: 76-138, Blue: 0-107 for the M30 Cytodeath detection, where 0 is a white colour and 255 is a black colour. The intensities of positive staining in the field were counted as a percentage of the positive staining pixels/summary of the positive staining pixels densities in the field. The number of neutrophils was evaluated using the AMPLIVAL light microscope (Carl Zeiss, Jena). 15 randomly selected viewing fields were evaluated in each preparation at a 640-fold original magnification.

Data Processing

In the data obtained the diameter ± SEM was calculated and Mann-Whitney's test was used for the statistical analysis.

Results

In a group examined 4 weeks after irradiation, 2 rats died (during the 1st week) and 1 rat within a group examined 12 weeks after irradiation (7 weeks).

No infectious agents were found during histo-

Table 1

Expression of Apoptosis and the Number of Neutrophils in Irradiated Lung Tissue

Time interval after irradiation	1 week	2 weeks	3 weeks	4 weeks	5 weeks	8 weeks	12 weeks
Average number of M30 positive cells	0.18±0.03	1.63±0.22[a]	0.12±0.04[b]	4.25±0.51[c]	17.05±0.67[c]	10.25±0.77[c]	8.43±0.48
Average intensities of positive staining	2.29±0.08	3.30±0.13[b]	2.54±0.06[b]	5.48±0.22[c]	8.90±0.31[c]	7.49±0.45[b]	7.93±0.29
Average number of neutrophils	0.09±0.01	0.07±0.00	0.11±0.01	0.07±0.01	1.24±0.07[a]	4.80±0.19[c]	5.95±0.14[a]
Non-irradiated control							0,35±0,07

Probability of value difference to the previous time interval: $p<0,05$ - [a], $p<0,01$ - [b], $p<0,001$ - [c]

logic examination of the laboratory animals lungs.

In the evaluated control non-irradiated group an average number of 0.35 M30 positive cells in the viewing field was found.

In the evaluated samples of irradiated animals we found significant differences in the quantity of apoptotic cells. Two weeks after irradiation we found a statistically significant elevation in comparison with the previous time interval, a significant decrease in apoptotic cells was measured 3 weeks after irradiation. A significant elevations in the number of apoptotic cells were observed 4 and 5 weeks after irradiation. A significant decrease was observed after 8 weeks and this decrease continues to the end of the experiment.

During the evaluation of average intensities of staining we observed a significant increase in comparison with the previous time interval 2 weeks after irradiation which was followed by a significant decrease 3 weeks after irradiation. A significant elevation was observed 4 and 5 weeks after irradiation. A significant decrease in this marker was measured 8 weeks after irradiation.

From the results of changes in the number of neutrophils in dependence on the time interval after irradiation, a significantly higher number of neutrophil granulocytes from 5 weeks after irradiation to the end of the experiment in irradiated animals than non-irradiated rats was found after irradiation. The highest number of neutrophils was found 12 weeks after irradiation.

Discussion

The positivity of immunohistochemical staining was found in type II pneumocytes, in bronchial epithelium and rarely we detected the presence of the slightly stained alveolar macrophages undetected in this study. The positivity of the alveolar macrophages seems to be connected with the phagocytar activity.

Our results show that exposure to ionizing radiation causes the increase of apoptotic cells. The temporary increase was found 2 weeks after irradiation. From 4 weeks after irradiation an increase in apoptotic cells was observed with maximum 5 weeks after irradiation when a significant difference in comparison with the control values was found. Perhaps the apoptosis of II. type pneumocytes is a mechanism which plays an important role in the development of changes after irradiation.

Up to now it has been unknown, if apoptosis is induced by neutrophils or if the presence of neutrophils in irradiated tissue is in the result of an unknown mechanism which is a probable response to the apoptosis of II. type pneumocytes. Up 4 weeks after irradiation the lung tissue without the significant infiltration of neutrophils was observed. From 4 weeks after irradiation the a significant increase in neutrophils was found. From our results we can assume that the presence of apoptotic cells precedes the increase of infiltration by neutrophils. Moreover, the cells starting the apoptotic process can indirectly cause the induction of a mechanism

which shares the increase of neutrophils infiltration in the lung tissue.

During the evaluation of the development of the apoptotic process we observed two markers - the number of apoptotic cells and the average intensities of positive staining. From the results identical trends in the values of both markers are apparent. For that reason it is necessary to measure these markers also in other studies.

The important study is the work of Ward et al. (11) who on the model of the irradiated mouse lung with 15 Gy demonstrated the initial increase in permeability 2 weeks after irradiation and a process of starting RP 4 weeks after irradiation. In our experiment we detected an increase in the quantity of the apoptotic cells in this time intervals. It corresponds to the results of the above mentioned authors. On the other hand it was found from our unpublished results that an increase in permeability of the alveolo-capillary membrane expressed by the measurement of the thickness of the alveolar septa came especially 3 weeks after irradiation. The fall of increased vascular permeability was found 4 weeks after irradiation, but no increase in RP. Ward et al. (11) used a dose rate of 1,4 Gy/min. In our experiment we used only a dose rate of 1,0 Gy/min. This could be manifested in slow expression of the histologic changes in the irradiated lung tissue.

In the next studies we will observe the postirradiation molecular changes of the bronchial epithelial cells and we will elaborate a model of the sublethal irradiated lung tissue.

Acknowledgements

The authors thank to Šárka Průchová and Stanislava Štefcová for their technical assistance during the experiment.

This study was supported by a grant from the Secretary of Defense MO 66 020398 127 and by the Grant Agency of Charles University, GAUK 227/98.

Translation Daniela Uhlířová

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Korespondence: Michal Králik
Vojenská lékařská akademie J. E. Purkyně
Třebešská 1575
500 01 Hradec Králové
e-mail: mikrálik@emeil.cz

Received 11. 4. 2000