ORIGINAL ARTICLE

Phosphorylation of histone H2AX in peripheral blood mononuclear cells after thoracic irradiation of rats

Radim Havelek[1], **Martina Řezáčová**[1], **Zuzana Šinkorová**[2], **Lenka Zárybnická**[2], **Jaroslav Pejchal**[2], **Jiřina Vávrová**[2]

[1]Department of Medical Biochemistry, Faculty of Medicine in Hradec Králové, Charles University in Prague, Czech Republic
[2]Department of Radiobiology, Faculty of Military Health Sciences in Hradec Králové, University of Defence, Czech Republic

Summary

Lymphocytes are among the most radiosensitive cells. After exposure of the organism to ionizing radiation, they promptly die by apoptosis at a rate proportional to the dose received. Because of this, they are frequently used in biodosimetry. We demonstrated that one hour after whole-body irradiation of rats, histone H2AX in the lymphocyte nuclei was quickly phosphorylated on serine 139, the phosphorylation process being directly dependent on the gamma radiation dose. In the work presented here, we studied the kinetics of lymphocyte depletion in the peripheral blood and phosphorylation of histone H2AX in the peripheral blood lymphocytes after local (thoracic) irradiation of rats. Twenty-four hours after whole-body irradiation of the rats at a dose of 5 Gy, the lymphocyte count declined to almost zero values, whereas after local irradiation of the thorax area, the counts of lymphocytes in the peripheral blood remained unaltered.

The authors employed two methods (flow-cytometric and microscopic) for the γH2AX determination in the peripheral blood lymphocytes, 1 h after thoracic irradiation of rats. Flow cytometry revealed a dose dependence on the increase in γH2AX in a dose range of 10–30 Gy. The microscopic method was more sensitive in the case of lower radiation doses, the dependence on the dose being obvious from a dose as low as 5 Gy. The methods are able, in the dose range 5–30 Gy, to differentiate between the type of irradiation, i.e. the whole-body or local.

Key words: γH2AX; biodosimetry; ionizing radiation; thoracic; lymphocytes

✉   Radim Havelek, Department of Medical Biochemistry, Faculty of Medicine in Hradec Králové, Šimkova 870, 500 38 Hradec Králové, Czech Republic

🖳   havelekr@lfhk.cuni.cz

☎   +420 495 816 177

INTRODUCTION

In radiation accidents, it is frequently impossible to establish doses in the people exposed based on physical dosimetry and thus, there is a need for new biodosimetric indicators which can be used in the retrospective analysis of the doses received. At the present time, the method frequently used is to

determine the dicentric chromosomes in lymphocytes isolated from the peripheral blood of the radiation exposed persons (Blakely et al. 2009). The method, however, has certain limitations, particularly the long time lapse between the exposure and dose determination due to the necessity to incubate the lymphocytes with phytohaemagglutinin.

Exposure of lymphocytes to increasing doses (under *in vivo* as well as *in vitro* conditions) results in their exponential cellular death by apoptosis. Under *in vivo* conditions, apoptotic cells are quickly phagocytosed and destroyed by the reticulo-endothelial system. The maximum decrease in the number of lymphocytes in the peripheral blood of whole-body irradiated animals has been observed 72 hours after irradiation (Vávrová and Filip 2002), and the predictability of the rate of lymphocyte depletion has made it possible to develop a model for purposes of biodosimetry based on lymphocyte depletion kinetics (Goans et al.1997).

Ionizing radiation (IR) produces DNA double-strand breaks (DSB) and, at the site of the DSB, so called ionizing radiation-induced foci (IRIF) are formed. After DSB generation, the neighbouring chromatin undergoes extensive modifications, initiated by the ataxia telangiectasia mutated (ATM) – mediated phosphorylation of histone H2AX (γ-H2AX) (Rogakou et al. 1998) followed by the recruitment of MDC1 adaptor and two ubiquitin ligases, RNF8 and RNF168 (Larsen et al. 2010). Thereafter, MRN complex repair proteins (NBS1, MRE11 a Rad50), BRCA1 and 53BP occur at the DSB (Bekker-Jensen et al. 2006). H2AX is a member of the H2A family and accounts for from 2% to 25% of the total histone H2A pool. Histone H2AX contains a distinct C-terminal extension with serine as the 4th C-terminal amino acid. This serine is phosphorylated after the DSB induction and the phosphorylated histone H2AX has been named gamma H2AX. In human H2AX, this C-terminal serine is serine 139 (Rogakou et al. 1998). Gamma H2AX could be specifically detected as early as a few minutes after the DSB induction (Huang and Darzynkiewicz 2006).

In our previous studies (Vilasová et al. 2008), we demonstrated the dose-dependent phosphorylation of H2AX 1 hour after *in vitro* irradiation of a human acute lymphoblastic leukemia cell line (MOLT-4) and human peripheral blood lymphocytes at doses of 0.5 to 5 Gy. We also demonstrated a dose dependence of the H2AX phosphorylation in lymphocytes isolated from the peripheral blood of whole-body irradiated rats one hour after irradiation. Specimens of peripheral blood may be stored for 23 hours on ice in a refrigerator (4 °C) to make possible a delayed

sample analysis with a minimum level of γH2AX decay (Havelek et al. 2011).

However, partial-body irradiation (from external sources) of humans is more common than whole-body irradiation. The lungs are organ sensitive to gamma radiation exposure. Lungs exposed to a gamma radiation dose over 8 Gy suffer from acute radiation pneumonitis as well as chronic radiation fibrosis in later periods of time after irradiation. Radiation pneumonitis is an exudative inflammation, which occurs in irradiated areas only (Travis 1980). Differences were described between the responses of different strains of mice and rats to thoracic irradiation (Down 1986, Jackson et al. 2010). Jackson et al. (2010) compared the responses of C57L and C57BL6 mice to thoracic irradiation at doses of 10–15 Gy. The C57L mice were very sensitive to the development of early pneumonitis 3–4 months after irradiation; in C57Bl6 mice, the response was delayed and many mice accumulated large amounts of the pleural fluid in the lungs 6–9 months after irradiation; Wistar rats developed a combination of early pleural effusions and pneumonitis. Österreicher et al. (2004) studied the dose-dependent response in rats three weeks after irradiation at doses of 1–25 Gy. A significant dose-dependent depletion of type II pneumocytes was found after thoracic irradiation of the rats at a dose of 1 Gy and above. Alveolar neutrophils increased in number after 1 Gy with a dose dependence observed after 10–25 Gy and alveolar septa thickening after 5–25 Gy.

In the work presented here we studied the possibility of using the γH2AX measurement as a biodosimetric indicator after partial-body irradiation. Gamma radiation exposure of the thoracic region was used as a model example of partial-body irradiation. Changes in γH2AX expression in the lymphocytes of rats after *in vivo* local thoracic irradiation at doses of 5–30 Gy, observed by flow cytometry and immunocytochemistry, were analysed to retrospectively estimate the gamma radiation doses received.

MATERIAL AND METHODS

Animals
SPF (specific pathogen-free) female Wistar rats (VELAZ-Lysolaje, Czech Rep.), body weight of 210–250 g, were used. Each experimental group included 6 animals. The animals were housed in a temperature- and humidity-controlled environment with a 12-hour light/dark cycle. Food and water were available *ad libitum*. All the procedures were

approved by the Ethical Committee supervising experiments performed on animals at the Faculty of Military Health Sciences Hradec Králové (MO 12-7/2008-3696).

Gamma irradiation
The female Wistar rats were exposed to whole-body (5 Gy) and local thoracic (5, 10, 20 and 30 Gy) irradiation by using a ^{60}Co γ-ray source (Chisotron Chirana, Czech Rep.) at a distance from the source of 0.5 m and dose rate of 3 Gy/min. The doses were measured by an ionization chamber (Dosemeter PTW Unidos 1001, serial No. 11057, with ionization chamber PTW TM 313, serial No. 0012; RPD Inc., USA); the set was validated by the Czech Metrology Institute – Inspectorate for Ionizing Radiation (protocol No. 9011-OL-U4124/2005). The animals were slightly anaesthetized before irradiation with a mixture of Rometar (10 mg/kg) and Narkamon (1.2 mg/kg) (Bioveta, Czech Rep.). The solution was injected intramuscularly. Local thoracic irradiation was performed in a field 3 cm wide; the body was otherwise shielded by a 10-cm lead layer, which reduced the dose in the remaining parts of the body to about 2–3 % of the lung dose (Österreicher et al. 2004). Control animals were treated in the same way, but were not irradiated.

Leukocyte differential count
A peripheral blood sample (5–7 ml) was taken from the rat myocardium (one sample from each animal) and the differential count was determined on the Sysmex XE-2100 haematological analyzer (Sysmex-Toa, Japan).

Flow-cytometric detection of γH2AX
The method of Huang and Darzynkiewicz (2006) was modified and used for γH2AX detection. The method was optimized for the detection of γH2AX in the rat peripheral blood mononuclear cells (PBMCs). The PBMCs of the rats exposed to thoracic irradiation and controls were isolated with Histopaque (Sigma Aldrich, Germany) according to the manufacturer's instructions. The red blood cells trapped in pellets were lysed with the EasyLyse^{TM} reagent (DakoCytomation, Germany) according to the manufacturer's instructions. The PBMCs were rinsed twice with PBS (Dulbecco's phosphate buffered saline, Sigma-Aldrich, Germany) and then fixed with an ice-cold 1% methanol-free formaldehyde solution, rinsed with PBS and suspended in ice-cold 70% ethanol. The cells were stored at 4 °C before further manipulation. In the next step, the cells were rinsed with 1% BSA-0.2%-Triton X-100 in PBS and stained with anti-phospho histone H2AX (Ser139)-FITC

conjugate primary antibody (Millipore, USA) at room temperature for 1 h in the dark. The cells (after rinsing) were suspended in a propidium iodide (PI) staining solution (0.1% RNase, PI 5μg/mL in PBS), incubated at room temperature for 30 min in the dark with PI staining solution and analysed immediately after incubation, on the FACS analyser CyAn DakoCytomation (Beckman Coulter, USA). At least 100 000 cells were analysed per each sample. List mode data were analysed using the Summit v 4.3 software (Beckman Coulter, USA). The cells in G0/G1 phase were gated and the mean of fluorescence of the cells in each sample was calculated. Blood samples from 6 animals were analysed per experimental dose.

Immunocytochemical detection of γH2AX
The PBMCs were isolated as described above and fixed with 4% freshly prepared paraformaldehyde for 10 min at room temperature, washed with PBS, permeabilized in 0.2% Triton X-100/PBS for 15 min at room temperature, and washed with PBS. Before the incubation with primary antibodies (overnight at 4 °C), the cells were incubated with 7% inactivated foetal calf serum + 2% bovine serum albumin in PBS for 30 min at room temperature. Murine monoclonal anti-phospho-histone H2AX (Millipore, USA) was used for γH2AX detection. For the secondary antibody, the affinity pure donkey anti-mouse-FITC-conjugated antibody was purchased from the Jackson Laboratory (USA). The secondary antibody was applied to each slide (after their pre-incubation with 5.5% donkey serum in PBS for 30 min at room temperature), incubated for 1 h in dark and washed (3 × 5 min) with PBS. The nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI). Images were obtained by the Nikon Eclipse fluorescence microscope; the exposure time and dynamic range of the camera in all the channels were adjusted to the same values for all the slides to obtain quantitatively comparable images.

Statistics
The bivariate histograms of γH2AX-FITC immunofluorescence intensity versus DNA content distributions obtained by the flow cytometer were analysed by using the Summit v 4.3 software (Beckman Coulter, USA) and the mean of immunofluorescence intensities of γH2AX-FITC signals in nuclei of lymphocytes in each sample was calculated by using this software. The descriptive statistics of the results was calculated and the charts were made in Microsoft Office Excel 2003 (Microsoft Inc., USA) or GraphPad Prism 5 biostatistics (GraphPad Software, Inc., USA). In this study, all the
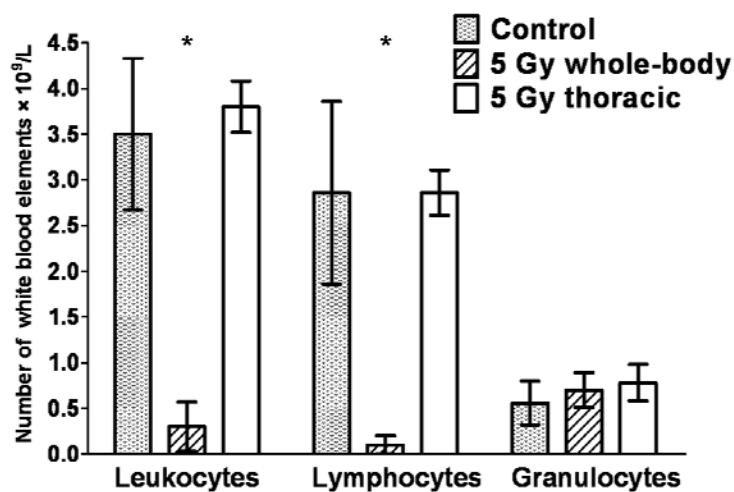
Fig. 1. Comparison of leukocyte differential counts in rats 24 h following *in vivo* whole-body and thoracic irradiation at a dose of 5 Gy. The figure presents arithmetic mean values ($\times 10^9$/l) with S.E.M of hexaplicates.

* whole-body irradiation at 5 Gy causes significant decline of leukocyte and lymphocyte numbers (to almost zero values in the case of lymphocytes), whereas local irradiation of the thorax area has insignificant effects on lymphocyte numbers in the peripheral blood; numbers of granulocytes remained unaltered in both experimental settings over the interval considered
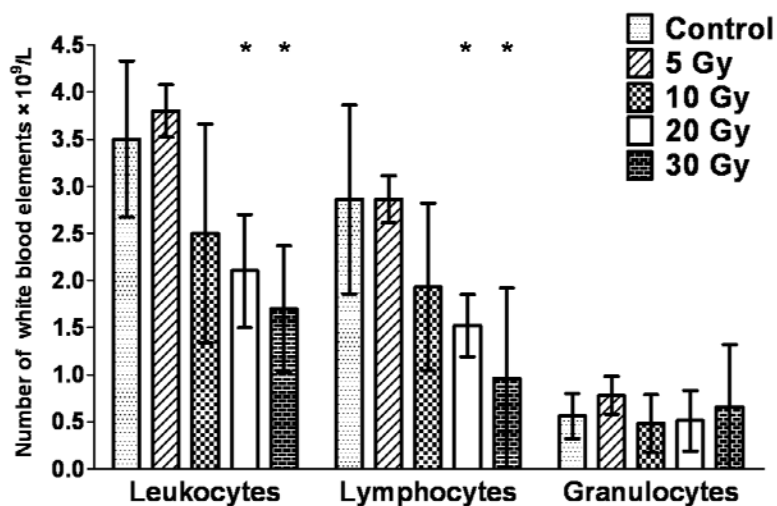

Fig. 2. Leukocyte differential count 24 h following the *in vivo* thoracic irradiation of rats. Columns show the number of white blood elements ($\times 10^9$/l) after exposure to different doses of ionizing radiation. The figure presents arithmetic means; error bars show S.E.M of hexaplicates.

* indicates statistically significant values in the Student's t-test for leukocyte and lymphocyte counts after 20 and 30 Gy

Fig. 3. **Microscopic detection of γH2AX in lymphocytes 1 h after *in vivo* thoracic irradiation of rats.** The peripheral blood lymphocytes were isolated from the heparinized peripheral blood of control and irradiated rats (doses 5–20 Gy) 1 h after irradiation, fixed and γH2AX was detected by the phospho-specific antibody. The figure shows representative projections of control (C) and irradiated samples obtained by fluorescence microscopy. Bright spots present γH2AX foci, the lines surrounding cell nuclei were drawn based on 4',6-diamidino-2-phenylindole (DAPI) counterstaining.
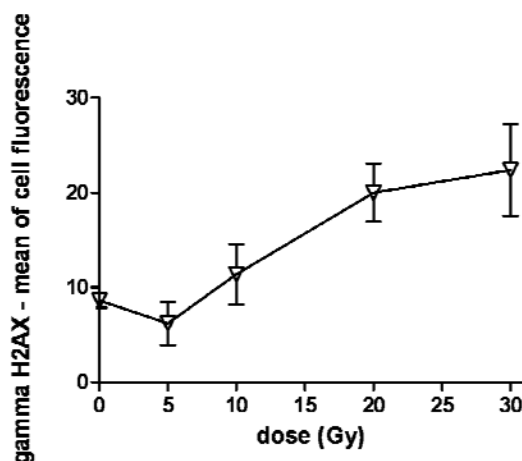

Fig. 4. **Flow-cytometric quantification of γH2AX-FITC immunofluorescence in lymphocytes 1 h following the *in vivo* irradiation.** The lymphocytes were isolated from the heparinized peripheral blood of control and irradiated rats (doses of 5–30 Gy) 1 h after irradiation, γH2AX was detected immunocytochemically, by the antibody specific for H2AX phosphorylated on serine 139 and simultaneously by analysis of the cellular DNA content. The mean of cellular fluorescence was measured by flow cytometry for G0/G1 cells. Samples from 6 rats were evaluated in each group. The graph presents means of γH2AX-FITC immunofluorescence depending on the gamma radiation dose. The results are statistically significant for doses of 20 and 30 Gy. The figure presents arithmetic mean; error bars show S.E.M of hexaplicates.

values were expressed as arithmetic means with S.E.M of hexaplicates unless otherwise noted. The significant differences between the groups were analysed by the Student's t-test at the significance level $2\alpha=0.05$.

RESULTS

Leukocyte differential count
Fig. 1 compares leukocyte differential counts in the peripheral blood 24 h after whole-body and local (thoracic) irradiation of rats. After whole-body irradiation at 5 Gy, a massive decline of the lymphocyte count was observed (almost to zero values), whereas thoracic irradiation at the same dose has only insignificant effect on the lymphocyte count compared to the control non-irradiated group.

Fig. 2 demonstrates the leukocyte differential count 24 hours after thoracic irradiation of rats at doses of 5–30 Gy. Declines in leukocyte and lymphocyte numbers were statistically significant against the non-irradiated group from a dose of 20 Gy. Numbers of granulocytes were not significantly changed 24 h after thoracic irradiation.
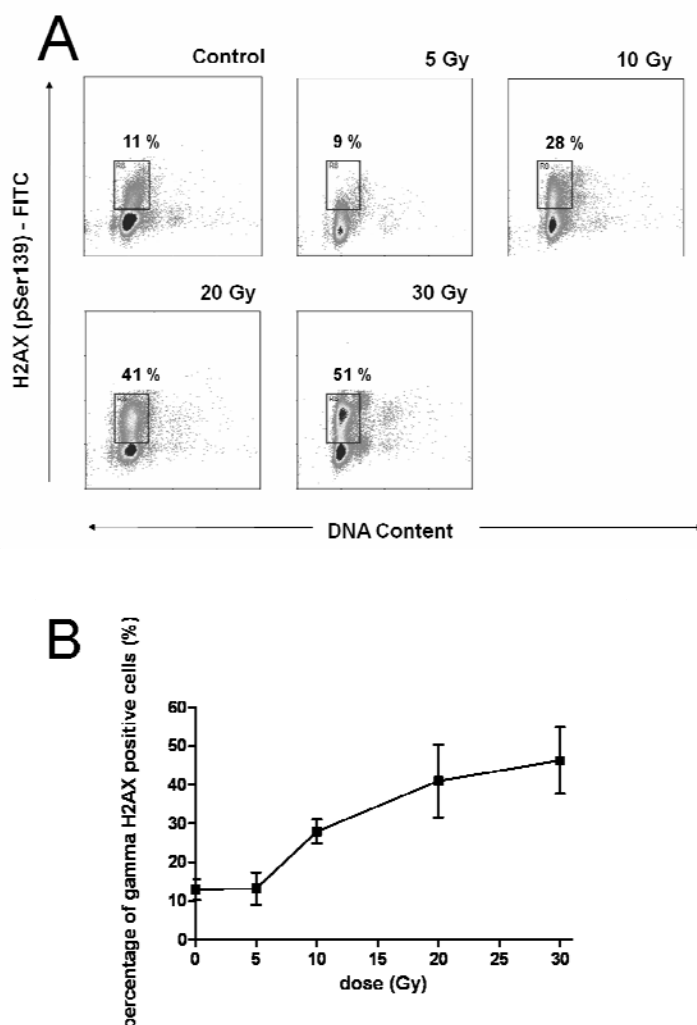
Fig. 5. **A. Flow-cytometric detection of the γH2AX protein expression in lymphocytes 1 h after the *in vivo* thoracic irradiation of rats (A) and γH2AX expression estimated according to gating analysis (B).** The data are shown as flow-cytometric bivariate histograms (dotplots) of the γH2AX-FITC immunofluorescence intensity on log scale (y axis) vs. DNA content distributions (x axis). Representative dot-plots for 1 of 6 independently evaluated samples from 6 rats in each group are shown. We used gating analysis to determine the IR-induced γH2AX expression. The gates were determined based on the intrinsic ("programmed") γH2AX expression of controls. The percentage of cells falling into this gate was defined as the percentage of γH2AX protein-positive cells. The graph of γH2AX expression estimated according to gating analysis described in Fig. 5A presents percentage of γH2AX-positive cells versus dose of ionizing radiation. Samples from 6 rats were evaluated in each group. The figure presents arithmetic means; error bars show S.E.M of hexaplicates.

Assessment of γH2AX

By using flow cytometry as a quantitative method of choice and fluorescence microscopy as a reference method, we investigated the phosphorylation of histone H2AX on serine 139 in lymphocytes of rats exposed to thoracic irradiation at 5–30 Gy.

The images obtained by fluorescence microscopy are shown in Fig. 3. We demonstrated increases in the number of cells containing γH2AX foci with increasing radiation doses (Controls 1.5%, 5 Gy 18%, 10 Gy 34%, 20 Gy 69%). However, even after the highest dose (20 Gy), not all the cells contained γH2AX foci; this implies that not all the cells were targeted by radiation. Individual γH2AX foci can be identified in the cells isolated from rats exposed to 5 Gy, while after irradiation at 20 Gy the individual
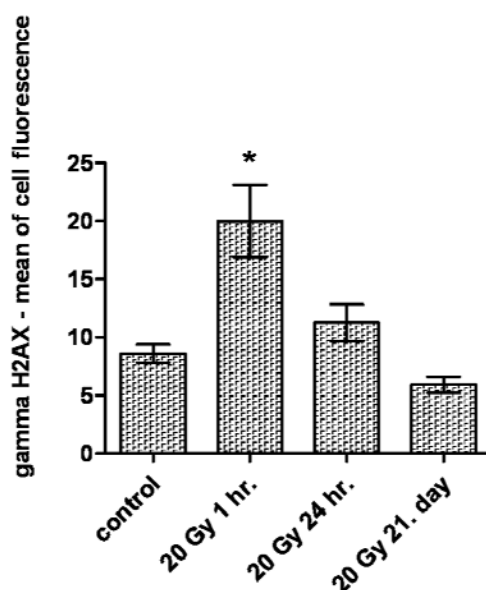
Fig. 6. **Time-dependent kinetics of the γH2AX immunofluorescence intensity in peripheral blood lymphocytes of thoracic-irradiated rats at a dose of 20 Gy.** The figure presents arithmetic mean; error bars shows S.E.M of hexaplicates.

* statistically significant increase in the γH2AX expression 1 hour after irradiation and subsequent decline to control group values within 24 hours, followed by additional decline under the level of control group on the 21st day after irradiation is shown

foci can no longer be distinguished and the positivity is observed through the whole nucleus.

The increase in γH2AX and the proportion of cells exposed to partial-body irradiation were further quantified by flow cytometry. Fig. 4 shows a graph representing the γH2AX quantification obtained by flow cytometry. Specimens taken from 6 rats 1 hour after thoracic irradiation were analysed in each group. By using the flow-cytometric method, we observed increases in the mean values of γH2AX immunofluorescence in lymphocytes from the dose of 10 Gy; statistically significant at 20 and 30 Gy.

Since microscopy revealed non-homogeneous γH2AX positivity among the lymphocytes, the flow cytometry data were also studied but using a different approach. Bivariate histograms (dotplots) of log γH2AX-FITC immunofluorescence vs. DNA contents were analysed to determine the percentage of γH2AX positive cells. A gating analysis was used; the gate was established to encompass the response of IR-treated cells. The gates were estimated according to the intrinsic γH2AX expression in the controls. The representative flow cytometry dotplots are shown in Fig. 5A. A graph, which shows the dose-dependence of the percentage of γH2AX positive cells in the whole set of the samples analysed (6 per group), is

shown in Fig. 5B. This method of data analysis also verified the IR-induced increase in the γH2AX expression after a dose of 10 Gy, which was statistically significant ($p \leq 0.05$) at 20 and 30 Gy.

The time-dependent kinetics of the γH2AX immunofluorescence intensity after thoracic irradiation at a dose of 20 Gy is shown in Fig. 6. As soon as 24 h after irradiation, the intensity of the γH2AX immunofluorescence drops significantly almost to the level of the control group. After 21 days, the level of γH2AX in the irradiated rats' lymphocytes is lower than that in controls. This indicates that lymphocytes with unrepaired IRIF are quickly eliminated from the blood circulation.

DISCUSSION

The phosphorylation of histone H2AX on serine 139 is a very early DSB marker. However, the majority of studies have been focused on γH2AX and IRIF detection after *in vitro* irradiation of isolated cells. In our previous work, we reported a dose-dependence of the integral optical density (IOD) of phosphorylated H2AX detected by confocal microscopy in *in vitro*

irradiated lymphocytes isolated from the peripheral blood of human donors. The increase in γH2AX IOD was dose-dependent up to 5 Gy and then reached a plateau (Vilasová et al. 2008). Andrievski and Wilkins (2009) compared the H2AX phosphorylation in different lymphocyte subpopulations after *in vitro* irradiation (0–10 Gy) of lymphocytes isolated from the peripheral blood. The phosphorylation process reached its maximum 1.5 h after irradiation and there were only minimum differences between particular lymphocyte subpopulations. Redon et al. (2009) described a linear dependence on the increase in γH2AX foci 30 min and 24 h after *in vitro* irradiation of human lymphocytes. Due to the DNA repair, the number of IRIF detected 24 h after irradiation was lower by a factor of ten compared to that 30 min after irradiation.

There are fewer data on H2AX phosphorylation after *in vivo* irradiation. Löbrich et al. (2005) evaluated changes in γH2AX 30 and 60 min after irradiation of patients exposed to very low doses during computerized tomography of the thorax and/or abdomen. The average damage level in lymphocytes from individuals exposed *in vivo* with DLP (defined as the product of the dose deposit within the exposure field and length of the body examined) of 150–1500 mGy/cm, achieved its maximum 30 min after irradiation. The damage was repaired and foci disappeared within 24 h after irradiation. At times of 30 and 60 min after irradiation, the average damage level in lymphocytes from individuals exposed *in vivo* to DLP of 1 000 mGy/cm is similar to that in lymphocytes irradiated *in vitro* at a dose of 20 mGy. Sak et al. (2007) studied the relationship between the integral total body radiation dose (this dose was estimated from the dose volume histogram of the patient's body corrected for the proportion of the body scanned by computerized tomography for 3D treatment planning) and the number of γH2AX foci. There was a strong linear correlation between the mean number of γH2AX foci per lymphocyte in the peripheral blood sample and integral total body radiation dose (0.0089–0.354 Gy). The kinetics of the *in vivo* γH2AX foci induction was studied by Redon et al. (2010) in peripheral lymphocytes of rhesus macaque after total-body gamma radiation exposure. By the use of laser-scanning confocal microscopy, they quantified mean values of γH2AX foci per cell (fpc) in *in vivo* irradiated peripheral blood lymphocytes depending on the time after the exposure and radiation dose. A dose-dependent increase in the γH2AX formation (fpc) was observed 0.3 day (doses of 1–3.5 Gy) and 1, 2 and 4 days (doses of 1–8.5 Gy) after irradiation. However, the numbers of fpc quantified for each radiation dose substantially decreased with increasing time after irradiation. The numbers of persisting residual foci were proportional to initial irradiation doses and statistically significant (p≤0.05) responses were obtained up to the 1st day after 1 Gy, 4 days after 3.5 and 6.5 Gy, and 14 days after 8.5 Gy. In our study (Havelek et al. 2011) we evaluated the dose dependence of the H2AX phosphorylation in peripheral blood mononuclear cells of rats exposed *in vivo* to whole-body irradiation at doses of 1–10 Gy. The data obtained by both methods – the modified flow-cytometric method published by Huang and Darzynkiewicz (Huang and Darzynkiewicz 2006) and the microscopic detection of γH2AX foci in individual cells – indicated a linear dose dependence. While microscopic detection is time-consuming and limited to hundreds of cells, the flow-cytometric method makes possible objective and relatively quick quantification of large numbers of cells. It can be used as soon as 1 h after irradiation to estimate the radiation dose received. Importantly, unprocessed blood samples can be stored at 4 °C for 24 h and the results are not significantly affected (Havelek et al. 2011). We can expect that the *in vivo* gamma radiation-damaged lymphocytes in the peripheral blood, which is sampled and *ex vivo* stored (in 4 °C on ice) after irradiation, cannot be naturally destroyed by the reticuloendothelial system of the body. Storage at 4 °C also (on ice) inactivates enzymes, which are phosphatases responsible for γH2AX foci loss in this particular case.

In the work presented here, we described dose-dependent increases in γH2AX in the lymphocytes of rats irradiated in the thoracic area occurring l h after the exposure to doses of 10 Gy and above. Seven weeks after irradiation of the thoracic area at doses of 15 and 20 Gy, based on histology, we demonstrated the development of radiation pneumonitis, an increase in the count of alveolar neutrophils and alveolar septa thickening (unpublished data). According to our knowledge, the results presented here offer the first report describing the γH2AX expression in lymphocytes isolated from the peripheral blood of rats exposed to local irradiation in the thoracic area. A maximum increase in the mean of the γH2AX fluorescence intensity was observed in a subpopulation of cells, which was apparent from both microscopic and flow-cytometric histograms. We can presume that the γH2AX positive lymphocytes were present in the irradiated area during the irradiation, while the γH2AX negative ones were not present there that time. It is of importance to note that only about 2% of all the lymphocytes are present in the blood. The others reside in other tissues, especially in the thymus, lymph nodes, tonsils, intestines, spleen and bone

marrow. Eighty percent of all the lymphocytes migrate between these tissues and the peripheral blood, with an overall recirculation time of about 12 hours. It was estimated that the average time period of the presence of a given lymphocyte in the peripheral blood is 30 minutes, over which it travels through the body (Westermann et al. 2001). This implies that blood samples taken within one hour after irradiation reflect the average dose given to the peripheral blood (Rothkamm and Horn 2009).

In our experiments, both the mean value of the γH2AX immunofluorescence in lymphocytes and the percentage of γH2AX positive lymphocytes 1 h after thoracic irradiation of rats exerted the same tendency and increased significantly with increasing doses. This was accompanied by dose-dependent lymphocytopenia 24 hours after thoracic irradiation at doses above 10 Gy. IR-induced lymphocytopenia is considered an established haemopoietic mark of the post-irradiation syndrome. In comparing flow-cytometric and microscopic methods the microscopic method is more sensitive in a range of low doses (exposure of the thorax to 5 Gy) compared to the method based on flow cytometry, since by the microscopic method, it was possible to reveal foci in 18% of cells irradiated at 5 Gy, whereas flow cytometry was not able to demonstrate any increase in γH2AX and the lymphocyte count also did not decrease in this group 24 h after irradiation compared to controls. In contrast to this, the detection of γH2AX is more precise after high doses (the foci cannot be counted by the microscopic method any more). From a long-term perspective, it would be of interest to count persisting foci in irradiated lymphocytes after the local exposure of the thorax.

ACKNOWLEDGEMENT

REFERENCES

Andrievski A, Wilkins RC. The response of gamma-H2AX in human lymphocytes and lymphocytes subsets measured in whole blood cultures. Int J Radiat Biol. 85: 369–376, 2009.

Bekker-Jensen S, Lukas C, Kitagawa R, Melander F, Kastan MB, Bartek J, Lukas J. Spatial organization of the mammalian genome surveillance machinery in response to DNA strand breaks. Cell Biol. 173: 195–206, 2006.

Blakely WF, Carr Z, Chin-May Chu, Dayal R, Fujimoto K, Hopmeir M. WHO 1st consultation on the development of a global biodosimetry laboratories network for radiation emergencies (BioDoseNet). Radiat Res. 171: 127–139, 2009.

Down JD. The nature and relevance of late lung pathology following localised irradiation of the thorax in mice and rats. Br J Cancer. 53 (Suppl. 7): 330–332, 1986.

Goans RE, Holloway EC, Berger ME, Ricks RC. Early dose assessment following severe radiation accidents. Health Phys. 72: 513–518, 1997.

Havelek R, Řezáčová M, Šinkorová Z, Zárybnická L, Bláha V, Vávrová J. Phosphorylation of histone H2AX as an indicator of received dose of gamma radiation after whole-body irradiation of rats. Acta Vet Brno. 80: 113–118, 2011.

Huang X, Darzynkiewicz Z. Cytometric assessment of histone H2AX phosphorylation: a reporter of DNA damage. Methods Mol Biol. 314: 73–80, 2006.

Jackson IL, Vujaskovic Z, Down JD. Revisiting strain-related differences in radiation sensitivity of the mouse lung: recognizing and avoiding the confounding effects of pleural effusions. Radiat Res. 173: 10–20, 2010.

Larsen DH, Poinsignon C, Gudjonsson T, Dinant C, Payne MR, Hari FJ, Danielsen JMR, Menard P, Sand JC, Stucki M, Lukas C, Bartek J, Andersen JS, Lukas J. The chromatin-remodeling factor CHD4 coordinates signaling and repair after DNA damage. J Cell Biol. 190: 731–740, 2010.

Löbrich M, Rief N, Kühne M, Heckmann M, Fleckenstein J, Rübe C, Uder M. *In vivo* formation and repair of DNA double-strand breaks after computed tomography examinations. Proc Natl Acad Sci USA. 102: 8984–8989, 2005.

Österreicher J, Pejchal J, Škopek J, Mokrý J, Vilasová Z, Psutka J, Vávrová J, Mazurová Y. Role of type II pneumocytes in pathogenesis of radiation pneumonitis: dose response of radiation-induced lung changes in the transient high vascular permeability period. Exp Toxicol Pathol. 56: 181–187, 2004.

Redon CE, Dickey JS, Bonner WM, Sedelnikova OA. Gamma-H2AX as a biomarker of DNA damage induced by ionizing radiation in human peripheral blood lymphocytes and artificial skin. Adv Space Res. 43: 1171–1178, 2009.

Redon CE, Nakamura AJ, Gouliaeva K, Rahman A, Blakely WF, Bonner WM. The use of gamma-H2AX as a biodosimeter for total-body

radiation exposure in non-human primates. PLoS ONE 5: e15544, 2010.

Rogakou EP, Pilch DR, Orr AH, Ivanova VS, Bonner WM. DNA Double-stranded breaks induce histone H2AX phosphorylation on serine 139. J Biol Chem. 273: 5858–5868, 1998.

Rothkamm K, Horn S. γH2AX as protein biomarker for radiation exposure. Ann Ist Super Sanita. 45: 265–271, 2009.

Sak A, Grehl S, Erichsen P, Engelhard M, Grannaß A, Levegrün S, Pöttgen C, Groneberg M, Stuschke M. Gamma-H2AX foci formation in peripheral blood lymphocytes of tumor patients after local radiotherapy to different sites of the body: dependence on the dose-distribution, irradiated site and time from start of treatment. Int J Radiat Biol. 83: 639–652, 2007.

Travis EL. The sequence of histological changes in mouse lungs after single doses of x-rays. Int J Radiat Oncol Biol Phys. 6: 345–347, 1980.

Vávrová J, Filip S. Radiosensitivity of the Haemopoietic System (in Czech). Galén, Praha ISBN 80-7262-200-5, 99 p., 2002.

Vilasová Z, Řezáčová M, Vávrová J, Tichý A, Vokurková D, Zölzer F, Řeháková Z, Österreicher J, Lukášová E. Changes in phosphorylation of histone H2AX and p53 in response of peripheral blood lymphocytes to gamma irradiation. Acta Biochim Pol. 55: 381–390, 2008.

Westermann J, Engelhardt B, Hoffman JC. Migration of T cells *in vivo*: molecular mechanisms and clinical applications. Ann Intern Med. 135: 279–295, 2001.