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Phosphorylation of histone H2AX as an indicator of received dose of gamma radiation after whole-body irradiation of rats

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Abstract

The aim of our study was to determine whether phosphorylation of histone H2AX can be used as an indicator of received dose of gamma radiation after whole-body irradiation of rats.

Wistar rats were irradiated by 1-10 Gy of gamma radiation by ^{60}Co source. Value LD50/60 was 7.37 (4.68-8.05) Gy. Histone H2AX is phosphorylated by ATM kinase on serine 139 (γH2AX) quickly after the irradiation. It forms microscopically visible foci in the site of double strand breaks of DNA. Flow-cytometric method was used for quantitative detection. This study is the first one that evaluated dose-dependency of H2AX phosphorylation in peripheral lymphocytes of rats irradiated by whole-body dose 1-10 Gy. Our data show a dose-dependent increase in γH2AX in rat peripheral blood lymphocytes 1 h after whole-body irradiation by the dose of 1-10 Gy. We proved that phosphorylation of histone H2AX is a prompt and reliable indicator of the received radiation dose suitable for rapid measurement before the number of lymphocytes in peripheral blood starts to decrease. It can be used already 1 h after the irradiation for an estimation of the received dose of radiation. Blood samples can be stored in 4 °C for 23 h without significantly affecting the result.

Gamma H2AX, biological dosimetry, whole body irradiation

High doses of radiation induce apoptosis of lymphocytes in peripheral blood and these are, after *in vivo* irradiation, quickly phagocytised. Maximal decrease in the lymphocyte number in peripheral blood after whole-body irradiation is observed after 72 h (Vávrová and Filip 2003). The decrease in lymphocyte number 24 h after irradiation is used as a simple marker of the received dose of radiation. Biodosimetric method based on detection of chromosomal aberrations is more precise but requires phytohaemagglutinin (PHA) stimulation of lymphocytes and manual counting of dicentric chromosomes. This method has its limitations, is time-consuming and after high doses it is not possible to find enough mitotic cells; therefore new methods are intensively studied (Blakely 2002). Of the many types of DNA damage that exist within the cell and probably the most serious ones are the double strand breaks (DSBs). Cytologic manifestation of nuclear remodelling in response to ionizing radiation is formation of ionizing radiation induced foci - IRIF (Bekker-Jensen et al. 2006). After *in vitro* irradiation of peripheral blood lymphocytes by the doses 0.2-5 Gy, the number of IRIF is maximal 0.5 -1 h post irradiation (Vilasová et al. 2008; Redon et al. 2009).

The key component of these foci is phosphorylated form of histone H2AX. H2AX variant comprises 2-25% of the histone H2A complement in mammalian cells (Rogakou et al. 1999). It has been proposed that H2AX may function as an anchor to maintain DSB ends in close vicinity (Bassing et al. 2004). The presence of histone H2AX phosphorylation on Ser-139 (γH2AX) can be detected within IRIF in a variety of cells after the exposure of ionizing radiation and is used as a marker of DSBs.

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The Mre11-Rad50-Nbs1 (MRN) complex acts as a DSBs sensor for ATM and recruits ATM to broken DNA molecules. It is also involved in activation and targeting of ATM protein kinase to initiate phosphorylation of the respective substrates (Lee and Paull 2005; Bekker-Jensen et al. 2006). ATM protein kinase is the key signal transducer in response to the induction of DNA DSBs by ionizing radiation (Banin et al. 1998). Co-localization of these proteins with γH2AX in the IRIF is very quick, within a minute after irradiation.

An initial formation of phosphorylated form of H2AX after DNA DSBs induction is extremely rapid and occurs within minutes. Rogakou et al. (1998) performed a study on human glioblastoma cell line SF268 using two-dimensional electrophoresis and they were the first ones who reported that H2AX becomes phosphorylated on serine residue 139 in the cells with gamma-radiation-induced DSBs. With IMR90 and MCF7 cells, foci were apparent 3 min after irradiation with 0.6 Gy still persisting at 15–60 min and then decreasing in number at 180 min (Rogakou et al. 1999).

Our previous studies on the *in vitro* irradiated cells of human acute lymphoblastic leukaemia MOLT-4 and human peripheral blood mononuclear cells (PBMCs) demonstrated dose-dependent phosphorylation of H2AX 1 h after the doses of 0.5-5 Gy (Vilasová et al. 2008; Řezáčová et al. 2008). In these studies, we evaluated integral optical density of γH2AX IRIF visualised by confocal microscopy. Huang et al. (2004, 2006) described flow-cytometric assessment of DSBs by detection of phosphorylation of histone H2AX on the basis of binding primary antibody together with indirect immnofluorescence using secondary antibody conjugated with fluorescein isothiocyanate dye (FITC) while DNA was counterstained with propidium iodide (PI). Intensity of cellular green (FITC) and red (PI) fluorescence was measured by flow-cytometry. Halicka et al. (2005) studied using this flow cytometric method the effect of UV-B radiation on human promyelocytic leukaemia cells HL-60 and correlated DNA damage (γH2AX) with cell cycle phase. The highest degree of H2AX phosphorylation induced by UV was seen in S-phase of cells.

The aim of our study was to detect changes in γH2AX on PBMCs of rats after whole-body irradiation of rats by the doses 1-10 Gy using flow-cytometry and fluorescence microscopy, look for a dose dependency in the increase of γH2AX in PBMCs of *in vivo* gamma irradiated rats and develop a method, which can be used for estimation of received whole body dose of gamma radiation.

Materials and Methods

Animals

For the experiment, female Wistar rats (VELAZ-Lysolaje) weighing 210-250 g and of SPF (specific pathogen-free) quality were used. For the survival experiments, each experimental group consisted of 10-16 animals, control group consisted of 10 animals. For γH2AX detection each irradiated group consisted of 6-9 animals, control group consisted of 6 animals. Animals used for experiments were housed at a temperature- and humidity-controlled environment maintained on a 12-h light/dark cycle. Food and water were available *ad libitum*. All procedures were approved by the Ethics Committee supervising experimental procedures performed in animals at the Faculty of Military Health Sciences Hradec Králové (MO 12-7/2008-3696).

Gamma irradiation

The rats were exposed to 1-50 Gy (γH2AX detection) or 7-50 Gy (survival) of radiation from a ^{60}Co source (Chisotron, Chirana, Czech Republic) from a distance of 1 m from source at a dose rate of 1 Gy/min.

Flow-cytometric assessment of γH2AX

Modified method published by Huang and Darzynkiewicz (2006) was used for the detection of γH2AX. The method was optimised for detection of γH2AX in peripheral blood mononuclear cells of rats. PBMCs of irradiated and control group of rats were isolated with Histopaque (Sigma-Aldrich, Germany) according to manufactures instructions. The red blood cells trapped in pellet were lysed using EasyLyse™ reagent (DakoCytomation, Denmark). PBMCs were rinsed twice with PBS (Dulbecco's phosphate buffered saline, Sigma-Aldrich) and then fixed with ice-cold 1% methanol-free formaldehyde solution, rinsed in 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 in 1% BSA-0.2%-Triton X-100 in PBS stained with anti-phospho-histone H2A.X (Ser139)-FITC conjugate primary antibody (Millipore, USA) at room temperature for 1 h in the dark. The cells (after rinsing) were suspended

in the PI staining solution (0.1% RNase, PI 5 μg/ml in PBS). Cells were incubated at room temperature for 30 min in the dark with PI staining solution and immediately after incubation analyzed on FACS analyzer CyAn DakoCytomation (Beckman Coulter, USA). List mode data were analysed using Summit v 4.3 software (Beckman Coulter). The cells in G0/G1 phases were gated and median of fluorescence of the cells in each sample was calculated. Blood samples of 6-9 animals were analysed per experimental dose.

Immunocytochemistry

The PBMCs were isolated as described above and fixed with 4% freshly prepared paraformaldehyde for 10 min at room temperature, washed in PBS, permeabilized in 0.2% Triton X-100/PBS for 15 min at room temperature, and washed in PBS. Before 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. Mouse monoclonal anti-phospho-histone H2AX (Millipore, USA) was used for detection of γH2AX. Secondary antibody: Affinity pure donkey anti-mouse-FITC-conjugated was purchased from Jackson Laboratory (Bar Harbor, 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) and incubation for 1 h in the dark was succeeded by washing (3 × 5 min) in PBS. The nuclei were counterstained by DAPI. Images were obtained by Nikon Eclipse fluorescence microscope; the exposition time and dynamic range of camera in all channels were adjusted to the same values for all slides to obtain quantitatively comparable images. A minimum of 100 cells was scored for each dose. Integral optical density (IOD) was measured using image analysis software NIS-Elements AR (Laboratory Imaging, Czech Republic).

Statistics

The LD50 was derived from the dose-response curve and represents the dose at which 50% of test organisms are killed. LD50 was calculated by the Probit Analysis using the maximum likelihood method of Finney (1947) and Roth et al. (1962). Briefly, the probability data is subjected to probit transformation. A weighted regression is then undertaken using the probits and the log of the test dose. The linear regression equation is then used to find the concentration for the desired level (e.g. 50%) of effect.

The descriptive statistics of the other results was calculated and the charts were made in Microsoft® Office Excel 2003 (Microsoft, Inc., Redmond, USA); the detailed statistical analysis was performed in STATISTICA 7 (StatSoft, Inc., Tulsa, USA).

We observed a non-normal distribution of the values in our results; therefore we chose to present these results as medians and ranges of the values. The Mann-Whitney U test for difference in medians was used to verify differences in the results between the controls and the various experimental groups. The results are shown as median with indicated first and third quartile.

Results

Survival

The Wistar rats were irradiated by the whole-body dose of radiation ranging from 7 to 50 Gy (10-16 rats in the group). All rats irradiated by the dose of 20 and 50 Gy died on the 3rd and 4th days; all rats irradiated by the dose of 12 Gy died between the 5th and 7th day and rats irradiated by the dose of 10 Gy died between the 9th and 11th day after irradiation. The dose of 8 Gy and 7 Gy caused death of 81% and 17% of rats, respectively, during 30 days after irradiation. The determined LD50/30 value was 7.37 Gy; with 95% confidence limits 4.68–8.05 Gy.

Assessment of γH2AX

Fluorescence microscopy of immunostained γH2AX in individual cells revealed formation of nuclear foci in all irradiated cells (Plate IX, Fig. 1, top) 1 h after irradiation. Samples obtained from 6 rats were analysed in each irradiated group. The positivity for γH2AX in nuclei increased with increasing the dose of radiation. Intensity of H2AX phosphorylation per nucleus was quantified by image analysis software as integrated optical density (IOD). IOD showed linear dose-dependence for the studied dose-range (Plate IX, Fig. 1, bottom).

Fig. 2 (Plate X) shows the results of flow-cytometric detection of γH2AX. Samples obtained 1 h after the whole-body irradiation from 6-9 rats were analysed in each irradiated groups. The fluorescence of γH2AX in PBMCs 1 h after the irradiation increases linearly in dose-dependent manners, and could be used as an indicator of received dose.

In order to evaluate the possibility of delayed sample analysis, it was also investigated how storage of blood in refrigerator (4 °C) affects subsequent flow-cytometric analysis of

γH2AX content. After irradiation of the rats by the dose of 10 Gy, part of the samples (n = 9) was analyzed immediately, whereas others (n = 6) were stored at 4 °C for 23 h. No significant decay of the γH2AX content was found (Fig. 3).

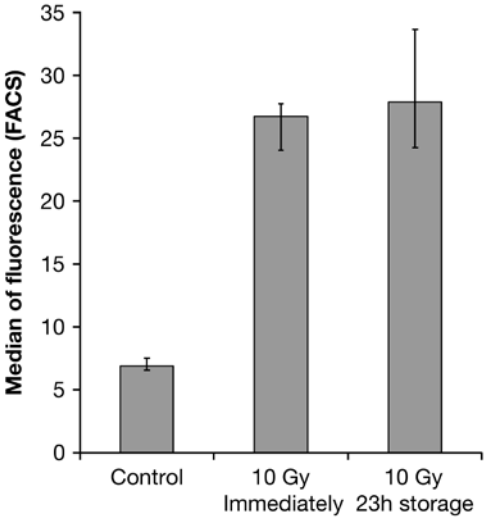

Fig. 3. Effect of sample storage on γH2AX content. The samples obtained after whole-body irradiation of rats by the dose 10 Gy were analysed immediately or after 23 h long storage at 4°C. The results are shown as median with indicated first and third quartile.

Discussion

Phosphorylation of histone H2AX on serine 139 is a very early marker of DSBs. In non-irradiated lymphocytes, the number of DSBs is very low (Löblich et al. 2005; Vilasová et al. 2008). However, γH2AX foci can be detected in cells undergoing meiosis or V(D)J recombination, as well as in senescent and apoptotic cells (Olive 2009). High amount of endogenous γH2AX foci was detected in many tumour cell lines, and it seems to be linked with telomere damage (Nakamura et al. 2009). In our previous work we employed confocal microscopy and we reported the dose-dependency of IOD of phosphorylated H2AX. The increase in γH2AX IOD was (after *in vitro* irradiation) dose-dependent up to 5 Gy and then reached plateau (Vilasová et al. 2008). Andrievski and Wilkins (2009) compared phosphorylation of H2AX in various lymphocyte subpopulations after *in vitro* irradiation (up to10 Gy) of lymphocytes isolated from peripheral blood. The phosphorylation reached maximum 1.5 h post irradiation and the differences between lymphocyte subpopulations were minimal. Redon et al. (2009) described linear dependence of increase in γH2AX foci 30 min and 24 h after *in vitro* irradiation of human lymphocytes. With regard to DNA repair, the number of IRIF detected 24 h post irradiation was 10 × lower compared to the number of IRIF 30 min post irradiation.

Considerably less information is available about H2AX phosphorylation after *in vivo* irradiation. Löblich et al. (2005) evaluated changes in γH2AX after irradiation of human patients by very low doses during computed tomography of the thorax and/or abdomen 30 and 60 min post irradiation. The average damage level in lymphocyte 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) 150-1500 mGy/cm was maximal 30 min post

irradiation. The damage was repaired and foci disappeared within 24 h post irradiation. At 30 and 60 min post irradiation, the average damage level in lymphocytes from individuals exposed *in vitro* to a DLP of 1 000 mGy/cm was similar to that for lymphocyte irradiated *in vitro* with a dose of 20 mGy (Löblich et al. 2005). Sak et al. (2007) studied the relationship of the integral total body radiation dose (this dose was estimated from the dose volume histogram of patient's body corrected for proportion of the body scanned by computed 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.

Based on our previous and present results, we can conclude that the amount of γH2AX is proportional to the received dose of radiation both *in vitro* and *in vivo*. Using suitable immunodetection method, the phosphorylation of H2AX can be quantified and used as a biodosimetric marker for retrospective radiation exposure assessment.

This study is the first one that evaluated dose-dependency of H2AX phosphorylation in peripheral lymphocytes of rats irradiated by whole-body dose 1-10 Gy. The data obtained by the flow-cytometric method and by the microscopic detection of γH2AX foci in individual cells, indicate linear dose-dependency. While the microscopic detection is time-consuming and limited on hundreds of cells, the flow-cytometric method allows objective and relatively quick quantification of large number of cells. It can be used already 1 h after the irradiation for an estimation of the received dose of radiation. Blood samples can be stored in 4 °C for 23 h without affecting the result significantly.

Fosforylace H2AX jako indikátor dávky gama záření po celotělovém ozáření potkanů *in vivo*

Cílem naší studie bylo stanovit, zda fosforylace histonu H2AX po celotělovém gama ozáření potkanů může být použita jako indikátor obdržené dávky záření.

Potkani (kmene Wistar) byli ozáření dávkami 1-10 Gy gama záření. Hodnota LD50/60 byla stanovena 7,37 (4,68-8,05) Gy. Histon H2AX je po ozáření rychle fosforylován ATM kinasou. V místě dvojitých zlomů DNA (DSB) se tvoří mikroskopicky viditelná ložiska. Ke kvantitativnímu hodnocení jsme použili flow-cytometrické stanovení. Tato studie jako první prokazuje dávkovou závislost fosforylace H2AX v periferních lymfocytech potkanů, ozářených celotělově dávkami 1-10 Gy. Prokázali jsme, že 1 h po celotělovém ozáření potkanů stoupající vysokou dávkou 1-10 Gy dochází k dávkově závislému vzestupu gama H2AX v lymfocytech izolovaných z periferní krve potkanů. Fosforylace histonu H2AX je tedy rychlý indikátor dávky záření v době, kdy ještě nedochází k poklesu počtu lymfocytů v periferní krvi. Může být detegována již 1 h po ozáření a využita ke zpětnému odhadu obdržené dávky. Krevní vzorky mohou být uchovány při 4 °C po dobu 23 h bez vlivu na výsledek analýzy.

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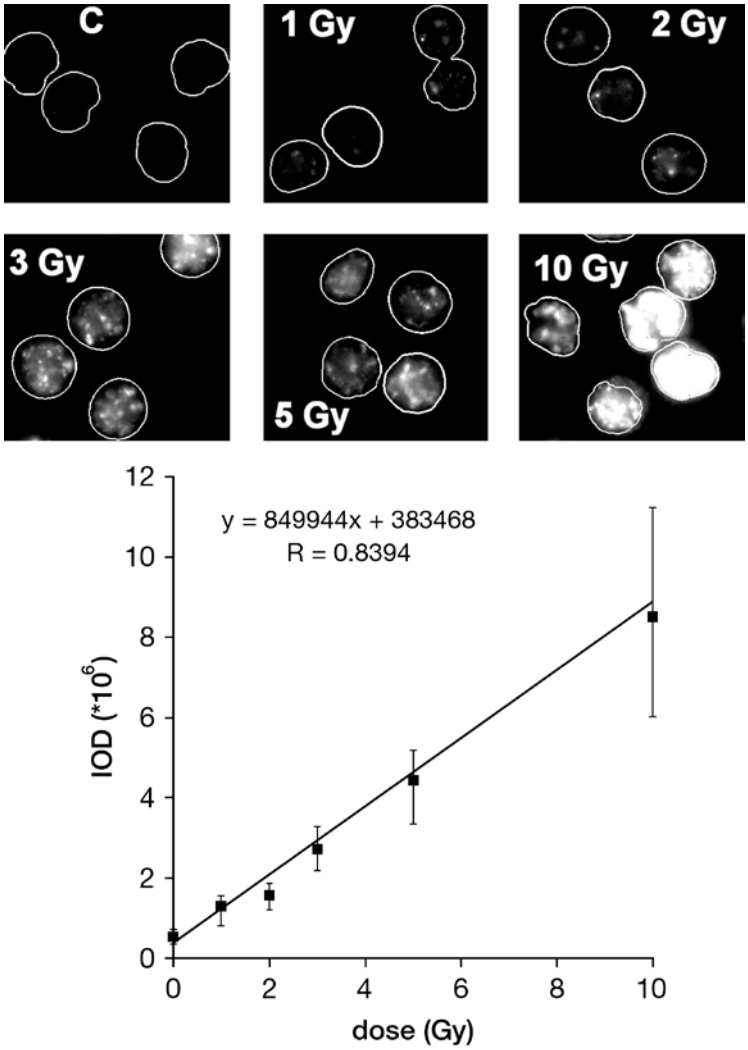

Fig.1. Microscopic detection of γH2AX in lymphocytes 1 h after irradiation of rats. The top panel shows cell nuclei (outlined according to 4',6-diamidino-2-phenylindole [DAPI] counterstaining) with visible γH2AX foci. The bottom panel shows linear dose-dependent increase in integral optical density (IOD) of γH2AX in cell nuclei. The results are shown as median with indicated first and third quartile.

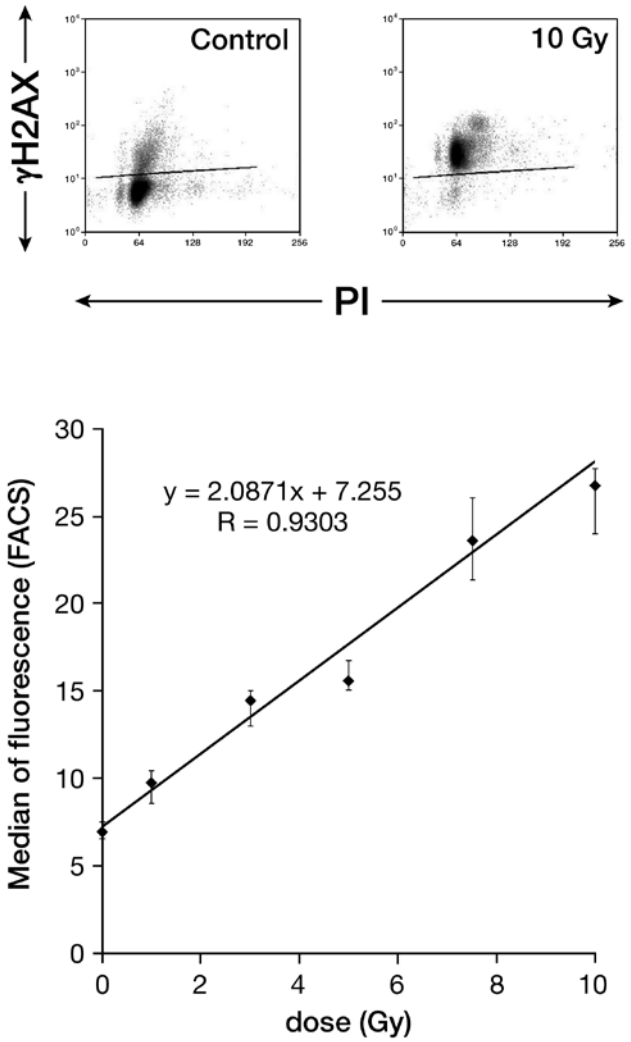

Fig. 2. Flow-cytometric detection of γH2AX in lymphocytes 1 h after irradiation of rats. The top panel shows representative dot plots of control and 10 Gy irradiated rats. The bottom panel shows linear dose-dependent increase in γH2AX fluorescence in cell nuclei. The results are shown as median with indicated first and third quartile.