In vivo Analysis of H2AX Phosphorylation Induced by γ-Radiation

Seyed Mostafa Mir (MSc)
Department of Clinical Biochemistry, School of Medicine, Babol University of Medical Sciences, Babol, Iran

Esmaeil Samadian (MSc)
Department of Molecular Medicine, Faculty of Advanced Medical Technologies, Golestan University of Medical Sciences, Gorgan, Iran

Seyed Hossein Sadeghi (MSc)
Department of Medical Genetics, School of Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran

Alireza Khoshbin khoshnazar (PhD)
Department of Biochemistry and Biophysics, Faculty of Medicine, Golestan University of Medical Sciences, Gorgan, Iran

Corresponding author: Alireza Khoshbin khoshnazar

Tel: +981732421651-3

Email: akhoshbin@yahoo.com

Received : 01 Dec 2014
Revised: 05 Dec 2014
Accepted: 16 Dec 2014

ABSTRACT

Background and Objectives: Exposure to ionizing radiation in modern societies is inevitable and can cause a variety of adverse health effects such as cancer and birth defects. Therefore, a reliable, repeatable and sensitive method is required for evaluation of radiation exposure. The aim of this study was to determine the amount of histone H2AX phosphorylation as an indicator of radiation exposure to evaluate the rate of double-strand DNA breakage in irradiated mice.

Methods: In this study, 15 mice were exposed to different doses of ionizing radiation. After extraction of total protein from bone marrow cells, γH2AX protein was measured by western blotting. Data analysis was performed using ANOVA, Tukey's post hoc test, and the Pearson's correlation test.

Results: The amount of γH2AX protein in the exposed groups increased significantly compared to the control group ($P<0.05$).

Conclusion: The results of this study indicate that exposure to ionizing radiation increases the amount of γH2AX protein in bone marrow cells during the early hours. The protein can be used as a biomarker for monitoring of acute radiation or suspected local radiation exposure.

Keywords: γH2AX Protein, Ionizing Radiation, Mouse.

This paper should be cited as: Mir SM, Samadian E, Sadeghi SH, Khoshbin khoshnazar A[In vivo Analysis of H2AX Phosphorylation Induced by γ-Radiation] . mljgoums. 2017; 11(2): 11-15

INTRODUCTION

The need for radiation in medicine is increasingly expanding. Identification of the biological effects of radiation and its diagnostic and therapeutic applications has attracted a lot of attention in the field of medicine (1). Exposure to ionizing radiation leads to cellular damage, which is due to direct ionization cause by radical attack (2). One of the most important effects of this exposure is DNA double-strand breaks (DSBs), a serious damage to the DNA that leads to destruction of cells or error-prone DNA repair (3). Several proteins such as histone H2AX are potential candidates in the early stages of response to ionizing radiation, specifically to DSB. Histone H2AX is a H2A histone derivative with molecular weight of 15KD, which is widely involved in eukaryotic nucleosomes (4). In case of chromosomal DNA breakage, H2AX becomes phosphorylated in vicinity of the break. In fact, the immediate consequence of DSB is the phosphorylation of this histone on serine 139 of its C-terminal. The phosphorylation generated in the histone H2AX megabase domains around the DSB is called γH2AX, which is a primary marker for the detection of DSBs (5). The histone plays a role in the stability of the genome by marking the damaged DNA. On the other hand, this phosphorylated histone returns to its natural conformation after the DNA repair [6]. Since ionizing radiation induces DSB, the rapid detection of DNA DSBs using this biomarker is essential (7). In addition, measurement of γH2AX can be considered as a fast biodosimetry method of evaluating the level of DNA damage or its repair in patients undergoing radiotherapy or chemotherapy (3, 8). This is of great importance in indirect evaluation of the extent of these injuries in in vivo studies on mice (9). Considering the importance of identification of a biomarker for the rapid detection of DNA DSBs, we aimed to determine the rate of DSBs in DNA by using γH2AX protein as a DSB biomarker in early hours after exposure to ionizing radiation.

MATERIAL AND METHODS

The present study was performed at the laboratory of the Laboratory Sciences Research Center at Golestan University of Medical Sciences in 2014. For this study, 15 healthy female mice (without any infections) were used. The animals used were kept under controlled temperature and humidity with access to water and food. All stages of the study were approved by the Animal Research Ethics Committee of the Golestan University of Medical Sciences, Iran. The animals were randomly divided into five experimental groups (each consisting of three mice) and a control group (n=3). Each experimental group was exposed to different doses of gamma radiation (1.2, 1.4, 1.6, 1.8, 2 Gy). The gamma ray was from cobalt 60 obtained from the Radiotherapy Center of the Golestan Province with a dose-rate of 67.55 cm/min. Exposure time was in the range of 2.13-3.55 minutes, irradiation field was 30 × 30 cm, and the source to sample distance was 80 cm. After one hour, femoral bone marrow of the mice was removed and placed in test tubes. Then, 150 µl of buffer lysis (containing 5% SDS, 100 mM Tris-HCl (pH=7.4), 150 mM NaCl, 5 mM EDTA, 5% sodium dodecylocyte and 10% glycerol) were added to the tubes. Later, 10 µl of protease inhibitor cocktail and phosphatase inhibitor cocktail were added to the samples. Pierce bicinchoninic acid assay (BCA) Protein Assay Kit (Thermoscientific Co.) was used for plotting the standard curve of bovine serum albumin for determining the concentration of cellular proteins. The basis of SDS-PAGE electrophoresis is to separate proteins based on their molecular weight. After preparing 15% gel, 5x sample loading buffer (10% SDS, 0.5% bromophenol blue, 60 mM Tris-HCL with pH 6.8, 50% glycerol, 14.4 mM 2-mercaptoethanol) were added to each tube. The tubes were placed at boiling temperature for 5 minutes. Then, 35 µg protein was loaded onto each well for blotting. Protein samples were separated on 15% polyacrylamide gel under 110 V for 48 minutes. The proteins were transferred to nitrocellulose membrane (Thermo Co.) using transfer buffer (15.6 mM Tris-base, 120 mM Glycine, 10% methanol, pH 8.4). The blots in 3% skim milk in phosphate buffer saline (PBS, final concentration of 0.1% Tween) were placed on a rotator for 2-3 hours at room temperature. The blots were then incubated at 4 ^{o}C with primary antibody [anti-phospho-histone H2AX (Ser139)] diluted 1:1000 in 3% skim milk and PBS-T. They were washed three times with

PBS-T for 5, 10 and 15 minutes at room temperature while on the rotator. In the next step, the blots were incubated on the rotator with the secondary antibody (HRP conjugated goat anti-mouse IgG) diluted 1: 2000 in 3% skim milk in PBS-T for 1-2 hours at room temperature. After washing, the blots were exposed to autoradiography films after 10-15 minutes of incubation with ECL. Finally, films related to the blots were printed, visualized and analyzed. Statistical analysis was performed using SPSS software (version 16). Teste were carried out at 95% confidence level and α risk of error <5%. ANOVA was used to evaluate the significance of the differences in amount of γH2AX protein between the groups. Tukey's test was used for pairwise comparison of groups. Due to the normality of data, Pearson correlation coefficient was used to determine the correlation between dose of radiation and level of γH2AX.

RESULTS

In order to determine the relationship between the dose and γH2AX protein formation, five appropriate doses of radiation (1.2, 1.4, 1.1, 6.2, 8) were selected based on previous studies. Three samples were irradiated in vivo at each point.

After determining the total concentration of protein by BCA, 35 µg of cellular protein from each sample was used for western blotting (35 µg was loaded into each well). Presence of γH2AX in bone marrow lysate was evaluated. The results of western blot analysis are shown in Figure 1.

No band was observed for the control group. Beta-actin was used as the loading control (Figure 2). At this stage, densitometric measurement of the western blot bands was converted into quantitative data (percentages) using the Image J software. Intensity of color and surface area of each band were given numerical scores. It should be noted that the numbers calculated by the Image J software are merely numbers and only represent a specific coordinate of the selected curves. In other words, they do not represent the amount of proteins in the bands in any unit. Results of ANOVA showed a significant difference in amount of γH2AX protein between the groups ($P<0.05$). Results of the Tukey's test showed a significant difference between all doses except for 1.6 and 1.8 Gy ($P<0.05$). On the other hand, the Pearson correlation coefficient showed a significant correlation between the dose of radiation and amount of γH2AX protein ($P<0.05$, $r = 0.833$).

Figure 1- Results of western blot analysis of γH2AX proteins derived from mice bone marrow cells after exposure to 1.2, 1.4, 1.6, 1.8 and Gy

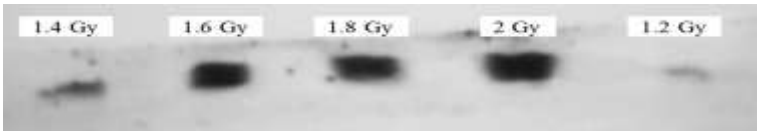

Figure 2- Results of western blotting analysis of beta-actin proteins derived from mice bone marrow cells

DISCUSSION

Studies have shown that DSB is the most important DNA damage caused by ionizing radiation that leads to genomic changes, cancer and mortality (5). There is limited information on the H2AX phosphorylation in dividing cells following in vivo radiation (10). The most sensitive stage of the cell cycle to ionizing radiation is mitosis (11). This different behavior during the cell cycle is due to the density of chromatin. There are two pathways for repairing DSBs in mammalian cells, homologous recombination (HR) and non-homologous end joining (NHEJ). HR is the pathway for the dividing cells (bone marrow cells) that repair the damages during

the S and G2 phases. In fact, the sister chromatids produced in the S phase are used as template for the proper repair of DSBs. As the cell cycle phases (S, G2) progress, the cell becomes more susceptible to DSBs and as a result, more γH2AX is formed in the DNA molecule. These cells are more sensitive to ionizing radiation compared to cells in the G0 phase (lymphocytes). In cells in the G0 and G1 phases of the cell cycle, the HR pathway is suppressed and most DSBs created are repaired by the NHEJ pathway (12). Recent studies have shown that the γH2AX level could be used as a biomarker for identification of doses of ionizing radiation (13-15). Our dosimetry studies showed that there are significant differences in the level of γH2AX formation in groups irradiated with different doses. Same findings have been reported by similar studies. Consistent with our findings, study of Havelek et al. on irradiated mice (1-10 Gy) showed that high-dose radiation induces γH2AX formation in peripheral blood lymphocytes of the irradiated mice (9). However, we were able to detect γH2AX in dividing cells in a narrower dose range (1-2 Gy). In a similar study, Redon et al. studied the effect of in vitro radiation (0.2-5 Gy) on 60 different blood samples and reported a linear increase in response to the radiation (7). In another study, Redon et al. evaluated the amount of γH2AX in lymphocytes of six groups of monkeys receiving whole-body irradiation (1-8.8 Gy) and found significant results (5). Lefevre et al. investigated the presence of γH2AX in peripheral blood lymphocytes after in vitro irradiation (0.02-2 Gy) and found a significant linear relationship (4). The results of our study are consistent with the mentioned studies, which all found a significant relationship between different doses of radiation and the amount of γH2AX formation. However, most of these in vitro studies investigated the issue in cells in the G0 phase. Contrary to other studies, the present study has indirectly evaluated a different pathway of DSBs repair (HR pathway).

CONCLUSION

The results of this study indicate that exposure to ionizing radiation increases the amount of γH2AX protein in bone marrow cells during the early hours. The protein can be used as a biomarker for monitoring of acute radiation. However, further studies on a larger population of mice using different doses are required to verify the results of the present study.

ACKNOWLEDGMENTS

The authors would like to thank the Clinical Biochemistry Laboratory of the Golestan University of Medical Sciences and the Shafa Radiotherapy Center of the 5th Azar Hospital in Gorgan, Iran.

CONFLICT OF INTEREST

We have no conflict of interest to declare.

REFERENCES

1. HoA SmAa. *Compare of Dosimetery Biologic with Dosimetery Physical at Radiation Worker City Babol.* Magazine Science Medical Mazandaran. 2006; 51(16): 43-50.

2. Lassmann M HH, Gassen D, Biko J, Meineke V, Reiners Ch, et al. *In vivo formation of γH2AX and 53Bp1 DNA repair foci in blood cells after radioiodine therapy of differenttiated thyroid cancer.* Nuclear medicin. 2010; 51(8): 1318-25.

3. Redon CE NA, martin OA, parekh PR, weyemi US, bonner WM. *Resent development in the use of γH2AXas a quantitive DNA double-strang break biomarker.* Agine. 2011; 3(2):168-74.

4. Roch-Lefèvre S, Mandina T, Voisin P, Gaëtan G, Mesa JEG, Valente M, et al. *Quantification of γ-H2AX foci in human lymphocytes: A method for biological dosimetry after ionizing radiation exposure.* Radiat Res. 2010; 174(2): 185-94. doi: 10.1667/RR1775.1.

5. 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. 2010; 5(11): e15544.

6. Pinto DMS, Flaus A .*Structure and function of histone H2AX.* Subcell Biochem. 2010; 50: 55-78. doi: 10.1007/978-90-481-3471-7_4.

7. Redon CE, Dickey JS, Bonner WM, Sedelnikova OA. *γ-H2AX as a biomarker of DNA damage induced by ionizing radiation in human peripheral blood lymphocytes and artificial skin.* Advances in Space Research. 2009; 43(8): 1171-8.

8. Bonner WM, Redon CE, Dickey JS, Nakamura AJ, Sedelnikova OA, Solier S, et al. *γH2AX and cancer.* Nature Reviews Cancer. 2008;12(8): 957-67. doi:10.1038/nrc2523.

9. Havelek R ŘM, Šinkorová Z, Zárybnická L, TichýA, Vávrová J. *Phosphorylation of histone H2AX as an indicator of received dose of gamma radiation after whole-body irradiation of rats.* Acta Veterinaria Brno. 2011;80(1):113-8.

10. Moroni M, Maeda D, Whitnall MH, Bonner WM, Redon CE. *Evaluation of the Gamma-H2AX Assay for Radiation Biodosimetry in a Swine Model.* International journal of molecular sciences. 2013;14(7):14119-35. doi: 10.3390/ijms140714119.

11. Rothkamm K, Horn S. *Gamma-H2AX as protein biomarker for radiation exposure.* Ann Ist Super Sanita. 2009;45(3):265-71.

12. Marti TM, Hefner E, Feeney L, Natale V, Cleaver JE. *H2AX phosphorylation within the G1 phase after UV irradiation depends on nucleotide excision repair and not DNA double-strand breaks.* Proceedings of the National Academy of Sciences. 2006; 103(26): 9891-6.

13. Wang Z, Hu H, Hu M, Zhang X, Wang Q, Qiao Y, et al. *Ratio of γ-H2AX level in lymphocytes to that in granulocytes detected using flow cytometry as a potential biodosimeter for radiation exposure.* Radiation and environmental biophysics. 2014; 53(2): 283-90. doi: 10.1007/s00411-014-0530-0.

14. Löbrich M, Rief N, Kühne M, Heckmann M, Fleckenstein J, Rübe C, et al. *In vivo formation and repair of DNA double-strand breaks after computed tomography examinations.* Proceedings of the National Academy of Sciences of the United States of America. 2005;102(25): 8984-9.

15. Paull TT, Rogakou EP, Yamazaki V, Kirchgessner CU, Gellert M, Bonner WM. *A critical role for histone H2AX in recruitment of repair factors to nuclear foci after DNA damage.* Current Biology. 2000; 10(15): 886-95.