Use of the H2AX activation response to investigate DNA repair process

Min-ho Lee and Eun-hee Kim*
Nuclear Engineering Dept., Seoul National Univ., 1 Gwanak-ro, Gwanak-gu, Seoul, 151-744, Korea
**Corresponding Author: eunhee@snu.ac.kr*

1. Introduction

The cellular response to DNA damage and repair mechanism involves numerous signalings and effector pathways. One of the most well-established DNA damage marker is the phosphorylation of the histone H2AX (γ-H2AX). The γ-H2AX directly indicates the sites of DNA-double strand break (DSB) occurrences and their repairs [1].

The cell cycle arrest is another response to DNA damage. Two distinct G2/M checkpoints are activated in the irradiated cells. One is the early G2 checkpoint, which prevents the progression of damaged cells into mitosis. The other is the G2/M accumulation, which blocks the cells in G2 phase. The G2/M arrest is regulated by numerous proteins involved in DNA damage repair, such as MDM2 and p53 effector molecules [2]. p53 is a tumor suppressor protein, which stimulates p21 for the decision of a cell to undergo cell-cycle arrest. These check-points during the cell cycle progression provide cells with sufficient time to repair the radiation-induced DNA damage [3].

In this study, we identified various factors involved in DNA damage repair following high dose exposure of cells. In particular, we investigated whether H2AX phosphorylation has a dependence on cell cycle distribution.

2. Methods

2.1. Cell culture

Unsynchronized rat normal diencephalon cells (RDCs) (Catalog No. CRL-2005, ATCC) were cultured in T-25 flasks with a cell growth medium which Dulbecco's modified eagle medium (DMEM) (GIBCO, Grand Island, NY, USA) supplement with containing 10% (v/v) fetal bovine serum (FBS) (GIBCO). Cells were incubated in a 37°C incubator (Sanyo, Wood Dale, IL, USA) under a humidified mixture of 10% CO_2 and 90% air condition.

2.2. Cell cycle analysis

The fractional cell populations of G1, S, and G2/M phases were measured as a function of elapsed time after radiation exposure. The analysis was performed by using the Muse™ Cell Analyzer (Millipore, Billerica, MA, USA) and Muse™ cell cycle reagent (Millipore). The Muse cell cycle software module performs automatic calculations. Data are displayed in two plots. One is a dot plot of DNA content index and cell size index and the other is the histogram of DNA content index with markers available to analyze the cell populations in each phase of the cycle.

2.3 Multiplex analysis (Luminex's laser-based fluorescent analysis)

After irradiation, the cells were lysed in milliplex® map lysis buffer (Millipore) containing protease inhibitors. Total 20 μg of proteins in each lysate diluted in milliplex® map assay buffer 1 (Millipore) were analyzed according to the appropriate assay protocol (lysate incubation at 4°C overnight). The milliplex® map 7-plex DNA damage/genotoxicity magnetic bead kit (Millipore) is used to detect changes in phosphorylated H2A.X (Ser139) and p53 (Ser15) as well as total protein levels of MDM2 and p21 in cell lysates using the Luminex system in the unit of median fluorescence intensity (MFI).

2.4 Irradiation

Cells were irradiated by operating the X-ray beam tube (YXLON, Germany, 450-D08) at 200 kV and 10 mA. Dose rate was 1.5 Gy/min.

3. Results

3.1. Cell cycle analysis

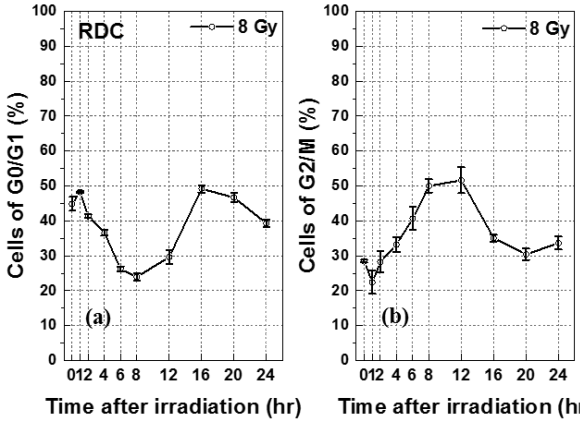

Fig. 1. The percentages of cells in each cell cycle phases with rat diencephalon cells as functions of the time (0, 1, 2, 4, 6, 8, 12, 16, 20 and 24 hours) elapsed after single irradiation at 8 Gy: (a) percent cells in G0/G1, (b) percent cells in G2/M.

Figure 1 shows the cell cycle distributions of RDCs as functions of the elapsed time after 8 Gy of exposure. The cell cycle arrest in the G2/M phase was maintained for 12 hours after irradiation.

3.2. Multiplex analysis

After RDCs were irradiated at 8 Gy, the multiplex analysis was applied to detect the protein expression and phosphorylation, which involved in DNA damage. The maximum value of the H2AX activation was reached right after irradiation. The H2AX activation level continuously decreased with the incubation time of up to 24 hours except at 12 hours after irradiation (Fig.2(a)). In ~~the~~ MDM2, p53 and p21 analyses, two distinct peak-values were noticed at 2 and 12 hours after irradiation (Fig.2(b-d)).

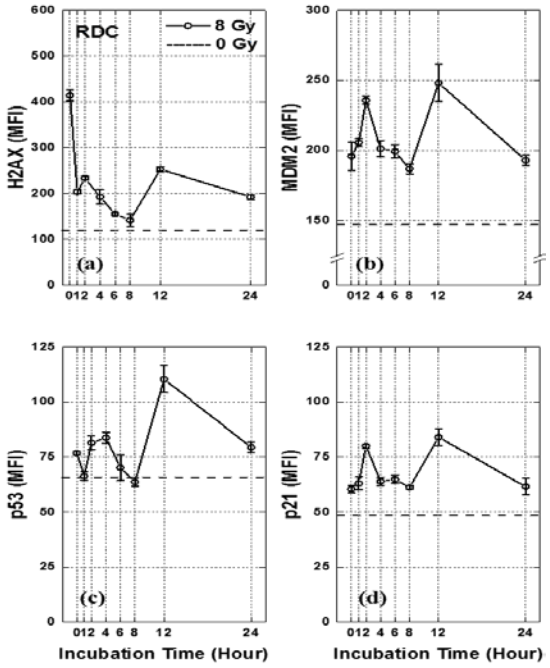

Fig. 2. The protein levels in MFI with rat diencephalon cells, varying with the incubation time (0, 1, 2, 4, 6, 8, 12 and 24 hours) after 8 Gy exposure. The figures represent the average and standard error of three replicate wells: (a) phosphorylated level of H2AX (Ser139), (b) total protein level of MDM2, (c) phosphorylated level of p53 (Ser15), and (d) total protein level of p21.

3.3. Cell cycle arrest and H2AX activation

To investigate cell cycle and various protein dependency of γ-H2AX, the MFI of protein and G2/M phase percentage were normalized to that of non-irradiation condition (Fig. 3). The normalized fractions of MDM2, p53, and p21 showed the fluctuation in similar patters to that of γ-H2AX.

In general model, the maximum level of γ-H2AX is reached at a certain time after irradiation, and it continuously decreases if the cells do not face additional exposure. However, in this study, a peak level of γ-H2AX was detected at 12 hours after irradiation. At the same elapsed time after irradiation, the cell cycle arrest in the G2/M phase was maximized. Since γ-H2AX is more clarified in cells at M-phase [4], we inferred that the peak level of γ-H2AX at 12 h after irradiation was attributed to the G2/M phase arrest at 12 h after irradiation.

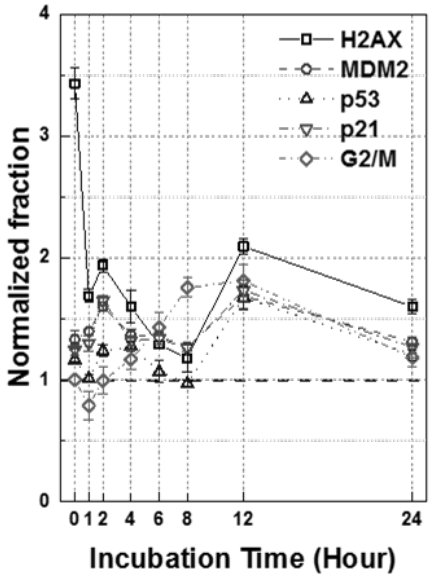

Fig. 3. The normalized fractions of protein (H2AX, MDM2, p53, and p21) levels and cell cycle (G2/M) to that under non-irradiation conditions for a varying incubation time (0, 1, 2, 4, 6, 8, 12 and 24 hours).

4. Conclusion

According to the observations in this study, the level of γ-H2AX may be perturbed by other repair factors of DNA damage, such as cell cycle arrest. We suggest that the γ-H2AX be carefully used as a DNA damage/repair marker.

REFERENCES

[1] LG. Mariotti, G. Pirovano, KI. Savage, M. Ghita, A Ottolenghi, KM. Prise, and G. Schettino, Use of the γ-H2AX assay to investigate DNA repair dynamics following multiple radiation exposure, PLOS ONE, Vol. 8, No. 11, p. e79541, 2013.
[2] S.A. Gatz, and L. Wiesmuller, p53 in recombination and repair, Cell Death Differ, Vol. 13, pp. 1003–1016, 2006.
[3] M. Fernet, F. Megnin-Chanet, J. Hall, and V Favaudon, Control of the G2/M checkpoints after exposure to low doses of ionizing radiation: Implications for hyper-radiosensitivity, DNA Repair, Vol. 9, pp. 48–57, 2010.
[4] Y. chijima, R. Sakasai, N. Okita, K. Asahina, S. Mizutani, and H. Teraoka, Phosphorylation of histone H2AX at M phase in human cells without DNA damage response, Biochem Biophys Res Commun, Vol. 336, No. 3, pp. 807-812, 2005.