Gamma irradiation induces DNA double-strand breaks in fibroblasts: a model study for the development of biodosimetry

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Abstract. Double-strand breaks (DSBs) of DNAs induced by ionizing radiation can pose detrimental damages on organisms which include genetic instability and cell death. It is necessary to be able to assess health risks associated with irradiation from both accidental and therapeutic exposures in a timely manner for proper medical treatments. This present study showed the first attempt to develop a biodosimetric measure in Thailand based on the quantification of phosphorylated histone H2AX (γ-H2AX) formed at DSB sites with an aim to establish a dose response curve using a two-dimensional (2D) cell culture model. Human dermal fibroblasts were grown into monolayers before irradiated by gamma rays from a Co-60 source in a custom-made lead chamber at doses 0, 0.2, 1, 2 and 4 Gy and a dose rate of 0.21 Gy/min. After 30 min post exposure, γ-H2AX proteins were immunofluorescently labelled for evaluation by confocal microscopy and flow cytometry. The accumulation of phosphorylated γ-H2AX proteins at DSBs appeared as nuclear foci with the most prominent intensity at 4 Gy. Linear regression analysis of flow cytometric data showed a linear response (R^2 = 0.9862) of foci intensity in proportion to irradiation dose. In addition, the fraction of cell viability was shown to decrease at higher doses. This technique can be further developed as a quick assessment tool to identify individuals subjected to accidental radiation in parallel to other established biodosimetric measures.

1. Introduction

The structure of DNA which stores the cells' genetic materials is constantly subject to lesions generated by both internal and external agents [1–3]. Internally, products released from normal metabolic activities inside the cells including reactive oxygen species can induce base damage, sugar damage and breaks on one of the DNA strands [1,3]. Unlike the single-strand break (SSB) damage, lesions that induce simultaneous breaks on both strands of the DNA helices at sites that are located sufficiently close to one another are known as double-strand breaks (DSBs). The primary source of external agents responsible for DNA DSBs is ionizing radiation. These DSB damages are much more lethal to the cells as they lead to genetic instability if incorrectly repaired or cell death when the damage remains unrepaired [1,3].

After sensing the damage, the cells rapidly respond to DSBs through various mechanisms including DNA repair, cell cycle checkpoints and apoptosis. The latter serves to eliminate severely damaged cells

that have lost the ability to regulate their normal growth [4]. The repair of DNA DSBs relies on two major processes, non-homologous end joining (NHEJ) and homologous recombination (HR) [1,2,4]. NHEJ is the major pathway in non-replicating cells, whereas HR functions in the late S/G2 phase [2,4]. Proteins that are involved in the early stages of cellular responses in sensing the damage and lead to proper repair processes have been the central focus of research in recent years. One of the key factors in such event is phosphorylation of the core histone variant H2AX on the serine-139 residue (γ-H2AX) [3,5,6] in the vicinity of DSBs, which in turn recruits other protein machinery such as MRN complex, ATM, 53BP1 and MDC1 proteins [4] to the damaged sites. The phosphorylation of γ-H2AX can be visualized through the formation of foci inside the cells' nuclei [3,7]. At low doses of radiation, each γ-H2AX focus approximately represents one DSB [5,6] and it has been noted that up to thousands of H2AX protein can be phosphorylated per DSB [3] at higher doses. The importance of H2AX in the DNA repair process was confirmed by previous study [3] which showed the high sensitivity and genomic instability of H2AX-deficient cells to ionizing radiation compared to control.

As a marker for DNA damage, γ-H2AX can be used as a biodosimetric tool to assess the extent of DSBs induced by accidental or therapeutic exposures to ionizing radiation. In the former case, quantification of γ-H2AX foci using blood samples has shown a promising potential [6] that supplements other standardized methods such as a dicentric assay for population triage. In therapeutic exposure, radiotherapy can potentially induce injuries to the skin [5], in which the γ-H2AX assay may be applied to estimate the resulting dose and the extent of such injuries. In this present study, we attempted to develop γ-H2AX foci as a biodosimetric measure as a part of the biodosimetry program initiated by the Office of Atoms for Peace, Thailand. Using the 2D cell culture model, we first established a dose response curve for gamma radiation in the range of 0 - 4 Gy.

2. Materials and methods

2.1 Cell culture

Normal human dermal fibroblasts (HDFBs) passages 8 - 12 were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 50,000 µg streptomycin and 50,000 U penicillin in 6-well tissue culture plates. The media was refreshed daily. HDFB monolayers at day 2 post confluence were used in all experiments.

2.2 Gamma irradiation

Lead containers with shelves were custom built as illustrated in figure 1 to accommodate samples during irradiation by gamma rays from the Co-60 source. Doses in the range of 0 - 5 Gy were first calibrated using optically stimulated luminescence dosimeters. HDFB monolayers were irradiated for the total doses of 0.2, 1, 2, and 4 Gy at the dose rate of 0.21 Gy/min. Non-irradiated HDFBs (0 Gy) were used as sham control. Within 30 min after exposure to gamma irradiation, HDFBs were washed with phosphate buffered saline and treated with trypsin for characterization of γ-H2AX foci by flow cytometry and laser scanning confocal microscopy. In addition, separate sets of HDFBs were subcultured and maintained until 90% confluence before harvesting for quantification of DNA fragmentation by gel electrophoresis.

Figure 1. Experimental setup inside the gamma radiation facility.

2.3 Immunofluorescence staining of γ-H2AX
All staining steps were done on ice. Following treatment with trypsin, HDFBs were fixed with 4% paraformaldehyde for 20 min, washed, centrifuged, and resuspended in 0.1% Triton-X 100 for 5 min. The cells were then incubated in 1% bovine serum albumin (BSA) solution for 30 min to block non-specific binding prior to incubation with mouse anti-human γ-H2AX clone JBW301 (EMD Millipore, USA) antibody at a dilution of 1:200 in 1% BSA solution for 1 h. After washing in phosphate buffered saline (PBS), the cells were incubated in a 1:500 goat anti-mouse Alexa Flour 488 (Invitrogen, USA) solution for 45 min, washed, and resuspended in 1% paraformaldehyde for analysis by flow cytometry. For visualization by confocal microscopy, a small drop of cells was mixed with antifade agent (ProLong Gold, Life Technologies, USA) and mounted on a glass slide.

2.4 Flow cytometry
Immunofluorescence intensity of γ-H2AX was measured using a BD FACSCaliber flow cytometer (BD Biosciences, USA). Data were collected from 3 independent experiments with at least 10,000 cells for each dose. Analysis was done using BD CellQuest and FlowJo softwares (BD Biosciences, USA; FlowJo, USA).

2.5 Visualization of γ-H2AX foci
For microscopy imaging, the cell nuclei were counterstained with Hoechst 33258 (Life Technologies, USA). Images of γ-H2AX foci were captured on a Zeiss LSM510 Meta microscope (Carl Zeiss, Jena, Germany). About 30 – 50 cells were imaged per irradiation treatment.

2.6 DNA fragmentation assay
Harvested HDFBs were incubated at 37 °C for 1 h in 10 mM Tris-HCl, pH 8.0, 20 mM EDTA, 200 mM NaCl, 0.2 % Triton X-100 and 100 µg/mL proteinase K. Genomic DNA was precipitated from the supernatant with an equal volume of isopropanol and 100 mM NaCl at -20 °C, and then dissolved in NanoPure water with RNase A. 1 µg of genomic DNA was then separated on 0.6% agarose gel in Tris-borate buffer and stained with ethidium bromide for visualization.

2.7 Cell viability assay
A portion of HDFBs harvested for DNA fragmentation assay was mixed with an equal volume of 0.4% Trypan blue. After 10 min, cells were counted on a hemocytometer. Viable cells did not uptake the dye and appeared transparent, whereas non-viable cells were permeable to the dye and appeared blue.

2.7 Data analysis
To construct a dose response curve, the collected γ-H2AX intensities at each irradiation dose were normalized to control. Data were collected from 3 independent experiments and presented as mean ± standard error of mean.

3. Results and discussion

3.1 Gamma radiation-induced DNA DSBs
As a result of radiation-induced DNA damage, the immediate repair of DNA DSBs can be detected by fluorescence imaging of γ-H2AX phosphorylation, which appeared as bright nuclear foci (figure 2). In our present study, the dose-dependent increase of γ-H2AX proteins was evident by the increase in foci intensity within 30 min after exposure to radiation. At the lowest irradiation dose of 0.2 Gy (figure 2a), a few bright foci appeared sporadically inside the cell nuclei, whereas the uniform distribution of foci with prominent intensity was observed at the highest dose of 4 Gy investigated in this study (figure 2b).

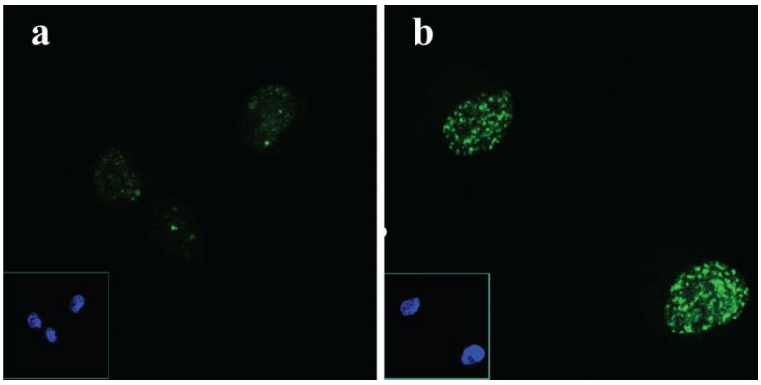

Figure 2. Confocal fluorescence images of γ-H2AX foci (green) formed inside the cells' nuclei after exposure to gamma irradiation at (a) 0.2 Gy and (b) 4 Gy. The nuclei (blue) were counterstained with Hoechst 33258 and shown on the lower left corner.

For quantitative measurement, the intensity of phosphorylated γ-H2AX foci at each irradiation treatment was analyzed by flow cytometry. By normalizing the collected flow intensity at all doses to the value at 0 Gy, our data showed a linear increase in foci intensity in proportion to irradiation dose (R^2 = 0.9862) (figure 3), indicating the extent of DSB damage at increasing doses. At the highest dose of 4 Gy, the phosphorylation of γ-H2AX showed a two-fold increase compared to sham control.

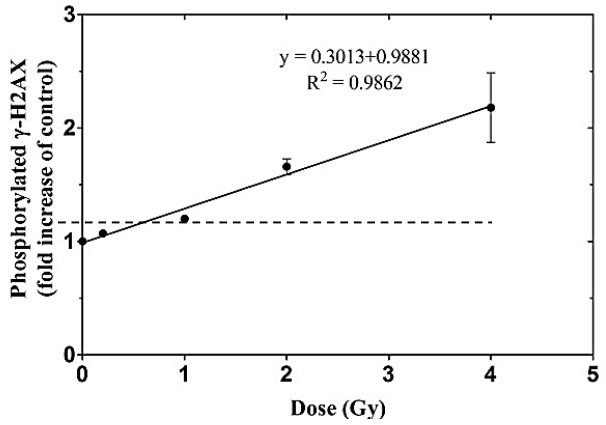

Figure 3. Graph represents the dose response relationship of γ-H2AX foci to gamma irradiation in HDFB cell model. Data were normalized to control (0 Gy) and shown as a fold increase in γ-H2AX intensity. Dashed line marks the background level.

This increase in foci intensity was in agreement with previous studies of X-ray [7,8] and gamma [5,6] irradiation-induced DNA DSBs of fetal fibroblasts [7], human lymphocytes [6,8], minipig skin tissues [5,9] and skin equivalents [6] that reported the linear relationship of γ-H2AX phosphorylation to irradiation dose. As an immediate cellular response to irradiation, the foci intensity peaked at 30 min post exposure [6,7] before attenuated over time as DNA repair proceeded [5-7,10]. However, this linear relationship of foci to irradiation dose was maintained at 48 h as reported in the case of lymphocytes, fibroblasts and keratinocytes [6] after gamma irradiation at doses 0 - 5 Gy. In addition, the residual of γ-H2AX foci was also reported in minipig skin biopsies at day 3 following 50-Gy exposure to gamma radiation [5]. This persistence in the formation of foci, which may indicate delayed or impaired repair of DSBs due to complex DNA damage, enables the use of γ-H2AX assay to supplement other biodosimetric measures for population triage during the first 48 h after accidental exposure for proper medical treatments. In addition, the assay has been shown to be implemented in the automated setup [11], which also facilitates the process of large-scale samples. Although only a 30-min time point was the focus in this present study to first establish the dose response curve, the levels of γ-H2AX

phosphorylation at longer time points and the use of other biological samples such as blood will be further investigated to fully complete the time-dependent, dose response relationship.

High dose radiation from radiotherapy can pose injury to the skin as well as the long-term effects such as fibrosis and skin cancer [5]. In this present study, we have used dermal fibroblasts in a 2D culture model to quantify the extent of DNA damage as an initial step towards the development of 3D culture model to substitute for the use of animals or biopsies in future study. Such results may provide the better estimation of cutaneous radiation reaction [5] in individuals subjected to therapeutic exposure.

3.2 Apoptosis and cell viability

Radiation-induced DNA DSBs can result in cell apoptosis if the damaged DNA is not repaired and the cells lose the ability to control their normal growth. During cell apoptosis, endonucleases are stimulated to induce fragmentation of DNA, which can be detected by agarose gel electrophoresis. In our study, HDFBs were first irradiated by gamma rays at the selected doses of 0.2 and 4 Gy, subcultured and then harvested for DNA fragmentation assay on day 4. Compared to the DNA ladder in the range of 0 - 20 kbp (figure 4), we did not observe any DNA fragments from HDFB extracts after exposure to both irradiation doses. However, DNA fragments in the range of 180-900 bp have been previously reported [10] in lymphocytes after exposure to X-ray irradiation at doses > 1 Gy. Such discrepancy in the data may be attributed to the apoptosis-resistance nature of fibroblasts. In comparison to keratinocytes, fibroblasts were two times more resistant upon exposure to 2 Gy soft X-ray [12]. Metabolically, HDFBs could recover their level of glutathione, which helps protect the cells from radiation-induced oxidative damage, within 72 h after 1 Gy gamma irradiation [13]. Gamma radiation-induced apoptosis in many cell types is mediated partly through Fas/FasL interaction [14,15]. Under normal culture conditions, HDFBs express FLIP protein which confers resistance to Fas-induced apoptosis [16] and therefore would also contribute to HDFBs' resistance to radiation-induced apoptosis. In addition, Bmi-1, a polycomb group RING finger protein, has also been shown to contribute to radioresistance in HDFBs especially at a low radiation dose [17].

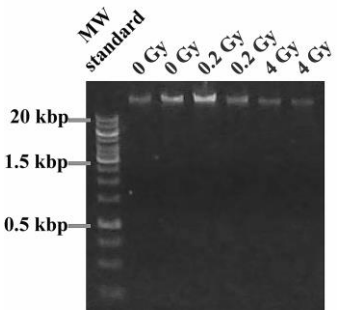

Figure 4. DNA fragmentation assay of HDFB extracts on 0.6% agarose gel

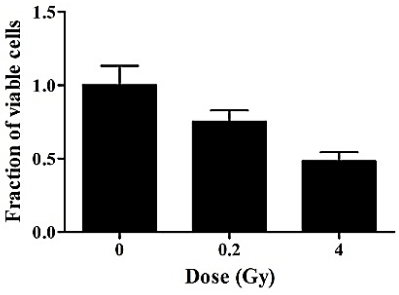

Figure 5. Fraction of cell viability analyzed by trypan blue assay

Although DNA fragments were not detected, the number of cells, represented by fraction of cell viability (figure 5), decreased from 1 to 0.5 as the irradiation dose increased from 0 – 4 Gy. This decline in the cell number suggests that some other cellular functions may be altered by irradiation and later affect the number of remaining, viable cells in subcultures. Similar observations have been reported for HDFBs [13], human pulmonary endothelial cells [18], SSC4 oral squamous cell carcinoma cells and MCF7 breast cancer cells [19] exposed to gamma radiation. In certain reports, reduction in cell viability has been shown to occur concomitantly with induction of apoptotic markers [18,20-22]. However, cell cycle arrest and reduction in clonogenic ability may also explain the reduction in cell counts upon a subculture [19,20]. To further investigate the mechanisms involved, apoptotic markers such as caspase-

3 activity, the distribution of cell population in various phases of the cell cycle and the clonogenic ability of irradiated HDFBs and will be investigated.

4. Conclusion

In this present study, we have established the dose-response relationship of DNA DSBs, measured from γ-H2AX phorphorylation, to gamma radiation using human-derived dermal fibroblasts as a cell model. The phosphorylation of γ-H2AX at DSB sites showed a linear increase with irradiation dose in the range of 0 - 4 Gy tested in this present study, supporting its application as a biodosimetric tool. Despite the absence of DNA fragmentation, the increase in irradiation dose had an effect on the number of live cells remained in subculture post irradiation.

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