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$Q_{\gamma\text{-H2AX}}$, an analysis method for partial-body radiation exposure using $\gamma\text{-H2AX}$ in nonhuman primate lymphocytes

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Abstract

We previously used the $\gamma\text{-H2AX}$ assay as a biodosimeter for total-body-irradiation (TBI) exposure (γ -rays) in a rhesus macaque (*Macaca mulatta*) model. Utilizing peripheral blood lymphocytes and plucked hairs, we obtained statistically significant $\gamma\text{-H2AX}$ responses days after total-body exposure to 1–8.5 Gy (^{60}Co γ -rays at 55 cGy min⁻¹). Here, we introduce a partial-body exposure analysis method, $Q_{\gamma\text{-H2AX}}$, which is based on the number of $\gamma\text{-H2AX}$ foci per damaged cells as evident by having one or more $\gamma\text{-H2AX}$ foci per cell. Results from the rhesus monkey – TBI study were used to establish $Q_{\gamma\text{-H2AX}}$ dose-response calibration curves to assess acute partial-body exposures. $\gamma\text{-H2AX}$ foci were detected in plucked hairs for several days after in vivo irradiation demonstrating this assay's utility for dose assessment in various body regions. The quantitation of $\gamma\text{-H2AX}$ may provide a robust biodosimeter for analyzing partial body exposures to ionizing radiation in humans.

Keywords

biological dosimetry; $\gamma\text{-H2AX}$; $Q_{\gamma\text{-H2AX}}$; $F_{\gamma\text{-H2AX}}$; rhesus macaque (*Macaca mulatta*); lymphocytes; ^{60}Co γ -rays; ionizing radiation; partial-body exposure

1. Introduction

Measurement of *in vivo* DNA damage responses to various treatments is providing useful information to clinicians for improving human health. Hundreds of H2AX molecules are rapidly phosphorylated on serine 139 ($\gamma\text{-H2AX}$) in response to the formation of each DNA double-strand break (DSB) (Rogakou et al., 1999; Rogakou et al., 1998). The most sensitive assays of $\gamma\text{-H2AX}$ utilize immunocyto- and immunohisto-chemistry techniques to stain for $\gamma\text{-H2AX}$ foci, intranuclear structures composed of the hundreds of $\gamma\text{-H2AX}$ molecules, which form at each DSB site in cells and tissues (Bonner et al., 2008). Exposure of humans to ionizing radiation during various radiological diagnostic or therapeutic treatments (Kuefner et al., 2009; Lohrich et al., 2005; Porcedda et al., 2006; Sak et al., 2007) and space

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travel (Ohnishi et al., 2009) lead to DSB formation that can be measured by counting the γ -H2AX foci in many tissues including lymphocytes, buccal cells, and skin biopsies; see reviews by (Redon et al., 2010b; Rothkamm and Horn, 2009).

Thus, counting γ -H2AX foci in cells of persons exposed to irradiation may be useful in determining the extent of exposure and in providing optimum medical care. In addition to radiation exposure from medical procedures and/or diagnostics, the risk of accidental irradiation to the general public and emergency responders has also increased in recent years. We recently utilized a non-human primate (NHP) model to further validate the γ -H2AX assay following TBI with both non-lethal and lethal radiation doses (Redon et al., 2010a). Measuring γ -H2AX foci in NHP lymphocytes and plucked hairs enabled us to assess the subject's exposure several days after irradiation at doses in the medical triage range, making γ -H2AX biodosimetry a robust tool for measuring TBI in macaques for several days after irradiation.

However, a serious consideration is that many radiation accidents are likely to involve inhomogeneous exposures or partial-body irradiation (PBI) (Prasanna et al.), the extent of which may be unknown both in terms of the fraction of the body irradiated and dose delivered to the irradiated fraction. In cases of acute exposure, the determination that a fraction of the blood volume had escaped irradiation would indicate that a portion of the body, and hence the bone marrow, may have also escaped lethal irradiation. Such knowledge may be critical for guiding decisions on optimal medical treatment for the victim, for example, whether a bone-marrow transplant is necessary for survival.

A method (Q_{dr}) to evaluate PBI in individuals was originally demonstrated by Sasaki and Miyata (Sasaki and Miyata, 1968) utilizing the frequency of chromosomal aberrations (dicentric and ring chromosomes) in lymphocyte metaphase spreads taken from exposed subjects. It was later adapted to utilize the frequency of chromosome fragments present in lymphocytes subjected to premature chromosome condensation (Blakely et al., 1995; Prasanna, et al.). Here, we extend this method to utilize the levels of γ -H2AX foci in the lymphocyte population of the exposed subject to assess dose and fraction of body exposed to PBI.

We also briefly introduce the use of plucked hairs as a mean to assess PBI. It may take a few minutes for blood to make a complete bodily circuit, hence, the fraction of the blood volume exposed and the dose received by that fraction may differ considerably from that received by other portions of the body, depending in large part on the dose rate and length of exposure. Exposure of sufficient duration to any part of a body could lead to irradiation of the complete blood volume. However, in these cases, measurements of γ -H2AX foci levels in plucked hair bulbs could help determine the extent of partial-body exposure.

2. Materials and Methods

2.1 PBI Calculations

The data used to develop the method for PBI calculations were obtained from a previous study by Redon et al. (2010) using γ -H2AX foci levels in macaque lymphocytes for TBI biodosimetry (Redon, et al., 2010a). This method, based on the Sasaki and Miyata method (Sasaki and Miyata, 1968), may provide useful estimates of the fraction of the body exposed to radiation. Considering a γ -H2AX focus to denote a damaged chromosome, the term $Q_{\gamma\text{-H2AX}}$, the mean number of γ -H2AX foci per cell in the cells containing foci, ignoring the foci-free cells, can be calculated. Similarly, we use the term $F_{\gamma\text{-H2AX}}$ to refer to the fraction of lymphocyte population containing damaged cells. $F_{\gamma\text{-H2AX}}$ and $Q_{\gamma\text{-H2AX}}$ can be calculated with the following formula:

$$Q_{\gamma\text{-H2AX}} = \frac{\sum f_{\gamma\text{-H2AX}}}{N_t - N_0}$$

$$F_{\gamma\text{-H2AX}} = \frac{N_{\gamma\text{-H2AX}}}{N_t}$$

$\sum f_{\gamma\text{-H2AX}}$ is the total number of $\gamma\text{-H2AX}$ foci in all cells observed; $N_{\gamma\text{-H2AX}}$ is the number of damaged cells (with at least one $\gamma\text{-H2AX}$ focus); N_t , the total number of cells observed and N_0 is the number of cells without foci.

We determined both $F_{\gamma\text{-H2AX}}$ and $Q_{\gamma\text{-H2AX}}$ values vs doses from the TBI data, values that allowed us to establish standard curves (Fig. 2A and 2B). Following PBI, both experimental $Q_{\gamma\text{-H2AX}}$ and experimental $F_{\gamma\text{-H2AX}}$ can be determined after $\gamma\text{-H2AX}$ examination in lymphocytes. Both experimental $Q_{\gamma\text{-H2AX}}$ and experimental $F_{\gamma\text{-H2AX}}$ and the standard curves obtained from TBI will allow the estimate of the irradiation dose and of the fraction of the body that was exposed.

As an example, shown graphically in Fig. 2A and 2B and mathematically here, we consider a hypothetical case in which a blood sample collected 2 days after a possible PBI was found to yield the experimental values $Q_{\gamma\text{-H2AX}} = 2$ and $F_{\gamma\text{-H2AX}} = 0.40$. Using the standard curve for 2 days after TBI in Fig. 2A, the value of $Q_{\gamma\text{-H2AX}}$ yields an estimated dose of ~4.4 Gy to the exposed portion of the body. In Fig. 2B, a dose of 4.4-Gy TBI would yield an $F_{\gamma\text{-H2AX}}$ value after 2 days of 0.63, considerably greater than the value in the hypothetical example of 0.4, a difference that would indicate PBI. PBI can be calculated by the ratios after subtracting the background values as follows:

$$PBI = \frac{\text{Experimental } F_{\gamma\text{-H2AX}}}{\text{TBI } F_{\gamma\text{-H2AX}}} = \frac{0.40 - 0.07}{0.63 - 0.07} = 0.59 = 59\%$$

- “Experimental $F_{\gamma\text{-H2AX}}$ ” is determined after lymphocyte examinations (= 0.40)
- “TBI $F_{\gamma\text{-H2AX}}$ ” is determined using the standard curve $F_{\gamma\text{-H2AX}}$ vs dose (Fig. 2B) (= 0.63)
- 0.07 is the $F_{\gamma\text{-H2AX}}$ value in sham-irradiated macaques.

2.2 $\gamma\text{-H2AX}$ detection in plucked hairs

Plucked hairs preparation for immunohistochemistry and $\gamma\text{-H2AX}$ detection were performed as previously described (Redon, et al., 2010a).

3. Results

3.1 $\gamma\text{-H2AX}$ foci in lymphocytes for acute partial-body irradiation (PBI) biodosimetry

The method utilizing $\gamma\text{-H2AX}$ foci distribution frequencies is modeled for TBI and simulated PBI in Fig. 1A. In an idealized acute PBI scenario, the lymphocyte population would have two components, one from the irradiated fraction exhibiting cells with foci (Fig 1A, blue line), and the other from the unirradiated fraction exhibiting cells with few if any foci (Fig 1A, red line). The fraction of each of these two components represented in the lymphocyte population would depend on the fraction of the blood volume irradiated. Figure

1B presents two modeled situations, one with 80% PBI (80% of cells from TBI samples and 20% of cells from unirradiated samples) and 20% PBI (20% of cells from TBI samples and 80% of cells from unirradiated samples). By first focusing on the damaged cell component of the TBI data, the number of foci per damaged cell, named $Q_{\gamma\text{-H2AX}}$ after the Sasaki and Miyata method, can be calculated. This term gives information on the dose received by the exposed body portion, largely independent of the fraction of the body exposed (Fig 2A). Second, the fraction of total cells with foci, $F_{\gamma\text{-H2AX}}$, when used in conjunction with $F_{\gamma\text{-H2AX}}$ calibration curves determined for TBI, gives information on the fraction of the body exposed (Fig 2B). Calibration curves for the application of the $Q_{\gamma\text{-H2AX}}$ and $F_{\gamma\text{-H2AX}}$ analysis method for the case of TBI are illustrated in Figure 2A and 2B.

Results from an illustrative example, as described in the Methods paragraph above, is shown graphically utilizing the TBI calibration graphs for $Q_{\gamma\text{-H2AX}}$ and $F_{\gamma\text{-H2AX}}$ (Figs. 2A and 2B). In this example, blood drawn from a putative acute PBI victim 2 days after exposure yielded values for $Q_{\gamma\text{-H2AX}}$ of 2 foci per cell, and a $F_{\gamma\text{-H2AX}}$ of 0.4, which is significantly less than that a TBI value of 0.63. The resulting estimate from these input values is that 59% of the body received ~4.4 Gy, and more importantly, 41% of the body was not exposed to radiation (see Materials and Methods paragraph for details of the calculation). To check for internal consistency, computer PBI simulations of $\gamma\text{-H2AX}$ foci distributions for 5, 12.5, 33, 55, 80 and 95% of total-body exposure to doses of 1, 3.5 6.5 and 8.5 Gy at 0.3, 1, 2 and 4 days post exposure were prepared as modeled in Fig. 3A. $F_{\gamma\text{-H2AX}}$ values were calculated for the simulated $\gamma\text{-H2AX}$ foci distributions and plotted vs the input PBI values (Fig. 3). When the result of the hypothetical example (59% of total body exposed to ~4.4 Gy) is plotted in the “2 d post-irradiation” panel (Fig. 3, 3rd panel from top), an $F_{\gamma\text{-H2AX}}$ value of 0.4–0.45 is obtained, in good agreement with the input value of 0.40. The 41% of the body that received little or no irradiation exposure may contain sufficient undamaged bone marrow to replenish the blood cell population without the need for a transplant.

3.2 $\gamma\text{-H2AX}$ in plucked hairs after TBI

We previously showed that plucked hairs may be a useful surrogate tissue for assessing exposures to ionizing radiation (Redon, et al., 2010a). Other studies show the use of hair follicles can be applied to monitor radiation exposure (Kyoizumi et al., 1998; Sieber et al., 1992) as well as to use $\gamma\text{-H2AX}$ as a marker for DNA damage in patients undergoing chemotherapy (Fong et al., 2009). $\gamma\text{-H2AX}$ residual foci in plucked hairs are dose and time dependent (Fig. 4). The incidence of $\gamma\text{-H2AX}$ foci after 1 Gy-TBI is low as signals disappear after 2 days TBI. In contrast $\gamma\text{-H2AX}$ signals were still detected after 2 days for 3.5 and 6.5 Gy-, and 8.5 Gy-TBI, after 9 days for 8.5 Gy-TBI.

4. Conclusion

Radiation accidents are more commonly characterized by acute partial-body or inhomogeneous rather than homogeneous TBI radiation exposures. Dose inhomogeneity can be a major confounder in dose assessment using biodosimetry (Kolanko et al., 2000; Prasanna, et al.). Two chromosome aberrations assays, metaphase-spread dicentric and ring and premature chromosome condensation or PCC, along with analysis methods, Q_{dr} (Sasaki and Miyata, 1968) and Q_{pcc} (Blakely, et al., 1995) respectively, for partial-body exposure assessment have been proposed for this purpose. Sak and colleagues evaluated the yield of $\gamma\text{-H2AX}$ foci formation to allow estimation of the applied integral body dose after local radiotherapy to different sites of the body (Sak, et al., 2007). In 2007, a study by Rothkamm et al. described the application of a related cytogenetic data analysis method, the contaminated Poisson method by Dolphin, to detect partial body exposure using $\gamma\text{-H2AX}$ -based biodosimetry in patients undergoing CT scans (Rothkamm et al., 2007). The authors observed an overdispersion of the $\gamma\text{-H2AX}$ focus distribution from the Poisson distributions

after CT examinations that revealed a partial body exposure to radiation. Here, we introduce a partial-body analysis methodology (i.e., $Q_{\gamma\text{-H2AX}}$, $F_{\gamma\text{-H2AX}}$), similar to that done for dicentric (Q_{dr}) and PCC (Q_{PCC}) assays, utilizing the gamma-H2AX foci assay. Utilizing $\gamma\text{-H2AX}$ foci levels for these calculations has the advantage over micronuclei and damaged chromosomes of not requiring cell culturing and stimulation, since the foci are formed in the quiescent lymphocytes. Representing an alternative and rapid approach both for the assessment of dose intensity and inhomogeneity following acute ionizing radiation exposures, this approach could be useful for several practical biodosimetry applications.

However, we are aware that this method is most useful when the partial irradiation time is much less than the rate of blood circulation so that the blood pool renewal in the irradiated part of the body is limited. In addition, further analysis will be necessary to evaluate the influence of potential confounders on our method (i.e., inter-individual variations in background foci levels, gradient dose distributions rather than just 'clean' partial body irradiation, number of cells scored, response of special populations, etc). Because of the fast signal lost, we believe that $Q_{\gamma\text{-H2AX}}$ could be used in the first hours but less than a day following low irradiation doses ($< 1\text{Gy}$). Thus, $Q_{\gamma\text{-H2AX}}$ could be limited to high PBI doses. PBI doses higher than 3.5 Gy would result in long lasting $\gamma\text{-H2AX}$ foci in irradiated lymphocytes with PBI doses $\geq 6.5\text{Gy}$ resulting in a population of lymphocytes with "stable" $\gamma\text{-H2AX}$ foci (non-repairable of DSBs)(Redon, et al., 2010a). With such high PBI irradiation doses, we could expect to use $Q_{\gamma\text{-H2AX}}$ for a few days after exposure (time that would also depend on the volume of irradiated body). Therefore, future studies will need to be performed to validate this model. Finally, because tissues from other parts of the body such as nails and teeth may be useful for biodosimetry (Fattibene and Callens, 2010; Romanyukha et al., 2010), their analysis together with $\gamma\text{-H2AX}$ detection in other parts of the body, i.e., hairs plucked from the scalp and elsewhere, may develop into a robust tool for PBI dose assessment. Since non-clinical personnel could pluck hairs, this tissue will be well suited for large scale sample collection.

Abbreviations

TBI	total-body irradiation
PBI	partial-body irradiation
NHP	non-human primate

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Fig.1. Scheme of γ -H2AX foci distributions for TBI and PBI

(A) The left panel illustrates foci distributions for samples from TBI (blue color) and sham-treatment (red line). Poisson distributions with averages of 0.1 and 5 fpc were used to simulate control and TBI populations. The insert panel in Fig. 1A represents the same data shown in the left panel but magnified to illustrate the distribution pattern for foci numbers less than 6 foci per cell. (B) Right panel illustrates foci distributions in samples from PBI. Two PBI conditions are illustrated based on data shown in Fig. 1A; PBI involving 20% (B, green line) or 80% (B, orange line) of the lymphocyte population irradiated. See manuscript for additional details. The insert panel in Fig. 1B represents the same data shown in the right panel but magnified to illustrate the distribution pattern for foci numbers less than 6 foci per cell.

Fig.2. The use of $Q_{\gamma\text{-H2AX}}$ and $F_{\gamma\text{-H2AX}}$ for partial-body irradiation (PBI)

(A) Graph of $Q_{\gamma\text{-H2AX}}$ (foci per damaged cell) vs TBI exposure. This value approximates the dose received by the lymphocytes in the fraction of the body subjected to irradiation, independent of the size of that fraction. (B) Graph of $F_{\gamma\text{-H2AX}}$ (fraction of damaged cells) vs TBI exposure. This value is used to approximate the PBI fraction. For example, assume values of $Q_{\gamma\text{-H2AX}} = 2$, and $F_{\gamma\text{-H2AX}} = 0.4$ were calculated from lymphocytes obtained from a subject 2 days after irradiation of unknown amount and bodily distribution. The $Q_{\gamma\text{-H2AX}}$ value indicates that the exposed portion of the subject received ~4.4 Gy (panel A, green dashed lines). If the subject had been exposed to TBI of 4.4 Gy, then an $F_{\gamma\text{-H2AX}}$ of ~0.63 would be expected in lymphocytes taken 2 days after irradiation (Solid horizontal violet

arrow in panel B). Thus, an $F_{\gamma\text{-H2AX}}$ of ~0.4 (horizontal dashed green line) indicates that the subject received less than TBI. Taking into account the control values, the PBI fraction is estimated as $(0.4-0.07)/(0.63-0.07) = \sim 59\%$ of the body being irradiated. Graph in panel **B** uses the same legends as Graph in panel **A**. Errors bars represent standard deviations of the mean (n=6).

Fig.3. Graphs of $F_{\gamma\text{-H2AX}}$ vs body percentage in a computer simulation of PBI

To test the consistency of the PBI estimate, various mixtures of total body and sham exposed $\gamma\text{-H2AX}$ foci distributions of lymphocytes were used to simulate situations in which 5, 12.5, 33, 55, 80, and 95% of the body was exposed to irradiation (as demonstrated for 20 and 80% in Fig 1B). $F_{\gamma\text{-H2AX}}$ values were calculated for these mixtures for the different irradiation exposures and phlebotomy times. In the example given above, on the graph of 2 days post irradiation (third panel from top), the calculated PBI percentage of 59% and the $F_{\gamma\text{-H2AX}}$ value of 0.4 intersect slightly above the line for 3.5 Gy and below the line for 6.5 Gy, consistent with the estimated value of 4.4 Gy (see text for further explanation).

Closed symbols: irradiated macaques; open symbols: sham-irradiated controls. Errors bars represent standard deviations of the mean (n=6).

Fig.4. Kinetics for γ -H2AX foci in macaque plucked hairs after total-body irradiation
(A) (Main graph) Kinetics for γ -H2AX foci formation and loss in plucked hairs for sham-irradiation (Sham IR) and at time points after the indicated TBI dose for up to 39 days. (Inset) Short-time course for γ -H2AX foci formation and loss. **(B)** Representative images of plucked hairs obtained (eyebrow) from a sham-irradiated (top panel) and 1 day after 8.5 Gy TBI (bottom panel). Inserted images in the right bottom of each main image are enlargements of the regions inside the white squares in these main images. Green, γ -H2AX; red, DNA stained with PI. Errors bars represent standard deviations of the mean ($n \geq 4$ for 1 Gy; $n \geq 5$ for 3.5 Gy; $n \geq 3$ for 6.5 Gy; $n \geq 7$ for 8.5 Gy; $n \geq 2$ for Sham-IR).