Chapter 12
Assessment of DNA Damage Induction in Farm Animals After the FNPP Accident

Asako J. Nakamura

Abstract Since the Fukushima Daiichi Nuclear Power Plant (FNPP) accident is a radiation accident which occurred as the aftermath of a devastating natural disaster, the Great East Japan Earthquake, people had to face radiation risks in the chaotic state. In the situation where general citizens, scientists and politicians were all confused, the importance of accurately assessing biological risks of radiation emerged. To understand the biological effects of radiation exposure by the accident, we measured the DNA damage level using the DNA double-strand break (DSB) marker phosphorylated histone H2AX in peripheral blood lymphocytes from farm animals left behind within a 20-km radius from FNPP (the ex-evacuation zone). As a result, statistically higher levels of DNA DSBs were detected from cattle in the ex-evacuation zone compared to non-affected control; however, it was not able to accurately evaluate the radiation dose from this accident with phosphorylated H2AX. This is thought to be caused by the fact that various changes of metabolism with the lapse of time and the living environment of individual organisms occurred. It may, therefore, be difficult to evaluate the exposure dose of chronic low-dose-rate (LDR) radiation by a single biomarker. However, in the inevitable modern society of radiation exposure and fear of nuclear accidents, our results showed that trying a certain dosimetric biomarker for the assessment of biological impacts of long-term LDR radiation exposure is effective and crucial for the protection from radiation.

Keywords Biodosimetry · DNA double-strand break · Phosphorylated histone H2AX · Biological effect

A. J. Nakamura (✉)
Department of Biological Science, College of Science, Ibaraki University, Mito, Ibaraki, Japan
e-mail: asako.nakamura.wasabi@vc.ibaraki.ac.jp

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12.1 Introduction

In 1998, it was first reported that histone H2AX, one of the core histones, is rapidly phosphorylated (phosphorylated histone H2AX is called γ-H2AX) at the time of induction of DNA double-strand breaks (DSBs) and plays an important role for recruitment of DNA damage repair proteins to the damage site [1–5]. As phosphorylation of H2AX occurs specifically at the site of DNA DSBs, it is possible to visualize the DNA DSB site as the focus of γ-H2AX by immunofluorescent staining method using an antibody specific to γ-H2AX [2, 5–7]. In fact, it was shown that the γ-H2AX focus number and the number of DNA DSBs are the same [8]. Though DNA DSBs had been detected by experimental methods such as the comet assay and pulsed-field gel electrophoresis previously, γ-H2AX is sensitive enough to detect radiation exposure equivalent to several mGy, and the operation is simple; therefore, it is widely used as a marker of DNA DSB [6, 9, 10]. Recently, the γ-H2AX assay is used as a method of monitoring in vivo DNA DSB level for clinical trials of novel anticancer drugs and for assessment of exposure dose of cancer patients who received radiation therapy [11–17]. Present when this manuscript is being written (June 2018), more than 40 clinical trials using the γ-H2AX assay seem to be performed in the United States (https://clinicaltrials.gov/). Since the September 11 attacks occurred in the United States in 2001, the risk of unexpected radiation exposure increased, and the possible application of the γ-H2AX assay as a biological biomarker for correctly evaluating exposure dose has also been studied [18–20]. However, the assessment of radiation dose by the γ-H2AX assay still has problems in case of a large-scale disaster like the FNPP accident because current method does not allow us to monitor the DNA DSB level by γ-H2AX assay on site, such as at a radiation accident site. On March 11, 2011, the FNPP accident occurred, and human beings faced an unexpected radiation accident. To understand biological effects of radiation exposure due to the accident, we measured the DNA DSB level based on the γ-H2AX assay in biospecimens obtained from farm animals living in the ex-evacuation zone set within a 20-km radius from FNPP [21]. In this review, we describe the first results of DNA damage monitoring in the cattle leashed in the ex-evacuation zone and the current situation of radiation biodosimeters.

12.2 Current Status of Biomarkers for Radiation Dose Assessment

The cellular response after irradiation is considered to correlate with radiation dose; the higher the dose is, the greater the biological effect is. In other words, to correctly understand the cellular response after radiation exposure and to predict the biological influence of radiation, biomarkers for accurate estimation of radiation dose are needed. Carcinogenic risk is one of the most concerned adverse effects of radiation exposure. Since radiation-induced DNA damage is one of the causes of cancer, it is

meaningful to know how much DNA damage is induced by radiation. Currently, a method of detecting chromosomal aberrations is generally used as an evaluation index of radiation exposure risk (see Chap. 20) [22]. Among chromosomal aberrations, the background level of dicentric chromosome (DC) is almost none, and there are almost no individual differences [22, 23], so the dicentric chromosome assay (DCA) is a standard protocol of the International Organization for Standardization (ISO) as a biodosimeter that can clearly evaluate the effect of radiation exposure [24]. However, in order to prepare metaphase spreads from the collected blood sample, it is problematic that in addition to the necessity of inducing blood leukocytes division and a culture period of several days, dose can be evaluated only if it is in a relatively high-dose range [22, 23]. In addition, although many automated analysis software for detecting chromosomal aberrations including DCs are currently being developed, experienced persons are necessary for final judgment [22, 23, 25]. As the assay is labor-intensive and time-consuming, the DCA is not adequate as a dose evaluation method in large-scale exposure but as an exposure dose evaluation method for a person who is at risk of high-dose radiation exposure at the time of emergency [26–28]. On the other hand, detection of the DNA DSB level by γ-H2AX does not require cell culture [29]. Detection of DNA DSBs by γ-H2AX in peripheral blood lymphocytes (PBLs) derived from patients who have received CT scans or CT angiography has been performed, which has shown the correlation of the DNA DSB levels with exposure doses in the low-dose (LD) range of 1 mGy [29–33]. In addition, dose evaluation experiments after whole-body radiation exposure using γ-H2AX have been performed using animal models [18, 20]. Bonner's group reported the monitoring of DNA DSBs in PBLs by the γ-H2AX assay of *rhesus macaque* after whole-body irradiation of 0, 1, 3.5, 6.5, and 8.5 Gy [18]. Correlations of the dose-dependent linear DNA DSB levels were detected in all dose ranges up to 4 days after irradiation [18]. Recently, they reported the result of analysis of the DNA DSB level in PBLs after whole-body irradiation using γ-H2AX using swine model, and similarly to the previous report, linear relationship between radiation dose and γ-H2AX level was found 3 days after irradiation [20]. These data demonstrate that it is possible to estimate the exposure dose by DNA DSBs monitoring using the γ-H2AX assay. It should also be emphasized that the γ-H2AX assay is not limited by animal species [5]. The amino acid sequence of H2AX and the phosphorylation of serine residues at the time of DNA DSB induction are highly conserved in almost all eukaryotes.

12.3 Detection of DNA DSBs In Vivo in Farm Animals by γ-H2AX

After the occurrence of the FNPP accident, the monitoring of radionuclides such as cesium-134 (^{134}Cs) and ^{137}Cs has been carried out from a relatively early stage [34–40]. In contrast, dose evaluation for the assessment of biological impacts to the ecosystem was not carried out comprehensively. As mentioned above, several

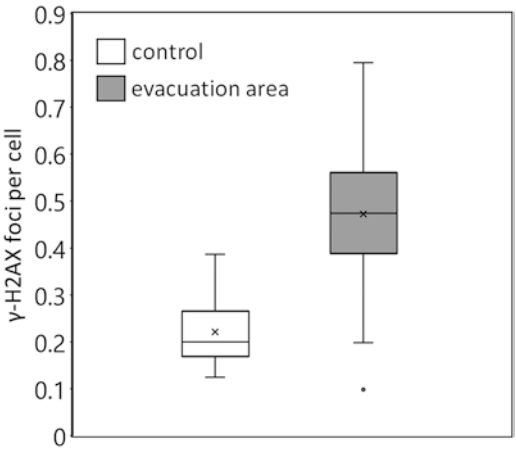

Fig. 12.1 γ-H2AX foci per peripheral blood lymphocytes from cattle living in control area and the ex-evacuation zone. The box plot of γ-H2AX foci per cell was shown. (Data modified from Ref. [21])

papers have been published, reporting that the radiation exposure dose assessment by the γ-H2AX assay can be conveniently performed for a wide range of animal species. Therefore, the γ-H2AX assay was performed on biospecimens derived from cattle in the ex-evacuation zone, and we monitored DNA damage level in vivo [21]. Briefly, peripheral blood samples obtained from cattle euthanized on site were shipped on ice conditions, and lymphocyte separation and detection of γ-H2AX were performed according to previously reported methods [6, 7, 21]. The γ-H2AX assay can be performed with the same antibody and the same experimental conditions even if the animal species is different. γ-H2AX detection was carried out in bovine cells under the same conditions as human cells [21]. As a result, a significantly higher level of γ-H2AX was detected compared to the control cohorts not affected by the FNPP accident, indicating that DNA DSBs were strongly induced in the cattle in the ex-evacuation zone (Fig. 12.1). Since there is a correlation between exposure dose and the DNA DSB level in vivo, the exposure dose was calculated based on the detected γ-H2AX level, and it corresponds to the radiation exposure equivalent to 20 mGy [21]. However, there was no correlation between estimated exposure dose and γ-H2AX level that is presumed from the radioactive Cs concentration remaining in the body of the same individual and/or ambient dose in the area animals were captured [21, 41]. It is the most important factor that DNA damage is repaired with time. Generally, after irradiation, the level of γ-H2AX reaches the maximum level from 30 min to 1 h after irradiation and decreases with DNA damage repair [5, 20, 42], which is restored to the level before irradiation within 24 h at the cell level [20, 42]. In other words, DNA double-strand breaks that occurred more than 72 hours ago have already been repaired and are not detectable by the γ-H2AX assay. Interestingly, as shown in Fig. 12.2, cells with many foci and cells with few foci are mixed. Although the γ-H2AX focus decreases with DNA damage repair, its rate of decrease is almost uniform, and γ-H2AX focus number shows the Poisson distribution, and there is no extreme heterogeneity [7, 20]. Repair process detected by disappearance of γ-H2AX foci occurs homogeneously if DNA DSB

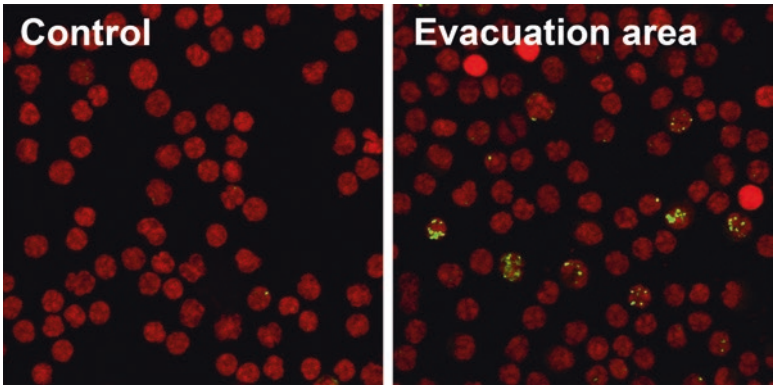

Fig. 12.2 Representative images of γ-H2AX immunostaining of peripheral blood lymphocytes from cattle living in control area and ex-evacuation zone. Green, γ-H2AX; red, DNA stained by propidium iodide

occurs simultaneously to all cells as in acute radiation exposure, but in the case of chronic exposure, DNA damage induction and damage repair might occur chronically and partly. Although the minimal DNA DSB level detected in our study is at the average about 20 mGy, more than 80% of the total cells were γ-H2AX negative, animals were exposed to chronic low-dose-rate (LDR) radiation, and the DNA DSBs induced more than 72 h before analysis could not be detected; the actual exposure dose might be higher. In fact, the γ-H2AX average value of only γ-H2AX focus positive cells was around 2.8 foci per cell (fpc), which was equivalent to radiation exposure to about 200 mGy [21]. In this study, we monitored the DNA DSB level in cattle in the ex-evacuation zone using the γ-H2AX assay and detected a high level of DNA DSBs compared to control animals [21]. Although it is difficult to evaluate accumulated dose of chronic radiation exposure by the γ-H2AX assay, our data first show biological effects of very complex radiation exposure environment such as internal and external chronic LDR exposure directly.

12.4 Perspective for Future Biodosimetry

In our previous study, to understand biological effects of the FNPP accident, we monitored DNA damage level in farm animals that were unleashed within the ex-evacuation zone of the FNPP accident and reported that higher levels of DNA DSBs were induced compared with non-affected control [21]. This indicates that DNA DSB induction by chronic LDR radiation exposure might be detected with γ-H2AX. In recent years, it has been reported that DNA DSB levels in inhabitants of high natural background radiation (HNBR) area can be detected by the γ-H2AX assay [43–45]. Although there was no significant difference, PBL derived from the HNBR population had trend to higher DNA DSB levels compared to PBLs derived

from residents in other areas. Interestingly, DNA damage repair capacity in PBLs derived from the HNBR population was elevated, suggesting the existence of adaptive response by chronic LDR radiation [43, 45]. Taken together, monitoring of DNA DSBs by γ-H2AX may not be suitable for the assessment of cumulative dose of chronic LDR radiation, but is very valuable for evaluating biological effects of chronic LDR radiation exposure.

Research on biological effects by radiation exposure has been conducted based on experiments using animal models and epidemiological investigations of atomic bomb survivors of Hiroshima and Nagasaki (Hibakusha) [22, 46, 47]. Although it is evident in middle to high dose that the DNA damage level and the risk of carcinogenesis increase along with the increase of radiation dose, there is still no clear answer on biological effects below 100 mGy. Long-term and large-scale research is necessary to understand the biological effect of LDR radiation because it takes time until the effect becomes evident. Recently, the importance of tissue microenvironment change due to radiation exposure in radiation-induced cancer has been pointed out [48]. For example, the rate of radiation-induced cell differentiation is several hundred to several thousand times higher than the frequency of radiation-induced mutations [49]. This indicates that radiation dose-dependent DNA damage might not merely induce tumorigenesis of cells but tissue microenvironmental changes. In fact, there are considerable evidences showing that radiation-induced inflammation and epigenetic changes promote carcinogenesis [48, 50, 51]. Thus, to understand the biological effect of radiation exposure, it is essential not merely to investigate the number of DNA damage and the mutation frequency but also to analyze microenvironmental changes in organisms for a long time using various biological markers. We need to continue the long-term and comprehensive analysis of the ecosystem around FNPP to understand correctly the effect of persistent LDR radiation.

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