Induction of somatic mutations by low-dose X-rays: the challenge in recognizing radiation-induced events

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ABSTRACT

It is difficult to distinguish radiation-induced events from spontaneous events during induction of stochastic effects, especially in the case of low-dose or low-dose-rate exposures. By using a hypersensitive system for detecting somatic mutations at the HPRT1 locus, we investigated the frequency and spectrum of mutations induced by low-dose X-rays. The mutant frequencies induced by doses of >0.15 Gy were statistically significant when compared with the spontaneous frequency, and a clear dose dependency was also observed for mutant frequencies at doses of >0.15 Gy. In contrast, mutant frequencies at doses of <0.1 Gy occurred at non-significant levels. The mutation spectrum in HPRT-deficient mutants revealed that the type of mutations induced by low-dose exposures was similar to that seen in spontaneous mutants. An apparent change in mutation type was observed for mutants induced by doses of >0.2 Gy. Our observations suggest that there could be a critical dose for mutation induction at between 0.1 Gy and 0.2 Gy, where mutagenic events are induced by multiple DNA double-strand breaks (DSBs). These observations also suggest that low-dose radiation delivered at doses of <0.1 Gy may not result in DSB-induced mutations but may enhance spontaneous mutagenesis events.

Keywords: mutations; low dose; HPRT

INTRODUCTION

The frequency of stochastic effects induced by radiation shows a wide scatter. This scatter makes it difficult to distinguish radiation-induced events from spontaneous events, especially in the presence of low-dose or low-dose-rate exposures. Therefore, the dose limit for radiation safety recommended by the International Commission on Radiation Protection (ICRP) is based on the linear no-threshold (LNT) model, a model in which ionizing radiation always causes detrimental effects [1]. This hypothesis argues that there is an elevation in the frequency of stochastic or random effects, regardless of the exposure dose, even at doses of <100 mSv.

In Japan, the health effects of low-dose radiation are a major public concern because of the disastrous accident at the Fukushima Dai-ichi Nuclear Power Plant that occurred in 2011. Concerns about low-dose radiation exposures are also relevant to medical radiation exposures. Several publications report elevation in the frequency of carcinogenesis induced by low-dose medical radiation or by high background radiation [2, 3], although a number of concerns have been raised concerning the analysis of these authors [4–6]. Clear experimental data is needed in order to address these concerns about the health effects of low-dose radiation exposures.

There are several hypersensitive detection procedures that are able to detect DNA damage induced by radiation, even at doses of a few milli-Grey (mGy) to up to ~10 mGy [7–11]. Among these methods, counting the number of radiation-induced foci (RIF) formed with gamma-H2AX or 53BP1 is considered to be the most
sensitive. Experimental work using these markers clearly indicates that radiation can induce DNA damage, regardless of the dose. However, DNA damage measured as the number of RIF does not correlate with cellular survival, especially in the low-dose region [11]. This is due to the presence of a cellular DNA repair system that acts to maintain genome stability. Thus, the most useful assessment of the stochastic effects of radiation must be able to detect any genetic alterations or epigenetic changes that remain following DNA repair, and which may lead to carcinogenesis.

Carcinogenic changes induced by radiation are thought to fit within the LNT model; however, the model is still hypothetical, due to the scatter in the frequency of events in the low-dose region. Only a limited number of experimental systems are available for analysis of the biological effects of low-dose or low-dose-rate radiation [12, 13], and at the present, there are no good experimental systems that can clearly show whether the LNT model is appropriate and valid.

In order to investigate the mutagenic effects of low-dose or low-dose-rate radiation, we developed a hypersensitive system for detecting somatic mutations at the HPRT1 (hypoxanthine-guanine phosphoribosyltransferase) locus [14]. This system utilizes a hamster cell line that was originally HPRT-deficient, but which has normal HPRT activity due to the transfer of an entire human X-chromosome into the cell line. In this cell line, cell viability is independent of any mutator phenotype. Indeed, this cell system was found to exhibit a more than 50-fold increase in the radiation-induced mutant frequency when compared with conventional assay systems [14, 18].

In the present study, we analyzed mutant frequencies and the mutation spectrum induced by low doses of X-rays. We found that there might be a critical dose affecting the type of the detected mutations, where spontaneous mutagenic events transition to radiation-type events.

**MATERIALS AND METHODS**

**Cells and irradiation**

GM06318–10 cells [14] were used in this study. The GM06318–10 cell line is a subcloned hamster cell line that carries a human X-chromosome and is hypersensitive for mutation induction. Cells were cultured in D-MEM (GIBCO, Thermo-Fischer) supplemented with 8% fetal bovine serum (HyClone) and 25 μg/ml gentamycin sulfate (SIGMA). Confluent G0/G1 cells were irradiated with 70 kVp X-rays (5 mA) using a Soft X-ray generator (OM-B205, OHMiC, Japan) at a dose rate of 0.46 Gy/min.

**Mutation assays**

HPRT mutation assays using GM06318–10 cells were performed as described previously [14, 18]. Immediately after X-irradiation, the surviving fraction was determined by using a portion of the cells; the rest of the cells (more than 1 × 10^6 cells) were divided into four dishes and then cultured for 9 days to allow the expression of mutant phenotypes. The cells on each dish were trypsinized and inoculated into medium containing 5 μg/ml of 6-thioguanine (6-TG, Wako) at a density of 1 × 10^4 cells per 100-mm dish (at least eight dishes were used). After 14 days of incubation, the cells were fixed with ethanol and stained with a Giemsa solution (Merck). The induced mutant frequency was calculated from the number of 6-TG–resistant colonies, as previously described [18]. At least 15 independent experiments were performed.

**Analysis of mutation spectra**

Total genomic DNA was extracted from each mutant clone, and a single independent colony was subcloned from each 6-TG dish. The existence of DXS markers on the human X-chromosome was analyzed with PCR, as previously described [14, 18]. Briefly, the primer sets used in this study were DXS86 (5′-CAATATTTCCTCCTCTGACAC, 5′-AGTTGAAAATGAAGATAAGGA), DXS1194 (5′-CACCTCTC- GCCCTCTCTCTCTATG-3′, 5′-TGGAAA-AGGACA-TACAGAT G-3′), and DXS1048 (5′-TTGGGT-GTACATTGT-CCAC- TTAT-3′, 5′-AAAAATTGTGAGATGACT-TTCG-3′). Genomic DNA (∼250 ng) was added to the mixture (15 μl) containing 1 unit of ExTaq polymerase (TaKaRa), 0.2 mM dNTPs, and reaction buffer supplied with the polymerase. The reactions were heated to 95°C for 2 min and 30 cycles of DNA denaturation (95°C, 40 s), annealing (58°C, 40 s) and DNA polymerization (72°C, 1 min). The PCR products were analyzed with 1% agarose gel electrophoresis.

**Statistical analysis**

Experimental data obtained from at least 15 independent experiments were used for statistical analysis. Each data point is represented as the mean ± SD. Statistical analysis was performed using Student’s t-test, and a value of P < 0.05 was considered statistically significant.

**RESULTS**

**Induction of HPRT-deficient mutants by low-dose X-rays**

Previously, we reported that the cell line and methods used in this study could be used to detect a significant increase in mutation frequencies after an exposure of 0.1 Gy from an Fe ion beam (290 MeV/n) [18]. Because the Fe ion beam consists of high-LET radiation, we attempted to find the lowest dose that could produce a significant elevation in mutation frequencies using low-LET radiation. In order to compare mutant frequencies with long-term low-dose-rate exposures, cells were cultured until they became confluent, and the confluent G0/G1 population was irradiated with 70 kVp X-rays. Figure 1 shows the results obtained from at least 15 independent experiments. A significant increase in mutant frequencies was detected for doses of >0.15 Gy. However, due to a slight increase in the spontaneous mutant frequency, we failed to detect statistical differences between the spontaneous frequency and the radiation-induced mutant frequency at doses of <0.1 Gy. It should be noted that the induced mutant frequency at 1 Gy (19.0 ± 6.1 per 10^4 survivors) was similar to those induced by 1 Gy of tritium beta-rays (22.0 ± 4.0 per 10^4 survivors) [18], suggesting that 70 kVp X-rays can induce mutations with an efficiency similar to other types of radiation.
Mutation spectrum analysis revealed two types of mutagenic events

Next, the mutation spectrum was analyzed using PCR for the STS markers DXS1048 (Xp11.22), DXS1194 (Xq11.12) and DXS86 (Xq26). It was found that >95% of 6-TG-resistant mutants had lost the DXS86 (Xq26) locus that is adjacent to HPRT1 (Table 1). The approximate deletion size was analyzed by using DXS1048 and DXS1194. In spontaneously induced mutants, ~40% of the mutants had lost both of the DXS markers, and this may have been due to the instability of the human X-chromosome in rodent cells. The percentage of double negative (–/-) mutants showed a slight decrease in a dose-dependent manner (Table 2). In contrast, the percentage of the double positive (+/+)-type of mutants increased significantly when cells were irradiated with 1.0 Gy.

In order to clarify the dose-dependent change in the mutation spectrum, we calculated the frequency of each type of mutation by using the average value for the gross mutant frequency and the percentage for the induction of each type of mutation. Figure 2 shows the dose dependence for the induction of each type of mutation. It can be seen that only the frequency of the double positive (+/+)-type mutation clearly increased at doses of >0.2 Gy, and that the frequency of the other types of mutations did not change, even though the radiation dose increased. The slope of the double positive type mutations correlated well with the increase in the net mutant frequency, suggesting that radiation-induced mutations in this cell line are likely to be mutations with limited or small deletions. This is a unique property of this hypersensitive system. In addition, the DXS1194 negative (–/+)-type mutations also showed a slight increase when the dose was >0.2 Gy.

**DISCUSSION**

It is clear that ionizing radiation induces DSBs, regardless of the dose. Many publications have described experimental systems that can detect DSBs induced by radiation, even when the doses are as low as several milli-Gray [7–11]. From these published results, one could imagine that radiation can induce genetic alterations even at a dose in the neighborhood of several milli-Gray. However, cells have multiple DNA repair systems, and almost all of the DSBs induced by low-dose radiation can be repaired by these repair pathways [19, 20]. As a result, the time-course for DSB induction that has been measured with methods such as gamma-H2AX foci formation does not correlate with cell viability [11], and possibly may not correlate with the frequency of genetic alterations. Therefore, the most critical analysis of the biological effects of low-dose radiation should examine the possibility of genetic alterations occurring due to errors in DNA damage repair.

In the present study, we analyzed the induction of HPRT-deficient mutations by low-dose X-rays in a hypersensitive system. This system allowed us to detect a significant increase in mutant frequencies at doses of >0.15 Gy, although we failed to see any differences between the controls with spontaneous mutations and the irradiated population at doses of <0.1 Gy in either the mutant frequency or mutation spectrum.

Mutation spectrum analysis revealed that the major mutations seen among the spontaneous mutations in this cell line were large deletions, where both the DXS1048 and DXS1194 markers were lost. This may be due to the instability of the human X-chromosome in rodent cells [15–17]. The mutant frequency with double-negative type mutations did not change, even when cells were irradiated (Fig. 2); as a result, the percentage of double-negative type mutations decreased slightly, in a dose-dependent manner (Table 2). However, a clear, dose-dependent increase was observed for mutations that maintained both of the DXS markers (a double-positive type), suggesting that DSB induction by radiation contributed to limiting the size of the deletions. Although a further detailed analysis of the STS markers on the human X-chromosome is necessary, we can propose a model for mutation induction in GM06318–10 cells. Under spontaneous conditions, where only a few or no DSBs are induced on the human X-chromosome, a major mutation event results from the instability of the human X-chromosome in rodent cells (Fig. 3b). In contrast, when cells are irradiated, DSBs are generated on the human X-chromosome in a dose-dependent manner. These DSBs on the X-chromosome may induce partial-deletion type mutations and result in a small deletion (Fig. 3c). Differences in the nature of the mutations, spontaneous and DSB-induced mutations, may allow us to distinguish radiation-induced events from spontaneous events.

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**Fig. 1.** X-ray dose dependence of induced mutant frequencies. Each point represents a mean ± SD obtained from at least 15 independent experiments. Asterisks (*) indicate $P < 0.05$ and double asterisks (**) indicate $P < 0.01$ versus unirradiated controls.

| Table 1. Deletion analysis of DXS86 that locates adjacent HPRT1 locus on human X-chromosome |
|-----------------------------------------------|
| Treatment               | Number of mutants analyzed | DXS86 negative Number of clones (%) |
|-------------------------|-----------------------------|-----------------------------------|
| Spontaneous             | 104                         | 100 (95.9)                        |
| X-rays (0.2 Gy)         | 110                         | 90 (81.8)                         |
Radiation-induced mutations (the double-positive type) start increasing when the dose exceeds 0.15 Gy. Because DSBs in genomic DNA can be generated, even when doses are below 0.1 Gy, this proposed model suggests that a small number of DSBs induced by low-dose radiation may not cause genetic alterations. Induction of radiation-type mutations requires DSB induction on the long arm of the human X-chromosome, where the \textit{HPRT1} locus is found. It was reported that 1 Gy of low-LET radiation can generate ~40 DSBs in human genomic DNA in a nucleus \cite{21}. Based on the size of the long arm of the human X-chromosome (98 Mb of a total of 158 Mb) in a Chinese hamster genome (2.4 Gb) \cite{22}, the percentage of DNA in the long arm of the human X-chromosome in GM06318–10 cells is ~3.8%. This ratio indicates that the number of induced DSBs on the long arm of the human X-chromosome is ~1.5 (40 \times 0.038) per Gray. Combining this calculation with our results, an average of 0.2 DSB on the long arm of the X-chromosome (at a dose of 0.15 Gy) might be required for a significant increase in radiation-type mutagenic events.

A slight increase in DXS1194 negative mutants was found in the irradiated population, and double-negative mutants were also found in the irradiated population, although the frequency decreased (Fig. 2). Thus, it is clear that spontaneous-type events occur even when cells are irradiated. However, especially with very low-dose irradiation, there are two possible mechanisms that could account for the induction of spontaneous-type mutants by radiation. First, low-dose radiation might simply stimulate the same type of mutagenic events that occur spontaneously. Second, some mutations induced by low-dose radiation mimic spontaneous mutations, but the fine structure of the deletions might be different due to the presence of additional DSBs. In the latter case, further higher resolution studies of the DXS markers on the short arm of the human X-chromosome might reveal fine differences in the deleted regions of the X-chromosome between spontaneously occurring DXS1194 negative and radiation-induced DXS1194 negative mutants (Fig. 3b and c).

Russell and Hunsicker (2012) analyzed the historical data for radiation-induced mouse-specific locus mutations and found that only the ‘large lesion’ (large deletion) events were dependent on the dose rate; they proposed a critical dose rate of ~10 mGy/min (0.8 R/min) \cite{23}. They also reported that the large lesions were radiation dose dependent, whereas ‘other lesions (events excluding large lesions)’ did not depend on either the radiation dose or the dose rate. In addition, there is a report showing that low-dose gamma-irradiation (>0.2 Gy) increases the Hprt-deficient mutant

**Table 2. Mutation spectra analysis of human X chromosome in 6-thioguanine–resistant mutants induced by X-rays**

| Dose (Gy) | Number of mutant clones analyzed | Double positive (+/+) | 1194 negative (+/-) | Double negative (-/-) | 1048 negative (-/+?) |
|-----------|-------------------------------|-----------------------|---------------------|----------------------|----------------------|
|           |                               | Number of clones (%)  | Number of clones (%)| Number of clones (%) | Number of clones (%) |
| Spontaneous | 114                           | 51 (44.7)             | 11 (9.6)            | 46 (40.4)            | 6 (5.3)              |
| 0.05      | 66                            | 35 (53.0)             | 9 (13.6)            | 21 (31.8)            | 1 (1.5)              |
| 0.1       | 114                           | 41 (36.0)             | 17 (14.9)           | 51 (44.7)            | 5 (4.4)              |
| 0.15      | 77                            | 36 (46.8)             | 12 (15.6)           | 27 (35.1)            | 2 (2.6)              |
| 0.2       | 114                           | 52 (45.6)             | 8 (7.0)             | 40 (35.1)            | 14 (12.3)            |
| 0.5       | 80                            | 34 (42.5)             | 19 (23.8)           | 24 (30.0)            | 3 (3.8)              |
| 1.0       | 57                            | 36 (63.2)             | 10 (17.5)           | 8 (14.0)             | 3 (5.3)              |

Chromosome location of \textit{hHPRT1} gene is Xq26.

**Fig. 2. Dose dependence of the mutation spectrum.** Mutants are classified into DXS1048 negative, DXS1194 negative, double negative and double positive, as shown in Table 2. The contribution of the mutation frequency for each type of mutation was calculated by using the average gross mutation frequency and the ratio of each type of mutant. The dotted line represents the dose dependence of the induced mutation frequency that was shown in Fig. 1.
with doses <1 Gy. Our results revealed a switching or critical dose of ~0.15 Gy, where the radiation-type events became apparent.

Previous studies of tritium beta-rays delivered at a low dose rate showed that mutagenic events induced by <0.2 Gy were the same as spontaneous events if the dose rate was <8 mGy/h, although a significant increase in the mutant frequency was observed at 0.2 Gy [18]. Consideration of these results suggests that a critical factor determining the number of radiation-type mutagenic events could be the number of DSBs on a chromosome per unit time. This hypothesis is supported by several publications that show the absence of a DSB response in cells irradiated with very low doses from ~1 mGy to ~10 mGy [25, 26].

Low-dose radiation exposure is a major public concern in Japan because of the unexpected public exposures that resulted from the Fukushima Dai-ichi Nuclear Power Plant (Fukushima Dai-ichi NPP) accident as a consequence of the great Tsunami in March 2011. In addition to external low-dose exposures of around several milli-Sieverts that occurred because of the accident [27, 28], tritium contamination also became a public concern because of the increasing amounts of contaminated water accumulating at the site of the Fukushima Dai-ichi NPP. Tritium at the Fukushima Dai-ichi NPP contaminated the water with HTO, and the concentration of tritium in the contaminated water is 0.5–5 kBq/ml, depending on the time of collection [29]. These concentrations of HTO can result in internal radiation exposures with a dose rate of 12–120 μGy/day when the water is orally consumed [30]. Yamamoto et al. (1998) reported the possibility of a dose-rate threshold based on their observation that the frequency of thymic lymphoma remained at nearly control levels when mice were orally administrated tritiated water throughout their lives at a dose rate of <12 mGy/day [30]. Our preliminary observations using this hypersensitive system also showed a significant decrease in mutation frequencies for dose rates lower than 10 mGy/day (unpublished).

Although our hypersensitive system can detect mutagenic effects at a 50–100-fold higher frequency than conventional systems [14], the slightly higher frequency of spontaneous mutations obscures any radiation-induced events at a dose of ~0.1 Gy. Establishing an improved system, in which the spontaneous mutation level is suppressed, may enable us to make a clearer determination of whether or not radiation doses of <0.1 Gy affect mutation frequencies, and to provide answers for public concerns about low-dose exposures.

In conclusion, the present results suggest that mutagenic events after exposure to low-dose radiation at levels of <0.1 Gy could occur at the same frequency as spontaneous events. In other words, low-dose radiation could merely enhance spontaneous mutagenic events, even when it causes a slight increase in mutation frequency. Alterations in the quality of mutagenic events in the GM06318–10 cell line might be dependent on the number of DSBs on the human X-chromosome, and on the critical dose, where spontaneous mutagenic events transition to radiation-type events. This transition could occur at between 0.1 Gy and 0.2 Gy in the case of acute exposures. Improving this experimental system to make it more sensitive and reducing the background rate may reveal more details of the mechanism involved in radiation-induced events leading to mutagenesis, as well as the relationship between mutagenesis and radiation-induced carcinogenesis.
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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest associated with this work.

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