Dose-rate-effects and dose and dose-rate effectiveness factor (DDREF) on frequencies of chromosome aberrations in splenic lymphocytes from mice continuously exposed to low-dose-rate gamma-radiation

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Abstract

Dose-rate effects on chromosome aberrations in the low-dose-rate range have not been evaluated. The incidences of chromosome aberrations were analyzed in splenic lymphocytes from female specific pathogen-free (SPF) C3H mice that were continuously irradiated with low- or medium-dose-rate (LDR, MDR) ^{137}Cs γ rays from 56 days of age to evaluate the dose-rate effects. The dose-response relationship for the frequency of dicentric chromosome aberration at each dose rate (400 mGy/22h/day, 20 mGy/22h/day and 1 mGy/22h/day) was obtained using age-adjusted multiple linear regression analysis assuming that the relationship can be represented by a linear or a linear quadratic model and a test for difference between the irradiation group and the non-irradiated group. Values of the linear term, shown as the slope, decreased as the dose rate was reduced from 400 mGy/22h/day(18.2 mGy/h) to 1 mGy/22h/day(0.045 mGy/h), indicating a positive dose-rate-effects at the dose-rate region. The incidences of dicentric chromosome and translocation for LDR (20 mGy/day) were compared with those for HDR (890 mGy/min) irradiation at each total dose to obtain the dose and dose-rate effectiveness factor (DDREF). The DDREFs were 4.5 for dicentrics and 2.3 for translocations at a total dose of 100 mGy based on the chromosome aberration rate. These results will be useful for estimating the risk of LDR radiation exposure and radiation protection.

1. Introduction

There is increasing concern about the biological effects of low-dose (LD) and low-dose-rate (LDR) radiation exposure in relation to human health. Chromosome aberrations in lymphocytes can be as a sensitive and convenient indicator for evaluating the biological effects of radiation exposure in humans. Chronically exposed individuals such as nuclear facility workers, medical radiologists, residents in high-background radiation areas and residents of the radio-contaminated apartments in Taiwan have been shown to have slightly higher frequencies of chromosome aberrations than non-exposed control individuals (Evans *et al.* 1979, Bauchinger *et al.* 1980, Lloyd *et al.* 1980, Kumagai *et al.* 1990, Hseh *et al.* 2002), but dose and dose-rate effects have not been investigated in the LDR range. Results obtained from epidemiological studies of large human populations are subject to uncertainty because confounding factors such as smoking, food and life style have make it difficult to clarify small biological effects of LDR radiation exposure. In this context, animal experiments conducted under fully controlled condition are necessary for evaluating the biological effects of long-term LDR-irradiations as well as for risk assessment based on epidemiological studies. The Institute for Environmental Sciences (IES) in Aomori, Japan, has been investigating the biological effects of LDR-γ-irradiation using unique irradiation facilities, which make it possible to continuously irradiate mice at 20 different LDR or medium-dose-rate (MDR) level of ^{137}Cs-γ-radiation under specific pathogen-free (SPF) conditions while the mice are being reared. In accordance with the 2010 UNSCEAR report, LDR and MDR are defined as 0.1 mGy/min (132 mGy/22h/day) or less and 0.1 to 99 mGy/min, respectively. The dose rates used for LDR are 20 mGy/22h/day (0.91 mGy/h), 1 mGy/22h/day (0.045 mGy/h) and 0.05 mGy/22h/day (2.25×10^{-3} mGy/h), being approximately 20, 400 and 8,000 times higher than background external radiation, respectively (Fig.1). The endpoints employed life span, cancer incidence, non-neoplastic disease, genetic effects and oncogene alterations, chromosome aberrations, mutations and cellular and tissue responses (Tanaka *et al.* 2003, Tanaka *et al.* 2007, Tanaka *et al.* 2008, 2009, Sugihara *et al.* 2011).

It is well known that LD- or LDR-irradiation induces biological responses different from those of high-dose-rate (HDR)-irradiation, including an adaptive response, hyper-radio-sensitivity, an inverse dose-rate-effect and bystander effects. The mechanisms responsible for induction of these phenomena have not been clarified, but can be explained by changes in DNA repair, alteration of the cell cycle-dependent cellular response, activation of reactive oxygen species and so on. Only a few reports

have documented the effects of dose rate on chromosome aberration rates and mutation rates in mice exposed to MDR to LDR radiation (Russell *et al.* 1958, Russell 1965, Lyon *et al.* 1972, Tucker *et al.* 1998). No differences have been observed in frequencies of translocation or dicentric chromosome（Dic）in peripheral blood lymphocytes of mice chronically exposed of γ-radiation at dose rates of 50, 200 and 400 mGy/day for 30, 60 and 90 days (Sorensen *et al.* 2000), this dose rate range being higher than that in our LDR groups (1 mGy/day and 20 mGy/day). There was also no difference in the mutation rate of the *HPRT* gene in splenic lymphocytes of mice chronically exposed to γ-radiation at dose rates of 1000 mGy/week (140 mGy/day) and 1000 mGy/day (Lorentz *et al.* 1994). Differences in the rates on mutation in spermatogonia and oocytes have been observed between dose rates of 900 mGy/min and 0.8 mGy/min (1,152 mGy/day) in mice chronically exposed to X-ray, the mutation rates being reduced by one third, but not between dose rates of 0.8 mGy/min (1,152 mGy/day) and 0.007 mGy/min (10.1 mGy/day), which were similar to those employed in our studies (Russell 1965, Russell and Kelly 1982). Vilenchik and Knudson (2000, 2006) summarized published data on the effects of dose rate on several endpoints and found inverse dose-rate effects, where the relationship with biological effects such as mutation rate was parabolic with a minimum dose rate of around 0.28 mGy/min (corresponding to the MDR range of 200-400 mGy/22h/day at our facility). This suggests that the dose-rate range producing minimal aberration rates may represent that in which DNA repair capacity is most efficient, consistent with that for which there is an equal capacity to repair endogenous double strand (ds) DNA damage and extrinsic radiation-induced ds DNA damage. On the basis of this theory, a lower-dose-rate of less than 200-400 mGy/day might carry a higher risk. In the present study, based on an examination of chromosome aberrations in mice, we investigated whether irradiation in the MDR (400 mGy/22h/day) to LDR (1 mGy/22h/day) range is associated with positive or inverse dose-rate effects.

2. Materials and Method

2.1. Exposure of mice to LDR radiation and chromosome analysis

Continuous exposure of female SPF mice (C3H/HeN) to ^{137}Cs γ-radiation was started from the age of 8 weeks (56 days). For observations of dicentric chromosome and translocation, 4 to 13 and 3 mice, respectively were grouped for irradiation at each total dose together with age-matched, non-irradiated mice as controls. Groups of mice were

irradiated with total doses of 100 to 8000 mGy at a LDR of 20 mGy/22h/day (0.91 mGy/h; abbreviated as 20 mGy/day thereafter) for 5-400 days, and with total doses of 125 to 615 mGy at a LDR of 1 mGy/22h/day (0.045 mGy/h; abbreviated as 1 mGy/day hereafter) for 125-615 days using a ^{137}Cs γ-rays irradiation device（Fig.1）. To allow animal care, mice were removed from exposure daily between 10 and 12 a.m.. For comparison, MDR exposure to ^{137}Cs γ-rays at 400 mGy/22h/day (18.2 mGy/h; abbreviated as 400 mGy/day hereafter) was also performed to achieve total doses of 400 to 8000 mGy for 1-20 days. Seven mice were also exposed to high-dose-rate (HDR) ^{137}Cs γ-irradiation to achieve total doses of 250 to 3000 mGy at a dose rate of 890 mGy/min to obtain the dose and dose-rate effectiveness factor (DDREF). Non-irradiated control mice were kept for the same period as the irradiated mice. For observation of translocation, three mice were used for irradiation at each point of 20 mGy/day and 1 mGy/day, and also for non-exposure at the same age (controls).

The mice were sacrificed, and their spleens sterilely removed under sterile conditions. For chromosome analysis, spleen cells were isolated and cultured in RPMI 1640 medium containing LPS (10 μg/ml), ConA (3 μg/ml) and 2-ME (50 μM) under a 5 % CO_2 atmosphere with 95% humidity at 37℃. Colcemide (0.02μg/ml) was added for the last 2h of cultures to allow collection of metaphase cells. The cells were treated with a hypotonic solution of 0.075M KCl, the supernatant was removed and the cells were fixed with Carnoy's solution. For the fluorescence *in situ* hybridization (FISH) method using a centromere probe (Cambio Ltd, UK), and multiplex fluorescence *in situ* hybridization (M-FISH) (Cambio Ltd, UK), the cells were stained with fluorescent dyes. Dicentric chromosome (Dic) detected by using the centromere FISH method and translocations by the M-FISH method were observed in 500-4000 metaphases per mouse in both the irradiated and age-matched control groups.

2.2. Statistical analysis for evaluation of dose and dose-rate effects

For statistical analysis, the 95% confidence interval (CI) for yields of chromosome aberrations was estimated by multiple linear regression analysis adjusted for age-related differences. Regression coefficient values of the linear regression lines or linear quadratic regression curves were estimated by multiple linear regression analysis. These values were compared with the 95% CIs among the four dose rates. The explanatory variables were 1) Intercept at age 56 days and 0 accumulated dose, 2) Age minus 56 days, 3) Accumulated dose for the 1 mGy/day group, 4) Accumulated dose for the 20 mGy/day group, 5) Accumulated dose for the 400 mGy/day group, 6) Accumulated dose

for the 890 mGy/min. The linear quadratic regression curve model for the 5 groups were expressed as non-exposed group: y=b1+ b2xT, 1 mGy/day group: y=b1+ b2xT+ b3xD+a3xD^2, 20 mGy/day group: y=b1+ b2xT+ b4xD+a4xD^2, 400 mGy/day group: y=b1+ b2xT+ b5xD+a5xD^2, 890 mGy/min group: y=b1+ b2xT+ b6xD +a6xD^2, where y is the number of chromosome aberrations per 100 cells as the response variable, bj, j=1,…,7 and aj, j=1,…..,6 are unknown regression coefficients, D is the accumulated dose in mGy, and T =Age-56. The linear regression model was also used for the 5 groups, same as previous analysis (Tanaka *et al.* 2009). In both models, values of b1+b2xT were common among the different dose rates in Dic. We estimated the unknown regression coefficients using the weighted least squares estimator with respect to the number of observed cells using SPSS version 15 software. Furthermore, tests for differences between the irradiated groups (20 mGy/day and 1 mGy/day) or 1 mGy/day and the non-irradiated groups were performed to allow comparison of the results from the LDR groups with those for non-exposed animals.

3. Results and Discussion

3.1. Dose-response relationships of Dic and translocation frequencies

Dose-response relationships between the incidences of Dic and total accumulated doses up to 3000 mGy, 8000 mGy and 615 mGy at different dose rates of 400 mGy/day, 20 mGy/day and 1 mGy/day were obtained, respectively. The dose-response relationships of 400 mGy/day and 20 mGy/day for doses of less than 8000 mGy are shown in Fig. 2. The dose-response curves for 890 mGy/min and 400 mGy/day were linear quadratic, and those for 20 mGy/day and 1 mGy/day increased almost linearly up to 8000 mGy and 615 mGy, respectively (Fig.4a ; Fig.3a, Left). For irradiation at 400 mGy/day, the rate of increase in the frequency of chromosome aberrations decreased with accumulating of dose (Fig.2). Furthermore, translocations detected by M-FISH, increased almost linearly up to a total accumulated of dose of 8000 mGy following irradiation for about 400 days at a LDR of 20 mGy/day (Fig.4b). The incidence of translocations was approximately 4 times higher than that of Dic under the present conditions of chronic irradiation. Translocations develop in an almost equal ratio to Dic just after irradiation (Kanda and Hayata 1996), but lymphocytes harboring Dic decrease soon after irradiation because of uneven cell division.

The present findings indicate that Dic accumulates in a dose-dependent manner during continuous irradiation at 20 mGy/day. We had expected that Dic would not

increase with accumulated dose, in contrast to the case of translocation, because about half of all lymphocytes harboring Dic are eliminated in each cell division. The increase in the frequency of Dic with accumulated total dose during chronic irradiation may possibly be attributed to LD or LDR-related biological phenomena such as retardation of DNA repair capacity (Rothkamm and Löblich 2003), persistence of p53 activation and an increase of anti-apoptotic function（Sugihara *et al.* 2011）, enhanced cell growth, and so on.

The frequencies of Dic in non-exposed age-matched mice showed no correlation with increasing age. However, the frequencies of translocation increased with age beyond 600 days after birth. Similar finding in human peripheral blood lymphocytes have also been reported (Tucker and Luckinbill 2011). These results will be of importance for establishing a suitable biodosimetry method using the frequency of Dic or translocation as an indicator of occupational or accidental exposure to chronic LDR radiation.

3.2. Dose-rate effects on frequencies of Dic and translocation

The equations obtained and parameters for the regression fits were obtained by multiple regression analysis. The dose-response relationship for chromosome aberration frequencies was obtained at each dose rate using age-adjusted multiple liner regression analysis on the assumption that the relationship could be represented by a linear or linear quadratic model. Values of the linear term, shown as the slope, decreased significantly with reduction of the dose rate from 400 mGy/day to 20 mGy/day (Fig. 2). To clarify whether the dose-response relationship for Dic differed significantly between LDRs of 1 mGy/day and 20 mGy/day (Fig.3a), or whether the linear dose-response relationship at 1 mGy/day differed significantly from the spontaneous background level (Fig.3b), the equations and parameters for the multiple linear regression fits were obtained. However, the regression coefficients (b3 and b4) in the equations for Dic at doses of less than 1000 mGy were not statistically significant, because their upper and lower limits overlapped at the 95% CI. The regression coefficients (b4 and b5) in the equations for Dic at 400 mGy/day and 20 mGy/day at doses of less than 8000 mGy were statistically significant (Fig.2), but not for low dose rates of 20 mGy/day and 1 mGy/day at doses of less than 1000 mGy (Fig.3a, Left), because the results for 1 mGy/day did not fit either the linear regression or the linear quadratic regression model. We therefore tested for differences between the 20 mGy/day and 1 mGy/day groups, and between the 1 mGy/day and non-irradiated groups, and this revealed significant

inter-group differences (Fg.3a, Right; Fig.3b, Right), respectively. These results indicated that there are positive dose-rate effects on the frequencies of Dic over a 400-fold dose rate range from 1 mGy/day to 400 mGy/day. No inverse dose-rate effects were found between 400 mGy/day and 1 mGy/day. The present results imply that DNA damage to splenic lymphocytes can be repaired in mice during continuous exposure to MDR or LDR radiation. According to the classical target theory, LDR radiation allows cells enough time to repair DNA damage during irradiation and the dose-rate effects disappear at dose rates less than the limiting one. However, if bystander effects occur in some non-irradiated cells adjacent to irradiated target cells during continuous exposure to LDR, then ongoing DNA repair might occur even in adjacent cells located within the zone influenced by cell to cell communication, or by released clastogenic factors, as well as in the target cells themselves (Brenner and Sachs 2002; Prise *et al.* 2003; Nuta and Darroudi 2008). These concepts imply that dose-rate effects on chromosome aberration might occur within a much lower dose-rate range and that there might be no threshold.

The difference in the frequency of Dic between irradiation at 20 mGy/day and that at 1 mGy/day was less than that between the 1 mGy/day and non-exposed groups, but still statistically different. In contrast, the frequency of translocation observed at a dose rate of 20 mGy/day was quite similar to that at 1 mGy/day (unpublished results), despite the 20-fold dose rate difference. Similarly, a previous study revealed that chromosome aberration rates in terms of Dic plus ring chromosomes for irradiation rates of 200 mGy/day and 400 mGy/day were also quite similar, despite the 2-fold dose rate difference (Tanaka *et al.* 2009). Dose rate-dependent biological effects might be induced by chronic irradiation at MDR and LDR, although the reasons are still unclear.

3.3. Evaluation of DDREF using frequencies of Dic and translocation

Extrapolation from a high-dose to a LD range, using a reduction factor known as the DDREF, is applied for estimating the overall risk of radiation exposure at LDR. Our present results, which showed that the α coefficient for the linear regression lines obtained over a radiation dose range of 20-400 mGy/day decreased significantly with reduction of the dose rate, indicating that calculation of the DDREF based on such a dose-rate-dependent repair model is not appropriate for estimating the risk of LDR radiation exposure. Accordingly, our results did not support the formula recommended by the International Commission on Radiological Protection (ICRP) and so on, $1 + (\beta/\alpha)D$ (ICRP1991, UNSCERE 1993, BEIR 2006) for calculation of DDERF. We

obtained DDREF simply as the ratio of HDR to LDR as $(\alpha 1D+\beta D^2)/\alpha 2D$, which is equal to $(\alpha 1+\beta D)/\alpha 2$. In this calculation, the value of the Y intercept (*c*), which was b1+b2T in the present multiple regression analysis, was neglected. DDREF values for Dic were calculated for each different dose (100 mGy, 125 mGy, 250 mGy, 500 mGy and 1000 mGy) at a LDR of 20 mGy/day, compared with a HDR of 890 mGy/min using the equation (Fig.4a). We obtained a DDREF value of 4.5 for Dic at 100 mGy, and the change in DDREF was dependent on the accumulated dose (Table 1). Similarly, the DDREF value for translocation at a dose of 100 mGy was 2.3 (Fig. 4b). Since a wide range of DDREF values for Dic and translocation were obtained at each different dose, it is suggested that DDREF is dose-dependent. DDREF varied from 4.5 to 17.8 for Dic and from 2.3 to 7.0 for translocation as the accumulated doses increased from 100 to 1000 mGy (Table 1). Many experimental studies have used a much higher dose rate within the MDR range than ours (20 mGy/day) for comparison with the high-dose-rate in order to obtain DDREF (Ullrich and Storer 1979; Ullrich *et al.* 1987; Thacker 1992; UNSCEAR 1994; Lorentz *et al.* 1994; Tucker *et al.* 1998).

The ICRP 1991 has recommended a reduction factor of 2 as the DDREF for low doses of less than 0.2 Gy at dose rates of less than 0.1 Gy/h for purposes of radiation protection based on the incidence of leukemia in atomic bomb survivors exposed to low and high doses. The National Council Radiation Protection (NCRP 1980) concluded from their data on cancer incidence and life-span shortening that the range of the dose-rate effectiveness factor (DREF) varies between 2 and 10.

Our preliminary study has indicated that chronic irradiation at a lower LDR of 0.05 mGy/day was associated with lower chromosome aberration rates in terms of both Dic and translocation than those at 1 mGy/day. Therefore, DDREF in the lower dose-rate range of less than 20 mGy/day became slightly higher than 2.3 for translocation and 4.5 for Dic. Because Dic is eliminated at each cell division after irradiation due to their unique morphology involving two centromeres on one chromosome, any increase of Dic has less biological significance than translocation. Cells with translocation can persist for a longer period after irradiation, and some of them can form a clone, which may lead to neoplastic transformation, suggesting the biological significance of continuous long-term exposure at LDR. Present results on dose-rate-effects and DDREF obtained at low-dose-rate range will be useful for radiation risk assessment and radiation protection.

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Figure legends

Figure 1. Irradiation procedure for HDR (0.89 mGy/min), MDR (400 mGy/day) and LDR (20 mGy/day and 1 mGy/day) in mice. Irradiation was started at 8 weeks of age (56 days after birth). Non-irradiated mice of the same age as the irradiated mice were used as controls. The accumulated doses and irradiation periods are shown.

Figure 2. Frequencies of Dic per 100 lymphocytes from mice irradiated with MDR (400 mGy/day, □, dotted line), and LDR (20 mGy/day; ◆, solid line) groups exposed to radiation within a dose range of 8000 mGy. Each symbol indicates the value for an individual mouse. All non-exposed 60 mice with different age (56 to 671 days) were used for 0 mGy value in multiple linear regression analysis. Equations and parameters for multiple linear quadratic fits obtained were as follows: 400 mGy/day; $y = (9.69\times10^{-2}\pm8.0\times10^{-2})+(2.73\times10^{-4}\pm3.41\times10^{-4})T+ *(3.37\times10^{-3}\pm3.05\times10^{-4})D+ (-2.21\times10^{-7}\pm4.12\times10^{-8})D^2$, 20 mGy/day; $y = (9.69\times10^{-2}\pm8.0\times10^{-2}) +(2.73\times10^{-4}\pm3.41\times10^{-4})T + *(6.66\times10^{-4}\pm1.12\times10^{-4})D+ (-2.8\times10^{-8}\pm1.60\times10^{-8})D^2$. * These two linear terms (α coefficients) were significant.

Figure 3a. (Left) Frequencies of Dic per 100 lymphocytes from mice in the LDR (20 mGy/day,□, solid line), and LDR (1 mGy/day; ◆, dotted line) groups exposed to radiation within the dose range of 1000 mGy. Each symbol indicates the value for an individual mouse. X axis is the total dose (mGy) and Y axis is the Dic rate (number of Dic per 100 metaphases). All non-exposed 60 mice with different age (56 to 671 days) were used for 0 mGy value in multiple linear regression analysis. Equations and parameters for multiple linear fits obtained were as follows: 20 mGy/day; $y = (-2.82\times10^{-1}\pm1.75\times10^{-1})+(1.22\times10^{-3}\pm8.32\times10^{-4})T +*(5.04\times10^{-4}\pm7.66\times10^{-5})D$, 1 mGy/day; $y = (-2.82\times10^{-1}\pm1.75\times10^{-1})+(1.22\times10^{-3}\pm8.32\times10^{-4})T +*(6.23\times10^{-4}\pm1.07\times10^{-3})D$. * Values of two linear terms (α coefficients) were not significant.

Figure 3a. (Right) Significant difference (P =0.013) in Dic frequency between the two LDR groups of 20 mGy/day and 1 mGy/day in the 0-1000 mGy dose region. Dotted horizontal line shows a distribution from maximum to minimum value in a cell population and upper and lower lines of the box shows 75% and 25% percentiles of cell population, respectively, where thick bar in the box shows median. Distance of notch in the box shows value of 95% confidence interval. Circle being far from upper line shows outlier.

Figure 3b. (Left) Frequencies of Dic per 100 lymphocytes from mice in the LDR group (1 mGy/day, □, solid line), exposed to radiation within the dose range of 700 mGy and those in non-irradiated mice within 700 days after the start of chronic irradiation (◆, dotted line). All non-exposed 60 mice with different age (56 to 671 days) were used for 0 mGy value in multiple linear regression analysis. Each symbol indicates the value for an individual mouse. X axis is the total dose (mGy) or period (days) after start of chronic irradiation and Y axis is the Dic rate (number of Dic per 100 metaphases). All non-exposed mice with different age (56 to 671 days) were used for 0 mGy value in multiple linear regression analysis. Equations and parameters for multiple linear fits obtained were as follows: 1 mGy/day; $y = (-2.82x10^{-1}\pm1.75x10^{-1})+(1.22x10^{-3}\pm8.32x10^{-4})T +*(5.04x10^{-4}\pm7.66x10^{-5})D$, Non-irradiated; $y = (-2.82x10^{-1}\pm1.75x10^{-1})+(1.22x10^{-3}\pm8.32x10^{-4})T$, where value T equals D in these formulae. * Values of the two linear terms (α coefficients) were not significant.

Figure 3b. (Right) Significant difference (P= 0.00011) in Dic frequency between the 1 mGy/day and non-exposed groups in the 0-700 mGy dose region or 0-700 days region. Explanation of the box pot is same as Figure 3a Right.

Figure 4a Changes in the frequencies of Dic in splenic lymphocytes from mice in the HDR (890 mGy/min) (□, dotted line) and LDR (20 mGy/day) groups (◆, solid line) *X axis is the total dose (mGy) and Y axis is the Dic rate (number of Dic per 100 metaphases). Each symbol indicates the value for an individual mouse. DDREF value for Dic was obtained using with these correlations.

Figure 4b Changes in the frequencies of translocation in splenic lymphocytes from mice in the HDR (890 mGy/min) (□, dotted line) and LDR (20 mGy/day) (◆, solid line) groups. *X axis is the total dose (mGy) and Y axis is the translocation rate (number of translocation per 100 metaphases). Each symbol indicates the value for an individual mouse. DDREF value for translocation was obtained using with these correlations. Equations and parameters for multiple linear regression and multiple linear fits obtained were as follows: HDR (890 mGy/min) ; $y = 0.083+0.0071D+ 7.0 x 10^{-6}D^2$, LDR (20 mGy/day); $y = 0.083+0.0019D$, where D is the total dose (mGy) and Y is the translocation rate (number of translocation per 100 metaphases).

Table 1. DDREFs obtained from incidences of dicentric chromosome（Dic） and translocation. DDREF= incidence at HDR（890 mGy/min)/incidence at LDR (20 mGy/day)

Dose (mGy)	Dicentric (Dic)	Translocation
1000	17.8	7.0
500	10.4	4.4
200	6.0	2.9
100	4.5	2.3

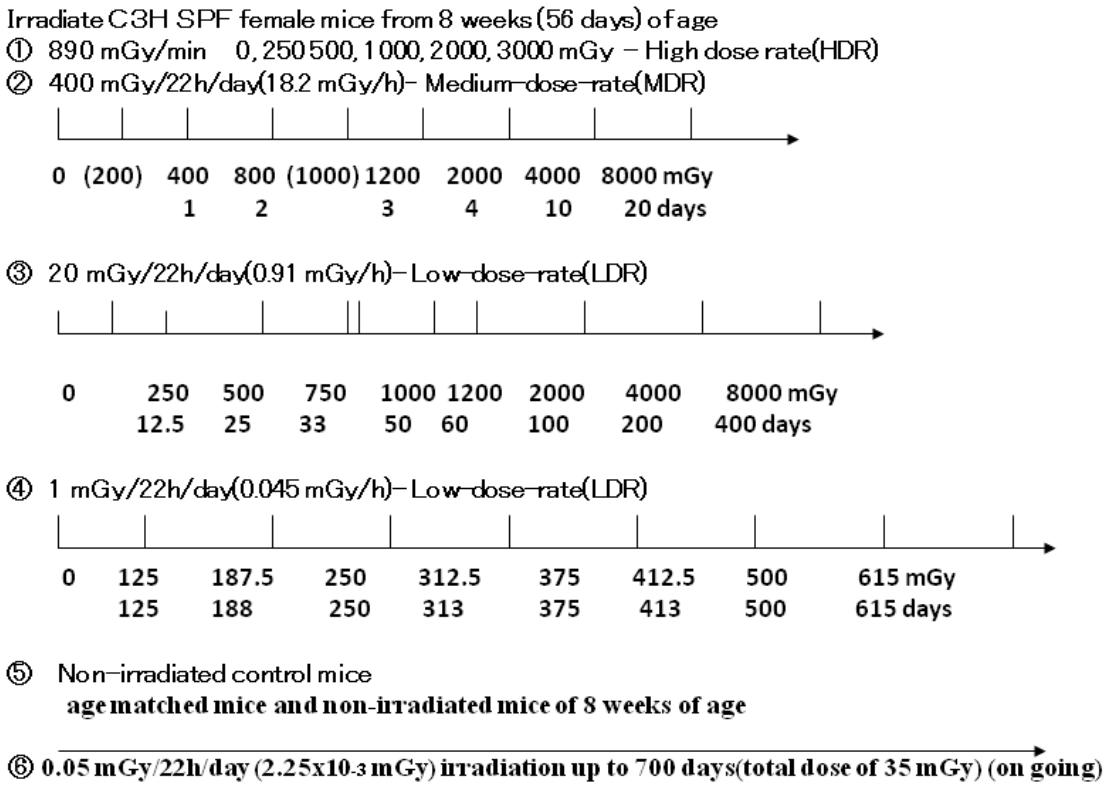

Figure 1 (figure1.TIF)

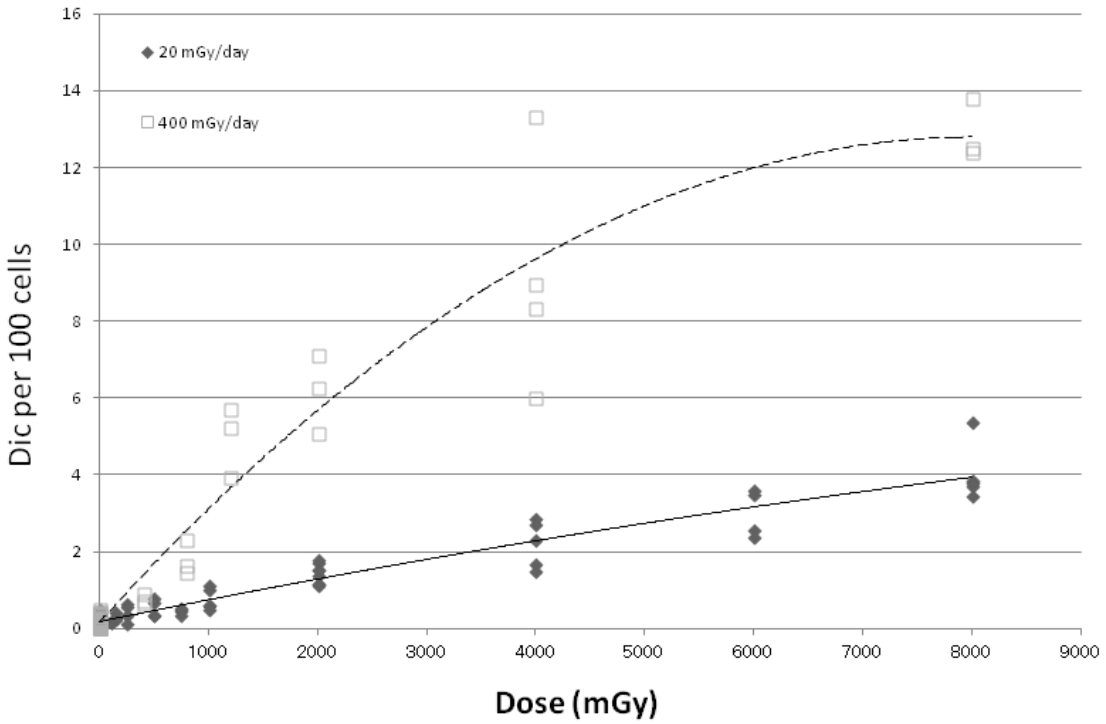

Figure 2 (figure2.TIF)

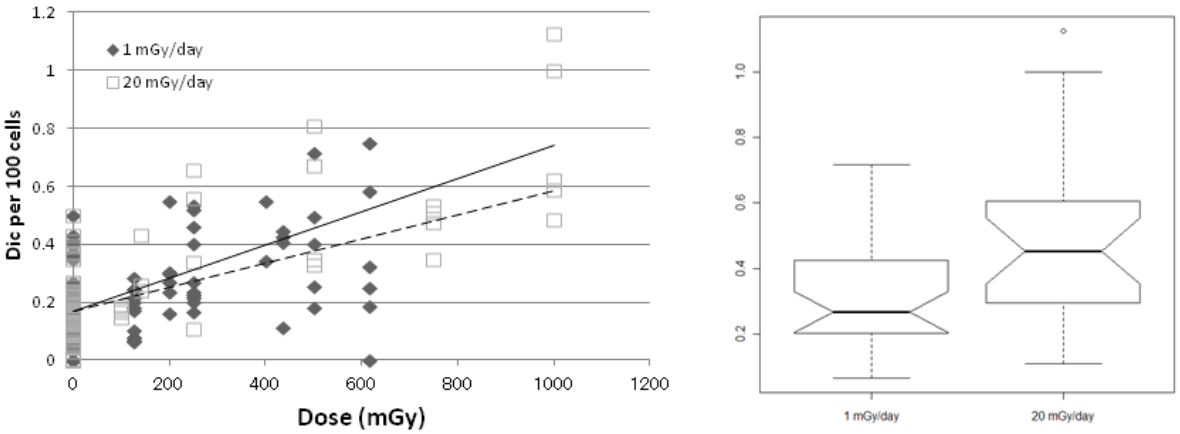

Figure 3a (figure3a.TIF)

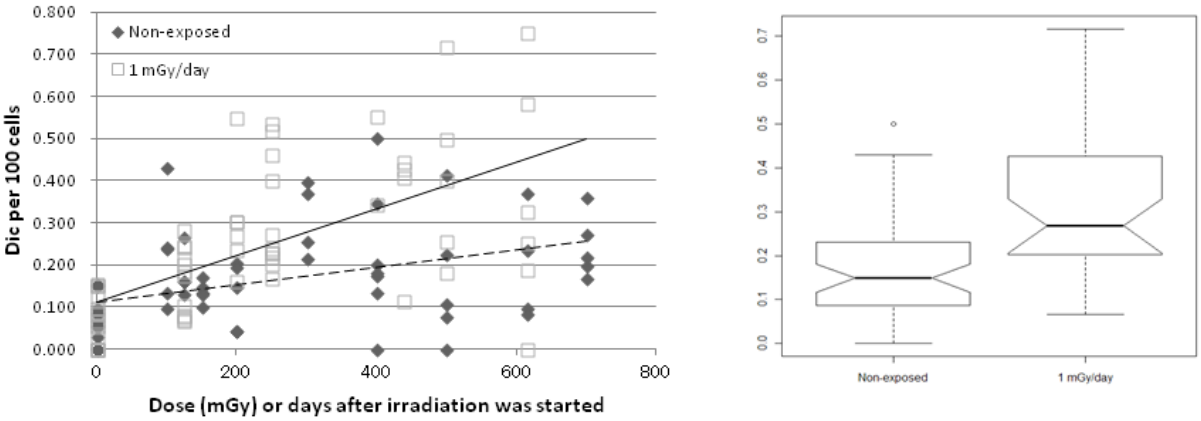

Figure 3b (figure3b.TIF)

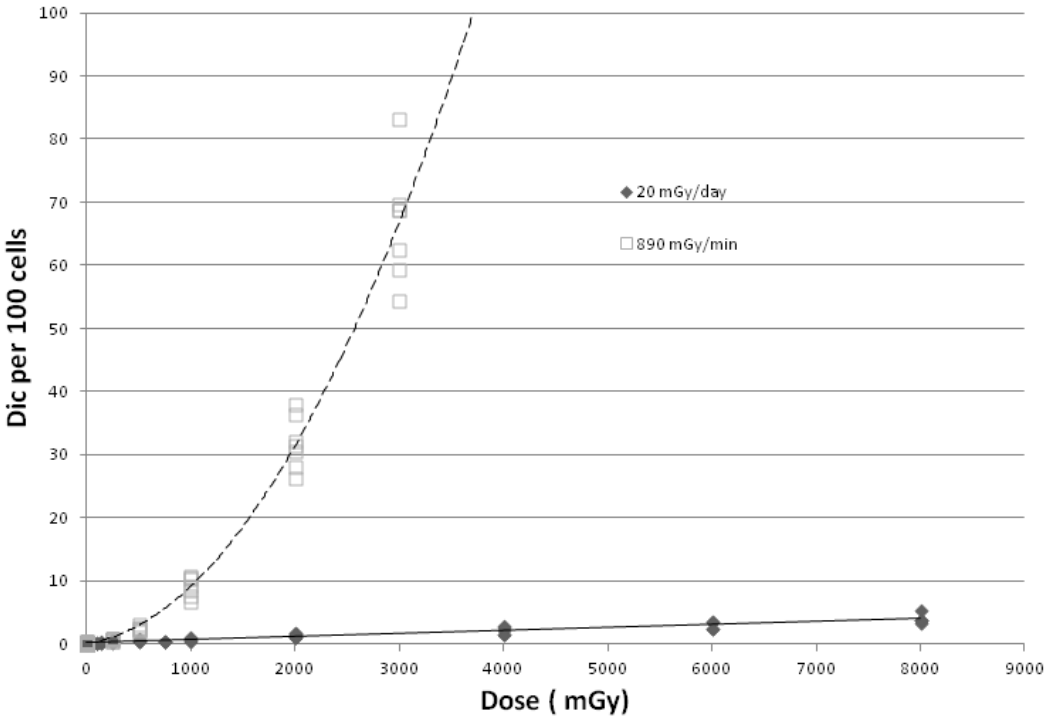

Figure 4a (figure4a.TIF)

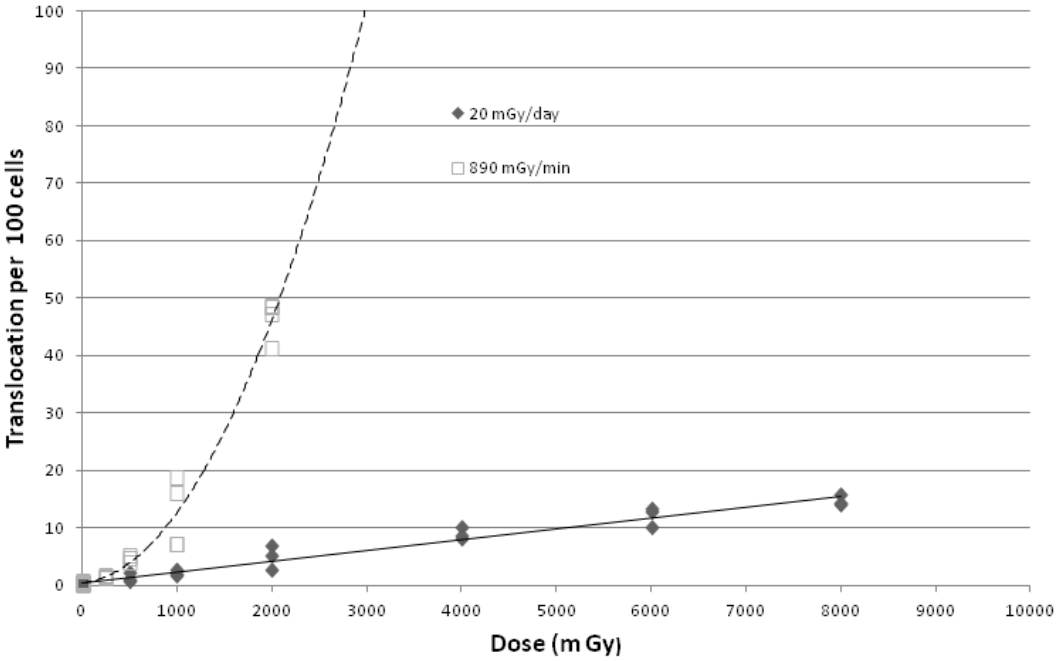

Figure 4b (figure4b.TIF)