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Chromosome aberration frequencies and chromosome instability in mice after long-term exposure to low-dose-rate γ-irradiation

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ABSTRACT

Chronological changes of chromosome aberration rates related to accumulated doses in chronically exposed humans and animals at a low-dose-rate have not been well studied. C3H female specific pathogen-free mice (8 weeks of age) were chronically irradiated. Chromosome aberration rate in mouse splenocytes after long-term exposure to low-dose-rate (LDR) γ-rays was serially determined by conventional Giemsa method. Incidence of dicentrics and centric rings increased almost linearly up to 8000 mGy following irradiation for about 400 days at a LDR of 20 mGy/day. Clear dose-rate effects were observed in the chromosome aberration frequencies between dose rates of 20 mGy/day and 200 mGy/day. Furthermore, the frequencies of complex aberrations increased as accumulated doses increased in LDR irradiation. This trend was also observed for the incidences of micronuclei and trisomies of chromosomes 5, 13 and 18 in splenocytes, detected by micronucleus assay and metaphase fluorescence *in situ* hybridization (FISH) method, respectively. Incidences of 2–4 micronuclei and trisomy increased in mouse splenocytes after irradiation of 8000 mGy at a LDR of 20 mGy/day. These complex chromosome aberrations and numerical chromosome aberrations seem to be induced indirectly after radiation exposure and thus the results indicate that continuous γ-ray irradiation for 400 days at LDR of 20 mGy/day induced chromosomal instability in mice. These results are important to evaluate the biological effects of long-term exposure to LDR radiation in humans.

1. Introduction

Medical radiologists and residents in radio-contaminated buildings in Taiwan, who have been chronically exposed, have a higher incidence of chromosome aberrations in peripheral blood lymphocytes than non-exposed individuals [1–3], however, chronological changes of chromosome aberration rates related to accumulated doses of a low-dose-rate of radiation have not been well studied in animals or humans. Moreover, residents in radio-contaminated buildings in Taiwan have been reported to have a higher rate of formation of clones containing chromosomal aberrations as compared with the non-exposed reference population [3], which suggests that chromosome instability is induced after prolonged low-dose-rate (LDR) γ-ray irradiation. Chromosome instability has been identified as a genetic instability induced by radiation after several cell divisions in cultured rodent and human cells [4,5], which is also expressed as hypoxanthine-guanine phosphoribosyltransferase (HPRT) mutations and apoptosis [6,7]. It is well known that delayed (or late-arising) chromosome aberrations are induced by acute irradiation at a high dose rate after several cell divisions in cultured human cells [8–10], and also in mice [11–13]. Radiation-induced genomic (chromosome) instability is defined as non-clonal DNA (chromosomal) damage that arises or increases in several cell generations after exposure to radiation. Genetic (chromosome) instability might be related to cancer, although the mechanism is not understood. However, little information is available regarding chromosome instability *in vivo* induced by long-term irradiation with γ-ray at LDR, although it will be important for the assessment of low-dose radiation in human health.

Cytogenetic methods, such as conventional chromosome analysis, micronucleus assay and the fluorescence *in situ* hybridization (FISH) technique have been used as suitable methods for evaluating delayed chromosome aberrations, because they can score the aberration rate at each cell level and measure its distribution among observed cells. The present study was performed in mice using ^{137}Cs-γ-rays irradiations at an LDR of 20 mGy/22 h/day (0.91 mGy/h) for several tens to hundreds of days, up to 400 days, to observe chronological changes of chromosome aberration rates related with accumulated dose, and also to observe whether chromosome instability can be induced *in vivo*. Also, chronic irradiation

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of 200 mGy/22 h/day (10 mGy/h), up to 40 days, was used for comparison. Micronucleus assay and interphase FISH analysis using centromere probes of mouse chromosomes 5, 15 and 18 were also performed to detect cells with numerical chromosome changes in splenocytes after long-term irradiation at the LDR.

2. Materials and methods

2.1. Mice

Female, 8-week-old specific pathogen-free (SPF) C3H/HeN mice (CLEA Japan, Inc. Shizuoka, Japan) were used for long- or short-term irradiation with ^{137}Cs-γ-rays irradiation starting from 8 weeks of age. They were kept in animal rooms under SPF barrier conditions at a room temperature of 23 ± 2 °C, humidity of 50 ± 10% and a 12 h light/dark cycle. These experiments were approved by the appropriate Ethics Committee of our institute.

2.2. Radiation exposure

At least 5–7 mice were grouped for irradiation with each total dose together with age-matched, non-irradiated mice as controls. Groups of mice were irradiated with total doses of 100, 140, 250, 500, 750, 800, 1000, 2000, 4000, 6000 and 8000 mGy at a dose rate of 20 mGy/22 h/day (0.91 mGy/h) for 5–400 days, and with total doses of 200, 400, 800, 1000, 2000, 4000 and 8000 mGy at a dose rate of 200 mGy/22 h/day (9.1 mGy/h) for 1–40 days using a ^{137}Cs γ-ray irradiation device (74 GBq and 740 GBq, respectively; Yoshizawa LA Co. Ltd., Japan). Mice were not exposed daily between 10 a.m. and 12 a.m. for animal care. The daily doses of 200 mGy/22 h/day and 20 mGy/22 h/day are designated as 200 mGy/day and 20 mGy/day, respectively, in the present paper.

2.3. Spleen cell cultures

Mice were sacrificed under ether anesthesia immediately after the cessation of irradiation, and spleens were sterilely removed. All reagents were obtained from Sigma, St Louis, MI, USA except where noted. For chromosome analysis, spleen cells were isolated and cultured in RPMI 1640 medium (Invitrogen Corp., Carlsbad, CA, USA) containing lipopolysaccharide (LPS, 10 μg/ml), concanavalin A (ConA, 3 μg/ml) and 2-mercapto-ethanol (2-ME, 50 μM) under 5% CO_2 atmosphere with 95% humidity at 37 °C. For micronucleus assay, cytochalasin B (0.5 μg/ml) was added 24 h before harvesting to obtain binucleated cells.

2.4. Conventional Giemsa staining method

The conventional Giemsa staining method was suitable for the analysis of large numbers of chromosome metaphases of LDR-irradiated mice, because the procedure is simple and rapid. Colcemide (Invitrogen Corp., Carlsbad, CA, USA) at a final concentration of 0.02 μg/ml was added for the last 2 h of culture to collect metaphase cells. Cells were treated by hypotonic solution with 0.075 M KCl for 8 min and centrifuged. The supernatant was removed and the cells were fixed with Carnoy's solution (methanol and acetic acid = 3:1). Fixed cell suspensions were stored at −20 °C before chromosome analyses. For the conventional Giemsa staining method, chromosome preparations were made using a device able to automatically control humidity (ADSTEC, Ciba, Japan), and stained with Giemsa solution. Chromosome aberrations such as dicentric chromosomes (Dic), centric ring chromosomes (Rc), acentric ring chromosomes (Ra), fragments, minutes (min) and deletions (del), hyperploids and chromatid-type aberrations such as gaps (ctg), breaks (ctb) and exchanges (exc) were observed in 500–1000 metaphases per mouse under a light microscope (Olympus Bx50, Olympus, Tokyo, Japan). At least 500–1000 metaphases were scored per mouse in both irradiated and age-matched control groups. Deletion was defined as a chromosome piece with centromere less than the size of mouse chromosome 20.

2.5. Micronucleus assay in spleen and bone marrow

Three to five mice were analyzed in each experimental group at the total doses of 5000, 6000 and 8000 mGy with irradiation at a dose rate of 20 mGy/day by micronucleus assay to observe micronucleus incidence in splenocytes. Non-irradiated mice of the same age were also analyzed as a control group. Another 5 mice receiving 8000 mGy at 20 mGy/day and non-irradiated control mice of the same age were analyzed for micronuclei in erythrocytes of bone marrow. Spleens and bone marrow were sterilely removed from irradiated mice. Bone marrow samples were taken from the femur and then resolved into single cells in 2 ml solutions of ice-cooled α-MEM (Invitrogen Corp., Carlsbad, CA, USA). Spleen cells were isolated and suspended in RPMI 1640 medium. Their homogenized solutions were filtered through a cell strainer. One hundred to 150 μl of cell suspensions from the spleen or bone marrow was used for each slide prepared by an auto-cytospin (Shandon, Pittsburg, PA, USA) and stained with May-Grünwald Giemsa solution (Wako, Osaka, Japan) for the micronucleus assay. In the spleen, 10,000 binucleated lymphocytes per mouse were enumerated to observe micronucleus-positive cells under the microscope (Olympus Bx50, Olympus, Tokyo, Japan). Micronucleus-positive cells in about 5000–7000 polychromatic erythrocytes only of the bone marrow cells per mouse were scored under the microscope. Cells with 2–4 micronuclei were also scored.

2.6. Interphase fluorescence in situ hybridization (FISH) analysis

Five mice were analyzed at 8000 mGy with irradiation of the 20 mGy/day dose rate by the FISH method using centromere probes of chromosomes 5, 15 and 18 to observe the numerical chromosome aberration rate in splenocytes. Five non-irradiated control mice of the same age as irradiated mice were used as a control. About 3000 cells were enumerated to observe cells with one and three signals in the interphase nucleus, which were scored as monosomy and trisomy, respectively.

For FISH analysis, slides were hybridized with a mouse centromere probe (STAR* Mouse Chromosome Probes, Cambio, Cambridge, UK) to detect the mouse centromere. One microlitre of mouse DNA probe was mixed well with 9 μl of hybridization buffer and denatured by heating at 80 °C for 10 min. The chromosome slide was treated by denaturing the solution (70% of formamide in 2× SSC solution) at 75 °C for 5 min and dehydrated in 70%, 85% and 95% ethanol. Formamide was purchased from Roche Diagnostic, Indianapolis, IN, USA. After air drying, the mouse denatured DNA probe solution was laid on a part of the slide and hybridized at 37 °C overnight in a humidity chamber. After hybridization, the slide was washed three times with washing solution I (50% of formamide in 2× SSC solution) at 43 °C for 5 min, and three times with washing solution II (0.8× SSC) at 61 °C for 5 min. The slide was then treated with 150 μl of blocking solution [3% of bovine serum albumin (BSA) in 4× SSC and 0.1% Tween 20 (Nakarai, Osaka, Japan)] at 37 °C for 30 min. To detect biotin, 150 μl of avidin-fluorescein (Oncor, Gaithersburg, MD, USA) diluted 400 times with 4× SSC and 0.1% Tween 20 was applied to the slide, incubated at 43 °C for 30 min, and then washed three times with washing solution (4× SSC and 0.1% Tween 20) at 43 °C for 5 min. The slide was stained with propidium iodide (Oncor, Gaithersburg, MD, USA) solution (100 ng/ml in 2× SSC) for 5 min at room temperature to stain the cell nucleus and chromosome, washed with 2× SSC, and coverslipped with 40 μl of anti-fading solution (Oncor, Gaithersburg, MD, USA). Microscopic observation was performed under propidium iodide staining, and/or fluorescein isothiocyanate (FITC) (Oncor, Gaithersburg, MD, USA) staining images of the same metaphases. The number of FISH signals was scored in about 3000 interphase nuclei of splenocytes per mouse under a fluorescent microscope (Olympus Bx50, Olympus, Tokyo, Japan).

2.7. Statistical analysis

The standard error for yields of chromosome aberrations was estimated by regression analysis. Alpha and beta coefficient values in the linear regression lines or linear quadratic regression curves were estimated by regression analysis. Incidences of chromosome aberrations and micronuclei were compared at each total dose between irradiated groups and non-irradiated control groups at 8 weeks of age, and were tested for statistical significance using Student *t*-test.

3. Results and discussion

3.1. Incidences of dicentric and centric ring chromosomes

Total aberrations of dicentrics and centric rings in mice exposed to γ-rays at a dose rate of either 200 mGy/day or 20 mGy/day, and age-matched, non-irradiated control mice were estimated. The frequencies of aberrations per 100 cells at 8000 mGy at 20 mGy/day were 17.2 times higher than those of non-irradiated mice, and increased as accumulated doses increased (Table 1, Fig. 1). Dose–response relationships between the incidences of dicentrics and centric rings and total accumulated doses up to 8000 mGy at both dose rates of 200 mGy/day and 20 mGy/day were obtained, respectively. The dose–response curves for both 200 mGy/day and 20 mGy/day were almost linear up to 8000 mGy, indicating that these aberrations increased with accumulated dose for both dose rates. The rate of chromatid-type aberrations also slightly increased with the accumulated dose of irradiation of 200 mGy/day and 20 mGy/day (Table 1). The equations obtained are shown in Fig. 1 for 20 mGy/day and 200 mGy/day. The value of 0.086 at 0 Gy was obtained as the mean number of aberrations of dicentric and centric rings per 100 cells from the observation of 25 non-irradiated 8 weeks old mice at the beginning of irradiation.

The incidence of aberrations as dicentric and centric ring chromosomes were compared at two different dose rates (200 mGy/day

Table 1
Chromosome aberrations in spleen cells from chronically irradiated mice at dose rates of 200 mGy/day and 20 mGy/day.

Dose (mGy)	Number of observed cells (number of mice)		Dic and Rc/100 cells mean ± S.E.		Complexity (Cu/cell)		Hyperdiploid cells(%) mean ± S.E.		Chromatid aberrations/100 cells mean ± S.D.	
	200 mGy/day	20 mGy/day	200 mGy/day	20 mGy/day	200 mGy/day	20 mGy/day	200 mGy/day	20 mGy/day	200 mGy/day	20 mGy/day
0	25549 (25)	25549 (25)	0.09 ± 0.02	0.09 ± 0.02	1.13 ± 0.03	1.13 ± 0.03	0.16 ± 0.16	0.16 ± 0.16	6.91 ± 0.63	6.91 ± 0.63
100		8663 (8)		0.24 ± 0.04		1.19 ± 0.04		0.08 ± 0.10		7.23 ± 0.92
140		7207 (7)		0.06 ± 0.02		1.27 ± 0.04*		0.10 ± 0.11		5.92 ± 0.50
200	4045 (8)		0.91 ± 0.04		1.32 ± 0.11*		0.15 ± 0.20		9.74 ± 1.53	
250		6985 (7)		0.16 ± 0.05		1.12 ± 0.04		0.19 ± 0.21		7.10 ± 1.00
400	4062 (8)		1.40 ± 0.06		1.33 ± 0.10*		0.17 ± 0.17		8.22 ± 0.67	
500		4602 (9)		0.35 ± 0.05		1.36 ± 0.06*		0.20 ± 0.20		6.26 ± 0.57
750		5209 (10)		0.38 ± 0.06		1.29 ± 0.08*		0.52 ± 0.76		4.78 ± 0.64
800	3522 (7)	3521 (7)	2.04 ± 0.21	0.31 ± 0.06	1.44 ± 0.06*	1.36 ± 0.10*	0.26 ± 0.10	0.28 ± 0.25	11.8 ± 1.20	7.16 ± 0.41
1000	3538 (7)	6609 (13)	2.43 ± 0.20	0.48 ± 0.09	1.39 ± 0.04*	1.23 ± 0.04*	0.17 ± 0.18	0.20 ± 0.41	8.14 ± 0.65	6.84 ± 0.97
1200		3626 (7)		0.44 ± 0.10		1.61 ± 0.11*		0.25 ± 0.15		9.40 ± 0.65
2000	3534 (7)	5675 (12)	3.17 ± 0.33	0.79 ± 0.14	1.49 ± 0.08*	1.34 ± 0.09*	0.34 ± 0.34	0.32 ± 0.24	9.48 ± 0.62	9.37 ± 0.73
3000		3479 (7)		1.41 ± 0.19		1.53 ± 0.07*		0.13 ± 0.15		9.23 ± 0.93
4000	3557 (7)	5633 (8)	5.03 ± 0.42	1.81 ± 0.35	1.56 ± 0.03*	1.40 ± 0.10*	0.56 ± 0.31	0.23 ± 0.13	10.3 ± 0.72	11.8 ± 1.11
6000		3878 (7)		2.01 ± 0.15		1.56 ± 0.02*		0.39 ± 0.34		9.77 ± 0.92
8000	3104 (7)	6193 (12)	9.12 ± 0.77	2.20 ± 0.23	1.58 ± 0.09*	1.54 ± 0.03*	0.52 ± 0.30	0.55 ± 0.40	13.5 ± 3.42	11.4 ± 0.86

Cu: Total numbers of aberrations of dicentric (Dic), centric ring (Rc), acentric ring (ace), fragment (Fg), minute (min) and deletion (del).
* The value was statistically higher than that of 0 mGy ($p < 0.05$ in t-test).

Fig. 1. Frequencies of dicentrics and centric rings from mice after irradiation of less than 8000 mGy at LDR of 20 mGy/day (■) and 200 mGy/day (□). Each dot indicates the value obtained from individual mice.

and 20 mGy/day) at several identical total doses (Table 1). The ratios of aberration frequencies between the two different dose rates at total doses of 8000 mGy, 4000 mGy, 2000 mGy, 1000 mGy, and 800 mGy were 4.15, 2.78, 4.01, 5.06 and 6.58, respectively. Clear dose-rate effects were observed within these two different dose rates in the frequencies of dicentrics and centric rings.

The present study revealed that dicentric and centric ring aberrations in splenocytes continued to increase almost linearly up to 8000 mGy at dose rates of 20 mGy/day and 200 mGy/h as accumulated total doses increased. Dicentric chromosome aberrations from peripheral blood lymphocytes and *Dlb-1* mutations induced in the small intestine in mice showed no statistical difference in the dose responses between three different dose rates (550 mGy/day, 185 mGy/day and 64 mGy/day) [14]. Inverse radiation dose-rate effects have been seen for somatic and germ line mutations, and DNA damage [15]. Dose-rate effects on mutation rates in the mouse spermatogonia and spermatid were observed at the dose rates of 900 mGy/min to 0.8 mGy/min (1056 mGy/22 h/day), but not at a much lower dose rate of 0.007 mGy/min (9.24 mGy/22 h/day) [16]. Since the dose rate of 20 mGy/day (0.91 mGy/min when calculated for 22 h in a day) is approximately 20–200 times lower than those used in most other laboratories to observe adaptive response and dose-rate effects on mutation [15,17,18], the obtained results will be valuable for risk assessment of low-dose radiation.

Splenocytes proliferated by ConA and LPS stimulation are considered to be mostly T- and B-lymphocytes, respectively. Lymphocytes with unstable-type aberrations could be eliminated in each cell division during long-term exposure to LDR γ-rays. It is possible that the spleen is a reservoir for lymphocyte populations, which could migrate into the spleen from other tissues, including the bone marrow, thymus and lymph nodes of mice continuously irradiated for a long period. Another possibility is that a long-lived lymphocyte subset may be relevant for the linear increase of dicentric and centric ring chromosomes.

Since it is well known that chromosome aberration rates increase with aging [19], we previously analyzed the relationship between unstable-type chromosome aberration rates and the age of mice in 109 SPF C3H/HeN female mice. In the analysis, unstable-type aberration was defined as total aberrations of Dic, Rc, Ra and Fg. Unstable-type aberration rates did not increase and were almost constant in younger or older SPF mice, as in the regression line shown by $y = -0.0008x + 1.014$, $R^2 = -0.019$, where y is unstable-type aberrations per 100 cells and x is age in days after birth. As there was no overall correlation, it is possible that aging does not raise the unstable-type aberration rate in C3H/HeN female mice under the SPF conditions. Then we compared directly observed chromosome aberration rates of dicentrics and centric rings between dose rates of 20 mGy/day and 200 mGy/day

Table 2
Micronucleus incidence in splenocytes and bone marrow erythrocytes from mice irradiated with the LDR at 20 mGy/day.

Dose (mGy)	Age (Days)	Number of observed cells (number of mice)	Number of cells with MN	Number of cells with MN per 1000 cells (mean ± S.D.)	Number of cells with 2–4 MN	Number of cells with 2–4 MN per 1000 cells (mean ± S.D.)
Splenocytes						
0	69	50,452 (5)	181	3.59 ± 0.39	14	0.14 ± 0.13
0	306	52,607 (5)	519	9.87 ± 2.98	72	0.72 ± 0.54
0	356	53,235 (5)	623	11.7 ± 3.45	32	0.30 ± 0.30
0	456	40,501 (4)	296	7.31 ± 1.24	12	0.15 ± 0.13
250	69	40,234 (4)	163	4.05 ± 0.93	13	0.15 ± 0.10
5000	306	52,607 (5)	670	16.7 ± 7.45	167	1.81 ± 0.82
6000	356	51,339 (5)	448	8.73 ± 0.94	78	0.76 ± 0.72
8000	456	52,256 (5)	702	13.4 ± 4.39	129	1.23 ± 0.71*
Bone marrow erythrocytes						
0	456	17,365 (3)	44	2.54 ± 1.49	0	0
8000	456	39,880 (5)	50	1.25 ± 0.66	3	0.01 ± 0.01

MN: micronucleus.
* The value was statistically higher than that of 0 mGy control group with the same age of irradiated mice ($p < 0.05$ by t-test).

at several doses to identify the existence of any dose-rate effect.

3.2. Chromosome instability detected by unstable-type aberrations

The complexity of chromosome aberrations in terms of the number of unstable-type aberrations per cell will be a suitable cytogenetic index for evaluating chromosome instability after prolonged LDR irradiation. Chromosome aberrations such as Dic, Rc, Ra, Fg, min and del were scored and the total number of aberrations per cell was calculated using only aberrant cells in each mouse. Complexity was determined as [total number of unstable-type aberrations; (Dic + Rc + Ra + Fg + min + del)/total number of abnormal cells with chromosome-type aberrations]. The results are summarized in Table 1 and Fig. 2a and b. Their complexity started to increase from more than 200 mGy in the irradiation at 200 mGy/day and from more than 500 mGy in the irradiation at 20 mGy/day, and both increased with the accumulated dose and their numbers became saturated to be almost identical at 8000 mGy (Fig. 2a). Then, with irradiation at both 20 mGy/day and 200 mGy/day, they started to increase from early days, 8 days and 1 day after initial irradiation, respectively (Fig. 2b). On the other hand, our preliminary observation of translocation in spleen cells from mice with prolonged irradiation at 20 mGy/day showed that the complexity increased after 200 days after initial irradiation (more than 4000 mGy). The number of aberrations per cell was not different between unstable-type aberrations and translocations. Chromosome complexity was also compared at the same irradiation period within 40 days between dose rates of 20 mGy/day and 200 mGy/day (Fig. 2b). Complex aberrations were enhanced by irradiation with a dose rate of 200 mGy/day than 20 mGy/day, indicating an influence of dose rate on chromosome instability.

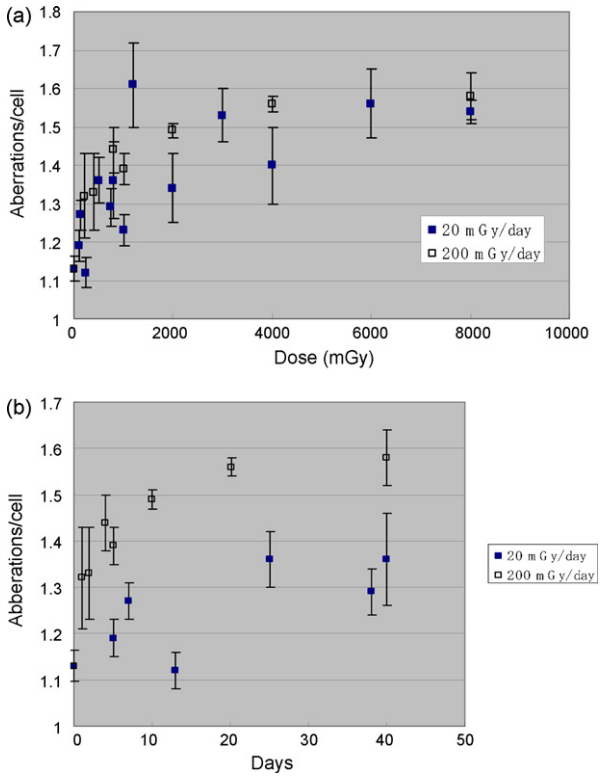

Fig. 2. (a) Chromosome complexity [total number of unstable-type (Cu) aberrations per cell in abnormal cells] from mice after irradiation of less than 8000 mGy at LDR of 20 mGy/day (■) and 200 mGy/day (□). Each value indicates the mean ± standard error (S.E.) of data from at least seven mice. (b) Chromosome complexity [total number of unstable-type (Cu) aberrations per cell in abnormal cells] from mice after irradiation for 40 days at LDR of 20 mGy/day (■) and 200 mGy/day (□). Each value indicates the mean ± standard error (S.E.) of data from at least seven mice.

On the other hand, the incidences of hyperploidy and complexity of chromatid-type aberrations, which are determined as [total number of (ctg + ctb + exc)/total number of abnormal cells with chromatid-type aberrations] did not increase with accumulated dose (Table 1), although these two types of chromosome aberrations have been used as suitable markers for establishing chromosome instability [4,5,20,21].

3.3. Chromosome instability detected by micronucleus assay

Then we observed micronucleus incidence in splenocyte and bone marrow cells in mice after long-term irradiation at an LDR of 20 mGy/day, and found that the pattern of chronological changes of micronucleus incidence was quite different from that of chromosome aberrations. Micronucleus incidence in splenocytes increased with age. Micronucleus incidence in mitogen-stimulated splenocytes started increasing after more than 306 days (Table 2), but the incidence was not significantly different between irradiated mice and age-matched non-irradiated mice. However, the numbers of cells with 2–3 micronuclei out of 10,000 observed cells were significantly higher in mice irradiated with 8000 mGy than age-matched non-exposed mice. Although the number of micronuclei in 5000–7000 polychromatic erythroblasts in bone marrow from

each mouse was not higher than in non-exposed mice (Table 2), the number of cells with 2–3 micronuclei in bone marrow erythroblasts was higher in 8000 mGy-exposed mice at LDR.

3.4. Chromosome instability detected by interphase FISH method

Trisomic splenocytes were also observed by the interphase FISH method to demonstrate chromosome instability in splenocytes from mice at a LDR of 20 mGy/day for 8000 mGy. The FISH method using three mouse centromere probes of chromosomes 5, 13 and 18 showed that splenocytes in the exposed mice had a significantly higher incidence of 3 signals in the nucleus, also indicating higher incidence of trisomies of chromosomes of 5, 13 and 18, than in non-exposed mice (Table 3). One signal in the nucleus, indicating monosomy of chromosomes 5, 13 and 18, was also observed at a higher incidence in irradiated mice, but cells showing one signal were excluded from the present analysis, because they are unconfirmed due to too much high background FISH signal (about 3%) in the nucleus from non-irradiated mice. FISH analysis of splenocytes showed that mice exposed to 8000 mGy by LDR irradiation had a higher percentage of trisomy cells. These numerical chromosome aberrations seem to be induced indirectly after radiation exposure and thus the results indicate the possibility that prolonged γ-ray irradiation at LDR of 20 mGy/day induces delayed (late-arising) chromosomal instability in mice. Numerical aberrations are induced directly by more than 2000–4000 mGy of acute X-ray irradiation with high-dose-rate in human peripheral blood [22]. These results will be useful for risk assessment of LDR radiation exposure.

3.5. Chromosome instability induced by chronic radiation at LDR

A micronucleus forms from either a broken chromosome or from chromosome loss during the step of chromosome segregation at the M phase, and induces cells with a chromosome deletion or aneuploid cells. Of general concern is that these numerical chromosome aberrations obtained by the FISH method and micronucleus assay are not induced directly by radiation exposure. These results indicate that prolonged ^{137}Cs γ-ray irradiation with LDR of 20 mGy/day for 5000–8000 mGy induced delayed chromosome instability in splenocytes and bone marrow cells from mice. We have, for the first time, demonstrated in mice splenocytes that LDR long-term irradiation with γ-rays can induce chromosome instability *in vivo*.

Cells with heavy DNA damage may be eliminated by apoptosis, whereas cells able to survive after exposure may have a higher probability of developing a mutation, and cells surviving after ionizing radiation lead to genomic (chromosome) instability. Radiation-induced genomic instability arising at a late stage is demonstrated by increased rates of mutation, decreased cell survival, and various chromosome aberrations, such as chromosome-type aberrations, chromatid-type aberrations, and micronuclei. An acute low dose to mice *in utero* induced high incidences of chromatid breaks and fragments and polyploid cells in bone marrow cells after birth [23]. Persistent chromatid-type aberrations were commonly observed in cells long-term cultured from haematopoetic and solid mouse tissues exposed to γ-rays [24–27]. Lung cells from rats injected with superoxide dismutase showed a reduced number of micronuclei, which indicates the involvement of oxygen radicals in chromosome instability [28]. Cells having chromatid-type aberrations result in one chromosomal deletion and one normal cell at the next cell division. Since unstable-type chromosome aberrations such as dicentric chromosomes are unstable and do not persist through the next cell cycle, a high percentage of these aberrations occurring by irradiation are eliminated after many cell generations. From these experiments, it was concluded that unstable-type aberrations, chromatid-type aberrations and polyploid cells are likely to be suitable markers for detecting chromosome instability *in vivo* after long-term LDR irradiation, although the precise classification of direct radiation-induced aberrations and delayed chromosome aberrations could be difficult. Complex chromosome aberrations having several aberrant chromosomes per cell are usually induced by high LET radiation and rarely induced by a low dose of low LET radiation. Peripheral blood lymphocytes from residents exposed to prolonged low-dose irradiation in radio-contaminated buildings in Taiwan had a higher frequency of variable type chromosome aberrations [3]. A clone with complex karyotype aberrations developed in a cultured human lymphocyte cell line during cell growth after γ-ray irradiation [9]. Thus, the complexity of unstable-type or stable-type chromosome aberrations will also be used as a suitable cytogenetic marker of chromosome instability.

Radiation-induced chromosome or genetic instability has been demonstrated mostly using acute irradiation at high-dose-rates. Few studies have addressed whether low-dose or LDR radiation can induce chromosome or genomic instability long-term after irradiation. Clones established from irradiated human lymphocytes by 1 Gy at a fairly LDR of 24 mGy/h showed chromosome instability with chromosome breaks and rearrangements [29]. The authors also stated that chromosome instability showed no dose-rate effects at 1 Gy with a high-dose-rate (4500 mGy/h) and LDR (24 mGy/h). On the other hand, low-dose radiation (100–500 mGy) did not induce chromosome instability in a rodent-human hybrid cell line after X-ray irradiation of at least 5.52 mGy/h by X-rays [30]. Abrahamsson-Zetterberg et al. [31] reported exposure to radiation with a dose rate of 20–2000 mGy/h of mouse embryos, which showed no increase of chromosome instability at 35 days after birth using a micronucleus assay combined with flow cytometry. This indicates that LDR radiation doses might permit sufficient DNA repair, however, our present observation of exposure to 20 mGy/day (0.91 mGy/h), which is much lower than reported by others, clearly showed chromosome instability. As reasons for the discrepant

Table 3
Trisomy of chromosomes 5, 13 and 18 in splenocytes in mice irradiated with the LDR at 20 mGy/day, detected by interphase FISH method.

Dose (mGy)	Number of mice	Age (Days)	Total of observed cells	Observed chromosomes	Observed cells	Total cells with FISH signals of three chromosomes					Number of cells with 3 signals per 1000 cells (mean ± S.D.)
						Number of signals on nucleus					
						1	2	3	4	8	
0	5	456	49,011	Chr.18 Chr.13 Chr.5	16,216 16,689 16,106	1945	46,784	205	78	0	4.18 ± 0.76
8000	5	456	49,354	Chr.18 Chr.13 Chr.5	16,597 16,272 16,485	3128	45,726	375	123	2	7.60 ± 2.47*

* The value was statistically higher than that of 0 Gy ($p < 0.05$ by t-test).

results, the much longer irradiation period (accumulated dose) and different developmental stage at the time of irradiation used in present or previous *in utero* studies [31–33], might have had an influence.

3.6. Plausible mechanisms for chromosome instability

The mechanisms for chromosome instability induced by long-term LDR irradiation *in vivo*, observed in the present study, are unknown. However, these observations raised the possibility that radiation-induced genomic (chromosome) instability *in vivo* might not only result from direct DNA (chromosomal) damage by radiation, but also that persistent production of damaged cells in each cell generation might result from the production of reactive oxygen (ROS) or reactive nitrogen species (RNS), or inflammatory cytokines released by macrophages or other cells in tissue [24,34–36]. This also shows that genomic (chromosome) instability can arise from interactions between irradiated cells and non-irradiated cells, which is now known as a bystander effect by radiation exposure. It is well documented that cells exposed extracellularly to superoxide and ROS display high levels of chromosome aberrations [37], particularly chromatid-type aberrations [38]. Further precise analyses will be necessary to elucidate *in vivo* conditions in mice irradiated long-term at a LDR.

Because dicentric and centric ring chromosomes are eliminated at each cell division after irradiation due to their unique morphology with two centromeres on one chromosome, the increase of unstable-type chromosome aberrations or dicentric chromosomes has less biological significance than translocations. Translocations develop in an almost equal ratio to dicentric chromosomes after irradiation [39], and there is a possibility that chromosome instability in cells having translocations increase with the increase of the total accumulated dose of LDR irradiation. Cells with chromosome instability can persist for a longer period after irradiation and some form a benign or malignant clone, which may develop into neoplastic transformation, implicating the biological significance of continuous, long-term exposure to LDR radiation. Information on the incidence of chromosome aberration frequencies and chromosome instability in persistent translocations will become more important for low-dose radiation-induced risk in humans.

Conflicts of interest

None.

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