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Recovery kinetics of micronucleus formation by fractionated X-ray irradiation in various types of human cells

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ABSTRACT

High-dose ionizing radiation is sufficient for breaking DNA strands, leading to cell death and mutations. By contrast, the effects of fractionated ionizing radiation on human-derived cells remain unclear. To better understand the genotoxic effects of fractionated ionizing radiation, as well as the cellular recovery rate, we investigated the frequency of micronucleus (MN) formation in various types of human cells. We irradiated cells with fractionated X-ray doses of 2 Gy at a rate of 0.0635 Gy/min, separated into two to eight smaller doses. After irradiation, we investigated the frequency of MN formation. In addition, we investigated the rate of decrease in MN frequency after irradiation with 1 or 2 Gy X-rays at various recovery periods. Fractionated irradiation decreased MN frequency in a dose-dependent manner. When the total dose of X-rays was the same, the MN frequencies were lower after fractionated X-ray irradiation than acute irradiation in every cell type examined. The rate of MN decrease was faster in KMST-6 cells, which were derived from a human embryo, than in the other cells. The rate of MN decrease was higher in cells exposed to fractionated X-rays than in those exposed to acute irradiation. Recovery rates were very similar among cell lines, except in KMST-6 cells, which recovered more rapidly than other cell types.

Keywords: fractionated X-rays; micronucleus (MN); recovery; human cells

INTRODUCTION

In our daily life, ionizing radiation is widely used for a variety of purposes [1–5]. Although the use of ionizing radiation confers a wide range of benefits, high-dose ionizing radiation can harm cells by inducing DNA damage. These effects have been studied since their discovery, but several questions remain unanswered. In particular, the effects of fractionated ionizing radiation on genotoxicity in human-derived cells remain unclear.

Previously, Koyama *et al.* investigated the effects of X-ray irradiation on micronucleus (MN) formation in human cells derived from an embryo, a newborn and a child [6]. The results revealed no significant increase in MN formation in cells irradiated with X-ray doses of 0.02–0.2 Gy. However, irradiation with 1 or 2 Gy was enough to significantly induce MN formation, in comparison with unirradiated controls.

In this study, we evaluated the accumulated effect of X-ray irradiation on MN formation, as well as cellular recovery from fractionated X-ray irradiation. First, we measured the recovery of MN-induced cells irradiated with 2 Gy X-rays. Second, we examined the effect of dose rate by dividing a total dose of 2 Gy into smaller doses given over 4 days. Finally, to investigate the relationship between the effect of dividing ionizing radiation into smaller discrete doses and allowing recovery periods after exposure, we administered the dose in two parts, followed by a recovery period of up to 3 days. In a previous study [6], Koyama *et al.* showed that at <0.2 Gy, the MN frequency did not differ from control levels,

suggesting a non-linear dose–response relationship. Accordingly, the lowest dose used in this study was 0.25 Gy.

MATERIALS AND METHODS

Cells and growth conditions

We used four human-derived cell lines in this study. HeLa, derived from cervical cancer, and KMST-6, derived from an embryo, were obtained from the RIKEN Bio Research Centre (Ibaraki, Japan). CCD32Sk, derived from a 1-month-old baby, and CCD42Sk, derived from a 4-year-old child, were obtained from the American Type Culture Collection (ATCC) (Manassas, VA, USA). These two cells were normal human fibroblasts. HeLa was used as a model of malignant cells, KMST-6 as a model of transformed cells, and the two others to represent normal cells.

Cells were cultured in Eagle's MEM (Wako, Osaka, Japan) supplemented with 10% fetal bovine serum (FBS, Bovogen Biologicals, Victoria, Australia) and maintained in an incubator at 37°C and 5% CO_2. Cell suspensions of 1×10^5 cells/ml in a volume of 4 ml were seeded on 6-cm culture dishes (AGC Techno Glass Co. Ltd, Tokyo, Japan) at least 24 h before exposure to X-rays to allow attachment to the bottom of the culture dishes. In this study, we performed experiments at passage numbers of ~3–10 for normal cells (CCD32Sk, CCD42Sk) and ~10–40 for the malignant (HeLa) and transformed cell lines (KMST-6).

X-ray irradiation system

Exponentially growing cells were exposed to a total dose of 2 Gy at a rate of 0.0635 Gy/min using an X-ray generator (MX-80Labo; MediXtech, Chiba, Japan) operating at 80 kV and 1.25 mA without a filter. Dishes were maintained at a constant temperature with a heating plate and sealed with Parafilm (Bemis Flexible Packaging, Oshkosh, WI, USA) to maintain the same humidity and atmosphere as inside the incubator. Negative control cells were concurrently incubated in a conventional incubator or the X-ray generator.

Fractionated irradiation

MN formation rate with variation of recovery period after 2 Gy irradiation

To investigate the capacity to recover from MN formation, the four types of cells were exposed to a continuous X-ray dose of 2 Gy (0 Gy for negative controls), and then incubated for up to 4 days as a recovery period. The cells were treated with cytochalasin B (Sigma-Aldrich, St Louis, MO, USA) and processed for MN testing immediately after 2 Gy irradiation ('2 Gy–0 days') or on the second or fourth day after irradiation ('2 Gy–2 days' or '2 Gy–4 days,' respectively). Control cells were treated and processed 4 days after seeding.

Recovery of cells irradiated with fractionated 2 Gy

The effect of dose rate on the long-term effects of exposure to fractionated ionizing radiation was examined by dividing the total dose of 2 Gy into smaller doses given over 84 h. The irradiation schedule was as follows: cells were irradiated twice with 1 Gy, separated by an interval of 72 h ('two-division irradiation'), four times with 0.5 Gy with an interval of 24 h ('four-division irradiation'), or twice a day for four days with 0.25 Gy, with an interval of 12 h ('eight-division irradiation'). After the last exposure, the cells were immediately treated with cytochalasin B and processed for MN testing. Control cells were treated and processed 84 h after seeding.

Recovery of the cells subjected to two-division irradiation of 2 Gy

To investigate the relationship between the effect of dividing fractionated ionizing radiation and the recovery period after exposure, we performed the two-division irradiation experiment with a recovery period of up to 3 days. The four kinds of cells were exposed twice to 1 Gy with an interval of 72 h. The cells were treated with cytochalasin B and processed for MN testing either immediately after the last irradiation or after a recovery period of 1 to 3 days. Control cells were treated and processed 3 days after seeding.

Micronucleus test

We evaluated the frequency of MN formation, an *in vitro* index of genotoxicity. Previously, Koyama *et al.* described the procedure for the MN test [6] and reported the effects of X-ray irradiation for longer durations. In this study, cells were incubated for a number of days as a recovery period after X-ray exposure. Following three washes with phosphate-buffered saline (PBS), cells were cultured in medium containing cytochalasin B at a concentration of 3 μg/ml for 24 h to prevent cell division and create binucleated cells. The duration of cytochalasin B treatment was set based on the cell cycle period of the cells used in this study. The cells were then collected and fixed with 70% cold ethanol for at least 30 min. After a careful wash with PBS, the cells were suspended in PBS and stained with propidium iodide (PI; Invitrogen, Carlsbad, CA, USA). A suspension containing ~2×10^4 cells was mounted on slides using a Cytospin centrifuge (Shandon Southern Ltd, Runcorn, UK) at 100 *g* for 5 min.

Scoring procedure and statistics

Cells were mounted with VECTASHIELD (Vector Laboratories, Inc., Burlingame, CA, USA) and kept in the dark prior to counting. To determine the frequency of MN formation, 300 binucleated cells in three individual experiments were scored on an AX-70 fluorescence microscope (Olympus, Tokyo, Japan). Cells were counted as MN-positive if they contained at least one MN. The procedure was performed in a double-blinded manner following the criteria described in previous studies [7–9]. Statistical analysis of the data was carried out by ANOVA (analysis of variance) followed by Dunnett's test in IBM SPSS Statistics (IBM, Chicago, IL, USA).

Calculation of the rate of MN decrease

The rate of decrease in MN (R) during the recovery period was calculated from the observed MN frequencies under each experimental condition, using the following formula:

$$R = \left(1 - \frac{D_X - C}{D_0 - C}\right) \times 100(\%),$$

where R = MN decrease rate; C = spontaneous frequency of MN (Control); D_0 = MN frequency immediately after Xray exposure; D_X = MN frequency after recovery period.

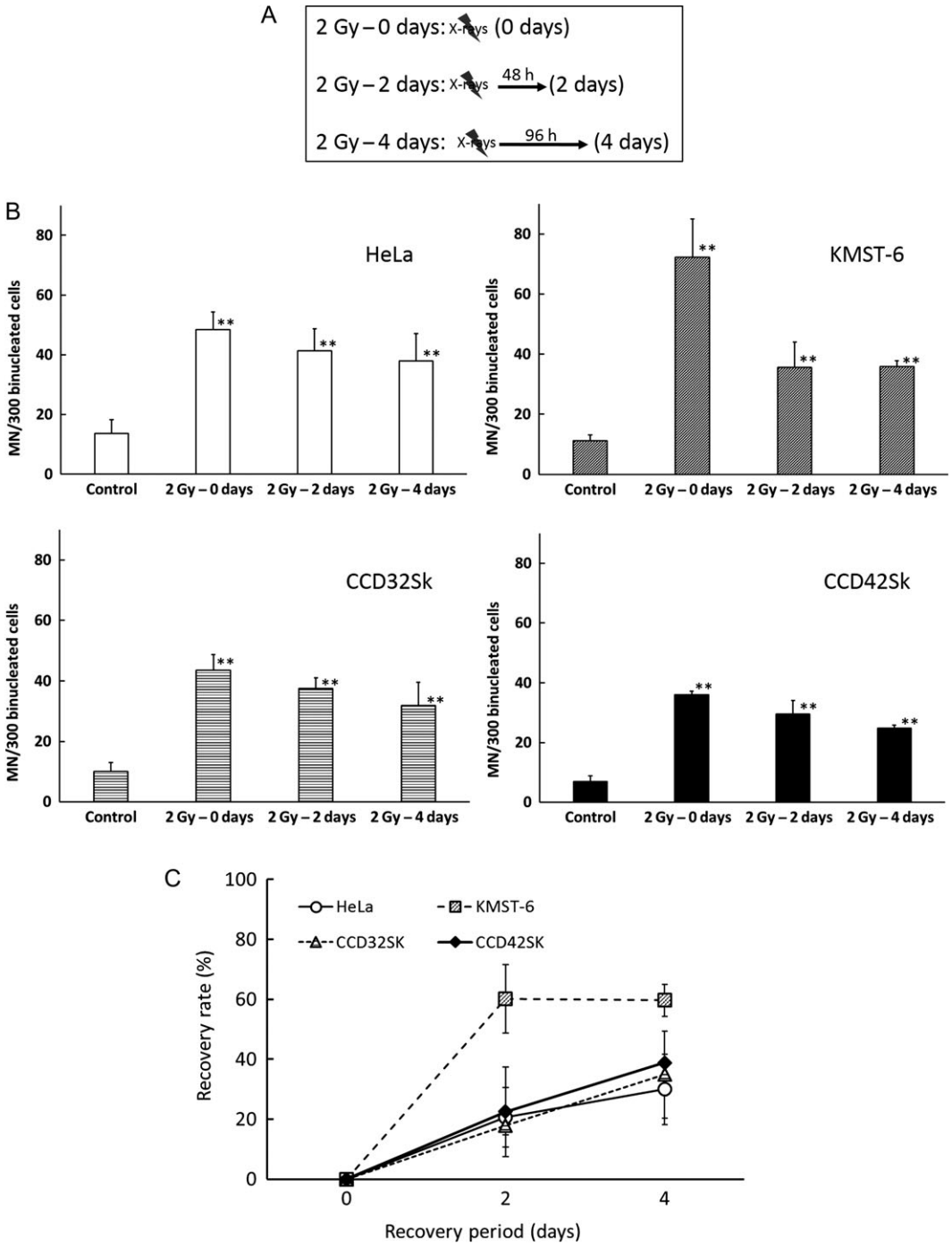

Fig. 1. (A). Brief illustration of the X-ray irradiation procedure for 2 Gy – 0 days, – 2 days and – 4 days experiments. A continuous X-ray dose of 2 Gy was administered to the four kinds of cells. The cells were treated with cytochalasin B and processed for MN testing immediately after the 2 Gy irradiation ('2 Gy — 0 days'), 48 h after irradiation ('2 Gy — 2 days') or 96 h after irradiation ('2 Gy — 4 days'). (B). Frequency of MN formation in HeLa, KMST-6, CCD32Sk and CCD42Sk cells exposed to X-ray irradiation at 2 Gy. Data are presented as means ± SD ($n = 3$). Asterisks indicate statistically significant differences in total MN between control (0 Gy) and X-ray irradiation ($P < 0.01$). (C). Recovery rate of HeLa, KMST-6, CCD32Sk and CCD42Sk cells exposed to X-ray irradiation at 2 Gy. Data are presented as means ± SD ($n = 3$). Vertical line indicates recovery rate (i.e. rate of reduction in MN frequency) relative to their respective controls. The horizontal line indicates the passage of time in days.**

RESULTS

Initially, HeLa, KMST-6, CCD32Sk and CCD42Sk cells were exposed to a continuous X-ray dose of 2 Gy. Figure 1A illustrates the procedure for the 2 Gy−0 days, −2 days, and −4 days experiments. The frequencies of MN formation in each experiment are shown in Fig. 1B. Figure 1C shows the rate of MN decrease in cells incubated for up to 4 days as a recovery period after X-ray exposure. In all experiments, MN frequency decreased with the number of recovery days. However, even in the '2 Gy-4 days' experiments, there were still statistically significant differences ($P < 0.01$) between irradiated and control samples in all cell types. The rate in embryo-derived KMST-6 cells was ~60% after 2 days, whereas the other cell lines exhibited lower rates. The actual frequency of MN formation in KMST-6 cells also differed from those in other cells.

Figure 2A shows the procedure for the fractionated irradiation experiment. The frequencies of MN formation in this experiment are shown in Fig. 2B. Figure 2C shows the rate of decrease of MN of cells exposed to 2 Gy divided into two, four or eight doses. A remarkable effect on the rate of MN decrease rate was observed in the two-division irradiation experiment of KMST-6 cells, even in its smaller number of fractions. By contrast, the rate of MN decrease in HeLa remained at ~25% after four-division irradiation. A larger effect on the rate of MN decrease rate, 80%, was observed in the eight-division irradiation experiment in all types of cells. In this experiment, the actual frequency of MN formation in KMST-6 cells also differed from that in other cells.

To investigate the relationships between the effect of dividing ionizing radiation and the recovery period after exposure, we performed the two-division irradiation experiment with a recovery period of up to 3 days. Figure 3A shows the procedure, and Fig. 3B shows the frequency of MN formation. Figure 3C shows the rate of MN decrease in cells incubated for up to 3 days as a recovery period after X-ray exposure when fractionated into two doses ($1\ \text{Gy} \times 2$). The rate of decrease in MN increased as the recovery period lengthened. There were still statistically significant differences, even in the '1 Gy × 2–3 days' experiments, in every cell type. The actual frequency of MN formation was also higher in KMST-6 than in the other cell lines.

DISCUSSION

In this study, we investigated the frequency of MN formation in human cells irradiated with fractionated X-rays.

We observed gradual recovery in three of four cell lines, and more rapid recovery in KMST-6 in the first experiment. The rate of MN decrease in KMST-6 was high (~60%) in the '2 Gy–2 days' experiment, and the actual frequency of MN formation was also higher in this cell line, suggesting that transformed embryonic cells have the ability to recover from irradiation. By contrast, the rate of MN decrease in the other three types of cells was relatively low.

In a previous study [6], Koyama *et al.* investigated the frequency of MN formation in cells irradiated with various dose of X-rays from 0.25 to 2 Gy. The frequency of MN formation in KMST-6 was the highest observed in the experiment, indicating that cells of newborns and children are more tolerant of X-ray irradiation than are embryonic cells. This means that newborn and child cells do not have as many recovery points compared with embryo cells. This may be relevant to the results of our current experiments. In this study, we investigated just one kind of embryonic cell; therefore, it will be necessary to validate and confirm these observations in other kinds of cells, including normal (i.e. non-transformed) cells. KMST-6 cells demonstrated a high frequency of MN formation at the 2 Gy–0 days point. The sensitivity of MN formation in KMST-6 cells might be higher than in the other cells. However, it appeared to be significantly unchanged on radiosensitivity between KMST-6 cells and CCD32Sk or CCD42Sk cells according to the surviving fraction at 2 Gy [6]. It will be necessary to investigate other factors, such as mitotic index or activation of the G2/M checkpoint.

Two-division irradiation resulted in a slight decrease in the MN frequency in comparison with single-dose irradiation. However, in KMST-6 cells the recovery rate was higher than in the other cell types. Four-division irradiation resulted in a further decrease in MN, and KMST-6 and CCD32Sk cells exhibited no differences in MN frequency relative to their respective controls. Eight-division irradiation exhibited no statistical difference from the control in any cell type. The results of the second experiment are reasonable and consistent with previous reports, but to date no detailed experiments have been performed. Here, we showed that cells exposed to a total dose of 2 Gy fractionated into eight doses did not differ from control cells. This experiment cannot reveal the underlying mechanism, but it could be suggested that all types of cells entirely recovered from eight-division irradiation or they were not affected by irradiation at 0.25 Gy.

In the third experiment, we examined the rate of decrease in MN in cells exposed to 1 Gy twice, and then incubated for 1–3 days as a recovery period after the last exposure. Although KMST-6 cells exhibited a relatively rapid recovery, the rate of MN decrease rate did not differ significantly between any cell type. This experiment demonstrates that a long-term interval might not lead to sufficient recovery of MN frequency.

These three experiments indicate that fractionation of X-rays might be effectively reduce MN; however, time-interval recovery did not seem to effectively decrease MN frequency. In addition, KMST-6 cells exhibited different behavior from that of the other cells, namely, relatively rapid recovery in the fractionated and time-interval recovery experiments.

As far as we know, few studies have examined the effect of fractionated X-ray irradiation on MN formation, although many papers have described its therapeutic effect in terms of killing cancer cells [10, 11]. For example, a comparison of acute and fractionated exposure was performed in adult mouse hippocampal neurogenesis [12]. Although the conditions differed from those used in our study, the doses were of similar magnitude. The results of that study indicated that acute and fractionated exposures of ^{56}Fe-particle irradiation were similarly detrimental to adult-generated neurons. However, there was a difference between acute and fractionated exposure revealed by stereological quantification of $BrdU^{+}$ cells 24 h post-irradiation. This result might correspond to the findings in this study.

Cervelli *et al.* [13] reported that single or fractionated low-dose irradiation does not affect cell viability or DNA repair in vascular endothelial cells. Specifically, they observed that exposure to fractionated doses resulted in higher endothelial activation, but did not

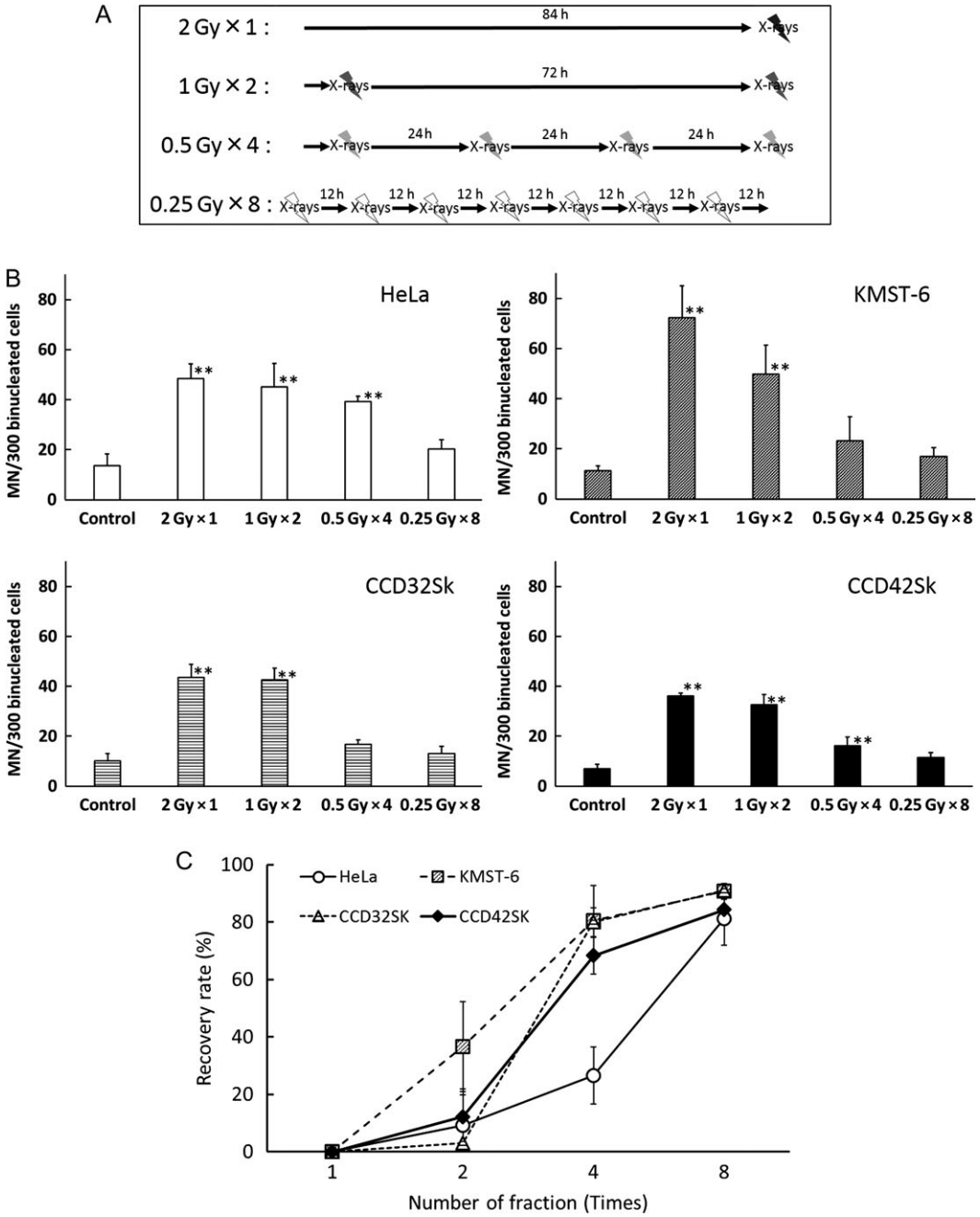

Fig. 2. (A). Brief illustration of the X-ray irradiation procedure for the fractionated irradiation experiment. Cells were exposed twice to 1 Gy with an interval of 72 h ('two-division irradiation'), four times to 0.5 Gy with an interval of 24 h ('four-division irradiation'), or to twice a day for 4 days to 0.25 Gy with an interval of 12 h ('eight-division irradiation'). (B). Frequency of MN formation in HeLa, KMST-6, CCD32Sk and CCD42Sk cells exposed to the indicated fractionated X-ray irradiation. Data are presented as means ± SD ($n = 3$). Asterisks indicate statistically significant differences in total MN between control (0 Gy) and irradiated cells ($P < 0.01$). (C). Recovery rate of HeLa, KMST-6, CCD32Sk and CCD42Sk cells exposed to fractionated X-ray irradiation. Data are presented as means ± SD ($n = 3$). The vertical line indicates the recovery rate (i.e. the reduction in MN frequency) relative to their respective controls. The horizontal line indicates the number of fractions.**

affect DNA repair. Our results showed that fractionated irradiation decreased the MN frequency in every cell type, as long as the total dose was the same (2 Gy). The difference in the results might be related to cell type. DNA damage was not detected directly, although other experiments indirectly suggested DNA damage. However Ojima *et al.* [14] demonstrated that frequencies of

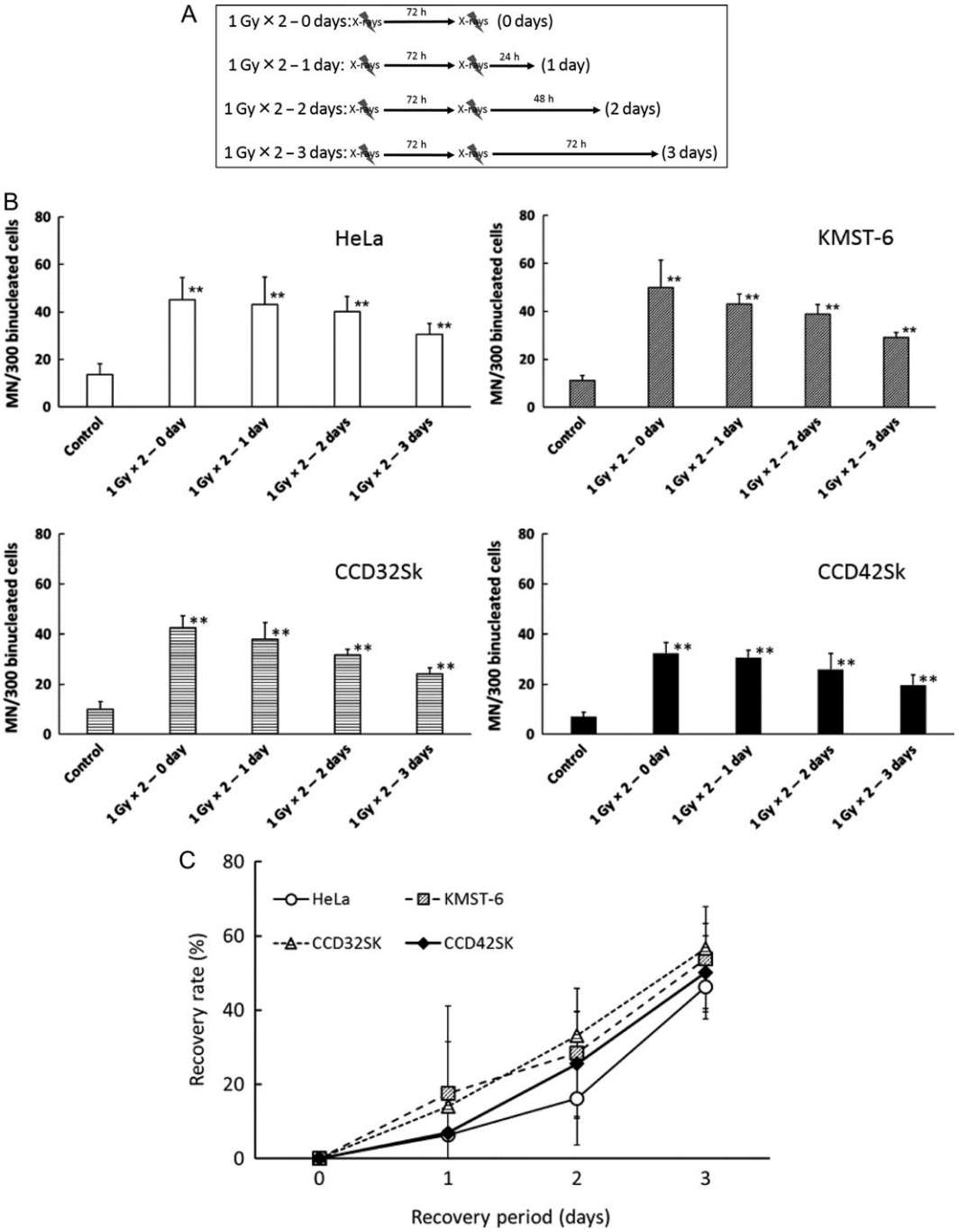

Fig. 3. (A). Brief illustration of the X-ray irradiation procedure for the two-division irradiation experiment. The four kinds of cells were exposed twice to 1 Gy with an interval of 72 h. The cells were treated with cytochalasin B and processed for MN testing immediately after the last irradiation or after a recovery period of 1–3 days. (B). Frequency of MN formation in HeLa, KMST-6, CCD32Sk and CCD42Sk cells exposed to X-ray irradiation at 1 Gy × 2 times with elapsed time from 0–3 days. Data are presented as means ± SD ($n = 3$). Asterisks indicate statistically significant differences in total MN between control (0 Gy) and irradiated cells ($P < 0.01$). (C). Recovery rate of HeLa, KMST-6, CCD32Sk and CCD42Sk cells exposed to X-ray irradiation at 1 Gy × 2 times with an elapsed time of 0–3 days. Data are presented as means ± SD ($n = 3$). Vertical line indicates recovery rate (i.e. rate of reduction in MN frequency) relative to their respective controls. The horizontal line indicates the passage of time in days after the second exposure.**

dicentric chromosomes in normal human fibroblast cells irradiated with fractionated X-rays were significantly reduced in comparison with those in acutely irradiated cells, though the interval between the fractionated irradiations must be short enough. In that study, the interval between fractions was from 1 to 1440 min, and the authors found that the frequency of dicentric chromosomes did not significantly differ when the interval was varied between 5 and 1440 min. The interval times in our experiments were very different, so it is difficult to make a comparison; our data indicated that the recovery rates were not higher when the interval time was extended to more days. Mariotti *et al.* [15] investigated the DNA repair dynamics of cells exposed to fractionated irradiation by assessing γ-H2AX foci in normal human fibroblast cells. They found that the number of foci induced was smaller after a split exposure than a single exposure when the second irradiation was performed within 5 h of the first. The interval times were different in each experiment. Overall, the results showed that fractionated exposure decreased DNA damage in comparison with acute exposure.

Park *et al.* [16] investigated the long-term effect of acute and fractionated irradiation on doublecortin-positive (DCX-positive) cells in hippocampal neurogenesis in mice. Their results showed that the number of DCX-positive cells was lower in the acute irradiation group than in the fractionated irradiation group, suggesting that hippocampal neurogenesis was more susceptible to being damaged by acute than fractionated irradiation. The irradiations were performed in mice, rather than cultured cells, and consequently the experimental conditions were totally different from ours; however, the results were still consistent with our data. Vral *et al.* [17] studied the effects of fractionated doses on the *in vitro* MN yield in human lymphocytes exposed to X-rays. In their fractionated-dose experiment, a continuous decrease in MN frequency was observed with fractionated irradiations.

In a recent *in vivo* study, Tsuruoka *et al.* [18] reported that the size of radiation-induced deletions was higher after acute gamma-ray exposure than after protracted exposure. These experiments used genetically radiosensitive *Ptch1* heterozygous mice. Many studies [19–21] have shown that protracted radiation induces significantly less cancer in mice than the same total dose of irradiation administered acutely. These results support our observations obtained using human cells.

At present, there are no clear-cut data regarding the increase in cancer risk due to low-dose irradiation (International Commission on Radiological Protection [ICRP]) [22]. In light of the uncertainty regarding the mechanism of low-dose effects, further study is required to clarify this issue.

Recent studies indicated that the MN test is a very accurate method for detecting genotoxicity, because the MN frequency is strongly correlated with cancer [23–26]. In our study, total MN frequency was lower following fractionated irradiation than after acute irradiation. In particular, the MN frequency in this study (0.25 Gy in eight doses = 2 Gy) did not differ from that in control cells. The MN test is recently indicated as an accurate method for detecting genotoxicity, however does not seem to elucidate the health risk issues of fractionated ionizing radiation, such as the doses in our experiments. The mechanisms underlying this phenomenon remain unclear; however, there seems to be a threshold for inducing MN formation at ~0.25–0.5 Gy or the recovery systems would function effectively at this dose range.

In conclusion, in this study we investigated cells from young humans (embryo, newborn and child), as well as the cancer line HeLa. Embryonic cells were more sensitive to MN formation, consistent with previous data. We speculate that sensitivity to elevated MN frequency depends on the developmental stage of the cell. In addition, the recovery rate was higher in the embryonic cells than in the other cell types. This might indicate that cells from an early stage of development have a relatively greater ability to recover from MN formation than other types of cells.

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CONFLICT OF INTEREST

The authors report that there are no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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