

# Equivalency of the quality of sublethal lesions after photons and high-linear energy transfer ion beams

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## ABSTRACT

The quality of the sublethal damage (SLD) after irradiation with high-linear energy transfer (LET) ion beams was investigated with low-LET photons. Chinese hamster V79 cells and human squamous carcinoma SAS cells were first exposed to a priming dose of different ion beams at different LETs at the Heavy Ion Medical Accelerator in the Chiba facility. The cells were kept at room temperature and then exposed to a secondary test dose of X-rays. Based on the repair kinetics study, the surviving fraction of cells quickly increased with the repair time, and reached a plateau in 2–3 h, even when cells had received priming monoenergetic high-LET beams or spread-out Bragg peak beams as well as X-ray irradiation. The shapes of the cell survival curves from the secondary test X-rays, after repair of the damage caused by the high-LET irradiation, were similar to those obtained from cells exposed to primary X-rays only. Complete SLD repairs were observed, even when the LET of the primary ion beams was very high. These results suggest that the SLD caused by high-LET irradiation was repaired well, and likewise, the damage caused by the X-rays. In cells where the ion beam had made a direct hit in the core region in an ion track, lethal damage to the domain was produced, resulting in cell death. On the other hand, in domains that had received a glancing hit in the low-LET penumbra region, the SLD produced was completely repaired.

**KEYWORDS:** sublethal damage (SLD), high LET, direct hit, glancing hit, track structure

## INTRODUCTION

The world's first heavy ion accelerator for therapy, the Heavy Ion Medical Accelerator in Chiba (HIMAC), was constructed in the National Institute of Radiological Sciences, Japan, in 1994. More than 10 000 cancer patients have been treated since then, demonstrating fruitful clinical results [1]. The most serious obstacle to worldwide availability of carbon ion radiotherapy (CIRT) is the high cost [2]. To reduce the cost, clinical and biological research into hypofractionation schedules should be a high priority. CIRT is optimized by dose escalation and hypofractionation, e.g. two fractions in 2 days or single-fraction were applied in the treatment of hepatocellular carcinoma or non-small-cell lung cancer. So-called

single-fraction CIRT at HIMAC [3] consists of a four-field exposure that takes 1–2 h in total, with several time intervals between the exposures. The intervals allow repair and affect the clinical results. In addition, it is generally believed that there is no damage repair, or only slight damage repair, after high-LET irradiation. Radiobiological knowledge about repair after high-LET particle irradiation could reduce big dose escalation study in clinical use.

It is well known that cellular repair systems are very capable of repairing DNA damage [4–6]. Radiation damage in cells can be operationally divided into three categories [7]. (i) Lethal damage (LD) refers to the irreparable and irreversible damage that leads to cell death. (ii) Potentially lethal damage (PLD) refers to damage

that can be modified by the post-irradiation microenvironment in the cell, especially under suspended-growth conditions. Such damage is lethal under ordinary conditions, but cells can survive when the environment is well manipulated. (iii) Sublethal damage (SLD) refers to repairable damage that allows survival within several hours under the ordinary environmental conditions of cells; however, if the cell is exposed to secondary radiation before repair of the damage can be completed, then LD ensues. In general, the SLD generated by low-linear energy transfer (LET) irradiation is believed to be better repaired than that caused by high-LET irradiation. SLD repair can be found when cells are given a split dose with an interval time for repair, known as split dose recovery. When the SLD has been completely repaired before the secondary irradiation, the radiobiological condition of the cells is the same as that of fresh and healthy cells, except for those cells that have LD or PLD. Survival curves with a shape analogous to that of healthy cells can be seen, with a shift in the survival level of the curve according to the number of cells with LD and PLD. However, in the case of high-LET irradiation, serious and complex cell damage is generated that is difficult to repair, and the SLD may not be repaired well in those cells.

In this study, cells were exposed twice (after the first and second doses) to observe SLD. After the first irradiation, cells were allowed a certain repair time before being exposed to the second irradiation. A point to note is that the first priming dose was with ion beams at different high-LETs, whereas the second test doses were all of X-rays. SLD repair is difficult to observe when cells are exposed to high-LET irradiation as the second test dose, because the survival curve from such irradiation has a small shoulder [8]. It is commonly accepted that SLD repair is only slightly observable, if at all, when high-LET radiation is used. In this study, we revealed that SLD repair occurs after priming irradiation with high-LET ion beams, and this can be seen when using X-rays as a test radiation.

## MATERIALS AND METHODS

### Cells

Chinese hamster V79 cells and human squamous carcinoma SAS cells (Cat. No. JCRB0260; Japanese Collection of Research Bioresources (JCRB) Cell Bank, Osaka, Japan) were cultured in Ham's F-12 medium (Gibco BRL, Tokyo, Japan), supplemented with 10% fetal bovine serum and antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin), at 37°C in a 5% CO<sub>2</sub> incubator. Cells at the exponential growth phase were harvested with 0.2% trypsin, split and diluted in the medium, and seeded at a density of  $2.5 \times 10^5$  cells in a T25 flask every 2–3 days for V79 cells or every 5–6 days for the SAS cells. At 1 day prior to irradiation, the harvested cells were seeded in T25 or T12.5 flasks at  $2.5 \times 10^5$  or  $1.5 \times 10^5$  cells with 5 or 3 ml of the medium, respectively.

### Radiation treatments

Two X-ray generators were used in this experiment, both having a tungsten target, equipped with a 0.5 mm Al and 0.5 mm Cu filter, and operated at 20 mA with 150 or 200 kVp. The generator operating at 150 kVp was installed in the biology cave of the Heavy Ion Medical Accelerator in Chiba (HIMAC; National Institute of Radiological Sciences, Chiba, Japan), and it was used for the SLD repair time kinetics

study. The other generator, operating at 200 kVp, was installed in the X-ray facility building, and it was used for the fractionated secondary irradiations in the survival curve study. The dose rate was ~1 Gy/min.

We used 290 MeV/u carbon (dose-averaged LET at 13, and 100 keV/µm on the sample), 490 MeV/u silicon (55 keV/µm), 500 MeV/u argon (85 keV/µm), 500 MeV/u iron (200 keV/µm) and 200 MeV/u iron (440 and 860 keV/µm) beams, accelerated by HIMAC, as high-LET ion beams. Excess energy reducers that were made from polymethyl methacrylate (PMMA) were inserted upstream of the sample to obtain 100 or 860 keV/µm beams. We also used 290 MeV/u carbon beams with a 6 cm spread-out Bragg peak (SOBP) at the centre (50 keV/µm) [9, 10]. Those beams were delivered to the biology cave in HIMAC, and the radiation field was expanded with a scatterer and wobblers to a 10 cm diameter within 5% of dose uniformity. The radiation doses were monitored by a monitoring ionization chamber placed upstream of the sample. The output of the monitor chamber was proofed by a calibrated ionization chamber placed at the sample position. The radiation doses were controlled with the accumulated output readings of the monitor chamber.

The priming doses chosen were based on an approximate one-tenth reduction of cell survival for each beam. The test doses for the repair kinetics study were also chosen to reduce cell survival by another one-tenth, meaning that when the total dose was given without repair time, the expected total cell survival was one-hundredth.

### Repair-time kinetics

For the kinetics study of SLD repair, V79 and SAS cells were again employed. Cells of each type were lined up on an automatic irradiation table at HIMAC. The temperature of the room was controlled at 25°C. All cell samples in each experimental group were exposed to the same beam at a 10% survival dose. After exposure of the priming dose, the cells were kept at room temperature for 180–600 min until the test doses of X-ray or SOBP beam were applied. The maximum repair time was limited by the machine schedule. The time between completion of the priming dose and initiation of the test radiation was defined as the repair time.

Immediately after the test dose, the cells were collected by trypsinization (0.2%) and resuspended in the medium. Adequately diluted cells of ~100 live cells were seeded in triplicates in each 6 cm dish with 5 ml of the medium and then incubated for 6 days (for V79 cells) or 13 days (for SAS cells). Cells from the resulting colonies were fixed with 10% formalin solution and stained with 1% methylene blue, and colonies consisting of more than 50 cells were counted as survivors in order to determine the surviving fraction (SF).

### Survival curve parameters

For the survival curve parameter study, only V79 cells were used. The cell samples were sequentially irradiated with a defined LET beam at different doses for acute single-shot survival. Cell samples of the pairing group for split doses were exposed to a priming dose of each beam that was expected to allow ~10% survival. The samples were kept at 25°C for 3–4 h (limited by HIMAC and X-ray machine schedules) to allow repair, and then subjected to test X-rays (200 kVp) of different doses. Immediately after the test dose, the colony-forming assay was performed as described above.

All survival data were normalized to the 0 Gy sample for the acute single shot for each LET group. The SF data were fitted with the modified linear–quadratic (LQ) equation (1), and the multi-target model equation (2) from the target theory as follows [11]:

$$SF = \delta \cdot \exp(-\alpha \cdot D - \beta \cdot D^2) \quad (1)$$

$$SF = 1 - [1 - \exp(D/D_0)]^n \quad (2)$$

where  $SF$  and  $D$  are the survival fraction of cells and the dose,  $\alpha$  and  $\beta$  are the parameters of the curve, as in as their definition in the LQ model, and parameter  $\delta$  is the survival shift value between the priming and test irradiations. For analysis of the data for an acute single shot experiments,  $\delta$  was set as 1 (because cell survival was not shifted according to the priming radiation). Coefficients  $D_0$  and  $n$  stand on mean lethal dose and extrapolation number in the multi-target model. The curves were fitted, and the parameters were obtained by a computer program with a least-square method. The  $D_{10}$  values were calculated from  $\alpha$  and  $\beta$ .

## RESULTS

### Time kinetics of repair

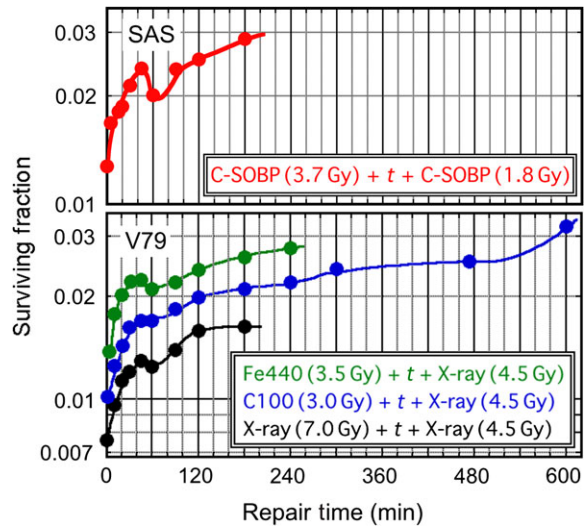
The repair kinetics of cell survival were observed as a function of repair time. A time-dependent increase in SF was clearly observed for all cases (Fig. 1). We could see the repair in hamster cells (V79) after use of monoenergetic high-LET beams or X-rays as the priming dose, with X-rays as the secondary dose. This was also seen in the human cells (SAS) after both priming and secondary doses with the therapeutic carbon SOBP beam. The dose-averaged LET of the SOBP beam, and monoenergetic carbon, iron and X-ray beams were 50, 440, 100 and 9.4 keV/ $\mu\text{m}$ , respectively [12].

Irradiated cells were quickly repaired from SLD at room temperature, showing peaks at 50 min of repair. An unexpected dip in survival was found at 60 min for all cases. The cell survival was  $\sim 2.5$  times higher after 180 min than that at time zero. The highest increase in the SF in V79 cells treated with 100 keV/ $\mu\text{m}$  carbon plus X-rays was found at 600 min.

### Survival curve parameters

Figure 2 shows the dose–response curves of V79 cells after treatment with reference X-rays (black), an acute single shot of each high-LET radiation (red), and test X-ray doses after the priming dose and repair time (green). Plots in black indicate the average and standard deviation (SD) from five independent experiments (as reference survival data) with the 200 kVp X-ray beam (Fig. 2A). All the other data, except for the reference X-rays, were produced from a single experiment. The same curves (black lines) were added to all the other panels as the reference. In this experiment, cells were exposed first to different radiations and then to 200 kVp X-rays as the test radiation following the repair time, to express the SLD repair. The horizontal axis indicates the acute single-shot dose for each radiation, or the test X-ray dose. The curves (red) became steeper with the increase of LET up to 100 or 200 keV/ $\mu\text{m}$ , and then decreased with the high-LET beams. Survival curves (green) with shapes analogous to those of the reference X-ray curve were found, with a shift in the plating efficiency.

The numerical parameters ( $\alpha$ ,  $\beta$ ,  $D_0$  and  $D_{10}$ ) of the curves in Fig. 2 are listed in Table 1. The  $\alpha$  and  $\beta$  values (mean  $\pm$  SD, from

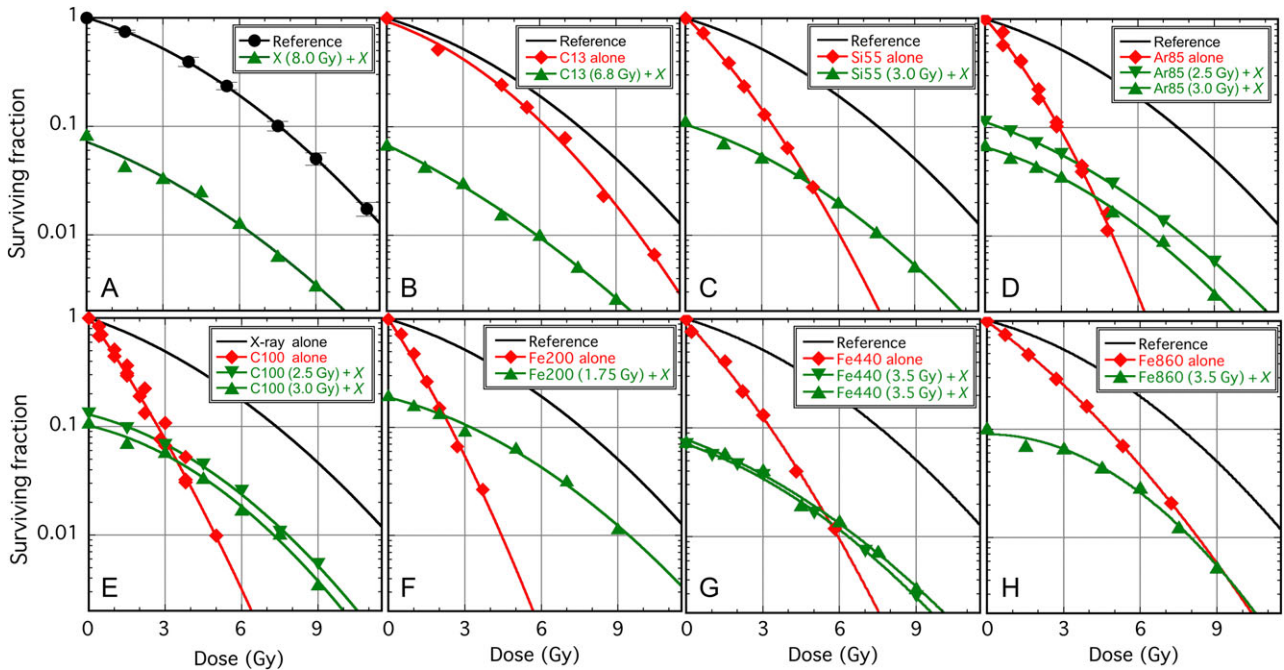


**Fig. 1.** Repair–time kinetics of the cell survival fraction after split dose irradiation. Upper panel: SAS cells irradiated with both primary and secondary radiations of 290 MeV/u carbon with 6 cm SOBP beams at the middle position, with an interval time. Lower panel: V79 cells irradiated with monoenergetic iron ions at 440 keV/ $\mu\text{m}$ , carbon ions at 100 keV/ $\mu\text{m}$ , or 150 kVp X-ray beams as the priming radiation, with an interval time, followed by secondary 150 keV X-rays. The dose for primary irradiation was selected to give a 10% survival rate, and that for secondary irradiation was selected to give another 10% survival at an interval time of zero (i.e. 1% survival in total). The cells were kept at different times ( $t$ ) at 25°C for repair.

five independent experiments) for the acute single shot (top right in Table 1) were  $0.192 \pm 0.029 \text{ Gy}^{-1}$  and  $0.0176 \pm 0.0027 \text{ Gy}^{-2}$ , respectively. Clear differences of  $\alpha$  and  $\beta$  values after an acute single shot with different LET beams were found between the groups. The  $\alpha$  values increased with increase in the LET, showing maxima at 100 or 200 keV/ $\mu\text{m}$ , and then decreased with higher LETs. However, no clear differences were found in the parameter values for the test X-rays. The values varied in a limited range around those for the reference X-rays. The averaged values of  $\alpha$ ,  $\beta$ ,  $D_0$  and  $D_{10}$  were  $0.177 \pm 0.020 \text{ Gy}^{-1}$ ,  $0.0184 \pm 0.0034 \text{ Gy}^{-2}$ ,  $2.95 \pm 0.17 \text{ Gy}$  and  $7.38 \pm 0.24 \text{ Gy}$ , respectively, for the test X-rays, showing only a slight discrepancy with respect to the reference X-ray data ( $0.192 \pm 0.029 \text{ Gy}^{-1}$ ,  $0.0176 \pm 0.0027 \text{ Gy}^{-2}$ ,  $2.62 \pm 0.22 \text{ Gy}$  and  $7.23 \pm 0.49 \text{ Gy}$ , respectively). Upon hypothesis and testing of the difference of the means, by X-rays and that by acute single shot experiments, the hypothesis was rejected by Student's  $t$ -test, with  $P$  values of 0.235, 0.635, 0.279 and 0.426, respectively. The test of equivalency was difficult to perform.

## DISCUSSION

The quick process of SLD repair must be taken into consideration in treatment planning for heavy-ion radiotherapy. We found clear time-dependent repair kinetics (Fig. 1, upper panel) of human cells



**Fig. 2.** Survival curves of V79 cells after irradiation with single (black or red) or split (green) doses. Ions and dose-averaged linear energy transfer (LET) values ( $\text{keV}/\mu\text{m}$ ) for each primary radiation with the doses are indicated in the graph keys. All secondary doses given were from 200 kVp X-rays. Cells were kept at room temperature ( $25^\circ\text{C}$ ) during the irradiations and repair interval times (see Table 1). A) Reference single-shot survival curve (mean  $\pm$  SD) for 200 kVp X-rays from five independent experiments; the same curve (black lines) is inserted in all the other panels. B–H) Red curves indicate results from the high-LET single-dose experiments on healthy cells (PE = 85–95%). Green curves are the secondary X-ray survival curves after repair from the split dose experiments; the PE of non-irradiated cells was normalized to a value of 1.

after the use of SOBP beams for cancer therapy at HIMAC. In the case of cell viability, the SF was 0.012 after 5.5 Gy ( $=3.7 + 1.8$ ) SOBP beam irradiation without intervals, increased to 0.02 at 20 min, and reached nearly 0.03 after 180 min. This quick increase in cell survival must affect the treatment results. The multifield method is applied for CIRT at HIMAC. The so-called single-fraction CIRT of lung cancer [3] consists of a four-field exposure, which takes time intervals between exposures. This consideration was, however, not taken into account in treatment planning at first, and a dose escalation study was performed instead to determine the treatment protocol. It was started with the relative biological effectiveness (RBE) dose of 28 Gy in 2003 and reached 50 Gy (RBE) in 2011. Similar kinetics (Fig. 1, lower panel) were also found in V79 cells, using monoenergetic 100 or 440  $\text{keV}/\mu\text{m}$  high-LET beams or X-rays. In this case, the 150 kVp X-ray machine installed in the biology cave was employed for the secondary irradiation in order to obtain data with short intervals. A clear incremental increase in survival with increase in repair time was found for all cases at approximately 3 h, and survival reached three times higher than that without the interval. Repair of SLD existed even after high-LET irradiation, as demonstrated by Dr Ngo using neutrons or neon ions [13, 14].

When the same ion beams were used for both the priming and test irradiations, the dose–response curves were different from those with the LET beams, and they became linear at high-LETs, making it difficult to compare the efficiency of SLD repair between different

LET beams. To test the repair efficiency after high-LET irradiations, we employed the same 200 kVp X-rays as the test radiation throughout the experiments. A similar method was used for a SLD experiment with fast neutrons [13]. Cells with LD will be killed by the priming dose and the SF will not be affected by the test dose. The SLD caused by the priming high-LET radiations will be well repaired during the interval, and the cells recover to become healthy cells. The shapes of the survival curves from the test X-rays must be analogous to one another and to that of the reference X-rays. It is generally believed that there is no damage repair, or only slight damage repair for high-LET radiations. However, efficient SLD repair was found after high-LET irradiations as X-rays in this experiment, even when the LET of the primary radiation was very high.

SLD repair kinetics have been determined by Elkind and Sutton [15] in V79 cells cultured under  $37^\circ\text{C}$ . A quick increase in the SF was observed within 1 h after X-ray exposure; it then showed a slight decrease at around several hours after the primary exposure, due to accumulation of cells at the radiosensitive cell cycle phase. This decrease at several hours after priming irradiation was not observed at  $24^\circ\text{C}$  [16]. The efficiency and the speed of the repair are slightly lower under room temperature than at  $37^\circ\text{C}$ ; however, clear repair is observed at temperatures under  $24^\circ\text{C}$  [16] (and even at temperatures under  $3^\circ\text{C}$ ). The repair kinetics in our experiments showed a small dip in the SF at 60 min for all cases. The time points for the former experiments were too coarse to detect this

**Table 1. Survival curve parameters for V79 cells**

Priming radiation		Parameters to test X-ray							Parameters to Single acute shot			
Ion & Energy (MeV/u)	LET <sup>a</sup> (keV/μm)	Dose (Gy)	δ <sup>b</sup>	α (Gy <sup>-1</sup> )	β (Gy <sup>-2</sup> )	D <sub>0</sub> (Gy)	D <sub>10</sub> (Gy)	Interval (h)	α (Gy <sup>-1</sup> )	β (Gy <sup>-2</sup> )	D <sub>0</sub> (Gy)	D <sub>10</sub> (Gy)
Reference (X-ray 200 kVp) (n = 5)		mean							0.192	0.0176	2.62	7.23
		sd							0.029	0.0027	0.22	0.49
X-ray (200 kVp)		8.0	0.072	0.182	0.0171	3.04	7.45	3.2	0.232	0.0147	2.56	6.90
C-290	13	6.8	0.068	0.197	0.0172	2.83	7.19	3.0	0.199	0.0267	2.12	6.28
Si-490	55	3.0	0.105	0.195	0.0161	3.00	7.35	3.0	0.521	0.0404	1.37	3.48
Ar-500	85	2.5	0.113	0.184	0.0163	3.03	7.50	3.0	0.620	0.0506	1.16	2.98
Ar-500	85	3.0	0.066	0.173	0.0193	2.93	7.33	3.0	0.593	0.0712	1.05	2.88
C-290	100	2.5	0.102	0.152	0.0245	2.73	7.08	3.0	0.800	0.0261	1.07	2.65
C-290	100	2.0	0.129	0.142	0.0241	2.80	7.26	4.0	0.823	0.0259	1.09	2.59
Fe-500	200	1.75	0.189	0.153	0.0171	3.31	7.96	4.0	0.822	0.0483	0.98	2.45
Fe-200	440	3.5	0.078	0.177	0.0183	2.89	7.37	4.0	0.612	0.0273	1.32	3.28
Fe-200	440	3.5	0.071	0.182	0.0198	2.78	7.13	4.0	-	-	-	-
Fe-200	860	3.5	0.090	0.208	0.0131	3.14	7.51	3.0	0.398	0.0193	1.88	4.71
		mean	0.098	0.177	0.0184	2.95	7.38					
		sd	0.037	0.020	0.0034	0.17	0.24					

<sup>a</sup>Dose averaged LET. <sup>b</sup>Shift value of survival curve at 0 Gy.

quick, small dip in cell survival. Because we scheduled much denser time points (i.e. 0, 10, 20, 30, 45, 60 and 120 min), this dip could be observed. We had thought that the cells may have accumulated in the sensitive phase to cause the dip, but evidence for this was not found by flow cytometry analysis (data not shown). If the pool size of some important DNA damage repair-related proteins gets reduced as a result of their concentration in the lesion by the priming dose, it may make repair of the damage produced by the test dose difficult. In one study [17], a part of cell nuclei in HeLa cells were irradiated at two separate times with a microbeam, and the cell nuclei at the earlier and later irradiated sites were immunostained. The fluorescent intensity of 53BP1 and Rad51 in the later irradiated site (at 50 min after the earlier irradiation) decreased to <50% of that in the earlier irradiated site, but no clear increase after 50 min was observed. On the other hand, the intensity of gH2-AX was not different before and after irradiation.

The data in green plots in Fig. 2 show the survival curves after test X-rays following priming with high-LET radiations and a repair interval. The survival curves were analogous to the reference X-rays for all cases. All numerical survival parameters after the repair interval showed similar values to those of the reference X-ray curve (Table 1). The  $\alpha$ ,  $\beta$ ,  $D_0$  and  $D_{10}$  values after the test X-rays were randomly spread in the range of 0.142–0.208 Gy<sup>-1</sup>, 0.0131–0.0245 Gy<sup>-2</sup>, 2.73–3.31 Gy and 7.08–7.96 Gy, respectively, and the respective averaged values for the reference X-rays were  $0.192 \pm 0.029$  Gy<sup>-1</sup>,

$0.0176 \pm 0.0027$  Gy<sup>-2</sup>,  $2.62 \pm 0.22$  Gy and  $7.23 \pm 0.49$  Gy. We could see no clear differences in these parameters for the test X-rays, even when the LETs of the priming radiations were different. Thus, we ignored the quality of the primary radiations, and calculated the average values of those parameters for the test X-rays (Table 1). The average values showed no significant differences to those for the reference X-rays.

The ion beam-mediated DNA lesion produced in a cell can be divided into two types: those from a ‘direct hit’ and those from a ‘glancing hit’. Ion beams have unique track structures; for example, the so-called ‘core’ and ‘penumbra’ having high and low ionization density parts around one ion traversal, respectively. We also considered the presence of a ‘domain’ (i.e. a small target volume of sub-micrometer size in a cell nucleus, such as is defined in the microdosimetric kinetic (MK) model [18, 19]), which has an important role in the killing of cells. LD (including PLD) will be produced by a direct hit on the domain at the core, whereas SLD will be produced by a glancing hit on the domain at the penumbra. This may be similar to the  $\gamma$ -kill and ion-kill theory [20]. LD causes cell killing after priming irradiation and will not influence the survival from the test irradiation, except for down-shifting of the curve. SLD is produced by glancing hits, where the LET is low and the dose will be given by  $\delta$ -rays. This lesion generated by  $\delta$ -rays must be the same as that caused by low-LET photons and is repaired as completely as the lesions from X-rays. Our results suggest that complete repair of SLD occurs even after high-LET

radiation exposure. In conclusion, a lesion of SLD is produced by glancing hit of a domain in a cell nucleus with an ion beam at a low-LET component in the track. The quality of the lesions that are caused by an ion beam must be the same to that caused by X-rays.

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#### CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

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