Cent. Eur. J. Biol. • 3(2) • 2008 • 121–133
DOI: 10.2478/s11535-008-0007-8

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Central European Journal of **Biology**

High yields of isochromatid breaks and successive formation of chromosome exchanges may lead to reproductive cell death following high-LET irradiation

Research Article

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Received 25 October 2007; Accepted 14 January 2008

Abstract: To clarify the relationship between cell death and chromosomal aberrations following exposure to heavy-charged ion particles beams, exponentially growing Human Salivary Gland Tumor cells (HSG cells) were irradiated with various kinds of high energy heavy ions; 13 keV/μm carbon ions as a low-LET charged particle radiation source, 120 keV/μm carbon ions and 440 keV/μm iron ions as high-LET charged particle radiation sources. X-rays (200 kVp) were used as a reference. Reproductive cell death was evaluated by clonogenic assays, and the chromatid aberrations in G2/M phase and their repairing kinetics were analyzed by the calyculin A induced premature chromosome condensation (PCC) method. High-LET heavy-ion beams introduced much more severe and un-repairable chromatid breaks and isochromatid breaks in HSG cells than low-LET irradiation. In addition, the continuous increase of exchange aberrations after irradiation occurred in the high-LET irradiated cells. The cell death, initial production of isochromatid breaks and subsequent formation of chromosome exchange seemed to be depend similarly on LET with a maximum RBE peak around 100 - 200 keV/μm of LET value. Conversely, un-rejoined isochromatid breaks or chromatid breaks/gaps seemed to be less effective in reproductive cell death. These results suggest that the continuous yield of chromosome exchange aberrations induced by high-LET ionizing particles is a possible reason for the high RBE for cell death following high-LET irradiation, alongside other chromosomal aberrations additively or synergistically.

Keywords: *Reproductive cell death • Chromosomal aberrations • High-LET radiation • Relative biological effectiveness (RBE) • Premature chromosome condensation (PCC)*

1. Introduction

Heavy ion irradiation in cancer therapy has been focused recently much more because of the unique physical aspects that are known as Bragg peak and the high relative biological effectiveness (RBE) [1-5]. Heavy ion particles deposit the energy in materials according to Bethe and Bloch formula [1,6] and the energy loss is roughly proportional to the square of the effective charge, and is inversely proportional to the square of the velocity of the particles. The particle velocity decreases as the energy diminishes, which promotes electron capture resulting in the decrease of effective charge. Consequently, maximum energy loss occurs at a certain depth of the incident path into the material, which is called the Bragg peak, and the ions deposit the whole residual energy at the Bragg peak and do not penetrate further [1]. The Bragg peak is thus unique to the heavy charged

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ion particle irradiation, but does not occur in X-rays or γ-rays that are utilized in conventional radiation cancer therapy [1]. The Bragg peak of heavy ion irradiation is therefore advantageous for cancer therapy, because the energy selectively attacks tumor cells located in deep body [7,8]. The charged particles also lose energy by ionization and excitation along the track. The ionizing range is defined by locally absorbed energy per unit distance (eV/μm), which is called linear energy transfer (LET). High energy, heavy and multi-ionized charged particles can cause dense ionization or excitation along their path, which subsequently generates many lower energy electrons and active radicals. Consequently, the heavy ion beams severely injures cancer cells along the ion beam pathway. Thus, heavy ion irradiation is more suitable for cancer therapy than conventional irradiation therapy, which utilize X-rays or γ–rays [4].

Many basic studies have been performed to show the effectiveness of the heavy ion irradiations on various biological samples *in vivo* and *in vitro*, *i.e.* animals, tissues, cultured cells, chromosomes and DNA, using various kinds of heavy ions and with various LET values. It is obvious from these studies that the RBE of heavy ion irradiation is dependent on LET values; heavy ion irradiation shows much higher RBE values in certain ranges of LET. These basic experiments have identified the relationship between the physical property of heavy ion irradiation and the biological effects.

Heavy ions have previously been shown to induce either cell death or chromosomal aberrations [9-17]. However, few studies have been published on the relationship between cell death and chromosomal aberrations [18-21]. In this study, we used a unique human salivary gland tumor cell line (HSG) and tried to analyze the relationship of cell death and chromosomal aberrations on the same cell line. We achieved the irradiations with several kinds of heavy ion charged particles with several LET values; 13 keV/μm Carbon (C) ions (290 MeV/nucleon), 120 keV/μm C ions (290 MeV/nucleon) and 440 keV/μm Iron (Fe) ions (200 MeV/nucleon). X-rays were used as a control. Cell death was evaluated by a colony formation assay and chromosomal aberrations were analyzed by the recently developed calyculin A-induced premature chromosome condensation (PCC) method [22]. PCC is usually achieved by a cell fusion method using either fusogeneic viruses or polyethylene glycol (fusion-mediated PCC) [23,24]. PCC is a very useful method and has been used widely in the cytogenetic field, particularly in radiation biology for analyzing chromosome aberration following ionizing irradiation [20,25-33]. However the use of viruses or polyethylene glycol is occasionally problematic [34]. The drug-induced PCC technique has several advantages over the fusion-mediated PCC [34] and is now becoming much more popular in a wide range of cytogenetic fields [35-43]. The drug-induced PCC method is particularly useful for analyzing the chromosomal aberrations following high-dose irradiation [35,40], which allows the chromosome aberrations to be observed immediately after the irradiation and enables the analysis of time dependent repair kinetics of chromatid break/rejoining [38,44].

The present study demonstrates that low-LET (X-rays and C 13 keV/μm ions) and high-LET (C 120 keV/μm ions and Fe 440 keV/μm ions) irradiation yield different quality and quantity of chromosomal aberrations. Different chromosomal aberration kinetics were also obtained between high- and low-LET irradiations. Accumulation of exchange formation aberrations in cells following high-LET irradiation might be a possible key event for the reproductive cell death associated with other chromosome damages.

2. Experimental Procedures

2.1. Chemicals

Calyculin A was purchased from Wako Chemicals (Osaka, Japan). 100 μg calyculin A was dissolved in 1 ml ethanol (100%) to constitute a 100 μM stock solution and stored at -20 °C.

2.2. Preparation of cells

Human salivary gland tumor cells (HSG cells) were used throughout the study. This cell line was originally established from an irradiated human submandibular gland which showed no neoplastic lesion [45]. HSG cells have several interesting features; the cells occasionally form a glandular arrangement *in vitro* and *in vivo*, and transplantation of cells into nude mice will result in adenocarcinoma [46]. Cells were maintained in growing exponentially in Eagle's minimum essential medium (E-MEM) supplemented with 10% fetal calf serum (FCS) at 37°C in 5% CO_2 atmosphere. HSG cells were grown in a monolayer with a doubling time of 24 hours.

2.3. Irradiation

Heavy charged ion particle irradiation was carried out at the Heavy Ion Medical Accelerator in Chiba (HIMAC) facility of the National Institute of Radiological Sciences (NIRS), Chiba, Japan. The ion sources, energy and LET values used for the irradiation experiment are listed in Table 1. The different LET was obtained by inserting a different thickness of Lucite degrader. The dose rates were measured at the sample position with a calibrated

ionization chamber, which ranged from 1 to 2 Gy/min. The LET values were determined by fitting a theoretical dose *versus* Bragg peak curve and the measured dose rates at water-equivalent depths [47]. Cells were irradiated by a computer-aided irradiation system. X-rays (200 kVp, 20 mA; tungsten target, 0.5 mm Aluminum + 0.5 mm Cupper filter; 0.856 Gy/min) was generated (Model Pantac-320S Shimadzu Co., Tokyo, Japan) and used as a reference.

ion sources	LET (keV/μm)
C ions 290 MeV/nucleon	13 *, 30**, 50**, 70**, 90**, 120*
Fe ions 200 MeV/nucleon	440*
Si ions 490 MeV/nucleon ***	50, 125, 150, 200, 250, 300, 400, 500
Ne ions 230 MeV/nucleon ***	45, 80, 100, 120

Table 1. Accelerated ion sources and the LET values used for either for the reproductive cell death study or the chromosomal aberrations study.

**: LET used for analysis both in reproductive cell death and chromosome aberration*

***: LET used single in reproductive cell death*

****: Study only in reproductive cell death*

Fifty thousand (5 x 10^5) cells were seeded in a 25 cm^2 bottle flask a day before irradiation. The culture flasks for analyzing cell death were placed on ice or in a cooling unit to maintain a low temperature during irradiation, whilst the flasks for chromosomal aberration analysis were kept at room temperature during irradiation.

2.4. Analysis of cell death using colony formation assay

To compare the effect of recovery process after irradiation, two identical culture dishes were prepared; one was for the cells without 12 hours repair (no recovery time, immediately plating, IP), another was for the cells that were permitted to recover for 12 hours before plating for the clonogenic assay (delayed plating, DP). For DP, the cells were cultured in FCS-free medium placed in the incubator at 37°C for 12 hours after irradiation. For plating, cells were trypsinized with 0.1% trypsin, and the cells were counted, diluted with serum free Eagles medium (MEM), and adjusted to an appropriate cell density that gave approximately 100 surviving colonies per dish. The cells were then seeded into each dish. Dishes were then incubated at 37°C for 13 days in MEM supplemented with 10% FCS. The colonies were fixed with 3.7% formaldehyde (10X dilution of commercially available formaldehyde solution) and stained with 1% methylene blue. Colonies were scored if they contained more than 50 cells.

2.5. Analysis of chromosomal aberrations using drug-induced premature chromosome condensation (PCC)

After irradiation, chromosomes were forced to condense prematurely with calyculin A. Details of the drug-induced PCC technique were as previously described [22,35,42]. 50 nM of calyculin A was added to the medium immediately after irradiation or at 12 hours after irradiation and incubated for 30 minutes at 37°C. Cells were harvested and centrifuged at 250 x *g* for 5 minutes. Cell pellets were re-suspended in 1.5 ml of 75 mM KCl for hypotonic treatment and kept at 37°C for 20 minutes. Then, the same volume of Carnoy's fixative (methanol:acetic acid 3 parts:1 part) was added and the cells were centrifuged again. The cells were then fixed three more times. Cells suspended in Carnoy's fixative were dropped on a glass slide, air-dried and stained with 5% giemsa in 1/15 M PBS (pH 6.8). At least 50 G_2/M PCC spreads were observed for each data point and chromatid breaks/gaps, isochromatid breaks or exchange aberrations were scored using standard scoring criteria [48]. The excess chromosome fragment number was scored as isochromatid breaks. Chromatid breaks in two different chromosomes can rejoin and form either complete or incomplete exchange involving two chromosomes. Complete exchange results from the rejoining of four broken ends between two individual chromosomes. This pattern was scored as one chromatid exchange with no residual breaks. Incomplete exchange results from the rejoining of two broken ends out of four. This pattern was scored as one chromatid exchange with two residual breaks. Usual exchange aberrations (T-shaped or cruciform-shaped exchange) were scored as one chromatid exchange. A complex exchanged aberration, which involves more than 3 chromosomes, was scored as n-1 chromatid exchanges (n is number of involved chromosomes). Repair kinetics of chromatid aberrations were analyzed as described previously [38], except that the kinetics were fitted to linear functions.

3. Results

3.1. Cell death

Figure 1 shows the surviving fractions (SF) of HSG cells irradiated with X-rays, 13 keV/μm, 120 keV/μm C ions, or 440 keV/μm Fe ions. SF of X-rays irradiation shows a typical shape with an obvious shoulder. In contrast, SF of 440 keV/μm Fe ion showed almost no shoulder and decreased simple exponentially with the dose. The SF seen in 13 keV/μm C ion or 120 keV/μm C ion irradiation showed intermediate characteristics between the SF of

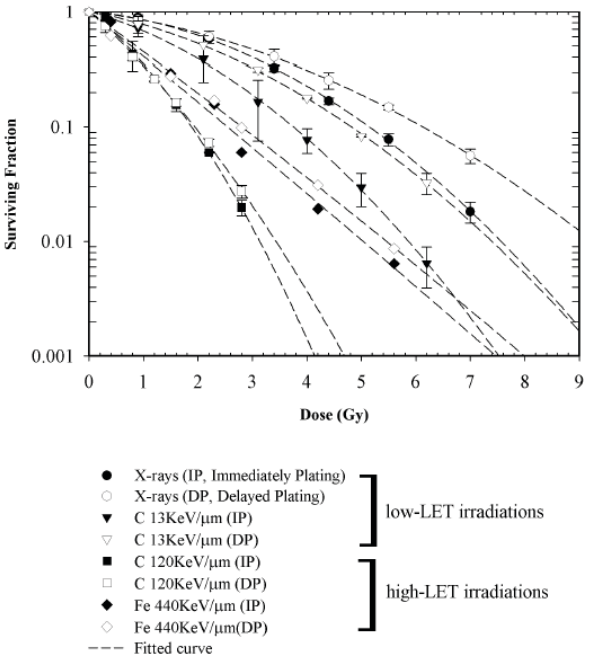

Figure 1. Surviving fractions (SF) as a function of irradiation dose (Gy) of HSG cells irradiated with different kinds of sources; 200 kVp X-rays, 13 keV/μm and 120 keV/μm C ions and 440 keV/μm Fe ions. SF for each irradiation sources was obtained from two different experiments either IP or DP following the irradiation.

X-rays and 440 keV/μm Fe ions. A linear-quadratic (LQ) model was applied to specify the individual SF curve, which is designated as follows:

$$SF = \exp(-\alpha D - \beta D^2)$$

where SF is the surviving fraction, D is the irradiation dose and the parameters α or β were fitted to the LQ model.

As shown in Table 2, the values α and β were maximal at 120 keV/μm C ions both in IP and DP except in the case of α for IP. C ion irradiation of 120 keV/μm was most effective for cell death of HSG cells in the case of DP. Conversely, the α/β ratio is far greater in the irradiation with 440 keV/μm Fe ions, as the β value was the lowest recorded. It seems that Fe ions may cause serious damage in a cell even after the hit of a single ion. Hence, this may be the reason why the SF of high-LET irradiations is linearly dependent on dose but not linearly dependent on the square of dose.

Table 3 shows the 10% survival dose (D_{10} values). The D_{10} value was minimal in the cells irradiated with 120 keV/μm C ions both in IP and DP. These data seems to suggest that the heavy ions of LET around 120 keV/μm kill the cells most effectively. Figure 2 shows the LET dependence of the D_{10} value both in IP and DP. The LET that gives the minimum D_{10} value ranged from 100 to 200 keV/μm either in IP or DP.

irradiation source	plating following irradiation	parameters		
		α	β	α/β
X-rays	IP (Immediately Plating)	0.11	0.066	1.7
	DP (Delayed Plating)	0.14	0.039	3.6
13 keV/μm C ions	IP	0.31	0.08	3.9
	DP	0.21	0.056	3.8
120 keV/μm C ions	IP	0.86	0.19	4.5
	DP	0.94	0.1	9.4
440 keV/μm Fe ions	IP	0.9	0.042	210
	DP	0.68	0.027	25

Table 2. Survival Curve Parameters obtained after irradiation with X-rays, 13 keV/μm C ions, 120 keV/μm C ions and 440 keV/μm Fe ions for IP (without 12 hours repair treatment) or DP (with 12 hours repair treatment) following irradiation.

irradiation sources	D_{10}- (Gy)	D_{10}+ (Gy)
X-rays	5.1	6.2
13 keV/μm C ions	4.2	5.3
120 keV/μm C ions	1.7	2
440 keV/μm Fe ions	2.5	2.8

Table 3. D10 dose of survivals for different irradiation source either in case of DP (D_{10}+) or IP (D_{10}-).

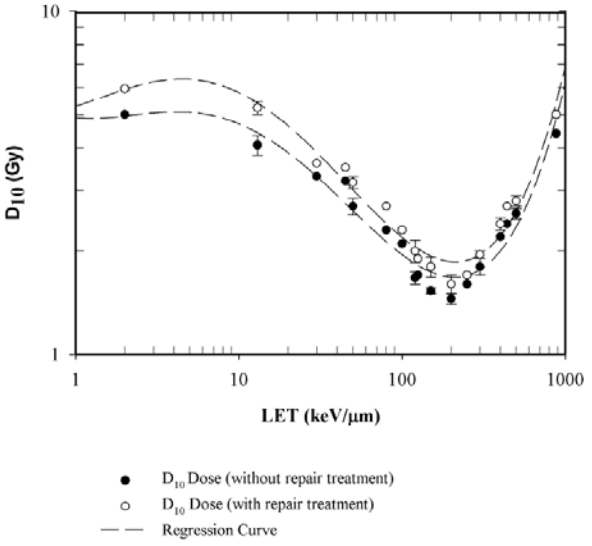

Figure 2. The LET dependences of the D10 value (Dose for 10% of surviving) either IP (Immediately Plating) or DP (Delayed plating) following irradiation.

3.2. Chromosomal aberrations

3.2.1. The dependence of chromosomal aberrations on the radiation quality

To clarify the relationship between cell death and chromosomal aberrations, exponentially growing HSG cells were irradiated in the same way as was performed for the colony survival assay. Forced condensed premature chromosomes were obtained by calyculin A. Irradiation dose was 2 Gy. We expected to obtain and analyze both chromatid type aberrations originating from G_2/M phase

irradiated cells and chromosome type aberrations originating from G_1 phase irradiated cells. However, we were able to obtain and analyze only chromatid type aberrations. For simplicity, unless stated otherwise, we classified low-LET irradiation as X-rays and 13 keV/μm C ions, and high-LET irradiations as 120 keV/μm C ions and 440 keV/μm Fe ions. Figure 3 shows a typical appearance of G_2 phase chromosomes of HSG cells irradiated with X-rays, 13 keV/μm C ions, 120 keV/μm C ions or 440 keV/μm Fe ions. PCCs of immediately after irradiation (0 hour) and at 12 hours after irradiation are shown. Immediately after irradiation, G_2/M PCCs seen in low-LET irradiations showed many chromatid breaks/gaps (Figure 3a-d). In G_2/M PCCs, following irradiation with 120 keV/μm C ions (Figure 3e), chromatid breaks/gaps were also produced, but the lesions seemed to be much more severe. G_2/M PCCs following further irradiation with 440 keV/μm Fe ions (Figure 3g) looked very badly damaged ("fragmented appearance of chromosome"). It was peculiar and should be noted that the PCCs irradiated with 440 keV/μm Fe ions showed the co-existence of non-damaged chromosomes and severely damaged chromosomes in the same spread, whilst low-LET irradiations damaged the chromosomes almost evenly. In G_2/M PCCs at 12 hours after irradiation, most of chromatid breaks/gaps aberrations rejoined and disappeared in most cells. However, some lesions still un-rejoined and additional exchange aberrations further appeared. The most complicated exchange aberrations were seen particularly in the cells irradiated with 120 keV/μm C ions (Figure 3f). It is also notable that severely damaged chromosomes induced by 440 keV/μm Fe ion irradiation still remained, even at 12 hours after irradiation (Figure 3h). This finding suggests that the chromosome damage introduced by 440 keV/μm Fe ions is less likely to be repaired.

3.2.2. Distribution of total chromosome fragment number

As HSG cell is an aneuploid cell line, as are many other cancer cell lines, the average chromosome numbers of the cells that were used as controls for irradiation with X-rays, C ions, and Fe ions were 68.6, 69.7, and 70.0, respectively. Figure 4 shows the PCCs distribution of the total chromosome fragment number immediately after irradiation or after 12 hours repair following irradiation with X-rays, 13 keV/μm C ions, 120 keV/μm C ions and 440 keV/μm Fe ions. Immediately after irradiation, the distributions of total chromosome fragment number in cells irradiated with low-LET showed a very similar pattern having a narrow peak. However, the distribution of chromosome fragment numbers in the PCCs irradiated with high-LET were fairly different and showed

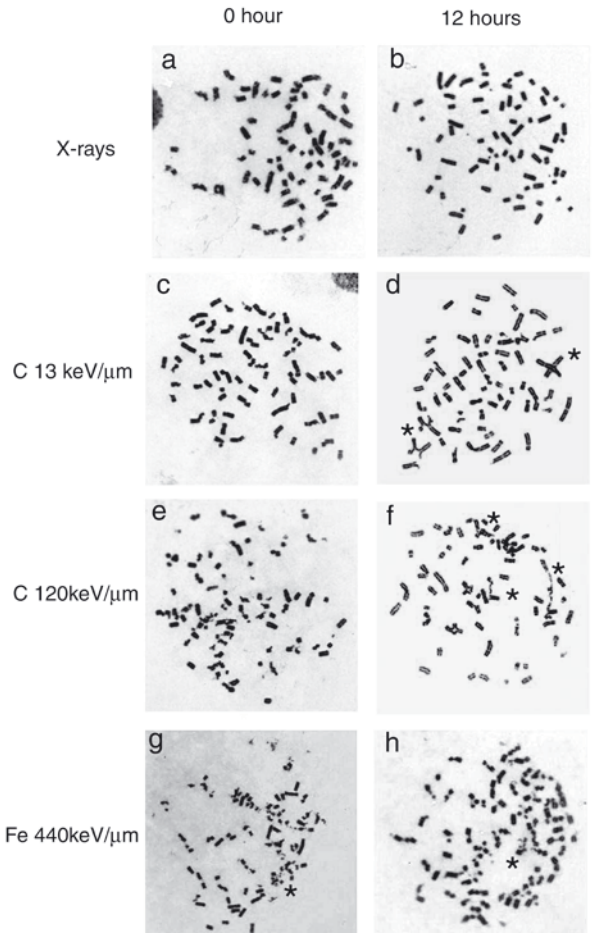

Figure 3. G2 PCCs of HSG cells irradiated with different kinds of sources; 200 kVp X-rays, 13 keV/μm and 120 keV/μm C ions and 440 keV/μm Fe ions. Chromosomes obtained immediately after irradiation (*i.e.* 0 hours) or 12 h after irradiation were shown. (a) Irradiated with 200 kVp X-rays immediately after irradiation and (b) 12 h after irradiation. The chromatid breaks seen at immediately after irradiations were mostly repaired at 12 h after irradiation. (c) Irradiated with 13 keV/μm C ions immediately after irradiation and (d) at 12 h after irradiation. The chromatid breaks seen immediately after irradiations were mostly repaired, as with X-rays, but some exchanges were instead produced (typical chromatid exchanges are shown with asterisks). (e) Irradiated with 120 keV/μm C ions immediately after irradiation and (f) at 12 h after irradiation. Many chromatid breaks were seen at immediately after irradiation, and at 12 h after irradiation, the breaks were mostly repaired but some exchange type aberrations were generated (shown with asterisks). (g) Irradiated with 440 keV/μm Fe ions immediately after irradiation and (h) at 12 h after irradiation. Chromosomes were very badly damaged (smashed appearance, shown with asterisks) immediately after irradiation, and the damages remains un-repaired at 12 h after irradiation (also shown with asterisks).

a broader distribution. The increase of total chromosome fragment number can be attributed to the production of isochromatid breaks. Questions maybe arise whether there is a possibility that some G_1 cells having chromatid breaks would pass through S phase and reach G_2 phase

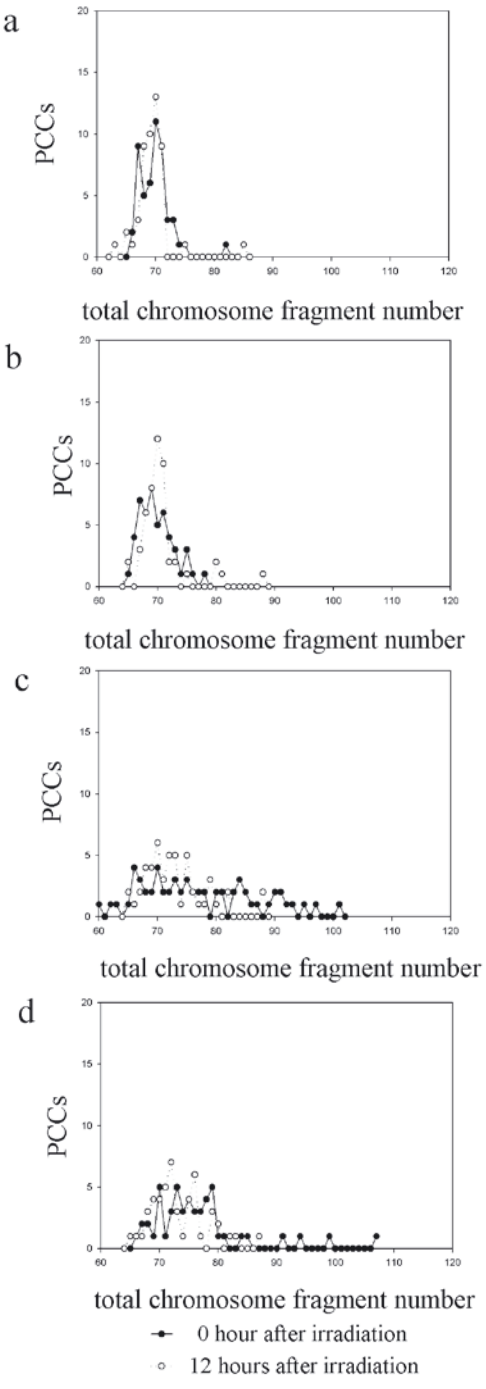

Figure 4. Histograms showing the distribution of total chromosome fragment number irradiated with (a) X-rays, (b) 13 keV/μm C ions, (c) 120 keV/μm C ions and (d) 440 keV/μm Fe ions either at immediately after irradiation (0 hour) and 12 after irradiation (12 hours).

after 12 hours incubation time, and would therefore be counted as fragments. However, this possibility can be ruled out because the fragments observed in this study were always coupled with other kinds of chromatid aberrations such as chromatid gap or break that occurred in G_2 irradiation. As shown in Figure 4 (distribution of total chromosome fragment number), low-LET irradiations rarely produced isochromatid breaks, whilst high-LET irradiations produced a significant number of isochromatid breaks, as previously reported [20,44].

3.2.3. Chromatid breaks/gaps

Chromatid breaks/gaps, which are thought to be produced primarily at the time of irradiation, were scored. Low-LET irradiation preferentially induces chromatid breaks/gap, whilst high-LET irradiation tends to produce isochromatid breaks rather than chromatid breaks/gaps. Therefore, we scored one isochromatid break as two chromatid breaks/gaps and evaluated as total chromosome break number per cell, as previously reported [44],

Total chromosome break number = chromatid breaks/gap + 2x isochromatid breaks.

Figure 5 shows the histograms of the total number of breaks/gaps generated in the cells immediately after irradiation (shown as 0h) and at 12 hours after irradiation (shown as 12h). In the 0 hour irradiation samples, the distribution seen in low-LET irradiation looked like a normal Poisson distribution. We tried to fit the data to the Poisson distribution ($f(x) = \exp(-\lambda)*\lambda^x/x!$, $\lambda>0$, x is positive integer), and obtained a sufficient fitting only in chromatid breaks at 12 hours after irradiation of X-rays (Figure 5a, shown by dashed dotted line, equivalent to $\lambda=0$). However, the distribution seen in high-LET irradiation seemed to be different from the Poisson distribution, consisting of two populations; one having less breaks (<10) and the other having >10 breaks. The distribution seen after 13 KeV/μm C ion irradiation seemed to be an intermediate pattern between X-rays and high-LET heavy ions irradiations. After 12 hours incubation, as shown in Figure 5, the number of breaks decreased to less than 10 in most of the cells irradiated with low-LET. This is not the case for high-LET radiation, the cells still having more than 10 breaks were often found and the number of such cells irradiated with 440 keV/μm Fe ions is higher than that with 120 keV/μm C ions. These results suggest that the breaks induced by high-LET irradiation were not likely to be repaired and this tendency is more pronounced in 440 keV/μm Fe ion irradiation than with 120 keV/μm C ions.

3.2.4. Repair Kinetics of chromosomal aberrations

In Figure 6, the kinetics of breaks/gaps rejoining, isochromatid breaks rejoining and exchange formation following irradiation are shown. All of data were fitted to a linear function, $f(t) = at + b$, where t is hours after irradiation. It was not possible to fit the data to other higher functions (such as an exponential decay) as in

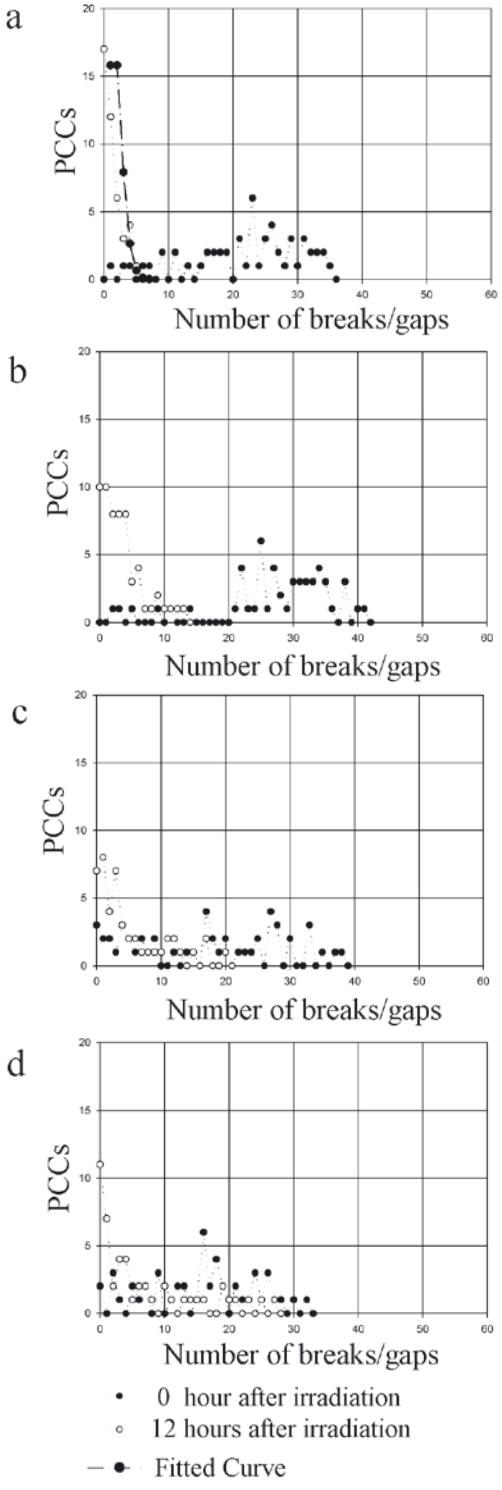

Figure 5. Histograms showing the number of total chromatid breaks/gaps per cell irradiated with (a) X-rays, (b) 13 keV/μm C ions, (c) 120 keV/μm C ions and (d) 440 keV/μm Fe ions in either at immediately after irradiation (0 hour) and 12 after irradiation (12 hours).

the previous studies [38]. This is presumably due to a requirement for more data points to obtain a sufficient

	a	b	R^2
breaks/gaps			
X-rays	-1.41	16.55	0.93
C 13KeV/μm	-1.85	25.05	0.98
C 120 KeV/μm	-1.06	17.98	0.98
Fe 440 KeV/μm	-0.85	17.40	0.93
isochromatid breaks			
X-rays	-0.07	1.54	0.80
C 13KeV/μm	0.03	0.63	0.71
C 120 KeV/μm	-0.31	6.64	0.75
Fe 440 KeV/μm	-0.24	6.44	0.73
exchange			
X-rays	-0.09	0.69	0.22
C 13KeV/μm	-0.037	0.77	0.24
C 120 KeV/μm	0.02	0.89	0.28
Fe 440 KeV/μm	0.04	0.31	0.37

Table 4. Kinetics of chromatid breaks/gaps, isochromatid breaks and exchange following X-rays, C 13KeV/μm, C 120 KeV/μm or Fe 440 KeV/μm heavy ion irradiation. The kinetics were fitted to the linear function, $f(t)=at+b$, where t is the hours after irradiations. R is coefficient value.

fit. The obtained parameters for individual kinetics are summarized in Table 4. The kinetics showed the clear difference between low- and high-LET irradiation. In kinetics of breaks/gaps rejoining, as shown in Figure 6a, the kinetics seemed to be dependent on LET value and are classified into two groups. In the case of low-LET irradiation, the number of breaks/gaps reduced rapidly. In the case of high-LET irradiation, the number of breaks did not decrease as rapidly as seen in the low-LET irradiation and un-rejoined chromatid breaks remained at a high level after 12 hours, in spite of the initial number of breaks remaining low. In the kinetics of isochromatid breaks rejoining, shown in Figure 7b, the number scored in the low-LET irradiations did not significantly change during 12 hours incubation time. The numbers of isochromatid breaks are kept as low; around 1 or 2 per a cell. However, high-LET irradiation caused more isochromatid breaks; around 6 or 7 per cell immediately after irradiation. The number of isochromatid breaks decreased during the incubation time, but at 12 hours after irradiation the number of un-rejoined isochromatid breaks still remained around 3 or 4 per cell.

Figure 6c shows the kinetics of exchange aberration formation. Although the obtained R^2 values were not high, the kinetics of exchange formation clearly showed a different tendency between high- and low-LET irradiations. In the case of low-LET irradiation, the exchange aberrations slightly diminished during the 12 hour repair time after irradiation. Whilst in the cases of high-LET irradiation, the increase of formation of exchanges continued and increased by 12 hours after

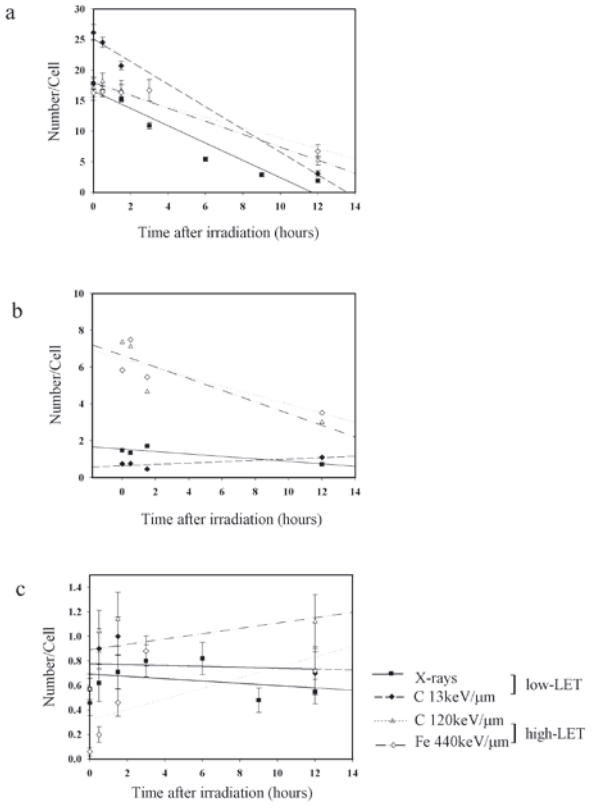

Figure 6. Kinetics of (a) breaks/gaps, (b) isochromatid breaks and (c) exchange formation irradiated with X-rays, 13 keV/μm C ions, 120 keV/μm C ions and 440 keV/μm Fe ions.

irradiation. Particularly, in the case of irradiation with 440 keV/μm Fe ions, the number of exchanges was the lowest immediately after irradiation. However, at 12 hours after irradiation the number of exchanges further increased more than the numbers seen in X-rays and 13 keV/μm C ions. The reason remains unclear why the exchange aberration produced by high-LET irradiation accumulated during the cell cycling, but un-rejoined chromatid breaks/gaps or isochromatid breaks may be more likely converted to the exchange aberrations during the repair process, particularly in high-LET irradiations.

3.2.5. The LET dependency of RBE for producing chromatid breaks/gaps, isochromatid breaks and exchanges

Figure 7 shows the LET dependency of RBE for the production of chromatid breaks/gaps, isochromatid breaks and exchange aberrations. As shown in Figure 7a, the RBE peak of the number of breaks/gaps induction was around 13 keV/μm. Conversely, the RBE peak of isochromatid breaks induction was around 120 keV/μm and a significant difference between the RBE values for low- and high-LET irradiation was observed. After 12 hours irradiation, the RBE for breaks/gaps and isochromatid breaks increased with the

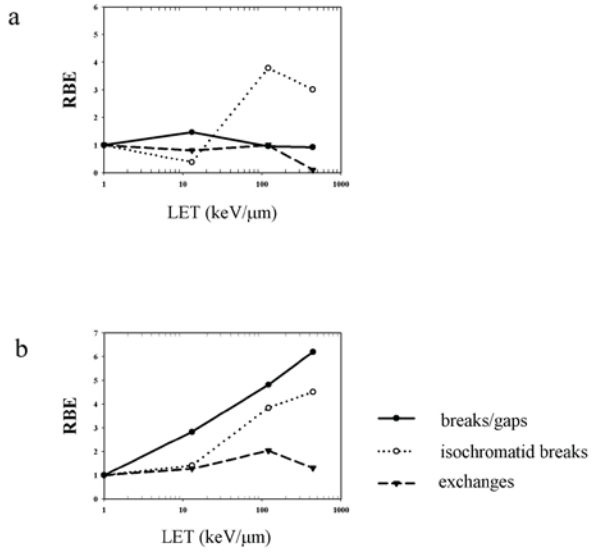

Figure 7. RBE for chromatid breaks/gaps, isochromatid breaks and exchange formation as a function of LET values either (a) immediately after irradiation (0 hour) or (b) at 12 after irradiation (12 hours).

increase of LET. Conversely, the exchange formation was at the highest RBE around 120 keV/μm of LET. As the LET dependence of RBE for the reproductive cell death was maximal around 120 keV/μm of LET, reproductive cell death is seemed to be well coupled with initially produced isochromatid breaks and successive formation of exchange aberrations induced by high-LET irradiations.

4. Discussion

It is widely accepted that X-rays or γ-rays irradiation (*i.e.* low-LET irradiation) is mainly responsible for cell death in G_1/G_0 phase cells because G_1/G_0 cells are particularly sensitive to X- or γ-rays irradiation, but G_2/M cells are less sensitive [10]. When cells are irradiated at G_2/M phase with X-rays or γ-rays, chromatid-type aberrations are most frequently induced. Regarding the chromatid breaks, one or both strands of a bivalent chromatid remains intact. The damaged strand will be eventually repaired referring the base sequences of the intact counterpart strand either using homologous recombination repair (HRR) or non-homologous end joining (NHEJ) (see reviews, for example [49]). These are the possible reasons why G_2/M cells are less sensitive to X-rays or γ-rays than G_1 cells. However, this will be not the case for heavy charged ion particle beam irradiation. The different track structure from X-rays or γ-rays, heavy ion beams can cause dense ionization and excitation along the pathway [50]. Accordingly, high- and low-LET irradiation sources may produce qualitatively and

irradiation sources	12 hours repair treatment		%unrepaired break/gaps (%repaired) [1]
	without	with	
X-rays	19.0 ±1.1	1.9±0.2	10.0±2.0 (90.0)
13 keV/μm C ions	26.1±0.4	3.1±0.3	12.0±1.0 (88)
120 keV/μm C ions	18.0±0.8	5.2±0.8	29.0±6.0 (71)
440 keV/μm Fe ions	17.5±1.5	6.8±0.9	39.0±9.0 (61)

Table 5. Average number of breaks/gaps per cell for each case of irradiation with and without 12 hours repair treatment.

[1] % unrepaired break/gaps is obtained as following calculation;
% unrepaired break/gaps = [break/gaps] with repair/[break/gaps]without repair x 100

quantitatively different types of chromosome aberrations in the cells. Thus, in case of high-LET irradiation, chromosome aberrations in G_2/M phase cells might be much more responsible for reproductive cell death as well as in G_1/G_0 phase chromosome aberration.

We analyzed the relationship between reproductive cell death and chromosome aberrations as LET dependency. As shown as G_{10} dose in Figure 2, cells were effectively killed at the LET of around 100 - 200 keV/μm. This result almost coincides with previous studies on the same HSG cells [51]. In the other studies using different cell lines and different irradiation sources, the LET dependence of cell death was reported to have a sharp increase or a peak in LET range of 100 - 120 keV/μm C-ion irradiation [21] or 120 - 300 keV/μm Ne-ion irradiation [52], which also seemed in accordance with the present study. Compared to the LET-dependence of the cell death with that of the chromosomal aberrations, the LET-dependence of the exchange formation after 12 hours irradiation is fairly similar to that of cell death, but less similarity of LET-dependence of isochromatid breaks or breaks/gaps at after 12 hours irradiation was obtained (shown in Figure 7b). These results suggest that chromosomal exchange formation induced by the high-LET irradiation might be one of the key steps leading to cell death, whilst un-rejoined isochromatid breaks or breaks/gaps are more tenuously coupled to cell death. Simple breaks like chromatid breaks/gaps might be easily repairable, and less liable to prove lethal to the cells.

Several studies have reported the relationship between cell death and chromatin breaks using high-LET irradiation [21,52-54]. In these studies, un-rejoined chromatin breaks following irradiation showed a strong correlation to the cell death. However, these results differ from our study, and the reason remains unclear. We speculate as follows; Chromatid breaks/gaps or isochromatid breaks generated by high-LET irradiations are much more un-repairable or mis-repairable than those generated by low-LET irradiation. During the repair process, chromatid breaks/gaps or isochromatid breaks are more liable to convert to the formation of exchange aberrations, particularly in the high-LET irradiation cases. Once exchange aberration chromosomes are introduced in cells, cell division or accurate segregation of chromosomes during mitosis might be profoundly disturbed, resulting in cell death.

Different quality and quantity of chromosome damage was found between the high-LET radiation and low-LET radiation. Regarding the primary aberrations seen at immediately after irradiation, high-LET irradiations frequently generated isochromatid breaks while low-LET radiations produced more chromatid breaks. After 12 hours incubation, most of the lesions seen in low-LET irradiation were rejoined while most of the chromatid breaks/gaps and isochromatid breaks produced by high-LET irradiations still remained un-rejoined with a comparatively high number. As shown in Table 5, 90% or 88% of the chromatid breaks/gaps induced by X-rays or 13 keV/μm C ions were rejoined within 12 hours after irradiation, respectively; but only 71% or 61% of breaks/gaps induced by 120 keV/μm C ions or 440 keV/μm Fe ions were rejoined, respectively. These findings may suggest the different quality of primary chromosome aberrations between high- and low-LET irradiation. The lesions generated by high-LET irradiations seemed to have severe damage that was difficult to be repaired. This observation was also reported previously at the level of chromosome or DNA [15,20,44,55]. Goodhead explained this *via* the basis of radiation track structure at the level of DNA [56,57]. In principle, for all cases of irradiation, there are large amounts of “minor damage” as single ionizations or excitations that can lead to simple base damage or single strand breakage of DNA. But, in the case of high-LET irradiation, there are also significant numbers of “more severe types of damage”, as initially highly localized clusters of ionizations and excitations that can lead to multiple strand breaks of DNA, associated multiple base damage, and cross-linking between DNA with protein. These more complex types of damage may be more difficult to be repaired and these un-repaired damages may be lethal to the cell. In addition, to produce an abundance of the small clusters *via* their delta-ray electrons, high-LET irradiation is also able to produce much more ionization clusters than low-LET irradiations. Therefore it is expected that high-LET irradiation will produce the more complex damages that are more difficult to be repaired. This hypothesis seems to be consistent with the results obtained in this study, although we analyzed the DNA damages in higher structure level of chromosomes rather than chromatin DNA.

The different effects observed between high- and low-LET irradiation is also shown in Figure 4 (histograms

of chromatid breaks/gaps). The distributions of the chromatid breaks/gaps produced by the irradiation with high-LET irradiation are broader and like a Neyman type A distribution as reported previously [20,58], while those with low-LET irradiations are narrow and show a typical Poisson like distribution. Since charged particles of high-LET produce more ionization/excitation along with these tracks compared with those of low-LET [56], thus the number of incident charged particles of high-LET ions is much smaller than that of low-LET radiation as to give the same absorbed dose in material. Small numbers of high-LET particles irradiate the cells in a more heterogeneous fashion than low-LET radiation. Hence, Neyman type A distributions may provide a more suitable explanation for the distribution of high-LET irradiation-produced chromatid breaks/gaps [58].

In the present study, we investigated the relationship of reproductive cell death and chromatid aberrations in G_2 cells as the LET dependency. We irradiated exponentially growing cells both for clonogenic assays and for chromosomal aberration analysis. It was expected that both types of aberration would occur (chromosome type aberrations originated from irradiated at G_1, chromatid type aberrations originated from irradiated at G_2/M). However, the aberrations we obtained were purely G_2 chromatid aberrations. Why no chromosome type aberrations were observed in this study remains unclear. The HSG cells irradiated in G_1 presumably did not enter in G_2 because of a cell cycle delay after irradiation, and the cells were not subjected to PCC at the sampling time. Hence, the relationship between cell death and G_2/M chromatid aberrations was analyzed. Future work will involve an in depth investigation of how both G_1/S- and G_2-aberrations contribute to cell death.

5. Conclusions

(a) According to the LET dependence study, production of the exchange type of chromosome aberration after high-LET irradiation could be one of essential causes of reproductive cell death, whilst the chromatid breaks/gaps produced by high-LET irradiations seem less likely to lead to cell death directly. However, this may just be specific for the HSG tumor cells in the current study.

(b) Some interesting differences in chromosomal damage was seen between high- and low-LET irradiation. Regarding the primary aberrations, high-LET heavy ions predominantly generated isochromatid breaks, while low-LET radiation mainly produced chromatid breaks/gaps rather than isochromatid breaks. At 12 hours after irradiation, most of the lesions produced by low-LET irradiations were repaired, whilst most of the lesions generated by high-LET irradiation of both chromatid breaks/gaps and isochromatid breaks still remained. About 90% of chromatid breaks induced by low-LET irradiations were rejoined within 12 hours after irradiation. Conversely, almost 70% of chromatid breaks induced by high-LET irradiation were repaired within 12 hours of irradiation. The lesions introduced by high- and low-LET irradiation differ qualitatively and quantitatively [59].

(c) The distributions of the isochromatid breaks and chromatid breaks produced by irradiation with high-LET heavy ions may be defined by a broader Neyman type A distribution as reported previously [20,58], while those with low-LET ions are narrow, with a Poisson like shape. This may reflect the different physical track structure between high-LET and low-LET irradiation [56,60].

Acknowledgement

We declare that there is no conflict of interest to influence the result in the present study. We would like to express our great thanks to Yoshiya Furusawa and Mizuho Aoki for technical assistance and all staff members for their excellent maintenance and operation of the Heavy Ion Medical Accelerator in Chiba (HIMAC). This study was performed under the approval of National Institute of Radiological Sciences (NIRS) Heavy Ion Radiation Therapy Project in the proposal number of 12B416.

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