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Chromosome Breakage and Cell Lethality in Human Hepatoma Cells Irradiated with X rays and Carbon-ion Beams

TORU OFUCHI[1,2], MASAO SUZUKI[1], YOKO KASE[1], KOICHI ANDO[1]*, KAICHI ISONO[2] and TAKENORI OCHIAI[2]

[1]Space and Particle Radiation Science Research Group, National Institute of Radiological Sciences, 9–1, Anagawa-4-chome, Inage-ku, Chiba-shi, Chiba 263–8555, Japan
[2]Second Department of Surgery, School of Medicine, Chiba University, 8–1, Inohana-8-chome, Chuou-ku, Chiba-shi, Chiba 260–8670, Japan
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Prediction of radiosensitivity would be valuable for heavy-ion radiotherapy. Premature chromosome condensation (PCC) technique has been a potential predictive assay in photon radiotherapy, but has not been investigated for hepatomas receiving heavy ions. Two human hepatoma cell lines, i.e., HLE and HLF, were irradiated with either 290 MeV/u carbon ions or 200 kVp X rays. Cell lethality was assayed by colony formation and compared with the unrejoined fraction of chromatin breaks as measured by PCC technique. Carbon ions at linear energy transfer (LET) of 76 keV/μm produced cell death more effectively than those of 13 keV/μm and X rays. For the cell killing, the relative biological effectiveness (RBE) of 13 and 76 keV/μm carbon ions compared with X rays was 1.10–1.24 and 2.57–2.59, respectively. Mean number of chromosomes in HLE and HLF cells was similar to each other, i.e., 60.48 and 60.28. RBEs for chromatin breaks of 13 and 76 keV/μm carbon ions were 1.30–1.31 and 2.64–2.79, respectively. A strong correlation between unrejoined chromatin breaks and cell killing for human hepatoma cells was observed irrespective of radiation quality. We conclude that PCC provides a potential predictor for the radiosensitivity of individual hepatoma that are treated with photon as well as heavy ion irradiation.

INTRODUCTION

Human hepatoma are commonly treated with either surgical resection/transplantation, intrahepatic arterial chemotherapy or percutaneous ethanol injection therapy (PEI)[1)]. Conventional radiotherapy produces severe adverse side-effects to the liver with cirrhosis. However, a remark-

*Corresponding author: Phone; +81–43–251–2111 ext. 6925, +81–43–206–3231, Fax; +81–43–251–4531, E-mail; ando@nirs.go.jp

able improvement has been achieved by new radiation modalities such as conformation radiotherapy[2] and proton therapy[3]. Several studies have previously demonstrated that high LET heavy ions are more effective than low LET photons in producing cell death[4,5], mutation and neoplastic transformation[4–6], chromosome aberrations[9–11], tumorigenesis[12]. Clinical trials of carbon-ion beams started in June 1994 at National Institute of Radiological Sciences[13]. Hepatoma has been included in the trial. However, the radiosensitivity of hepatoma cells irradiated with heavy ions is not known yet. The chromosome breakage detected by premature chromosome condensation (PCC) during interphase is one of the most sensitive biological indicator of radiation damage. The induction of chromosome breakage as measured by PCC method shows a linear dose-response in irradiated human cells[14], and unrejoined chromatin breaks after post-irradiation incubation correlated closely with cell death[15]. However, no report has demonstrated such correlations after carbon ions irradiation.

In this paper, we report RBE of carbon ions for two hepatoma cell lines, and demonstrate the relationship between the efficiencies of cell killing and induction of unrejoined chromatin breaks.

MATERIALS AND METHODS

Cell lines

Two human hepatoma cell lines, HLE (JCRB0404) and HLF (JCRB0405)[16], were obtained from the Japanese Cancer Research Resources Bank (JCRB). Mitotic XP2OS cells used as inducers of PCC were also obtained from JCRB. HLE, HLF and XP2OS cells were grown as monolayer in Eagle's medium (minimum essential MEM), supplemented with 10% fetal bovine serum (FBS). Incubator settings were 37°C and 5% CO_2.

Irradiation

For carbon-ion irradiation, hepatoma cells were seeded into 25 cm^2 plastic flasks (Nunc 1 52094) that were fitted to the sample holder of irradiation device. The hepatoma cells at log phase were irradiated with carbon ions accelerated by Heavy Ion Medical Accelerator in Chiba (HIMAC) at the National Institute of Radiological Science (NIRS). The details of the irradiation procedures and dosimetry have been described elsewhere[17]. Briefly, the particle fluence of the carbon-ion beam was measured using a plastic scintillator and the value of LET was measured using a proportional counter filled with tissue equivalent gas. The irradiation dose at the sample position was determined by multiplying the fluence by LET values of carbon ions. We also monitored and controlled the radiation dose using a parallel plate ionization chamber. The mean energy of the carbon-ion beam was 290 MeV/u. LET was adjusted by changing Lucite absorbers to obtain either 13 or 76 keV/μm. The dose rate was about 2 Gy/min for all of the carbon-ion beams. For comparison, we irradiated hepatoma cells with an X-ray machine operated at 200 kVp, 20 mA using a filter consisting of 0.5 mm Al and 0.5 mm Cu with a dose rate of 0.8 Gy/min. Irradiation was carried out at room temperature, and the experiments were repeated at least three times.

Cell survival assay

For cell survival assay, cells were replated immediately after irradiation into 60 mm plastic dishes to finally form 60 to 80 colonies per dish for cell survival assay. After 14-day incubation at 37°C, colonies were stained with a solution of crystal violet. The number of colonies per dish was counted and the surviving fractions were calculated as the ratio of plating efficiencies for irradiated and unirradiated cells. Plating efficiency is defined as the colony number divided by the number of cells plated.

Detection of residual unrejoined chromatin breaks

Residual unrejoined chromatin breaks were measured as fragmentation of prematurely condensed chromosomes allowed to undergo repair for 24 hrs after irradiation. The detailed procedure for the induction of PCC has been described elsewhere[10,18]. Briefly, $1 \infty 10^6$ mitotic XP2OS cells (PCC inducer), obtained by 6 h of incubation in the presence of 0.1 mg/ml demecolcine (Wako Pure Chemical Industries Ltd, Osaka), were mixed with an equal number of hepatoma cells in a polypropylene tube (Falcon 2059). The cell mixture was centrifuged at 200 g for 5 min and the pellet washed in 4 ml PBS. After centrifugation at 200 g for 5 min, the cell pellet was exposed to 0.15 ml of 50% (w/v) polyethyleneglycol (PEG; M.W. = 1540, Boehringer Mannheim GmbH, Germany) in 75 mM Hepes for 1 min. Then, 4 ml of PBS was slowly added to the tube and the cell suspension centrifuged at 200 g for 5 min. The cell pellet was resuspended in 5 ml of MEM containing 0.1 mg/ml demecolcine, and the tube was placed in the incubator at 37°C and 5% CO_2 for 1 h. By the time, cell fusion and induction of PCC was completed. Subsequently, these cells were treated with 75 mM KCl solution for 20 min at room temperature, and fixed in Carnoy's solution (methanol : acetic acid = 3 : 1). The cell suspension was dropped onto cleaned slides, air dried and stained with 5% Giemsa solution. PCC in 20 cells were scored under the light microscope. Because of the difficulty in distinguishing between intrachromosomal breakage and misrejoining, or ring formation , we cannot exclude a possible inclusion of some intrachromosomal asymmetric rejoinings into unrejoined or residual breaks, which also result in addition of chromosome number.

RESULTS

Plating efficiency and cell survival

Plating efficiency of the hepatoma cell lines was 0.48 and 0.44 for HLE and HLF, respectively. The dose-response curves for cell kill are shown in Fig. 1. HLE showed a trivial shoulder on the survival curve after X-ray irradiation (Fig. 1a). Surviving fraction at 2 Gy, i.e., SF2, was 0.28, 0.22 and 0.038 after X rays, 13 keV/μm carbon ions and 76 keV/μm carbon ions, respectively. HLF exhibited a clear shoulder and pronounced curvature (Fig. 1b). SF2 was 0.37, 0.38 and 0.047 after X rays, 13 keV/μm carbon ions and 76 keV/μm carbon ions, respectively. D_{10} dose that reduces surviving fraction to 10% was graphically obtained, and used to compare effectiveness of different radiation qualities (Table 1). The RBE of carbon-ion beams compared with X rays was calculated as a ratio of D_{10} doses between carbon ions and X rays. Carbon-ion beams

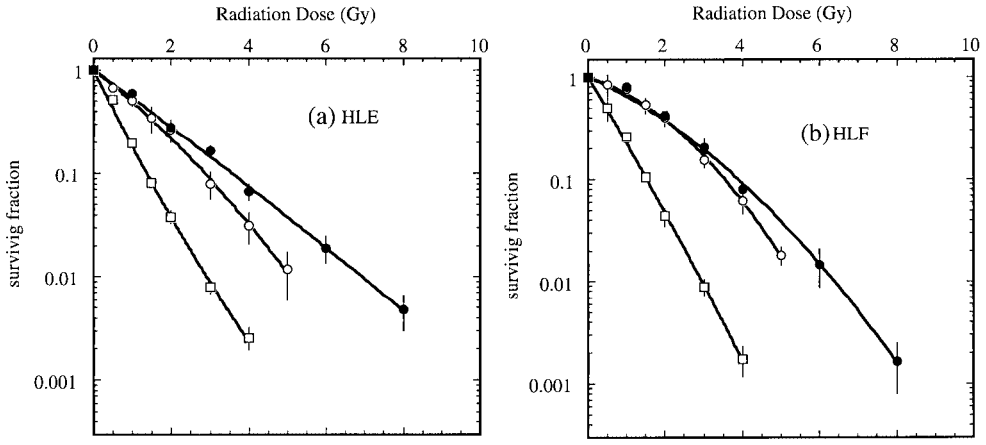

Fig. 1. Dose-response curves for cell death following irradiation with X rays (closed circles), carbon ions at 13 keV/μm (open circles) and 76 keV/μm (open squares). Each value represents the mean ± SE for three independent experiments. The curves are fitted by the linear-quadratic method; a: HLE, b: HLF.

Table 1. Relative biological effectiveness (RBE) of carbon-ion beams for colony formation and PCC breaks

LET	HLE cells				HLF cells			
	colony formation		PCC		colony formation		PCC	
(kev/μm)	D_{10}(Gy)	RBE[a]	isoeffect dose (Gy)	RBE[a]	D_{10}(Gy)	RBE[a]	isoeffect dose (Gy)	RBE[a]
X-ray 200 kVp	3.57		3.57		3.90		3.90	
Carbon ions								
13	2.87	1.24	2.73	1.31	3.55	1.10	2.99	1.30
76	1.38	2.59	1.35	2.64	1.52	2.57	1.40	2.79

[a] The RBE for colony formation was calculated by (D_{10} of X rays) / (D_{10} of carbon ions) where D_{10} is a dose to reduce cell survivals to 10%, while the RBE for PCC were calculated by (D_{10} of X rays) / (Isoeffect carbon dose to produce an equal number of chromatin breaks per cell by D_{10} of X rays).

of 13 and 76 keV/μm were more effective than X rays to produce cell death in each hepatoma cell line. Carbon ions at 76 keV/μm were more effective than at 13 keV/μm.

Chromosomes in unirradiated hepatoma cells

Number of chromosomes in individual cells were scored by PCC technique (Fig. 2). One-hundred fused interphase cells were counted for each cell line. The observed number of chromosomes for HLE and HLF was 60.48±1.35 (mean and SD) and 60.28±1.07, respectively.

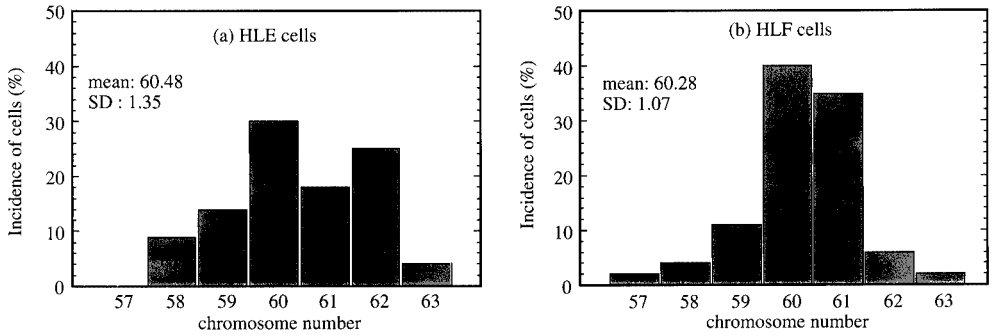

Fig. 2. Frequency distributions of chromosome number detected by PCC method for unirradiated hepatoma cells. One-hundred fused interphase cells were counted for each cell line; a: HLE, b: HLF.

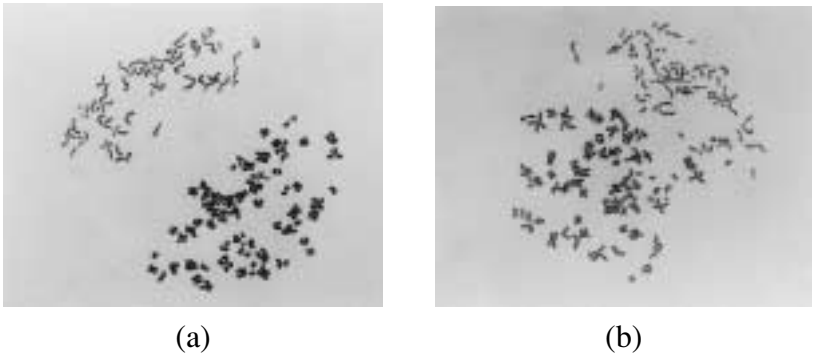

Fig. 3. Photomicrographs of PCC in HLE cells; a: unirradiated control, b: 24 h after receiving 2 Gy of 76 keV/μm carbon ions.

Residual unrejoined chromatin breaks

Figure 3 shows photographs of PCC in HLE cells. The unirradiated HLE cell (Fig. 3a) possessed 60 prematurely-condensed G_1/G_0 chromosomes, whereas the HLE cell developed 71 fragments 24 h after receiving 2 Gy of 76 keV/μm carbon ions (Fig. 3b). The yield of chromatin breaks per cell is estimated as the mean number of PCC fragments in excess of mean number of control cells; for Fig. 3, the yield was 71 – 60 = 11. The dose-response relationships for the residual chromatin breaks that were allowed to undergo repair for 24 h after irradiation are shown in Fig. 4. All showed a linear-quadratic dose-dependency. Carbon-ion beams were more effective than X rays in producing residual breaks in both hepatoma cell lines. Carbon-ion beams at 76 keV/μm were more effective than at 13 keV/μm. The RBE was calculated as a ratio of doses that produced equal breaks per cell at D_{10} dose of X rays. The RBE for the residual breaks thus obtained was 1.30 and 2.79 for 13 keV/μm and 76 keV/μm beams, respectively (Table 1).

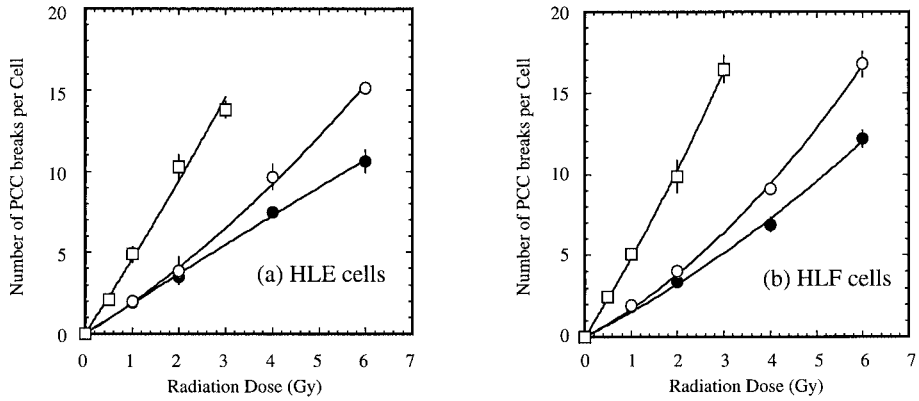

Fig. 4. Dose-response curves of chromatin breaks. Excess fragmentation of prematurely condensed chromosomes detected by PCC method was determined 24 h after irradiation with either X-rays (closed circles), carbon ions of 13 keV/μm (open circles) or of 76 keV/μm (open squares). A symbol and a bar are the mean ± SE for three independent experiments. The line is fitted by the linear-quadratic method; a: HLE, b: HLF. The regression line with mean and SE is as follows: For HLE, $Y = (1.8989 \pm 0.4221)X + (-0.4221 \pm 0.0080)X^2$ (closed circles), $Y = (1.8882 \pm 0.0758)X + (0.1076 \pm 0.0144)X^2$ (open circles), $Y = (5.2958 \pm 0.2265)X + (-0.2127 \pm 0.0863)X^2$ (open squares). For HLF, $Y = (1.4199 \pm 0.0763)X + (0.0978 \pm 0.0146)X^2$ (closed circles), $Y = (1.4920 \pm 0.0627)X + (0.2145 \pm 0.0120)X^2$ (open circles), $Y = (4.4960 \pm 0.1515)X + (0.3164 \pm 0.0578)X^2$ (open squares). Y and X are the number of PCC breaks and radiation dose in Gy, respectively.

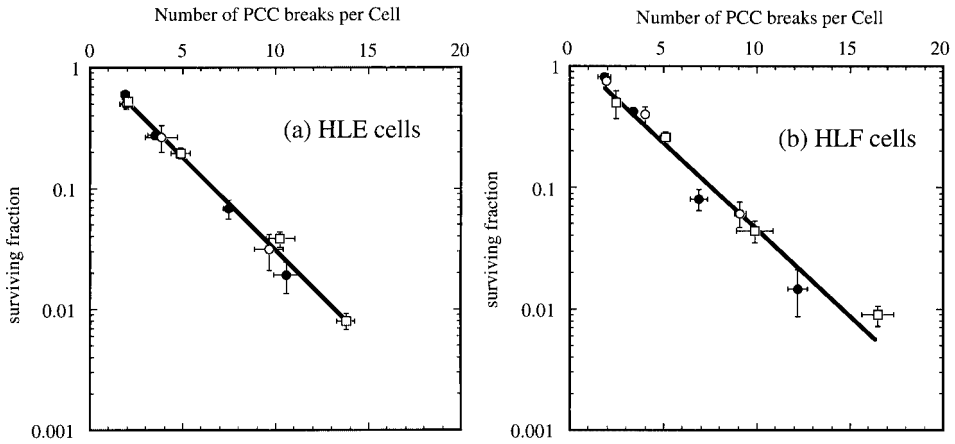

Fig. 5. Relation between surviving fraction and PCC breaks. Data in Fig. 1 and 4 are used to construct the relationship for HLE (a) and HLF (b). Symbols are same as those in Fig.4. The regression lines with mean and SE are as follows: HLE; $Y = (1.0723 \pm 0.0575)*\exp((-0.3581 \pm 0.0114\ X))$, $R = 0.99537$. HLF; $Y = (1.2418 \pm 0.1296)*\exp((-0.3284 \pm 0.0193\ X))$, $R = 0.98772$; where Y and X are surviving fraction and the number of PCC breaks, respectively.

Correlation between surviving fraction and residual unrejoined chromatin breaks

The correlation between the residual unrejoined chromatin breaks after irradiation and surviving fraction for each cell line was calculated from data in Figs. 1 and 4, and plotted in Fig. 5. As the correlation for three radiation with different LET apparently overlaps to each other, all data were combined and fitted to an exponential function. The correlations were best fitted with a negative slope of 0.3581 and 0.3284 for HLE and HLF cells, respectively. The slope of regression line for HLE was significantly steeper than that for HLE ($p<0.05$).

DISCUSSION

We reported here the radiosensitivity of two human hepatoma cell lines, HLE and HLF. A human hepatoma cell line HepG2 possesses SF2 of 0.4 and D_{10} of 5.0 Gy after ^{137}Cs γ-ray irradiation[19]. This value is close to that we obtained here for X rays in HLE and HLF cells. Biological characteristics of HLE and HLF have been reported for growth factor[20] and gene expression[21], but not for the radiosensitivity.

The relationship between RBE and LET for several biological assay is very similar to that for cell lethality[22]. A good correlation between the cell lethality and the induction of unrejoined chromatin breaks detected by PCC technique has been reported for several human cells that received either X rays[14], gamma rays, alpha particles[9] or carbon ions[15]. Recently, Suzuki et al. reported an in vitro dose-response curve for unrejoined chromatin breaks in human embryonic fibroblast-like cells exposed to neon-ion beams[23]. In their experiments, RBE for the induction of unrejoined chromatin breaks are higher than those of cell death, particularly for 100–300 keV/μm beams. However, for relatively low LET (63 keV/μm) beams, RBE for the frequency of unrejoined chromatin breaks and that of cell death is almost equal.

Predictive assays provide not only the likely outcome of standard radiotherapy, but also a basis for the selection of treatment options. The clonogenic survival assay is regarded as a good measure of radiosensitivity[24]. As test samples often mingle with fibroblasts in culture, and as the assay needs a long incubation time of 1-2 weeks, the applicability of this assay as a predictive test for radiosensitivity is limited. The micronucleus frequency after irradiation seems to well correlate with cell lethality[25]. This assay requires to culture cells for several days. The lack of a correlation between micronucleus frequency and radiosensitivity has been reported for malignant melanomas and ovarian cancers[26]. Surrogate assays for cell lethality have been also reported as pulsed-field gel electrophoresis (PFGE) and single-cell gel electrophoresis (comet assay), both of which measure DNA double-strand breaks[27]. Recently, some investigators reported the lack of correlation between radiosensitivity and DNA double-strand break induction or rejoining in human tumor cell lines[28]. Contrary to these assays, the scoring of chromosome breakage in interphase using PCC technique holds considerable promise for predicting the radiosensitivity of normal and tumor tissues. As this assay does not require cell division, results can be obtained rapidly. In this study, a strong correlation between unrejoined chromatin breaks and cell survival in human hepatoma cells was found for any LET values lower than 80 keV/μm (Fig. 5). As the RBEs for survival and PCC fragments dissociate in human embryonic cells that receive carbon

irradiation at LET higher than 100 kev/μm[23], the LET independency may hold only for radiations with low LET where the hits by charged particles are randomly distributed among cells. Therefore, the PCC technique would be a reliable method for detecting cell lethality caused by relatively low LET radiation. Moreover, it should be noted that the correlation between residual unrejoined chromatin breaks and surviving fraction was significantly different between the two hepatoma cells. Further study is required to detect cellular factors that would modify processing of chromatin breaks leading to cell death.

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