LET Dependency of Heavy-ion Induced Apoptosis in V79 Cells

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We investigated the relationship between the LET values and cell death, defined as either apoptosis or loss of reproductive integrity (reproductive death), using Chinese hamster V79 cells. The cells were irradiated with X-rays or carbon-ion beams from the Heavy Ion Medical Accelerator in Chiba (HIMAC) at the National Institute of Radiological Sciences (NIRS). Apoptosis was defined based on the morphological change upon treating of cells with caffeine. The apoptotic index, the ratio of apoptotic cells to the total, after exposure to 2 Gy of X-rays was 0.043. Upon irradiation with carbon-ion beams, the index was gradually increased with increasing LET values, reaching a maximum of 0.076 at 110 keV/µm, and then decreased to 0.054 at 237 keV/µm. An analogous pattern of the LET dependence was observed between reproductive death and apoptotic death. The cell-survival values obtained after 2 Gy exposure (SF₂) were 0.64, 0.13, and 0.24, respectively. A similar trend was found for the RBE values calculated from the initial slope for both apoptosis and reproductive death. These results strongly suggest that the target for both types of cell death is the same.

INTRODUCTION

Heavy-ion radiotherapy is a highly interdisciplinary field, which unifies diverse disciplines from accelerator physics to medicine, including nuclear physics, biophysics, radiobiology and radiation oncology. Knowledge concerning the interaction of high-LET radiation with biological matter is of great importance for applying of heavy ions to radiotherapy. The use of heavy-ion particles in radiobiological experiments also provides a fundamental tool for study-
ing the effects of different ionization densities, and proves the physical basis of radiation action. High-LET radiation is more effective than low-LET X- or γ-radiation to induce biological damage. The Bragg-peak of an accelerated charged particle beam provides good dose localization in a critical cancer tissue; high-LET radiation gives a higher RBE value for cell killing, a reduced oxygen effect, and a reduced dependence on the cell-cycle, of which, the effects are superior to low-LET radiation in cancer treatment.

Reproductive cell death has been used as a conventional index of cell killing. In addition, apoptosis is another pathway to cell death, and is worth attention. Although it is already known that high-LET radiation is more effective to induce apoptosis, only a few detailed studies on the LET dependence of cell killing through apoptosis have been carried out. Apoptosis is thought to be an important factor for cancer therapy, because in some tumors a substantial fraction of the malignant cells undergo post-radiation apoptotic death. Cancer cells disappear without inflammation if apoptosis is induced in cancer tissue by irradiation or a treatment with anticancer drugs.

Although DNA damage is believed to cause cells to undergo reproductive death, little is known about the target cellular molecules that can trigger apoptosis after heavy-ion beam irradiation. If the targets for both types of the cell death are the same, the LET dependency must be the same. In order to clarify the validity of this hypothesis, we examined the LET dependence of apoptosis and reproductive death using accelerated carbon-ion beams with varying LET values (13–237 keV/µm) from HIMAC (Heavy Ion Medical Accelerator in Chiba) at NIRS (the National Institute of Radiological Sciences), Japan.

MATERIALS AND METHODS

Cell culture

Chinese hamster V79 cells were used throughout the experiments. The cells were cultured in Eagle’s minimum essential medium (E-MEM, Nissui, Tokyo) supplemented with 10% fetal bovine serum (Lot. No. 5D2073, JRH, Lenexa) and incubated under humidified air with 5% CO₂ at 37°C. Exponentially growing V79 cells were harvested by trypsinization (Trypsin 1:250 (GIBCO BRL, Tokyo), 0.2% in PBS), and seeded in T25 culture flasks (152094, Nunc, Tokyo) one day before irradiation at a density of about 1 × 10⁴ or 8 × 10⁵ cells/flask for apoptosis analysis and cell-survival assays, respectively.

Irradiation

We used a 290 MeV/u heavy-ion carbon beam for the experiments, which was selected for the first clinical trial at NIRS. The irradiation system for heavy ions at HIMAC and the physical characteristics of the beam have been described elsewhere. Dosimetry of the beam at the biological experiment port was carried out by two independent methods: one using an ionization chamber, and the other using a plastic scintillator for fluence measurements. We monitored and controlled the irradiation dose using a monitoring ionization chamber placed upstream of the sample position, which was calibrated by a standard ionization
chamber placed at the sample position. The LET values were obtained by fitting the Bragg curve obtained by measurement to an already established theoretical reference. Polymethyl methacrylate and/or plastic film absorbers were used to reduce the beam energy in order to obtain optimum LET beams. The dose-averaged LET values in the sample selected for the experiments were 13, 30, 60, and 110 keV/µm, which were obtained from 290 MeV/u beams, and 152 and 237 keV/µm from 135 MeV/u beams. Samples were sequentially irradiated by selected doses and LET values using a computer-aided automatic irradiation device. Exponentially growing cells in culture-flasks were exposed to carbon beams through the bottom of the flask at room temperature. Screw caps of the culture-flasks were tightly closed just after removing from a CO2 incubator, and set vertically into each exposure holder of a sample changer equipped in the biological experiment port of HIMAC.

As reference radiation to calculate the RBE value of carbon beams, X-rays were used by a Shimadzu (Tokyo) generator operated under 200 kVp and 19 mA, with 0.5 mm Al and 0.5 mm Cu filters.

**Colony formation assay**

Cell survival (reproductive death) was assayed by the conventional colony-formation assay. Cells were collected by trypsinization and re-suspended in E-MEM. The concentrations in the cell suspension were determined by a cell counter (Coulter, model Z1 with a 100 µm aperture tube). The cells were diluted with E-MEM and seeded in 60 mm Petri dishes (3002 Falcon) to provide approximately 100 surviving cells per dish. Dishes were incubated for 6 days, then fixed with 10% formalin in PBS and stained with 1% methylene blue. Colonies containing more than 50 cells were scored as survivors under a stereomicroscope. The α and β parameters were obtained from survival data by curve fitting using

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

where $SF$ is the survival fraction, and $D$ is the dose. The parameters were calculated by a least-squares method of a computer program (KaleidaGraph, Synergy Software). The cell sensitivity on reproductive death was described as the inverse value of survival for a given dose of exposure from the α and β parameters for each survival data set.

**Detection of apoptotic cells**

We have developed a simple and quick method to detect and count “apoptotic cells” due to limitations of the allotted accelerator beam time. After cells were treated with caffeine for 1 hour after the 18 hour post-incubation, round-shaped cells appeared in a time- and dose-dependent manner. In subsequent incubation, most of these round-shaped cells began to exhibit the characteristic features of apoptosis, such as chromatin condensation, the formation of DNA ladders and apoptotic bodies. On the basis of these observations we identified those round-shaped cells as being “apoptotic” cells, although they appeared after a 1-hour-caffeine treatment, and were thought to be more exactly “pre-apoptotic”. We thus established dose-effect curves in relation to the induction of apoptosis.
Irradiated cells were post-incubated for 18 hours, being determined by preliminary experiments (data not shown), and treated with 10 mM caffeine for 1 hour at 37°C. The numbers of the round-shaped cell were scored under a phase-contrast microscope both before and after the caffeine treatment. The number of round-shaped mitotic cells before the caffeine treatment was subtracted from the number obtained after the treatment. The apoptotic index (AI) was defined as the fraction of round-shaped cells in a population, and the $\alpha$ and $\beta$ parameters of dose-response curves on AI were estimated using the same equation as that for survivals.

**Chromatin condensation**

To examine the apoptosis which occurred in V79 cells, a chromatin condensation analysis was performed with cells irradiated by 10 Gy of carbon-ion beams under a fluorescence microscope. Cells were post-incubated for 18 hours, and treated with 10 mM caffeine for 1 or 5 hours at 37°C, then collected by trypsinization and centrifugation (1000 rpm, 3 min). The cells were re-suspended in 200 $\mu$l PBS, and then collected again by centrifugation. After removing the supernatant, the pellets were suspended, fixed with 1% glutaraldehyde, and stored for over 24 hours at 4°C. After being stained with 1 mM bis-benzamide (Hoechst 33342), they were observed under a fluorescence microscope.

**Cytofluorometric analysis**

Cytofluorometric analyses were performed using a flow cytometer (FACScan, Becton-Dickinson). The cells were treated, washed as described above, and fixed with 70% ethanol to store for over 24 hours at –20°C. Following centrifugation and removing the supernatant, the cells were treated with 100 $\mu$g/ml-RNase A (DNase free, Sigma Co.) for 30 minutes at 37°C. After precipitation by centrifugation (4°C, 2500 rpm, 5 min) they were re-suspended in 1ml PBS and stained with 50 $\mu$l propidium iodide (1 mg/ml, Sigma). Cell suspensions were filtered by 50 $\mu$m nylon mesh and used for flow cytometer analysis.

**DNA ladder assay**

DNA ladder formation was confirmed as follows. The caffeine-treated cells were first washed and re-suspended in PBS, and then collected by centrifugation; the pellets were treated with a 100 $\mu$l cell lysis buffer (10 mM Tris pH 7.4, 10 mM EDTA pH 8.0, 0.5% Triton X-100) for 10 minutes at 4°C. The lysate was centrifuged (4°C, 16000 rpm, 20 min) in order to remove any cell debris, and then added to 2 $\mu$l of 20 mg/ml RNase A and incubated for 1 hour at 37°C. Cells were further incubated for 1 hour at 37°C with 2 $\mu$l of 20 mg/ml Proteinase-K. Extracted DNA was collected in 25 $\mu$l of 5 M NaCl and 125 $\mu$l Isopropanol for 4 days at –20°C, and then re-suspended in a TE buffer (10 mM Tris-Cl pH 7.4, 1 mM EDTA pH 8.0). The DNA samples or DNA size marker, with a loading solution (0.25% Bromophenolblue (BPB), 40% Sucrose, (5:1)), were applied in a 2% agarose gel (LO-3 Agarose (Takara Shuzo Co., Kyoto) in TBE buffer (89 mM Tris, 89 mM Borate, 2 mM EDTA pH 8.0)). The samples were electrophoresed for 2 hours in TBE buffer with 0.5 $\mu$g/ml ethidiumbromide using a Mupid II electrophoresis tank (Cosmo Bio Co., Tokyo) operated at 50 volts.
RESULTS

Detection of apoptosis, morphological changes and DNA ladder

Major signs of apoptotic changes, such as chromatin condensation, apoptotic bodies and

Fig. 1. Apoptosis in V79 cells exposed to 10 Gy of carbon ions (110keV/µm), post-incubated for 18 h, and treated with 10mM caffeine for various periods of time. Unirradiated cells without a caffeine treatment (1), irradiated and treated with 10mM caffeine for 0 h (2), 1 h (3), and 5 h (4). Morphological changes were observed under a phase-contrast microscope (a) or a fluorescent microscope (b), and cellular DNA content was analyzed by a flowcytometer (c).
DNA fragmentation, were observed in V79 cells exposed to 10 Gy carbon-ions (110 keV/µm).

The morphology of unirradiated cells without a caffeine treatment are shown in Fig. 1-1a (phase-contrast microscope; x 10 magnification) and in Fig. 1-1b (fluorescent microscope). By the irradiation of 10 Gy carbon beams, morphological changes as characteristics of apoptosis became clear after a caffeine treatment. The irradiated cells (Fig. 1-2a) as well as the nucleus (Fig. 1-2b) began to increase in size before 1 h of the caffeine treatment. Many round-shaped cells (pre-apoptotic cells) appeared after a 1-hour caffeine treatment (Fig.1-3a). Those cells, however, disappeared after 5 hours of the caffeine treatment (Fig. 1-4a).

Chromatin condensation was observed by applying fluorescent staining to nuclei. After a 1 hour treatment with caffeine, the reticulated cell nuclei were observed (Fig. 1-3b). The round-shaped cells under a phase-contrast observation, therefore, were assumed to be the same as the reticulated cells in the fluorescent observation, and all of those cells were considered to be apoptotic.

The induction of apoptotic bodies caused by DNA degradation was observed by cytofluorometric analysis (Fig. 1-c). An extensive accumulation of the cells at the G2/M phase was observed during 18 hour post-incubation regardless of a 1 hour caffeine treatment (Figs. 1-2c and 1-3c). A fraction of cells having a large amount of DNA were also observed. When cells were treated for 5 hours with caffeine, those accumulated in the G2/M phase were re-

| Lane | Caffeine (hr) | Dose (Gy) |
|------|--------------|------------|
| 1 2 3 4 5 6 7 8 9 | 0 1 1 1 5 5 10 10 | 0 0 3 10 3 10 3 10 |

Fig. 2. DNA fragmentation of V79 cells exposed to carbon ions (LET 110 keV/µm, 10 Gy). Exposed cells were incubated for 18 h and treated for various periods of time (1, 5, 10 h) in 10 mM caffeine prior to DNA extraction.
duced, and those having a small amount of DNA appeared in turn, significantly (Fig. 1-4c). A large number of cells were assumedly released from the G₂ block, and divided into small fragments to form apoptotic bodies.

DNA ladder formation resulting from DNA fragmentation was examined by electrophoresis (Fig. 2). Only those cells treated for 5 hours with caffeine showed the typical DNA ladder pattern (Lanes 6 and 7). A one or 10-hour caffeine treatment, which rendered cells the apoptotic change in morphology (Fig. 1), failed to produce ladder formation, but possessed smeary DNA. These results suggest that morphological changes, such as chromatin condensation, appear earlier than DNA fragmentation, and that a caffeine treatment longer than 5 hours causes alterations in DNA, leading to smeary DNA on electrophoresis.

**Dose response for cell killing and induction of apoptosis**

Cell-survival curves (reproductive cell death) for each of the carbon beams with different LET values and X-rays are shown in Fig. 3. The D₁₀ (the dose required to reduce survival to 10%) was 7.1 Gy for X-rays; the survival curve as well as that of 13 keV/µm carbon beam show a pronouncedly large shoulder. The shoulder was reduced and the curve became steeper as the LET value increased. For LET values higher above 110 keV/µm, the survival curves became purely exponential with no shoulder. The dose-response curves for apoptotic indexes (number of apoptotic cells in total cells) are shown in Fig. 4. Each curve shows saturation at a high-dose region. The slope and the saturation level increases along with the increase in the LET value up to 110 keV/µm, and then decreases as the LET values become higher. A similar change in the radiosensitivity is observed.

![Survival curves determined by the colony-formation assay for V79 cells exposed to X-rays ( ), 13 ( ), 30 ( ), 60 ( ), 110 ( ), 152 ( ), and 237 keV/m ( ) carbon-ion beams, respectively.](image)
LET dependency of RBE in two types of cell death

The biological effects by carbon beams together with X-rays were estimated using two types of cell death, reproductive death and apoptosis, as the biological endpoints; further, the LET dependencies of both were examined (Fig. 5). The cell sensitivity to 2 Gy exposure (SF2) by each of radiation was described as the inverse value of the survival value. The sensitivity
of cell killing increased with increasing LET value to reach a maximum at 110 keV/µm, and then decreased as the LET value increased. The apoptotic index for 2 Gy exposure was 0.043 with X-rays, and increased within a lower (< 100 keV/µm) LET region, reaching a maximum at 110–150 keV/µm, and then decreased within a higher LET region. The RBE values on the initial slope of the dose-response curves for cell killing and apoptotic indexes are also plotted in Fig. 6. The LET-RBE relationship shows a similar spectrum: i.e., RBE vs. LET shows a maximum, but then decreases in a higher LET region for 2 Gy exposure.

DISCUSSION

Induction of apoptosis and caffeine treatment

We have observed the induction of apoptosis in V79 cells by 10 Gy exposure with high (110 keV/µm carbon beam) as well as low (X-ray) LET radiations. We confirmed chromatin condensation (Fig. 1-b), DNA fragmentation (Fig. 2) and the appearance of apoptotic bodies (Fig. 1-c) in the irradiated V79 cells. Apoptosis in V79 cells was observed by other groups following X-ray\textsuperscript{14} or γ-ray exposeure\textsuperscript{15}.

Observations under a phase-contrast microscope revealed an increase in the volume of the irradiated cells (Fig. 1-2a), suggesting the continued synthesis of DNA and protein in spite of impaired mitosis. Neonatal rat aortic smooth muscle cells exposed to 50 Gy γ-irradiation have been reported to cease cell division, but to continue the synthesis to accumulate elastin\textsuperscript{16}. Ionizing radiation has been known to alter gene expression\textsuperscript{17}, although the regulatory factors to block the cell-cycle are not yet completely understood.

It is well known that the cell-cycle progression is blocked in the G₂/M phase by irradiation\textsuperscript{9}, and that a caffeine treatment can release the G₂/M arrest in irradiated cells\textsuperscript{18}. Therefore,
we applied caffeine to release the G2/M block. Indeed, caffeine was confirmed to release the radiation-induced G2/M block and to enhance DNA fragmentation (Fig. 1) as well. A radiation-induced G2/M block is considered to play a role in gaining time for DNA repair and/or cell recovery. Release from the cell cycle block in the G2 phase by caffeine is known to increase chromosome breaks, chromosomal aberrations and aberrant mitoses. Then, those cells with unrepaired or irreparable damage undergo apoptosis. Consequently, the release of the G2/M block by caffeine might lead the cells to die via the apoptotic process. In other words, caffeine just promotes the apoptotic change by releasing the G2/M block.

Several pharmacological agents which reduced the radiation-induced G2/M phase arrest (caffeine, theabromine, theophylline and 2-aminopurine) are known to enhance the degree of DNA fragmentation at 24 hours. Studies on EL4 lymphoma cells are favorable with the view that the cells with radiation-induced genetic damage causes apoptosis after release from the G2/M block. Chromosomal breaks, chromosomal aberrations and aberrant mitoses can be induced even in surviving cells. In other words, unrepaired or irreparable DNA damage can trigger either the induction of apoptosis or the fixation of DNA damage to induce chromosomal breaks, chromosomal aberrations and aberrant mitoses. In the mitotic catastrophe model, commitment to self-killing may result from premature activation of the mitotic machinery. Also, this model proposes a concept of a “G1/G2 death circuit” in which the cells dying in the G1/G2 phase go to the M phase by a short circuit. Therefore, even though apoptosis was induced in some parts of damaged cells (Fig. 4), the surviving cells with unrepaired or irreparable DNA damage were able to proceed to the S phase (Fig. 1).

**Dose response curves and LET**

The radiosensitivity of V79 cells to carbon beams having various LET values was determined in the present study by either reproductive death or apoptotic death. The SF2 in reproductive death by X-rays was 0.64, and the sensitivity was 1.57 (= 1/0.64). It increased with increasing LET values, and showed a maximum of 7.9 (= 1/0.13) to 110 keV/µm beams. Such a type of LET dependency has been well known as the LET-RBE relationship, and has been reported concerning HSG (human salivary grand tumor cells) cells, human kidney T1 cells, Syrian hamster embryo cells and a variation of chromosome breakage, or theoretical calculations. Those RBE values were varied depending on the examined survival level. Particularly, in the case of apoptosis induction, the dose-response curves (Fig. 4) which showed saturation at a high-dose region (β<0) are difficult to obtain RBE values in somewhat higher levels of the effect. We simply calculated the apoptotic index for the same doses (2 Gy) of all radiations. The apoptotic index for X-rays was only 0.043, which increased with the LET to 0.076 at 110 keV/µm, and then decreased.

There was a study in which the initial slope of the dose-response curves was used to calculate the radiobiological effectiveness under different radiations. In the report, apoptosis in human peripheral G0 lymphocytes exposed to high LET (140 keV/µm) nitrogen ion beams was studied; the RBE was obtained from a comparison of the initial slope of the dose-response curve, 2.7. In the same way, we plotted (Fig. 6) the RBE value vs. LET values for both cell killing and the apoptosis induction to compare the effectiveness at very low dose.
The RBE values obtained by comparing of the initial slope for cell killing and apoptotic index were plotted; the maximum was found at around 110 keV/µm, and its values were 5.5 and 1.6, respectively (Fig. 6). The difference of the two maximum values probably depends on the efficiency of cell killing and apoptosis as well as on the difference in the shape of the dose-response curve.

The LET values that give the maximal RBE values were, however, the same between both endpoints, but the effectiveness were different (Fig. 6). This agreement concerning the peak in the fluctuation RBE suggests that the target of radiation may be the same in both of cell killing and the induction of apoptosis in V79 cells, or that both effects can be triggered by the same damage (DNA breaks). If DNA damage triggers apoptotic changes, $p53$ may play important roles in apoptosis\textsuperscript{29,30}. It is reported that V79 cells have a mutant-type $p53$\textsuperscript{31}. On the other hand, a $p53$-independent pathway of apoptosis is also known\textsuperscript{32}, and it is claimed that the human promyelocytic leukemia cell line HL-60, which is known to be deficient in $p53$ because of large deletions, can undergo apoptosis following X-irradiation. Our results also assume that the apoptosis induced here was independent from $p53$.

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