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Low-dose Hypersensitivity in Nucleus-irradiated V79 Cells Studied with Synchrotron X-ray Microbeam

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X-ray microbeam/Cell survival/Low-dose radiation/Low-dose hypersensitivity.

This study aims at elucidating the cellular responses induced by energy deposition in the cell nucleus or cytoplasm in the low-dose (< 1 Gy) region. We compared the survival fraction of V79 cells irradiated with X-ray microbeams of different sizes. Entire cells or cell nuclei were targeted with 5.35 keV monochromatic X-ray microbeams using a synchrotron radiation (SR) X-ray microbeam irradiation apparatus. Using a threshold of 30 cells/colony after 60 h of incubation, conditions that had been proven to give results equivalent to those of the conventional method, we determined the survival fraction of the microbeam-irradiated cells. When cell nuclei were irradiated with 10×10 μm^2 X-ray beams, the survival fraction was almost the same as that obtained with 50×50 μm^2 beams except in the low-dose region. In the low-dose region irradiated with 10×10 μm^2 beams, hyper-radiosensitivity (HRS) was clearly observed in the nucleus-irradiated cells, and the survival curve exhibited a minimum of about 60% at 0.5 Gy. This may be the most distinct HRS reported thus far when an asynchronous population is used. Difference in observed HRS phenomena is solely due to the irradiated part in the cell. These results suggest that energy deposition in the cytoplasm might suppress the HRS.

INTRODUCTION

The evaluation of risks to human health from low-dose radiation has become a great concern because of the potential risks from exposure to environmental radiation and radiation in the clinical setting, among others. Direct experimental data in the low-dose region with statistical significance are difficult to obtain because of the need for a large number of samples. In the absence of experimental data, the risks from low-dose radiation exposure have been estimated by observing the various biological effects in the high-dose region, e.g., in atomic bomb survivors, and extrapolating them linearly to the low-dose region. However, some data have indicated that biological responses to low-dose radiation appear to contradict this assumption based on the Linear-No-Threshold model.[1] The low-dose hypersensitivity to radiation (abbreviated as HRS) is an example of such phenomena. HRS has been observed in dose regions below 0.5 Gy, a clear deviation from the standard linear-quadratic (LQ) behavior of the cell survival curve extrapolated from higher doses, and it is accompanied by a subsequent increase in radioresistance (abbreviated as IRR). An HRS/IRR-type phenomenon has been observed in many cell lines.[2]

To elucidate the induction mechanisms of biological responses to low-dose radiation, the need to study the intracellular responses in irradiated cells and the intercellular responses from irradiated cells to nonirradiated cells (bystander effect)[3] has been pointed out. However, after low-dose irradiation with a broad irradiation field, it is not possible to distinguish between individual cellular responses and the cellular responses resulting from intercellular signaling, since the number and localization of track traversals in each cell cannot be controlled. In addition, it is difficult to have intracellular organelles as irradiation targets. The recent development of microbeam cell irradiation systems, which enable the irradiation of each cell, or part of a cell, and the monitoring of individual cellular responses, has provided the opportunity to elucidate the underlying mechanisms of HRS or bystander effects. Some microbeam irradiation systems using charged particles have been developed at several institutes.[4–7] The minimum dose in these systems is the value when one particle traverses through the target, and this value might be as high as ~0.1 Gy depending on the liner energy transfer (LET) and the geometrical size. If photoelectrons are used, we can better control the irradiation doses to a lower region. A microbeam irradiation system

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using focused C_K X-ray beams has been developed at the Gray Cancer Institute.[8] It is difficult, however, to irradiate through the cells with this system since the penetration depth of the C_K X-rays is about 2 μm. From this rationale, an X-ray microbeam irradiation apparatus, which is the first microbeam irradiation apparatus to exploit the synchrotron radiation X-ray for radiation biological research, has recently been developed.[9,10] Cells grown and irradiated on specially designed Mylar-based dishes can be identified after various treatments (e.g., incubation and immunostaining) for the observation of cellular responses. In this system, the cells are kept in a liquid medium during irradiation, since they are irradiated by X-ray microbeams from below, through the bottom of the dish. All the experimental sequences, such as the detection of targets, the recording of coordinates, and irradiation are automated. This irradiation system can provide arbitrarily sized X-ray microbeams larger than 5 × 5 μm^2 with a four-bladed slit system. Therefore, we are able to irradiate an arbitrarily defined target region with the desired number of X-ray photons. In this study, by taking advantage of this feature, we compared the survival fractions of Chinese hamster lung V79 cells irradiated with X-ray microbeams of different sizes. As an interesting result, the observed HRS seems to be dependent on the irradiated domain of the cells. The relationship between biological responses and intracellular energy-deposited sites will be discussed in this paper.

MATERIALS AND METHODS

Cell Culture

The V79 Chinese hamster lung cell line (provided by Dr. Kasai-Eguchi[11]) was used in this study, since it is one of the most commonly used cell lines for radiation biological research. V79 cells were grown in minimum essential medium alpha medium (Invitrogen Corp., Carlsbad, California, USA) containing 10% fetal bovine serum (FBS; SAFC Biosciences, Lenexa, Kansas, USA) and 0.1 mg/ml kanamycin sulfate (Invitrogen Corp., Carlsbad, California, USA), then incubated in a humidified incubator maintained at 37°C in an atmosphere containing 5% CO_2. The doubling time of V79 cells was about 9 h under these conditions. The averaged horizontal cross section of the nucleus and the entire cell, from microscopic measurement of about 400 cells, were 168 μm^2 and 360 μm^2, respectively.

Broad-field (broadbeam) irradiation

Sample preparation

A 0.25-ml cell suspension containing 1.0×10^5 cells was spread over a 30-mm diameter area at the center of 35-mm cell culture dishes. The dishes were incubated for 4 h to allow the cells to attach to the bottom of the dish. Just prior to X-ray irradiation, the medium in the dishes was removed and the dishes were sealed with a 5-μm sterilized Mylar film (polyethylene terephthalate; Teijin DuPont Films Japan, Ltd., Chiyoda-ku, Tokyo, JAPAN) to prevent the cells from drying and contamination.

Irradiation

Broadbeam irradiation with monochromatic X-rays was performed at the beamline 27B (BL-27B) of the Photon Factory (PF), High Energy Accelerator Research Organization (KEK), Tsukuba, JAPAN.[12,13] In this beamline, a double-crystal monochromator was used to obtain monochromatic X-rays; the energy resolution (ΔE/E) was less than 0.1%. Monochromatic X-rays were extracted from the vacuum beamline into the atmosphere through a thin Kapton film (polyimide; Du Pont-Toray Co., Ltd., Chuo-ku, Tokyo, Japan). In broadbeam irradiation experiments, the X-ray beam size was adjusted to 0.6 mm vertical and 37 mm horizontal. Sample dishes were scanned vertically and the entire plate was irradiated uniformly with 5.35 keV X-rays. The exposure rate was measured with a specially designed free-air ionization chamber.[14]

Determination of cell survival with conventional clonogenic assay

The irradiated cells were immediately harvested using trypsin-EDTA (ethylene diamine tetraacetic acid) solution (0.05% trypsin, 0.53 mM EDTA•4Na; Invitrogen Corp., Carlsbad, California, USA), and plated in a 60-mm cell culture dish after appropriate dilution to obtain approximately 150 viable colonies per dish. After incubation for 7 days, the cells were fixed in 10% formalin solution (Wako Pure Chemical Industries Ltd., Chuo-ku, Osaka, Japan) in phosphate buffered saline (PBS; Sigma-Aldrich Corp., St. Louis, Missouri, USA) and stained with 1% methylene blue (Wako Pure Chemical Industries Ltd., Chuo-ku, Osaka, Japan) solution. Colonies containing more than 50 cells were counted as survivors.

Calculation of absorbed dose in broadbeam experiments

The absorbed dose for V79 cells, D (in Gy), was calculated from the exposure, X (in C/kg), using equation [1], where μ_{en}/ρ is the mass energy absorption coefficient (m^2/kg); W, the energy required to generate an ion pair; and *e*, the elementary electric charge. We used $(\mu_{en}/\rho)_{lung\ tissue}$ for V79 cells.[15]

$$D = X \times \frac{W}{e} \times \frac{(\mu_{en}/\rho)_{V79}}{(\mu_{en}/\rho)_{air}} \quad [1]$$

Microbeam irradiation

Sample preparation

In microbeam experiments, V79 cells were plated on custom-designed Mylar based (34 mm in diameter) dishes whose bottoms were composed of a thin (5 μm) Mylar film

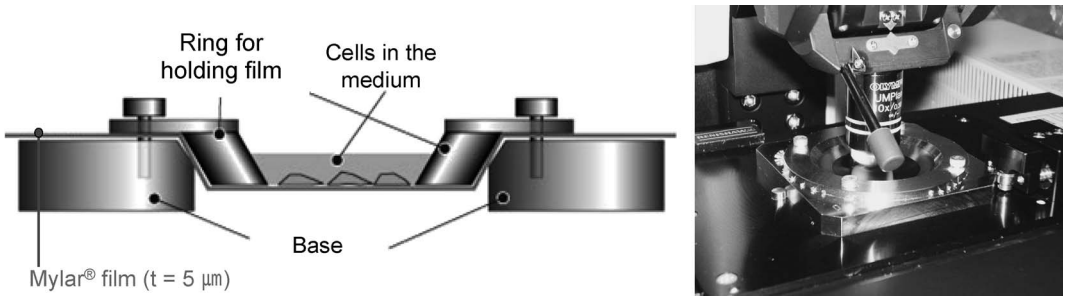

Fig. 1. Schema of Mylar-based dish designed for microbeam irradiation (left) and the dish on the irradiation stage (right). Even if this dish is removed from the stage for various treatments, the targeted cells can be identified precisely (less than 1μm) when it is set back on the stage.

(Fig. 1). To avoid colonies originating from single cells to overlap, the cells were seeded at a very low density (2×10^3 cells/dish). The dishes were incubated for 4 h to allow the cells to attach to the base film. Prior to X-ray irradiation, cell nuclei were stained with a 2-μM solution of Hoechst 33258 (Dojindo Molecular Technologies Inc., Kamimashiki-gun, Kumamoto, JAPAN) for 1 h. We have confirmed that staining with 2-μM Hoechst 33258 and short-period (less than 1 second) UV exposure to obtain fluorescence images do not affect cell survival, by comparing the survival with that in the case of non-processed cells. At the time of irradiation, the Hoechst solution was replaced with 5 ml of fresh medium (without FBS) containing 20-mM HEPES (4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid; Invitrogen Corp., Carlsbad, California, USA).

Irradiation

Monochromatic X-ray microbeam irradiation was performed using the synchrotron X-ray microbeam irradiation apparatus installed in KEK-PF BL-27B.[10] Using this system, we were able to irradiate an arbitrarily defined target region with the desired number of X-ray photons. Considering the cross section of the nucleus (168 μm^2) and the entire cell (360 μm^2), we irradiated cell nuclei with 10×10 μm^2 beams, which could irradiate much of the nucleus, and an entire cell with 50×50 μm^2 beams, which are sufficient in size to irradiate the entire single cell. The energy of the X-rays was 5.35 keV and the exposure rate was about 7.7×10^{-3} C/kg/s (9.3×10^3 photons/s/100 μm^2) as measured using an AXUV-100 absolute XUV silicon photodiode (International Radiation Detectors Inc., Torrance, California, USA).

After each sample dish (containing HEPES medium) was placed horizontally on the irradiation stage, the position of each cell nucleus in a 6-mm square area at the center of the dish was determined automatically using a computer-controlled irradiation stage, a fluorescence microscope, and a cooled charge coupled device (CCD) camera. The coordinates of the target cell nuclei (centers of mass) were automatically calculated and stored on a computer and used to observed the irradiated cells after incubation. Among the targets detected, we selected isolated single cells as targets to exclude doublet cells. The distribution of irradiated cells in the cell cycle, as analyzed from the fluorescence intensity, was as follows: 47% in the G_1 phase, 35% in the S phase, and 18% in the G_2 phase. All the target cell nuclei or cells were irradiated automatically with microbeam X-rays under an aerobic condition.

Determination of cell survival with single-cell clonogenic assay

In microbeam experiments, the survival fraction cannot be measured using the conventional clonogenic assay used for a uniformly irradiated population, since irradiated and non-irradiated cells are located in the same sample dish, where colonies are located at places where seeding before irradiation. Therefore, we employed and determined a threshold to determine the life-or-death of microbeam-irradiated cells. This threshold must give results equivalent to those in the conventional clonogenic assay. To state briefly, we irradiated V79 cells with soft X-rays using SOFTEX M-60W (50 kV, 20 mA; SOFTEX Corp., Ebina-shi, Kanagawa, JAPAN) and measured the survival fractions using the conventional clonogenic assay and the single-cell clonogenic assay with several candidates for the life-or-death threshold. We defined, as threshold, the value that gives the same survival fraction in both the measurement methods and we used it in the microbeam experiments hereafter.

After irradiation, the medium was removed and the cells were twice washed in PBS. Then, 5 ml of the fresh medium was added to the dishes immediately and the dishes were then incubated for 60 h. This incubation period was defined to minimize the overlap of neighboring colonies and to guarantee that the number of cell divisions was sufficient for showing immortality. The medium was then removed and the cells were twice washed in PBS and fixed with HC Tissue Fixative MB (Amresco Inc., Solon, Ohio, USA) for 25 min at 4°C and twice rinsed for 5 min with PBS. The cell nuclei were again stained with 2-μM Hoechst 33258 for 1 h. Images of all the irradiated cell colonies were captured

using a CCD camera and the number of cells per colony was analyzed sequentially. The colonies that included more cells than the threshold were counted as survivors.

Calculation of absorbed dose in microbeam experiments

In radiation biology, we generally use the absorbed dose "Gy," which is defined as J/kg. Gy can be used when the cell population or the entire cell is uniformly irradiated with an X-ray beam, since the entire cell uniformly receives energy from the X-rays. For irradiation using 50×50 μm^2 X-ray microbeams, the absorbed dose was calculated by equation [1] as that used in the broadbeam experiment. However, in microbeam experiments using 10×10 μm^2 X-ray microbeams, the beams irradiated only part of the cell. The X-ray energy is absorbed mainly by the targeted area in the cell nucleus since the range of photoelectron produced by 5.35

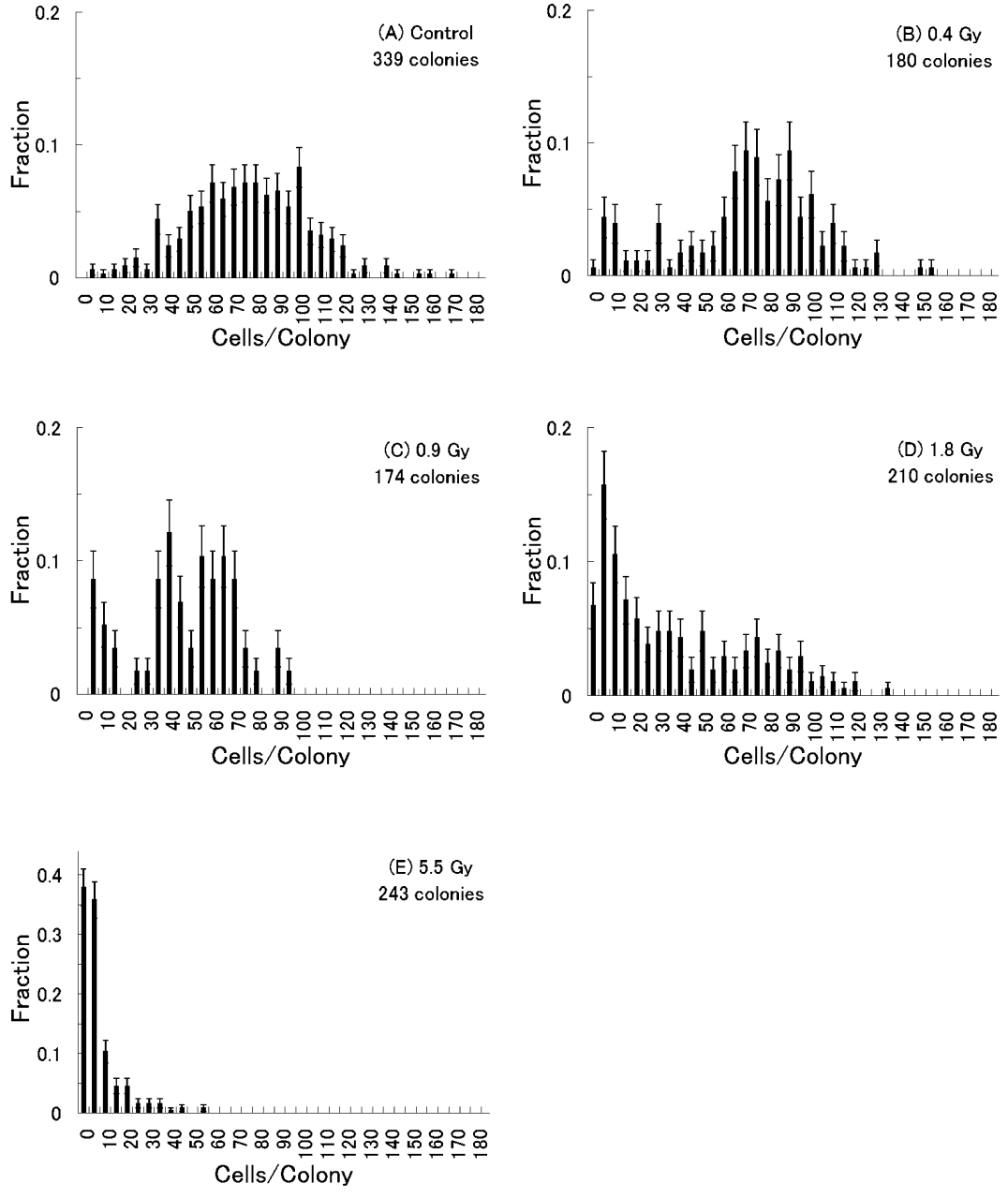

Fig. 2. Distribution of number of cells per colony after incubation for 60 h. Data were grouped into 5 cell intervals (from 1 cell per colony) and fractions in total are shown in the histogram. Panel (A) shows the histogram of the nonirradiated control group, and panels (B) to (E) show the histograms for the cells that were irradiated with soft X-rays of 0.4 Gy, 0.9 Gy, 1.8 Gy, and 5.5 Gy, respectively. Each histogram was drawn based on three independent experiments, and the total numbers of actually observed colonies are indicated in each panel. The error bars represent standard errors.

keV X-rays is less than 1 μm. In this research, we used the nuclear-averaged dose as a measure by which we could compare the biological effects of irradiation using 10×10 μm^2 X-ray beams with those using broadbeams and 50×50 μm^2 X-ray beams (see *Discussion*).

RESULTS

Life-or-death threshold for V79 cells for single-cell clonogenic assay

In the case of the conventional clonogenic assay, the V79 cells were incubated for 7 days after irradiation to form a colony. However, in the microbeam experiments, we needed to observe many colonies sequentially in a small irradiation area. To avoid interference between colonies originating from individual cells in a restricted area of the dish, we set the postirradiation incubation time to 60 h, a period sufficient for more than 6 cell cycles. In the conventional clonogenic assay with plastic dishes, we generally use 50 cells/colony as the life-or-death threshold of the cell.[16] This threshold can be used with sufficient incubation time, such as 7 days in the case of the V79 cells. Then, we determined the most appropriate threshold that gave us the same survival fractions as those obtained with the conventional assay.

We irradiated the samples prepared for the broadbeam experiments with 0.4-Gy, 0.9-Gy, 1.8-Gy, and 5.5-Gy soft X-rays using SOFTEX. The corresponding average survival fractions of the V79 cells obtained from three independent experiments with the conventional clonogenic assay were 0.89 ± 0.07, 0.83 ± 0.04, 0.50 ± 0.01, and 0.04 ± 0.003. Next, we prepared the samples in a microbeam dish, irradiated them with soft X-rays of the same dose as above, and incubated them for 60 h. Then, all the colonies originating from the irradiated cells were reobserved and the numbers of cells in each colony was determined. Figure 2 (A–E) show the histograms of the numbers of cells per colony that were drawn from three independent experiments in each dose. Two peaks, one at approximately 40 cells/colony and the other at approximately 60 cells/colony were observed in the histogram of 0.9 Gy-irradiated cells, as shown in Fig. 2-C.

Table 1. Survival fractions (*SFs*) determined using various thresholds of death in single-cell clonogenic assay. Deviations (*ΔSF*) are also shown from the survival fraction obtained using conventional clonogenic assay (values are shown in parentheses).

Threshold	*0.4 Gy* (0.89)		*0.9 Gy* (0.83)		*1.8 Gy* (0.50)		*5.5 Gy* (0.04)	
of cell death	*SF*	*ΔSF*	*SF*	*ΔSF*	*SF*	*ΔSF*	*SF*	*ΔSF*
10	0.92	0.03	0.87	0.05	0.68	0.4	0.16	3
15	0.91	0.02	0.84	0.01	0.61	0.2	0.12	2
20	0.91	0.02	0.85	0.02	0.55	0.1	0.07	0.7
25	0.92	0.03	0.84	0.01	0.52	0.04	0.05	0.3
30	0.88	0.01	0.82	0.01	0.48	0.04	0.04	0
35	0.91	0.02	0.78	0.06	0.45	0.1	0.02	0.5
40	0.92	0.03	0.66	0.2	0.42	0.2	0.02	0.5
45	0.92	0.03	0.60	0.3	0.41	0.2	0.01	0.8

SF in the Table 1 stands for the survival fraction. *ΔSF* refers to the difference in the survival fraction and was calculated as | (*SF* single-cell clonogenic assay) - (*SF* conventional clonogenic assay) | / (*SF* conventional clonogenic assay). Each data point in the table was calculated from three independent experiments. The smallest *ΔSF* was observed when the threshold was set to 30 cells/colony.

Table 2. Survival fractions of V79 cells irradiated with 50×50 μm^2 5.35 keV X-ray beams, as determined by the single-cell clonogenic assay.

Exposure ($\times 10^{-2}$ C/kg)	Control	1.2	2.5	3.8	4.9	9.7
Living cells	208	189	86	78	65	35
Dead cells	12	48	31	32	46	84
Total	220	237	117	110	111	119
Survival fractions	1	0.84	0.78	0.75	0.62	0.31

Data for the control, 1.2×10^{-2} C/kg, and 4.9×10^{-2} C/kg were pooled from four independent experiments and the rest from three independent experiments.

The former could be attributed to the cell group recovered from the division arrest induced by irradiation and the latter to the group dividing normally after irradiation. Using these histograms, we calculated the survival fractions using several possible life-or-death thresholds, from 10 cells to 45 cells in 5-cell steps, and compared the survival fractions thus calculated with those obtained from the conventional clonogenic assay. It was observed that the difference between the survival fractions from the two measurement methods was minimum when the threshold of 30 cells/colony was used for determining the survival fraction (Table 1). With this threshold, colonies containing less than or equal to 30 cells were counted as dead cells and those containing more than 30 cells were counted as living cells. The survival fractions of V79 cells irradiated with 0.4-Gy, 0.9-Gy, 1.8-Gy, and 5.5-Gy soft X-rays were 0.88 ± 0.02, 0.82 ± 0.03, 0.48 ± 0.03, and 0.04 ± 0.01, respectively. It must be mentioned that the division-delayed cells obtained in Fig. 2-C were counted as survivors using this threshold, as in the conventional clonogenic assay. From these results, it could be concluded that the survival fraction calculated in the single-clonogenic assay employed in this study gives results equivalent to those obtained using the conventional assay.

Survival fractions of V79 cells when entire cells were individually irradiated with 5.35-keV X-rays

We measured the survival fraction of the V79 cells irradiated with 5.35 keV X-rays using two methods, namely, the conventional clonogenic, and single-cell clonogenic assays, to confirm the validity of the single-cell clonogenic assay. In the former, the sample dishes were irradiated with broadbeams; in the latter, each cell was individually irradiated with 50×50 μm^2 beams, which are sufficiently large to irradiate entire cells.

The dose-survival relationship obtained after the broadbeam irradiation is shown in Fig. 3 (●). In the low-dose region below 1 Gy, the dose-survival curve showed slight HRS. The survival fractions of the cells irradiated with 50×50 μm^2 beams and assayed with the single-cell clonogenic assay are tabulated in Table 2 and also shown in Fig. 3 (■). The error of the survival fraction of the cells in the single-cell clonogenic assay is given in equation [2].[17] Large numbers of cells, as shown in Table 2, were irradiated with a microbeam, so that errors would be sufficiently small for comparison with the statistical significance.

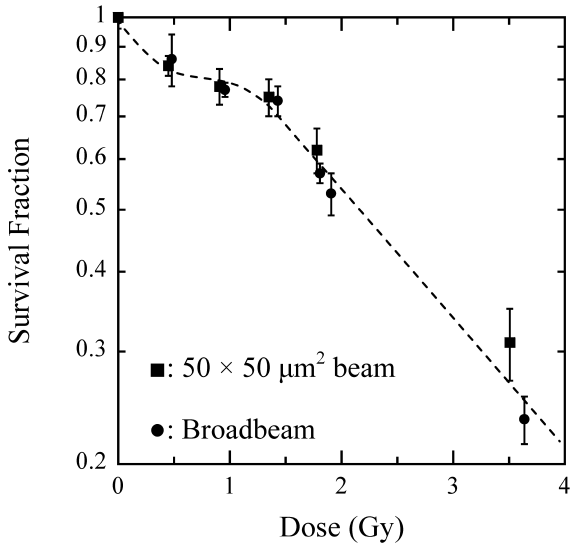

Fig. 3. Dose-survival curves of V79 cells irradiated with broadbeams (●) and 50×50 μm^2 X-ray beams (■). In the former case, survival fraction was calculated using conventional clonogenic assay (mean of six independent experiments); in the latter case, the survival fraction was calculated using single-cell clonogenic assay (mean of more than three independent experiments). The error bars represent standard errors. The dotted line is only a guide for the eyes.

$$Error = \pm\sqrt{\frac{SF(SF_0 + SF - 2SF \bullet SF_0)}{N \bullet SF_0^3}} \quad [2]$$

(N: total number of cells investigated; SF: survival fraction; SF_0: survival fraction of control)

The survival fraction decreased as the radiation dose increased, similar to the case of the broadbeam irradiation. Furthermore, a slight HRS, similar to that in the broadbeam experiments, was also observed in the low-dose region. The dose-survival relationship obtained with the 50×50 μm^2

Table 3. Survival fractions of V79 cells whose nuclei were irradiated with 10×10 μm^2 5.35-keV X-ray beams, as determined by the single-cell clonogenic assay.

Exposure ($\times 10^{-2}$ C/kg)	Control	1.3	2.6	3.8	5.1	10	15	21
Living cells	618	197	175	182	429	112	47	92
Dead cells	30	81	140	110	292	116	172	557
Total	648	278	315	292	721	228	219	649
Survival fractions	1	0.74	0.58	0.65	0.62	0.52	0.23	0.15

The data were accumulated from 5 to 12 independent experiments.

beams irradiation was almost the same as that obtained with the broadbeam irradiation. Under both experimental conditions, entire cells were irradiated with X-rays, with no difference in energy deposition in the cells between both conditions. Therefore, it can be concluded that, for survival studies, microbeam irradiation combined with single-cell clonogenic assay is an experimental method as reliable as the broadbeam irradiation combined with conventional clonogenic assay.

Survival fractions of V79 cells irradiated with 5.35-keV X-ray microbeams aimed at their nuclei

We irradiated the nuclei of the V79 cells using 10×10 μm^2 beams and determined the cell survival fractions with a single-cell clonogenic assay (Table 3). Note that values of the exposure in Table 3 are those inside the 10×10 μm^2 beams, not on the nucleus or on the entire cell. For this reason, we cannot simply plot the survival fractions from Table 3 in Fig. 3.

DISCUSSION

Estimation of dose for nuclear irradiation with 10×10 μm^2 X-ray microbeams

When broadbeams and 50×50 μm^2 beams irradiations we employed, the X-ray energy was uniformly absorbed in the entire cell. In these cases, we can determine the dose absorbed in the cell with the mass energy-absorption coefficient of the sample, as described above (equation [1]). However, in microbeam experiments using 10×10 μm^2 beams, only a part of the cell nucleus was irradiated. Therefore, we must determine the dose in order to compare the results of the microbeam and broadbeam irradiations under an appropriate assumption. One method of estimating the absorbed dose is by averaging the energy absorbed over the entire cell, i.e., "cell averaged dose," since we observe responses at a cellular level. According to this assumption, the cellular dose can be determined by dividing the total energy absorbed in the cell with the mass of the entire cell, regardless of the irradiated regions. It has already been reported, however, that cells whose cytoplasm are irradiated by alpha particles are killed much less efficiently than those whose nuclei are irradiated.[18,19] This suggests that the above method is not suitable for comparison. When 10×10 μm^2 beams were used for irradiation, the X-ray energy was absorbed mostly in the targeted area of the cell nucleus, since the horizontal cross section of the V79 cell nucleus was 168 μm^2 and the range of photoelectrons generated by the 5.35-keV X-ray photons was 0.78 μm. Therefore, we can consider that X-ray energy is absorbed by only a part (about 60%) of the cell nucleus when irradiation is performed using 10×10 μm^2 beams. Here, as the second method, we propose a "nuclear-averaged dose" at which the absorbed energy is divided by the mass of the nucleus. In whole-cell irradiation, the dose in the nucleus and that in the cytoplasm can be considered to be almost equal. Now, we can compare the survival fractions in nucleus-irradiated cells with those in whole-cell-irradiated cells using the nuclear-averaged dose. The nuclear-averaged absorbed dose in 10×10 μm^2 beams irradiation was calculated using equation [3].

$$D_{nucleus} = \frac{X \times W}{e} \times \frac{(\mu_{en} / \rho)_{V79}}{(\mu_{en} / \rho)_{air}} \times \frac{V_{beam}}{V_{nucleus}} \quad [3]$$

[V_{beam}: volume of nucleus traversed by beam; $V_{nucleus}$: total volume of nucleus. The values given in *Materials and Methods* were used for other parameters.]

In calculating the nuclear-averaged dose, we assumed that the cell has a thin, double-cylindrical structure, neglecting the cytoplasm located below and above the nuclei in the beam path and the variation in the thickness of the cells attached to the dish. The inner cylinder is the cell nucleus and the outer shell is the cytoplasm. In this case, the volume factor ($V_{beam}/V_{nucleus}$) in equation [3] was equivalent to the ratio of the beam size to the sectional area of the inner cylinder (nucleus), and it was measured as 0.60. We calculated the nuclear-averaged dose using this value*. Figure 4 shows the survival fraction of the nucleus-irradiated cells plotted as a function of the nuclear-averaged dose, along with the dose-survival curve obtained in whole-cell irradiation with 50×50 μm^2 beams. In the dose region above the 2 Gy, the dose-survival curve obtained in whole-cell irradiation was almost the same as or slightly higher than that obtained in nucleus irradiation. Considering that in cells irradiated with 50×50 μm^2 beams, radiation energy was deposited not only in the nucleus but also in the cytoplasm, these results may support the idea that the energy deposited in the cytoplasm is not highly efficient in inducing cell death.[18,19]

*Note: It is necessary to consider a three-dimensional structure of the cell in order to estimate doses to nuclei more accurately. We preliminarily measured the dimensions of V79 cells using an FV300 laser confocal microscope (Olympus Corp., Shinjuku-ku, Tokyo, JAPAN). The averages of the nucleus volume, the maximum thickness of the nucleus, and the thickness of the cytoplasm located between the nucleus and the surface of the Mylar film were approximately 1.0×10^3 μm^3, 8.7 μm, and 1 μm, respectively. For the nucleus irradiation with 10×10 μm^2 beams, the beam traversal volume in a nucleus was calculated to be about 760 μm^3 with the assumption that the center of mass of nucleus was irradiated by the beam. The volume factor in equation [3] ($V_{beam}/V_{nucleus}$) is 0.76 when these values are used. Considering this value, the nuclear-averaged dose in 10×10 μm^2 beams irradiation would be about 1.27 times higher than the dose plotted in Fig. 4. Therefore, the survival curve of the nucleus-irradiated cells would be magnified horizontally and

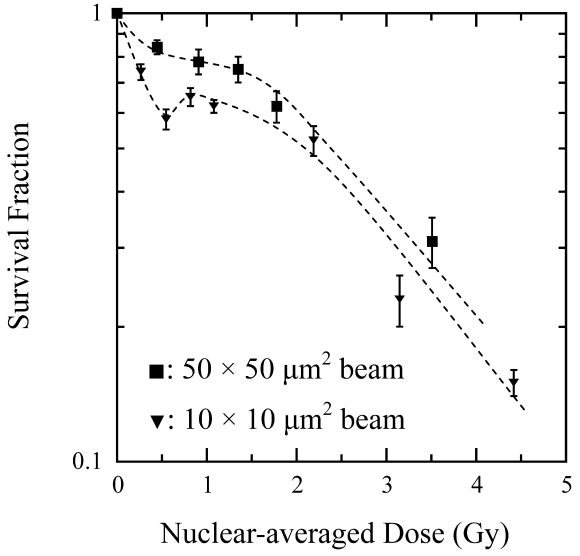

Fig. 4. Dose-survival curves of V79 cells irradiated with 10×10 (▼) and 50×50 (■) μm^2 X-ray beams. Standard errors are indicated by error bars. The survival fraction of the cells whose nuclei were irradiated with 10×10 μm^2 X-ray microbeams is plotted as a function of nuclear-averaged dose (mean of more than five independent experiments). The survival fraction of the cells irradiated with 50×50 μm^2 X-ray microbeams is plotted as a function of the absorbed dose. The dotted lines are only guides for the eyes.

become more similar to the survival curve obtained from whole-cell irradiation. Note, however, that even after the dose was corrected, the difference in shape between both survival curves in the low-dose region did not become obscure. Further measurements using a laser confocal microscope would give more reliable values statistically, but no precise description of absorbed dose at the cellular level would be attainable.

Magnitude of HRS is related to radiation-exposed domain in nucleus

Several reports have described HRS in survival studies using an asynchronous V79 population. When cell nuclei were irradiated with focused C_K X-rays, the survival fraction decreased to 85% in the HRS phase.[20] For a 3.2-MeV proton microbeam, the survival fraction decreased to about 80%.[17] In broadbeam irradiation, however, the survival fraction of V79 cells irradiated with 250-kVp X-rays or ^{60}Co γ-rays decreased to approximately 90%.[21–23] HRS was less evident in broadbeam experiments than in microbeam experiments. In our experiments, HRS was clearly observed in the V79 cells when only the nuclei were irradiated with 10×10 μm^2 beams, and the cell survival fraction decreased to approximately 60%. This may be the most distinct case of HRS in cell lethality reported thus far in microbeam experiments. On the other hand, for entire-cell irradiation, the survival fraction decreased to approximately 80%. These data show that HRS is enhanced only when the cell nucleus is irradiated.

With regard to the nucleus-irradiated case, we will consider in greater detail the energy-deposited volume in the nucleus. For the focused C_K X-ray irradiation, the beam size at the bottom surface of the nucleus had a radius of 0.25 μm, and the penetration depth of the C_K X-rays was about 2 μm. In proton beam irradiation, the energy-receiving region could be considered as a thin, needlelike volume, the diameter of which could be estimated to be approximately 10 nm. The volume that absorbs energy is the volume of the thin cylinder multiplied by the number of particle traversals. In both cases, the energy-receiving volumes are very small fractions of the entire nucleus; roughly, less than 1%. On the other hand, in our experiment, X-ray energy was rather uniformly absorbed in about 60% of the volume of the cell nucleus. These results suggest that the magnitude of HRS induction in nucleus-irradiated cases depends on the distribution of energy deposition in the cell nucleus. We can say that the distribution of DNA damage affects HRS induction in nucleus-targeted irradiation.

Possible interaction between radiation exposure of cytoplasm and HRS/IRR induction mechanism

HRS/IRR has been reported in many cell lines, and the dose-survival curve typically looks similar to the survival curve of the V79 cells irradiated with a broadbeam, as shown in Fig. 3 (●).[2] From the study of the cell cycle dependence of lethality, it was reported that cells irradiated in the G_2 phase exhibit more distinctive HRS than those irradiated in the G_1 or S phase.[24] HRS/IRR observed in dose survival curves is considered to be induced by DNA damage in cells irradiated in the G_2 phase as follows: Cells in the G_2 phase, with little amount of DNA damage, proceed to mitosis, passing through G_2 arrest and leading to cell death after division. Further, an increase in DNA damage at higher doses induces G_2 arrest and secures time for repair activity. As a result, HRS/IRR was observed in the dose-survival curve.

Figure 4 shows an obvious difference in the magnitude of HRS between nucleus irradiation and whole-cell irradiation, although the irradiated cell populations were the same. In the whole-cell irradiation with 50×50 μm^2 beams, the survival fraction decreased to 85% for an absorbed dose of 0.5 Gy, followed by a slow decrease in the next dose region up to 1 Gy. In the irradiation of the nucleus with 10×10 μm^2 beams, the survival fraction decreased to 60% for an absorbed dose of 0.5 Gy, and recovered to about 65% in the next dose region to approximately 1 Gy. In our experiments, we cannot ascribe this difference to the cell stage in the population, because the cell populations were prepared in the same methods. The only difference in the experimental procedure is the irradiated region in the cell: the entire cell or the cell nucleus. In whole-cell irradiation experiments with 50×50 μm^2 beams, the entire cytoplasm, as well as the

nucleus, was uniformly irradiated with X-rays, while most of the cytoplasm (roughly more than 90%) was not irradiated under cell nucleus irradiation with 10×10 μm^2 beams. The amounts of energy deposited in the nuclei were almost equal in both experiments at the same nuclear-averaged dose. These results suggest a possibility that the energy deposition in the cytoplasm can suppress HRS or enhance IRR. We consider that radiation-sensing mechanisms exist in the cytoplasm, which might induce some kind of intracellular signaling to control HRS/IRR, such as repair-related factors.

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