Comparison between Fractionated High Dose Rate Irradiation and Continuous Low Dose Rate Irradiation in Spheroids

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Recent interest in clinical brachytherapy focuses on the possible radiobiological equivalence between fractionated high dose rate (HDR) and continuous low dose rate (LDR) irradiations. This study is designed to compare the radiobiological effects between the two in vitro using multicellular spheroids of human tumor. Both HDR and LDR irradiations were delivered by ^{137}Cs source, the dose rates of which were as 1.18 Gy/min and 5.5 mGy/min, respectively. Fractionated HDR irradiation of various fraction sizes was applied twice a day. We found that: (1) The fractionated HDR irradiation (8 Gy/2 fr/day) was more effective radiobiologically than continuous LDR irradiation (8 Gy/day) and the ratio of radiobiological effects of these irradiations was estimated as 0.82, based on the 50% spheroid cure dose (SCD_{50}); (2) the radiobiological effectiveness was independent of the fraction size of HDR irradiation administrated, and the repair of sublethal damage (SLD) was absent, suggesting that the sparing effect of fractionated HDR irradiations was absent in spheroids. Our findings could provide important information for the clinical usage of the fractionated HDR radiotherapy to replace continuous LDR radiotherapy.

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Fractionated high dose rate (HDR) brachytherapy has the following advantages over the continuous low dose rate (LDR) brachytherapy: a) The applicators tend to remain in place during irradiations because the treatment time is shorter than with LDR irradiation, and thus the dose distribution can be estimated more accurately; b) the applicator of the HDR is easier to manipulate for better dose distribution at the target; and c) the personnel administrating the treatment is not exposed to radiation (1). Because of these advantages, fractionated HDR brachytherapy using ^{192}Ir is replacing the conventional LDR brachytherapy as a common treatment for a variety of malignant tumors. Therefore, recent interest in clinical brachytherapy focuses on the radiobiological equivalence between these two types of irradiation but there are few clinical and experimental data available comparing the two therapies. This study aims at providing much needed data to develop and support a clinical study, by comparing the radiobiological effectiveness of fractionated HDR irradiation with that of continuous LDR irradiation using the human tumor model and anti-tumor endpoint.

We used the multicellular spheroid system of human malignant tumor cells. This system is useful as an experimental model of solid tumors because of its unique characteristics—the cell cycle distribution, radiosensitivity and drug sensitivity—that are absent in monolayer cultures (2). Furthermore, spheroids can be maintained in culture for a relatively longer period than monolayer cultures and thus appear to be suitable for studies to investigate the radiobiological effect of multiple fractionated irradiation and continuous irradiation (3, 4).

The radioresistant hypoxic cells in malignant tumors pose major problems in radiotherapy. In addition, hypoxic cell fraction in tumor affects its radiosensitivity to fractionated radiotherapy because hypoxic cells reoxygenate during irradiations (5–7) and the oxygen level in the tumor affects the repair of sublethal damage (SLD) of tumor cells (8, 9). Since large spheroids contain radiobiological hypoxic cells (10, 11), we used them as in vitro models to approximate the condition of solid tumor exposed to fractionated radiation.

We show in this study the effective dose of fractionated HDR irradiation equivalent to that of the continuous LDR using multicellular spheroids.

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MATERIAL AND METHODS

SQ5, a human squamous lung cancer cell line was donated by Dr Ohara of Okayama University and has been maintained in our laboratory for years. The cell line was cultured in α-MEM (Flow Laboratories, Scotland) supplemented with a mixture of 20 mM HEPES, 10% fetal calf serum (Xavier Investments, Australia), penicillin (50 units/ml) and streptomycin (50 μg/ml). Cell cultures were maintained in a humidified incubator at 37°C filled with a mixture of 98% air and 2% CO_2.

For experiments with monolayer cells, 5×10^4 cells in 1.5 ml medium were plated in a 15-mm well (Nunc, Denmark) and incubated for 24 h before treatment. Cells were nearly confluent at the start of irradiation.

Multicellular spheroids were obtained by a spinner culture method, as described in our previous study (12). On day 1, spheroid formation was initiated by incubating the 10^6 cells in 100-mm Petri dishes (Corning, NY) coated with 1% agar. On day 3, small spheroids were transferred from these dishes to a spinner flask (Bellco, Vineland NJ) with a fresh medium supplemented with 5% serum. Spheroids in the spinner flask were stirred continuously at 190 rpm on a magnetic stirrer placed inside the CO_2 incubator. The medium was changed every other day until the spheroids grew to a diameter of 500 μm on days 13–15. At that point, about 75 spheroids were transferred to a 15-mm well and irradiated. For all experiments with spheroids, the medium containing 0.2% agar was used to keep the spheroids from disaggregating during the period of overall treatment of up to 6 days.

Continuous LDR irradiation experiments were carried out in a humidified CO_2 incubator at 37°C using ^{137}Cs-tube sources. Four tubes with a total activity of 2.96 GBq (80 mCi) were placed, two by two, with no space between the tubes, in a slot of a piece of Styrofoam and taped in place. Another piece of 2 cm-thick Styrofoam and a 1-mm acryl plate were layered on the Styrofoam board with sources to give an appropriate dose to cells and ensure a homogeneous energy deposit. The well containing the cells was placed on the acryl plate exactly above the center of the sources.

Irradiations of single dose, fractionated HDR and split dose were delivered by a Gamma cell 40 ^{137}Cs source (Atomic Energy of Canada, Ltd.). The well containing the cells or spheroids was placed in a Styrofoam box to maintain the temperature at 37°C during irradiation. For fractionated HDR irradiation, multiple fractions of 4, 5 or 6 Gy were given twice a day at intervals of at least 6 h and the well was stored inside the CO_2 incubator between irradiations.

The actual dose rates for LDR and HDR irradiation were measured by both thermoluminescent dosimetry (TLD) and ionization chamber-type dosimetry (Markus chamber PTW-Freiburg, Germany). TLD chips were calibrated using the Gamma cell 40 ^{137}Cs source, the activities of which were estimated with the chamber dosimetry. The calibrated TLD chips were placed inside the well under the same conditions as the cells for experiments. For the estimation of dose rate of LDR and HDR irradiations, 10 TLD chips were exposed for 24 h and 10 min, respectively, and were analyzed 24 h after the irradiations. All measurements were repeated at least twice. The instantaneous dose rates of HDR and LDR irradiations were estimated by the chamber dosimetry. The dose rates for LDR and HDR were measured as 5.5 mGy/min (about 8 Gy/day) and 1.18 Gy/min, respectively. The difference between the dose rates estimated by TLD and chamber dosimetry was not significant.

To assess the dose distribution uniformity of LDR irradiation to the bottom of the 15-mm well, the difference in instance dose rate between three points—at the center of sources, at 5 mm and 1 cm horizontally away from the center—were estimated by the chamber dosimetry. Alternatively, an enveloped x-ray film was exposed under the same experimental conditions. The homogeneity of density of the film was measured by densimeter (Fujitec, Japan). The dose inhomogeneity of the bottom of the well was determined to be below 8% by these methods.

For the single dose and split dose irradiation experiments, surviving fractions of both monolayer cultures and spheroids were estimated by a conventional clonogenic colony formation assay. After irradiation, monolayer cells and spheroids were digested with a 0.05% trypsin solution containing 0.02% EDTA. Cells were counted, plated in 60-mm Petri dishes (Corning, NY) containing 5 ml complete medium at appropriate cell concentrations and incubated at 37°C in the humidified CO_2 incubator for 12 days, at which time colonies were stained with crystal violet. Colonies with 50 or more cells were counted as survivors. Control plating efficiencies were around 80–95% for both monolayer cultures and spheroids.

For the fractionated HDR and continuous LDR irradiation experiments on monolayer cultures, relative numbers of clonogenic cells per well were calculated after exposure by counting the numbers of total cells and colony forming cells. For the spheroids, the cure probability was measured by the method of Durand (13). After irradiation, each spheroid was transferred to an individual well of a 48-well plate (Corning, NY), incubated for 3 weeks in a CO_2 incubator. Spheroids were not considered cured if any outgrowth was evident while cure of spheroids was defined by a lack of outgrowth (14). We calculated the dose required to cure 50% of the spheroids (SCD_{50}) and analyzed the significant differences between HDR and LDR irradiation using the statistical analysis devised by Litchfield & Wilcoxon (15). All experiments were repeated at least twice.

RESULTS

To determine intrinsic radiosensitivity, cell survival curves for SQ5 monolayer cells and spheroids after single dose irradiations were obtained (Fig. 1). Spheroids were irradiated in the medium containing 0.2% agar at 37°C. Spheroids were clearly more resistant to radiation than monolayer cultures. The survival parameters for monolayer cells were: α, 0.243; β, 0.030, and for spheroids: α, 0.089; β, 0.005.

The dose-response curves (the relationship between total dose and relative numbers of clonogenic cells per well) for SQ5 monolayer cultures after fractionated HDR and continuous LDR irradiations are shown in Fig. 2. The radiobiological effectiveness was defined by the ratio of LD_{98} (lethal doses required to reduce the relative numbers of clonogenic cells per well to 0.02). The ratio between the doses of HDR (8 Gy/2 fr/day) and LDR (8 Gy/day) in the same overall time was 0.77 (see Table 1). As the size of fraction of HDR irradiation increased, so did the decline of the response curves and the radiobiological effectiveness, indicating that monolayer cultures had a sparing effect on the fractionated irradiation.

The relationship between the total dose and cure probabilities for SQ5 spheroids after the exposure to fractionated HDR and continuous LDR irradiation is shown in Fig. 3. The SCD_{50} of fractionated HDR irradiations was significantly smaller than that of continuous LDR irradiations. The ratio between SCD_{50} for HDR (8 Gy/2 fr/day) and LDR (8 Gy/day) in the same overall time was 0.82 (see Table 1). Differences among three sigmoid curves

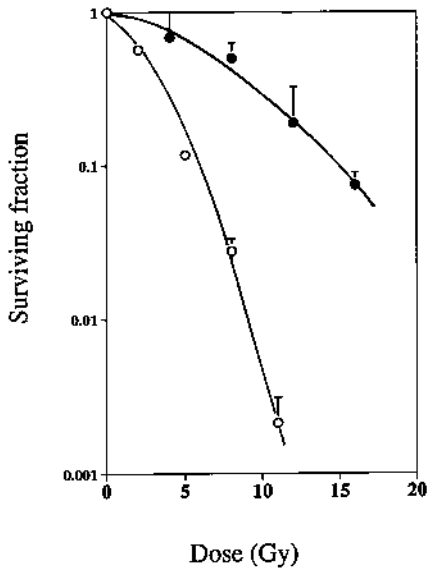

Fig. 1. Cell survival curves for SQ5 monolayer culture and spheroids after high dose rate (HDR) irradiation as a single dose. Monolayer culture (○); 500 μm spheroids in a 15-mm well containing 0.2% agar medium at 37°C (●). Each point represents the mean of two or three experiments with standard error.

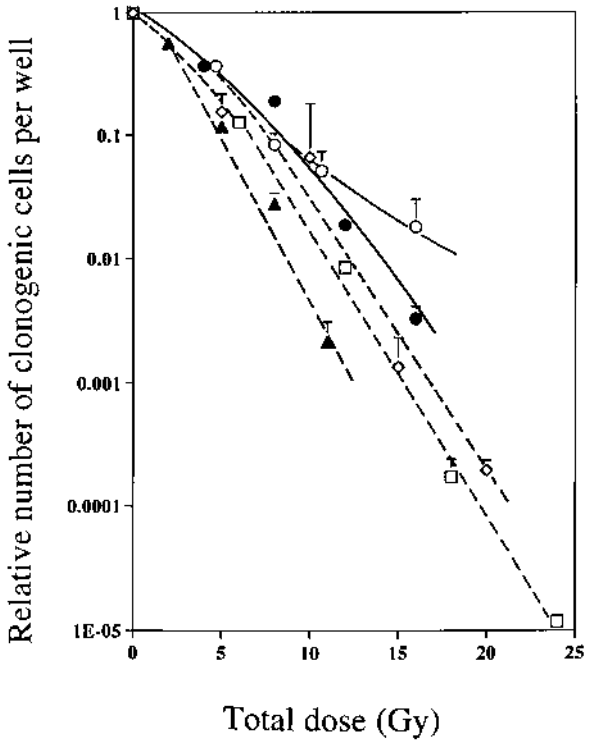

Fig. 2. Survival curves (relationships of radiation dose and relative number of clonogenic cells per well) for SQ5 monolayer cells after fractionated high dose rate (HDR) irradiation and continuous low dose rate (LDR) irradiation. Single dose (▲); LDR continuous irradiation (○); HDR fractionated irradiation, 4 Gy/fr (●); 5 Gy/fr (◇); 6 Gy/fr (□). Each point represents the mean of two or three experiments with standard error.

showing the radiobiological effectiveness of HDR irradiations at fraction sizes 4, 5, and 6 Gy (Fig. 3), were not statistically significant. Thus, the isoeffective dose (the total dose required to induce an identical biological effect) was not dependent on the fraction size. Data suggested that spheroids lacked the sparing effect of fractionated HDR irradiation and this led us to focus on the repair of SLD of spheroids.

Next, we compared the repair of SLD of monolayer cultures to that of spheroids. The result of split dose experiments, in which two doses of 6 Gy were separated by increasing intervals of time, is shown in Fig. 4. The results are expressed in terms of the recovery ratio, i.e. the ratio of the surviving fraction resulting from two-dose fraction to the survival from a single equivalent dose. In monolayer cultures, a marked repair of SLD was observed in the first hour and completed in 4 h with a recovery ratio of 2.5. In spheroids, however, no recovery was observed even after 8 h, and the recovery ratio remained less than 1.

DISCUSSION

Our data indicate that the fractionated HDR irradiation (8 Gy/2 fr/day) is significantly more effective than the contin-

Table 1

The ratio of radiobiological effectiveness between HDR and LDR

	Monolayer cultures		Spheroids	
	LD_{98}(Gy)*	LD_{98}(HDR)/LD_{98}(LDR)	SCD_{50}(Gy)**	SCD_{50}(HDR)/SCD_{50}(LDR)
LDR (8 Gy/day)	16	—	38	—
HDR				
4 Gy × 2 fr/day	12.3	0.77	31	0.82
5 Gy × 2 fr/day	11.3	0.70	30	0.79
6 Gy × 2 fr/day	9.8	0.61	32	0.84

LDR = low dose rate; HDR = high dose rate.
* The lethal doses required to reduce the relative numbers of clonogenic cells per well to 0.02.
** The dose required to cure 50% of the spheroids.

uous LDR irradiation (8 Gy /day) in SQ5 spheroids when both the total dose and the overall time are held constant. Several studies have demonstrated results that support our results to some degree, while their tumor models and experimental protocols were not identical to ours. Chen et al., for example, using monolayer culture cells, have demonstrated that the pulsed high dose rate irradiation became more effective than continuous LDR irradiation as the pulse interval increased and that there were significant differences for pulse intervals of 6 h and 12 h (16). Sethi et al. have shown that in the transplant rodent tumor model, the pulsed dose irradiation was significantly more effective than continuous LDR irradiation when the same daily average dose rate was maintained in both irradiation regimens (17). Inoue et al. have demonstrated in their clinical study that fractionated HDR interstitial brachytherapy (60 Gy/10 fr/5 day, twice daily irradiation) with ^{192}Ir can be equivalent to conventional continuous LDR (70 Gy/4–9 days) for an early tongue cancer (1). The irradiation protocol of the clinical study is similar to ours. In their study, the ratio of total dose in these isoeffective treatments is 0.86, close to the 0.82 which we determined as the SCD_{50} ratio between HDR (8 Gy/2 fr/day) and LDR (8 Gy/day). This finding suggests that the total dose used for fractionated HDR interstitial brachytherapy in the clinical study may be radiobiologically appropriate.

In the present study, we obtained the following results in spheroid in contrast to those of monolayer cultures: (a) In spheroids, the radiobiological effectiveness of fractionated HDR irradiation is independent of the fraction size; and (b) the spheroids are incapable of repairing the SLD. According to Ling et al., the capability to repair SLD appears to be dependent on the cellular energy status, which in turn is dependent on the oxygen concentration (8). Hence, we suggest that cells in spheroids lose the capability to repair SLD because spheroids contain more radiobiological hypoxic cells compared with monolayer cultures. We obtained the following two findings to suggest the existence of radiobiological hypoxic cells in spheroids. First, spheroids of 500 μm in diameter in the medium containing 0.2% agar at 37°C were clearly more resistant to irradiation than monolayer cultures (Fig. 1). This suggests that the spheroids kept in this condition may have a significant radiobiological hypoxic cell fraction, while other factors may also contribute to the differences in sensitivity to radiation. Second, the spheroids of the same diameter that were irradiated in a spinner flask with stirring at 190 rpm at 37°C and in a well at 4°C became significantly more radiosensitive than spheroids in the agar at 37°C. Their survival curves were close to those of monolayer cultures (data not shown). These results suggest that the continuous stirring of the medium supplies oxygen of a constant amount to the cells and that the oxygen consumption rate of spheroids at 4°C declines because the metabolite state of the cells decreases at this temperature. Under these conditions there may be no significant radiobiological hypoxic cells in spheroids. According to Moore et al. (10) and Rofstad et al. (18), the larger the spheroid (diameter 500 μm <), the greater the fraction of radiobiologically hypoxic cells it tends to contain. We suggest that not only the size of the spheroid but also the condition in which the spheroid is kept can influence the fraction of hypoxic cells. In summary, we suggest that the hypoxic fraction in spheroids may induce its unique radiobiological feature in contrast to monolayer cultures, i.e., lack of sparing effect of fractionated irradiation and SLD repair.

Studies using animal model have also demonstrated the lack of SLD repair. Vogler et al. have investigated the radiobiological effectiveness of fractionated HDR irradiation on rat rhabdomyosarcoma containing approximately 50% of radiobiological hypoxic fraction (19). In their study, the cellular response depended on the total dose and showed no significant difference between the different fraction schedules. Sethi et al. have found the apparent isoeffectiveness of tumor response to single (acute) and pulsed dose irradiations maintaining the same daily dose rate using a rat sarcoma (17). These data suggest that either the repair capacity is impaired or the effect of rapid repair is offset by the reoxygenation of hypoxic tumor cells. In addition, our data on split dose irradiations show that the

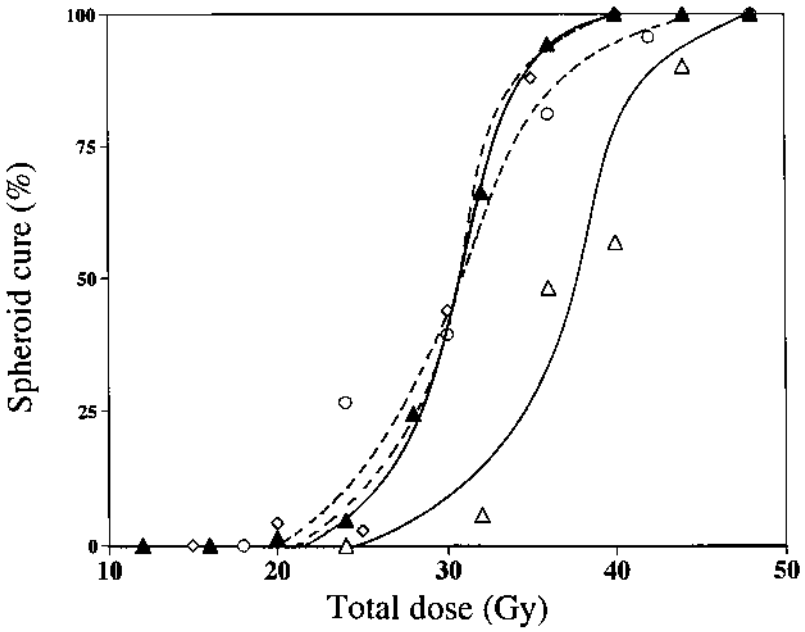

Fig. 3. Spheroid cure curves for SQ5 spheroids after fractionated HDR irradiation and LDR continuous irradiation. Continuous LDR irradiation (△); HDR fractionated irradiation, 4 Gy/fr (▲); 5 Gy/fr (◇); 6 Gy/fr (○).

survival fraction of spheroid cells decreases when the time interval between the two split doses increases. This indicates that the spheroid cells not only lose the capability to repair SLD, but also become more radiosensitive to the second dose exposure. Durand et al. reported a similar result using V-79 spheroids (20). They demonstrated that the spheroids tended to be sensitized to the second dose of irradiation during the first few hours after the first of the large doses. They suggested, therefore, that as the oxygen consumption of radiation-damaged cells decreased, the oxygen availability to the previous hypoxic cells increased. They also suggested that a balance between repair and sensitization resulting from reoxygenation determined the cell survival fractions. Hence, we conclude that the fraction of radiobiological hypoxic cells in a solid tumor influences the radiobiological effectiveness in fractionated HDR irradiation and that it should be considered in the clinical practice of fractionated HDR brachytherapy. Our conclusion, however, is drawn from studies using the one tumor type of spheroids. Radiobiological features of spheroids including SLD repair capacity differ according to tumor types as well as the size, proliferation status, the microenvironmental conditions and the distribution of oxygen (21). Further radiobiological studies using other tumor models will be necessary to establish the standard protocol of HDR-fractionated brachytherapy.

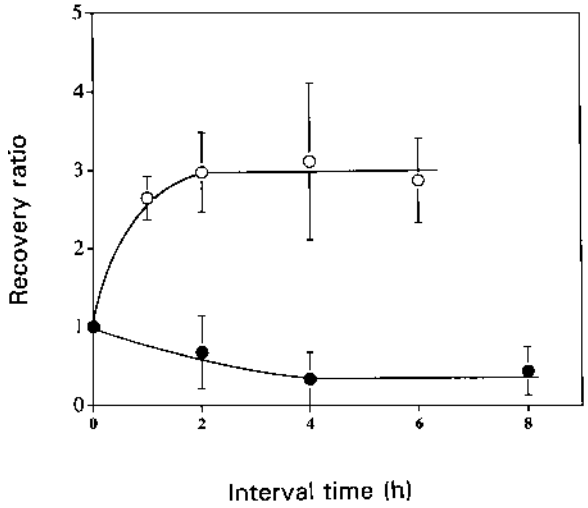

Fig. 4. Split-dose recovery ratio plotted as a function of time interval the split doses. Monolayer culture (○); spheroids (●). Each point represents the mean of two or three experiments with standard error.

We have shown that the radiobiological effectiveness of fractionated HDR irradiation is higher than that of continuous LDR irradiation with a corresponding ratio of 0.82 in SQ5 spheroids. In addition, the radiobiological effectiveness of fractionated HDR irradiation is independent of the fraction size, indicating that the sparing effect of fractionated irradiation is absent in the spheroids. Our study provides the information on the dose correlation between fractionated HDR and continuous LDR radiotherapy to determine the equivalent effect of the two. In the light of the current trend in which fractionated HDR radiotherapy is replacing continuous LDR radiotherapy, this information is vital for clinical practice.

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