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Impact of time interval and dose rate on cell survival following low-dose fractionated exposures

Shingo Terashima[1,*], Yoichiro Hosokawa[1], Eichi Tsuruga[1], Yasushi Mariya[2] and Toshiya Nakamura[3]

[1]Department of Radiation Science, Hirosaki University Graduate School of Health Sciences, 66-1 Hon-cho, Hirosaki, Aomori 036-8564, Japan
[2]Department of Radiology, Mutsu General Hospital, 1-2-8 Kogawa-cho, Mutsu, Aomori 035-8601, Japan
[3]Department of Bioscience and Laboratory Medicine, Hirosaki University Graduate School of Health Sciences, 66-1 Hon-cho, Hirosaki, Aomori 036-8564, Japan
*Corresponding author. Department of Radiation Life Science, Hirosaki University Graduate School of Health Sciences, Hirosaki University, 66-1, Hon-cho, Hirosaki 036-8564, Japan. Tel: +81-172-39-5525; Fax: +81-172-39-5912; Email: s-tera@hirosaki-u.ac.jp
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ABSTRACT

Enhanced cell lethality, also known as hyper-radiosensitivity, has been reported at low doses of radiation (≤0.5 Gy) in various cell lines, and is expected to be an effective cancer therapy. We conducted this study to examine the impact of time interval and dose rate of low-dose fractionated exposures with a short time interval. We evaluated the cell-survival rates of V79 and A549 cells using clonogenic assays. We performed fractionated exposures in unit doses of 0.25, 0.5, 1.0 and 2.0 Gy. We exposed the cells to 2 Gy of X-rays (i) at dose-rates of 1.0, 1.5 and 2.0 Gy/min at 1-min intervals and (ii) at a dose-rate of 2.0 Gy/min at 10-s, 1-min and 3-min intervals by fractionated exposures. Apoptosis and cell cycle analyses were also evaluated in the fractionated exposures (unit dose 0.25 Gy) and compared with single exposures by using flow cytometry. Both cell-type survival rates with fractionated exposures (unit dose 0.25 Gy) with short time intervals were markedly lower than those for single exposures delivering the same dose. When the dose rates were lower, the cytotoxic effect decreased compared with exposure to a dose-rate of 2.0 Gy/min. On the other hand, levels of apoptosis and cell cycle distribution were not significantly different between low-dose fractionated exposures and single exposures in either cell line. These results indicate that a stronger cytotoxic effect was induced with low-dose fractionated exposures with a short time interval for a given dose due to the hyper-radiosensitivity phenomenon, suggesting that dose rates are important for effective low-dose fractionated exposures.

KEYWORDS: low dose hyper-radiosensitivity, dose rate, time interval, low-dose fractionated exposure

INTRODUCTION

Hyper-radiosensitivity (HRS), at low doses of radiation (≤0.5 Gy), can increase cell lethality more than the linear–quadratic model (L–Q model) in various cell lines [1–3]. Biologically, hypersensitivity in this low-dose area is interpreted as a strategic mechanism, by which a transmutated cell is eliminated by cell death rather than kept with DNA damage. Increased radioresistance (IRR) is attained during the transition from sensitivity to resistance at approximately 0.2–0.8 Gy [4].

A number of laboratories have studied HRS with high cytotoxic effects for application in radiation therapy. The HRS response has been confirmed with not only tumors but also normal cells. However, it is thought that this response is not important for late-responding connective tissues such as the skin and other normal cells with slow growth [5, 6]. Additionally, HRS/IRR is more prominent in cells displaying genomic instabilities, including radioresistant cells, and is therefore more profound in tumors and some transformed normal cells [1–3]. It is reported that human and rodent tumor cells with a high probability of metastasis exhibit HRS by priority [7–10], and thus HRS is expected to be an effective cancer therapy. Marked HRS was shown at doses below 0.5 Gy. However, enhanced cytotoxic effects showing HRS is originally low, and it is difficult for radiation therapy to use a dose showing HRS by a single low-dose fraction. Therefore, HRS irradiation for

clinical applications is modified to low-dose fractionated radiation therapy (LDFRT).

It has been reported that a radioresistance-like adaptive response is observed after LDFRT with short (1–2 h) intervals and HRS recovers, provided there are >3-h intervals between successive doses [11]. Most laboratories performed LDFRT in low-dose fractions (≤1 Gy) at intervals of several hours twice a day or more [9, 12, 13]. In addition, it has been reported that there is the possibility of benefit from the effects of chemotherapy when LDFRT is used in conjunction with chemotherapy [12–14]. However, some studies have reported that LDFRT at intervals of several hours does not enhance the cytotoxic effect [15–17]. On the other hand, other studies have reported that LDFRT at short time intervals (2 or 3 min) shows enhanced cytotoxic effects [18–20]. Depending on the unit dose and the total dose, LDFRT alone takes at least 15 min or more, even when short, 3-min intervals are used. Discomfort to the patients (due to their restricted movement) and the burden to the medical staff are obvious in the context of the time schedule involved in the LDFRT-based treatment regime. In addition, sublethal damage (SLD) repair, leading to a decreased effect of radiation, occurs during prolonged radiation delivery in intensity-modulated radiotherapy with multiple low doses and is a cause for concern [21–23]. As mentioned above, little is known about whether various combinations of unit doses, time intervals, dose rates, and number of fractions influence the proportion of cells surviving in LDFRT. In this study, we investigated the effect of LDFRT administered at short time intervals (10 s, 1 min and 3 min) in an attempt to simplify practical clinical application. For example, if the efficacy of LDFRT using 1-min intervals could be established, this might reduce treatment time as well as ameliorate the burden on both patients and the medical staff. We evaluated the impacts of a unit dose, dose rates, and time intervals on the cytotoxic effect of LDFRT.

MATERIALS AND METHODS

Cell culture

Chinese hamster V79 lung cells (ATCC, USA) and human lung A549 cells (RIKEN Bio-Resource Center, Japan) were used for our study. These cells exhibit HRS and have been conventionally used for experiments involving fractionated exposures [1, 3, 13, 24–26]. Additionally, the parameters of the HRS/IRR responses of human cells and rodent cells do not differ significantly [27]. Cells were cultured in DMEM/Ham's F-12 (A549) and Ham's F-12 (V79) media (Wako, Japan) supplemented with 10% fetal bovine serum, and were maintained at 37°C with 95% air and 5% CO_2.

X-irradiation and irradiation schedule

X-irradiation was delivered using an MBR-1520R-3 X-ray machine (Hitachi Medico Technology, Tokyo, Japan) at 150 kVp through a 0.5 mm Al and a 0.1 mm Cu filter. Before exposure to X-rays, the cell-culture dishes were taken out of the incubator and equilibrated at room temperature for ~5 min to avoid possible temperature shock. The unirradiated samples were left in the room outside the X-ray machine while experimental samples were irradiated. Radiation dose was monitored by an ionization chamber, and irradiation was terminated automatically when the prescribed dose was achieved. Error was <1 cGy even with a maximum estimate by one irradiation since <1 cGy of the indicated values by the ionization chamber are omission of fractions. We performed exposure experiments using unit dose(=fractionated dose) of 0.25, 0.5, 1.0 and 2.0 Gy. First, we exposed the cells to 2 Gy of X-rays at dose rates of 1.0, 1.5 and 2.0 Gy/min at 1-min intervals by fractionated radiation (Irradiation Method 1). The dose rates were adjusted to 1.0, 1.5 and 2.0 Gy/min by changing the X-ray tube current to 10, 15 and 20 mA, maintaining a constant distance between the focus and the culture dishes. Next, we exposed these cells to a radiation dose rate of 2.0 Gy/min at 10-s, 1-min and 3-min intervals by fractionated radiation (Irradiation Method 2). Finally, total doses of 1, 2, 4, 6 and 8 Gy were given by fractionated radiation at a dose rate of 2.0 Gy/min for 10 s (Irradiation Method 3). As an example, a scheme of the radiation schedule (Irradiation Method 1) is shown in Fig. 1.

Clonogenic assay

In brief, exponentially growing V79 (6.0×10^1 – 8.0×10^3) and A549 (1.5×10^2 – 7.0×10^4) cells were plated onto four 60-mm culture dishes (IWAKI, Japan) for each exposure. Next, cells were irradiated 6 h later according to the above irradiation schedules before cells started replicating [28]. After incubation of V79 and A549 for 6–9 and 10–14 days, respectively, the resulting colonies were stained with 4% Giemsa in PBS (−). Colonies containing >50 cells were counted as surviving cells. The surviving fraction at each exposure was calculated as the ratio of the plating efficiencies for irradiated and unirradiated cells, and was normalized to that of a single exposure at the same conditions (dose rate or time interval) as relative survival ratio.

The surviving fraction data (Irradiation Method 3) were fitted to the L–Q model, $lnS = -\alpha D - \beta D^2$, where S is the surviving fraction and D is the radiation dose using the Origin 8J program (Lightstone Corp., Japan). The parameters α, β and D_{10} were calculated for each curve, and the relative biological effect at 10% survival (RBE_{10}) was calculated from (D_{10} of single exposure)/(D_{10} of fractionated exposures).

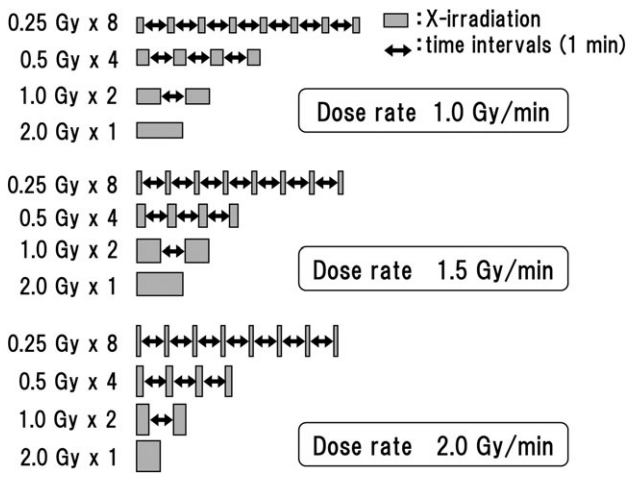

Fig. 1. Schematic diagram of the irradiation schedule. Cells were exposed to low-dose fractionation at three different dose rates with 1-min intervals (Irradiation Method 1).

Analysis of apoptosis by flow cytometry

V79 (5×10^4) and A549 (3×10^5) cells were plated onto 60-mm culture dishes. Twenty-four hours after plating, cells were exposed to total doses of 2, 4 and 8 Gy by single exposures or fractionated radiations in unit doses of 0.25 at a dose rate of 2.0 Gy/min at 10-s intervals. Forty-eight hours after irradiation, cells were stained with 5 μg/ml Annexin V (BioLegend, USA) and propidium iodide (PI) (Sigma, USA) in Annexin V Binding Buffer (BioLegend), and apoptotic cells were analyzed using a flow cytometer (Cytomics FC500; Beckman Coulter, USA). Sample data were analyzed using the FlowJo 7.6.5 software (Treestar, Inc., USA).

Cell cycle analysis by flow cytometry

V79 (7×10^4) and A549 (2×10^5) cells were plated onto 60-mm culture dishes. Twenty-four hours post plating, cells were exposed to 2, 4 and 8 Gy doses, either by single exposure or fractionated radiation in unit doses of 0.25 Gy and dose rate of 2.0 Gy/min at 10-s intervals. After irradiation for 12 and 24 h, cells were fixed in 70% ethanol and stored at −30°C. Ethanol-fixed samples treated with 250 μg/ml RNaseA PBS (−) and stained with 30 μg/ml PI/PBS (−) were analyzed by using flow cytometer. Sample data were analyzed using the FlowJo 7.6.5 software.

Statistical analysis

Statistical comparisons were performed using the Tukey–Kramer test for multiple comparisons (clonogenic assays) and Welch's *t*-test for two comparisons (analysis of apoptosis and cell cycle). Results are presented as means ± standard deviation from the results of at least three independent experiments. $P < 0.05$ was considered to indicate a statistically significant result. Statistical analysis was performed using the Excel 2013 software program (Microsoft, USA) with Statcel 3 add-in software (OMS Inc., Japan).

RESULTS

Clonogenic assays

In the initial experiment, we evaluated the relative survivals (normalized to 2 Gy single exposure) of V79 and A549 cells in fractionated exposures using clonogenic assays. First, we exposed the cells to 2 Gy of X-rays at dose rates of 1.0, 1.5 and 2.0 Gy/min at 1-min intervals (Irradiation Method 1) by fractionated radiation. A significant decrease in relative survivals was observed when both cell lines were exposed to low-dose fractionations with a unit dose of 0.25 Gy (0.25 Gy × 8) at 2.0 Gy/min, compared with a 2 Gy single exposure ($P < 0.01$) (Fig. 2). Furthermore, a significant decrease in relative survivals was observed when V79 cells were exposed to 0.5 Gy × 4 at 2.0 Gy/min, compared with that under a 2 Gy single exposure. Similarly, a significant decrease in relative survivals was observed in A549 cells when exposed to LDFRT (0.25 Gy × 8) at 1.5 Gy/min, compared with that under a 2 Gy single exposure. However, no significant differences in relative survival were observed in V79 cells between LDFRT (0.25 Gy × 8) and single exposures at 1.0 and 1.5 Gy/min. Similarly, in A549 cells, no significant differences in relative survivals were observed between LDFRT (0.25 Gy × 8) and a single exposure at 1.0 Gy/min. At lower dose rates (1.0 and 1.5 Gy/min), cytotoxicity caused by LDFRT (0.25 Gy × 8) was lesser than that at exposures of 2.0 Gy/min.

Next, we evaluated the relative survival of fractionated exposures at 2.0 Gy/min at 10-s, 1-min and 3-min intervals (Irradiation Method 2). In V79 cells, significant decreases in relative survival were observed between LDFRT (0.25 Gy × 8) and single exposures at 10-s and 1-min intervals ($P < 0.01$) (Fig. 3a). However, the enhanced cytotoxic effect decreased when V79 cells were exposed to low-dose fractionation (0.25 Gy × 8) at 3-min intervals, compared with that for LDFRT using shorter time intervals (10 s and 1 min). On the other hand, significant decreases in relative survival were observed when A549 cells were exposed to LDFRT (0.25 Gy × 8) using 10-s, 1-min and 3-min intervals compared with single exposures and 1 Gy × 2 ($P < 0.05$ or $P < 0.01$) (Fig. 3b).

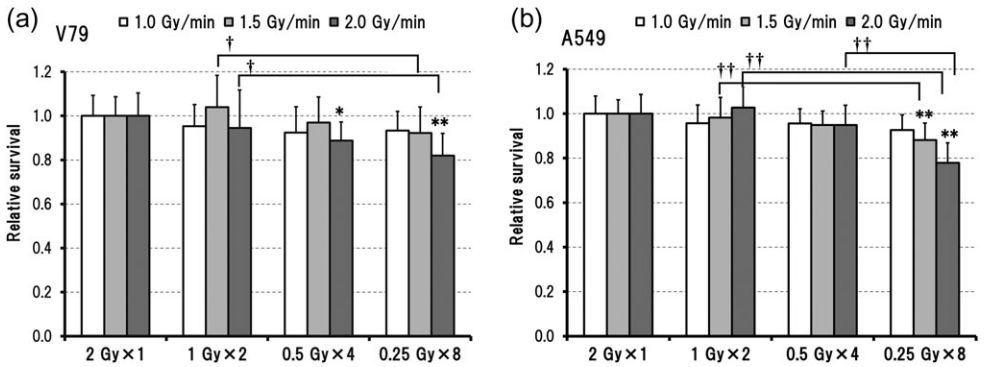

Fig. 2. Relative survivals of V79 (a) and A549 (b) cells after fractionated exposures with varying dose rates. Total doses of 2 Gy were administered by fractionated radiation in unit doses of 0.25, 0.5, 1.0 and 2.0 Gy at 1.0, 1.5 and 2.0 Gy/min with 1 min intervals (Irradiation Method 1). *$P < 0.05$ vs 2 Gy × 1 (each condition), **$P < 0.01$ vs 2 Gy × 1 (each condition). A single dagger (†) indicates $P < 0.05$. Double daggers (††) indicate $P < 0.05$. Data represent the mean ± standard deviation of the results of three to five independent experiments, each with four samples.

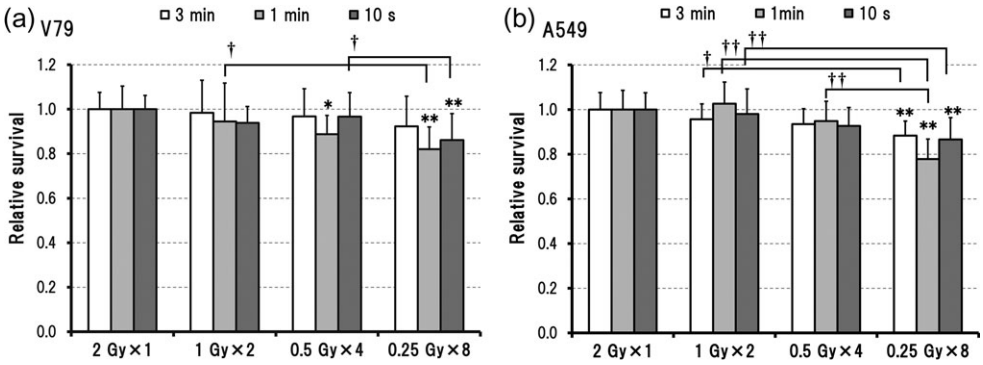

Fig. 3. Relative survivals of V79 (a) and A549 (b) cells after fractionated exposures with varying time intervals. Total doses of 2 Gy were administered by fractionated radiation in unit doses of 0.25, 0.5, 1.0 and 2.0 Gy at 2.0 Gy/min with intervals of 10 s, 1 min and 3 min (Irradiation Method 2). *$P < 0.05$ vs 2 Gy × 1 (each condition), **$P < 0.01$ vs 2 Gy × 1 (each condition). A single dagger (†) indicates $P < 0.05$. Double daggers (††) indicate $P < 0.05$. Data represent the mean ± standard deviation of the results of three to five independent experiments, each with four samples.

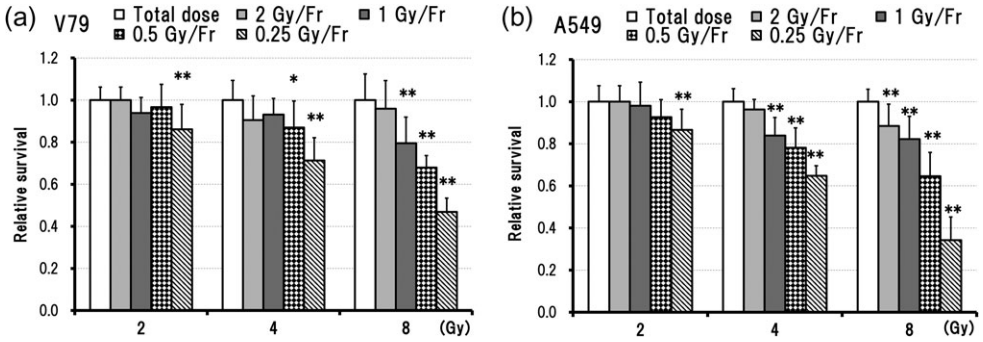

Fig. 4. Relative survivals of V79 (a) and A549 (b) cells after fractionated exposures with varying total doses. Total doses of 2, 4 and 8 Gy were administered by fractionated radiation in unit doses of 0.25, 0.5, 1.0 and 2.0 Gy at 2.0 Gy/min with 10-s intervals (Irradiation Method 3). *$P < 0.05$ vs Total dose (each condition), **$P < 0.01$ vs Total dose (each condition). Data represent the mean ± standard deviation of the results of three to five independent experiments, each with four samples.

Finally, we evaluated the relative survivals of 1, 2, 4, 6 and 8 Gy caused by fractionated exposures at a dose rate of 2.0 Gy/min for 10-s intervals (Irradiation Method 3). The average decrease in relative survival post irradiation at 1-min intervals was lower than that at 10 s. Therefore, it may be appropriate to select the interval of 1 min at conventional 2 Gy irradiation. However, LDFRT using 1-min intervals at a total dose of 8 Gy, as in the case of stereotactic irradiation, demands a long duration. A significant decrease in relative survival was observed in LDFRT using either 10-s or 1-min intervals; therefore, we chose a short, 10-s interval, considering its application in stereotactic irradiation. When exposed to total doses of 2, 4 and 8 Gy by fractionated radiation at the unit dose of 0.25, both cell lines showed a significant decrease in relative survival as the total dose increased, as compared with single exposures ($P < 0.01$) (Fig. 4). When exposed to total doses of 6 Gy by fractionated radiation at the unit dose of 0.25, both cell lines also showed a significant decrease in relative survival (V79: 0.56 ± 0.09, A549: 0.38 ± 0.07) compared with that of cell lines subjected to single exposures ($P < 0.01$). Furthermore, A549 cells exposed to 0.25 Gy × 4 exhibited a significant decrease in relative survival (0.84 ± 0.15) compared with that of cells subjected to 1 Gy single exposures ($P < 0.05$).

As the total dose increased, a significant decrease in relative survival was also observed at unit doses of 0.25, 0.5, 1.0 and 2.0 Gy, compared with single exposures. Furthermore, when both cell lines were exposed to total doses of 8 Gy, a significant decrease in relative survivals was observed at unit doses of 0.25 Gy compared with unit doses of 0.5 and 1.0 Gy ($P < 0.01$).

Survival curves fitted to the L–Q model are shown in Fig. 5. Table 1 shows parameters of α and β, and α/β, D_{10} and RBE_{10} calculated from these parameters. As indicated in Fig. 5 and Table 1, D_{10} decreased and α, β and RBE_{10} increased with lower unit doses. Pertinent to note is the fact that the rate of increase of the α value was larger than that of the β value for V79. On the contrary, the rate of increase of the β value was larger than that of the α value in A549. This may be forwarded as a plausible reason for the observed increase and decrease in the α/β value for V79 and A549, respectively, compared with that of each single exposure. RBE_{10} at a unit dose of 0.25 Gy for V79 and A549 cells was 1.14 and 1.18, respectively.

Analysis of apoptosis

Apoptosis was analyzed by flow cytometry at 48 h after irradiation (Fig. 6). In both cell lines, as the total dose increased, apoptosis increased. However, no significant differences in apoptosis were observed between LDFRT at a unit dose of 0.25 Gy and single exposures in either cell line.

Cell cycle analysis

Cell cycle distribution was analyzed by flow cytometry at 12 and 24 h after irradiation. In both cell lines, as the total dose increased, cell cycle arrest at G_2/M also increased. Cell cycle distribution was not clearly altered between LDFRT at a unit dose of 0.25 Gy and single exposures in either cell line (Fig. 7). SubG1 as an indicator for cellular apoptosis was also analyzed, and no significant differences were observed between LDFRT at a unit dose of 0.25 Gy and single exposures in either cell line.

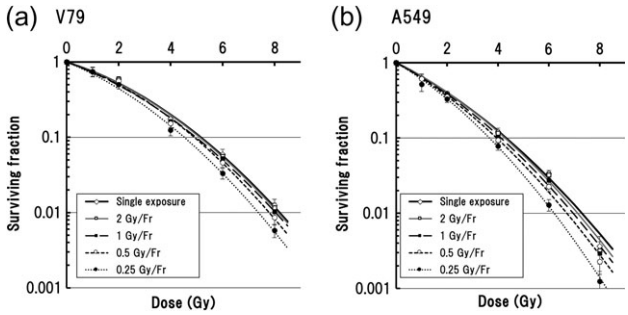

Fig. 5. Cell survival curves of V79 (a) and A549 (b) cells after fractionated exposure with varying unit doses. Total doses of 1, 2, 4, 6 and 8 Gy were administered by fractionated irradiation in unit doses of 0.25, 0.5, 1.0 and 2.0 Gy at 2.0 Gy/min with 10-s intervals. Curves were fitted to the linear–quadratic model. Data represent the mean ± standard deviation of the results of three to five independent experiments, each with four samples.

DISCUSSION

It is unusual to study low-dose fractionated exposures in the order of second intervals. Generally, in clinical trials, LDFRT at 0.4 Gy twice daily (intervals of 6 h or more) in combination with chemotherapy is feasible [29, 30]. In the present study, we examined the potential of low-dose fractionated exposures with a short-time interval for clinical applications by manipulating time intervals, dose rates, unit doses and total doses. We demonstrated that significant enhanced cytotoxic effects were observed when cells were exposed to LDFRT (0.25 Gy/Fr) using very short time intervals (10-s intervals) compared with single exposures. The α and β values for both the cell lines showed a slight increase with lower unit doses, suggesting their enhanced sensitivity to radiation. Even when the total amount of the irradiated dose was the same, a decrease in the relative survival rates were observed when lower doses were administered to the cells by fractionated radiation compared with by single exposures. It is suggested that enhanced cell lethality of HRS was detected indirectly.

It is well known that 'repair' or 'redistribution' (two of the 5Rs of radiotherapy) in response to radiation changes depends on the time interval of secondary irradiation after the first irradiation [31]. The time of intervals was as short as 10 s in our LDFRT radiation schedule; therefore, in our experiment with 10-s intervals it is unlikely that the cells would have been affected by a radiosensitivity change due to the timing of exposure. Incidentally, exposures of 2 Gy × 1, 1 Gy × 2, 0.5 Gy × 4, 0.25 Gy × 8 and control were conducted as one group in the experiment with a total dose 2 Gy. The

Table 1. Values of the parameters obtained from survival curves of V79 and A549 cells using the linear-quadratic model

		α (Gy^{-1})[a]	β (Gy^{-2})[a]	α/β (Gy)[a]	D_{10} (Gy)	RBE_{10}[b]
V79	Single exposure	0.25 ± 0.04	0.038 ± 0.006	6.4 ± 1.5	5.2	1.00
	2 Gy/Fr	0.25 ± 0.05	0.039 ± 0.008	6.5 ± 1.9	5.1	1.01
	1 Gy/Fr	0.28 ± 0.03	0.036 ± 0.005	7.8 ± 1.5	5.0	1.04
	0.5 Gy/Fr	0.26 ± 0.04	0.042 ± 0.006	6.1 ± 1.3	4.9	1.05
	0.25 Gy/Fr	0.32 ± 0.04	0.041 ± 0.006	8.0 ± 1.5	4.5	1.14
A549	Single exposure	0.39 ± 0.05	0.033 ± 0.008	11.8 ± 3	4.3	1.00
	2 Gy/Fr	0.38 ± 0.05	0.038 ± 0.009	10.0 ± 3	4.3	1.01
	1 Gy/Fr	0.43 ± 0.04	0.036 ± 0.007	11.8 ± 3	4.0	1.08
	0.5 Gy/Fr	0.44 ± 0.03	0.037 ± 0.005	11.7 ± 2	3.9	1.10
	0.25 Gy/Fr	0.46 ± 0.02	0.045 ± 0.004	10.4 ± 1.2	3.7	1.18

[a]The values are shown as the mean ± standard error.
[b]RBE_{10} = relative biological effect at 10% survival.

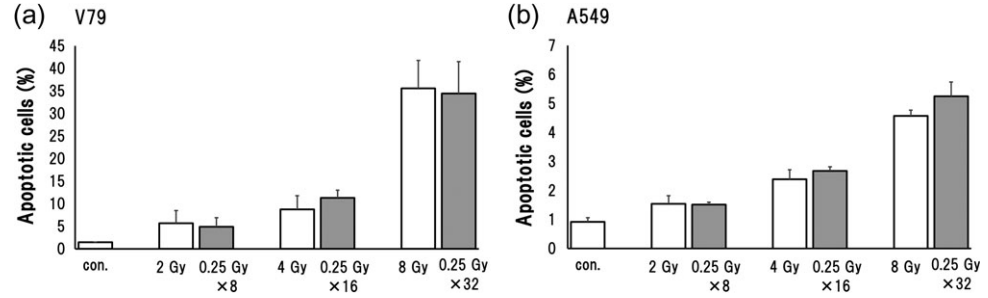

Fig. 6. Apoptosis was analyzed by flow cytometry using Annexin V and PI in V79 (a) and A549 (b) cells at 48 h after irradiation. No significant differences in apoptosis were observed between low-dose and single fractionated exposures in either V79 or A549 cells. Data represent the mean ± standard deviation of the results of three independent experiments.

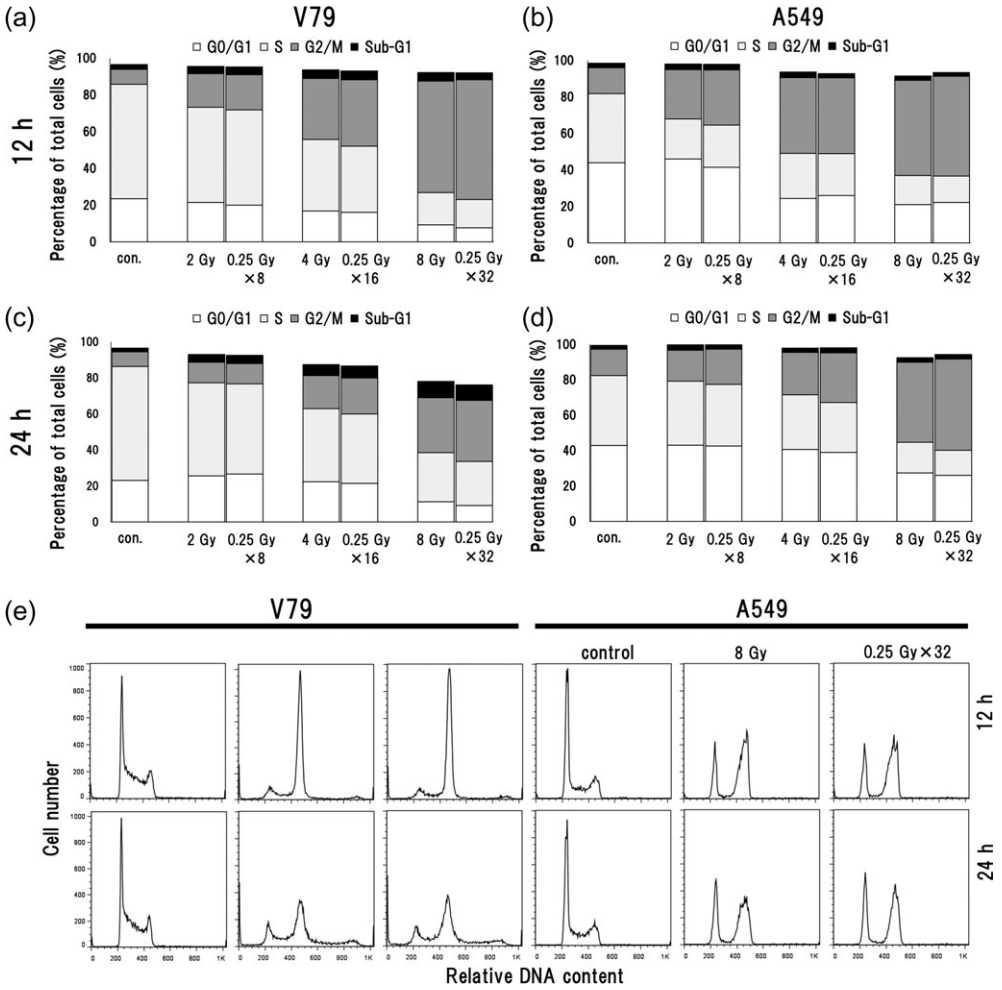

Fig. 7. Cell cycle analysis of V79 (a, c) and A549 (b, d) cells by flow cytometer using PI at 12 and 24 h after irradiation. Each value in the distribution is the mean of four to eight independent experiments. (e) A representative flow cytometric histogram of PI at 12 and 24 h after irradiation. The cell cycle distribution was not significantly altered between low dose and single fractionated exposures in either V79 or A549 cells.

group that had a radiation schedule with a dose rate 2 Gy/min at 10-s intervals was out of the 37°C incubator for the shortest time, ~20 min or less. Furthermore, significant differences in relative survival rates were observed between LDFRT (0.25 Gy × 8) and a single exposure. We presumed that the effects of differences in temperature or pH were limited.

In V79 and A549 cells, a decrease in relative survival rates was observed at higher dose rates (2.0 Gy/min) by LDFRT, whereas at lower dose rates (1.0 and 1.5 Gy/min), enhanced cytotoxicity caused by LDFRT was lesser than that at exposures at 2.0 Gy/min. The temporal difference from irradiation initiation to termination between 1.0 Gy/min and 2.0 Gy/min (at a unit dose of 0.25 Gy, total dose of 2 Gy) was only 1 min; therefore, the differences in the enhanced cytotoxic effect cannot be attributed to DNA repair during irradiation. No dose-rate effect was considered since surviving fractions of LDFRT were normalized to that of single exposures at the same dose rate. The report that dose rate can affect the cytotoxic effect of LDFRT is unprecedented. Generally, dose level is regarded as an important factor for controlling HRS/IRR, but only a few studies have evaluated dose rates for HRS. Thomas *et al.* reported that irrespective of the dose rates, the HRS/IRR response was observed systematically (although not at the same dose range), and that there is a linear relationship between D_{HRSmax} (maximal HRS response observed at a given dose) and dose rate [27]. In this study, we inferred from raising the dose rate that D_{HRSmax} reached ~0.25 Gy of unit dose. In fractionated exposures at a total dose of 8 Gy, we observed enhanced cytotoxic effects at the unit dose of 1.0 Gy compared with single exposures. We hypothesize that by raising the dose rate, the unit dose of 1.0 Gy functioned as a HRS/IRR dose level. The dose rate of 2.0 Gy/min is in the category of moderately high for an *in vitro* experiment. However, since irradiation is at a higher dose rate for tumors in the clinical setting, it is not a limitation in clinical settings to raise the dose rate as a condition for establishing LDFRT. In fact, we may be able to regulate D_{HRSmax} depending on the depth of the tumor in a human body.

Figure 3 shows the enhanced cytotoxicity of LDFRT (0.25 Gy × 8) with each interval (with one exception for V79 3-min intervals) compared with a total single dose of 2 Gy. Therefore, it can be concluded that all these three intervals are good and there is less time interval effect. In LDFRT at several-hour intervals (3–8 h), the cytotoxic effect tends to be enhanced under a high dose rate (>2 Gy/min) [9, 12, 13, 32]. However, under a low dose rate (1 Gy/min), such a tendency has not been established in various *in vitro* and *in vivo* models [15–17]. At intervals of several minutes, a cytotoxic effect was observed for LDFRT at 2-min intervals at dose rates of 1.3–1.5 Gy/min in tumor cell lines [18]. The efficacy of pulsed reduced-dose-rate radiotherapy for *in vitro* and *in vivo* studies has been demonstrated using human recurrent glioblastoma cell lines. It involves exposure to ten 0.2 Gy pulses separated by 3-min intervals over 38 min, creating an apparent dose rate of 0.0667 Gy/min [19, 20]. However, in this treatment technique, the dose rate was as low as 0.25 Gy/min, unlike that of our proposed method. In addition, in EMT-6 and SCCVII cell lines that do not exhibit HRS, the effects of radiation are decreased by imposing intervals of 10 s to several minutes of fractionated irradiation (<2 Gy/min, 0.2 Gy/Fr; total dose 2 or 8 Gy) [21, 33]. These results suggest that SLD repair may occur in between irradiations when cells are exposed to fractionated radiations. However, even if a tumor does not exhibit HRS, the influence of SLD repair in LDFRT using 10-s intervals is considered small for a single-treatment session. The effects of time intervals in LDFRT are not clearly understood; thus, it will be necessary to examine the effects of time intervals in LDFRT when considering dose rates in the future. Since the time interval of 10 s is very short, the burden of radiation therapy will be minimal for both patients and the medical staff if LDFRT using 10-s intervals proceeds to clinical application. As an example, when considering the application of the time schedule of this study to four-field irradiation of equal weights, the method seems to be quite simple. This would simply involve setting the irradiation dose per field to 0.25 Gy × 2, 10 s after irradiating with 0.25 Gy. The remaining 0.25 Gy could be irradiated without changing the radiation field, and repeating the remaining fields. Furthermore, computer-controlled LDFRT using 10-s intervals is expected to be an application for stereotactic irradiation, since cytotoxicity was enhanced as the total dose increased to 8 Gy, and the total time of using LDFRT at 10-s intervals was <10 min.

Despite having being addressed by a number of studies, the HRS mechanism is not clearly understood. It was notably suggested that the HRS response is due to the apoptosis of cells that failed to arrest the cell cycle [24, 34]. On the other hand, studies have reported that mitotic death and growth arrest, not p53-dependent apoptosis, is involved in HRS [10, 35, 36]. In this study, no significant difference was observed between single exposure irradiation and LDFRT in the analysis of apoptosis and the cell cycle. However, it is well known that apoptosis or other programmed cell death pathways may not affect overall survival after irradiation [37]. Since enhanced cytotoxic effects were observed in LDFRT by clonogenic assay, senescence-like growth arrest and/or mitotic death may be involved. It will be necessary to examine the mechanisms of LDFRT in further studies.

In the present study, we found that enhanced cytotoxic effects were easily achieved by LDFRT (0.25 Gy/Fr) using very short time intervals (10-s intervals), and that dose rate is an important factor for establishing LDFRT. RBE_{10} of LDFRT at a unit dose of 0.25 Gy in V79 and A549 cells increased slightly to 1.14 and 1.18, respectively. However, HRS has been confirmed in many cell types, such as glioma cell lines [1–3], thus LDFRT is expected to be an effective radiation therapy for radioresistant cells. Although further adjustments such as to unit doses and time intervals are necessary, LDFRT using very short time intervals may have high clinical application feasibility.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest in connection with this paper.

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