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Potential lethal damage repair in glioblastoma cells irradiated with ion beams of various types and levels of linear energy transfer

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ABSTRACT

Glioblastoma (GBM), a Grade IV brain tumour, is a well-known radioresistant cancer. To investigate one of the causes of radioresistance, we studied the capacity for potential lethal damage repair (PLDR) of three altered strains of GBM: T98G, U87 and LN18, irradiated with various ions and various levels of linear energy transfer (LET). The GBM cells were exposed to ^{12}C and ^{28}Si ion beams with LETs of 55, 100 and 200 keV/μm, and with X-ray beams of 1.7 keV/μm. Mono-energetic ^{12}C ions and ^{28}Si ions were generated by the Heavy Ion Medical Accelerator at the National Institute of Radiological Science, Chiba, Japan. Clonogenic assays were used to determine cell inactivation. The ability of the cells to repair potential lethal damage was demonstrated by allowing one identical set of irradiated cells to repair for 24 h before subplating. The results show there is definite PLDR with X-rays, some evidence of PLDR at 55 keV/μm, and minimal PLDR at 100 keV/μm. There is no observable PLDR at 200 keV/μm. This is the first study, to the authors' knowledge, demonstrating the capability of GBM cells to repair potential lethal damage following charged ion irradiations. It is concluded that a GBM's PLDR is dependent on LET, dose and GBM strain; and the more radioresistant the cell strain, the greater the PLDR.

Keywords: GBM; LET; PLDR; ^{12}C ion; ^{28}Si ion

INTRODUCTION

In this study, we examined the capability of glioblastoma (GBM) cells to repair potential lethal damage induced by charged ion beam radiation of various levels of linear energy transfer (LET). The current standard of care for GBM patients is surgery, where possible, followed by radiation therapy plus concomitant and adjuvant chemotherapy [1]. Unfortunately, GBM is a highly aggressive and persistent hypoxic tumour, it often recurs and patients generally

survive less than 2 years after initial diagnosis. GBM is known to be radioresistant to photon therapy. Radioresistant tumours have the ability to repair DNA damage induced by photon irradiation or by other low-LET irradiation [2, 3]. Possible causes of treatment failure have been proposed: hypoxic portions of tumour; stem (tumour-initiating) cells; genetic alterations; and many other factors [4–10]. To overcome radioresistance, new advances and improved radiotherapy strategies have been described [4, 7]. A potential alternative therapy to overcome radioresistance in GBM is charged ion radiotherapy. Charged ion radiations are forms of densely ionizing radiation with high LET, which are less dependent on oxygen for cell inactivation, are less affected by variation in cell cycle–related radiosensitivity, have a higher relative biological effectiveness (RBE) than X-rays [11], and which are less affected by the '5 Rs' of fractionated radiotherapy (repair, redistribution, reoxygenation, repopulation and radiosensitivity) [12].

The three categories of radiation damage produced by ionizing radiations, as described by Hall *et al.* [13, 14], are lethal damage (LD), sublethal damage (SD) and potentially lethal damage (PLD). Lethal damage is irreversible and irreparable, leading to cell death. Under normal circumstances, sublethal damage can be repaired in a matter of hours when no additional sublethal damage is added (e.g. due to a second dose of radiation) that could lead accumulatively to lethal damage [15, 16]. This type of damage has been taken advantage of by using fractionated treatments in radiotherapy [15, 16]. Potential lethal damage under normal circumstances can cause cell death, but may be prevented, and is influenced by appropriate post-irradiation environmental conditions, such as delayed subculture of irradiated cells, incubation at suboptimal temperature, minimal medium, or treatment with inhibitors of protein synthesis [3, 14, 17–20]. In other words, when the PLD is not repaired, it is lethal. Potential lethal damage repair (PLDR) has been observed in *in vivo* and *in vitro* experiments with radiation and chemotherapeutic drugs [18, 21–23]. This repair takes place post irradiation when cells are allowed time to repair instead of being allowed to proliferate (through to mitosis/division). Hence, PLDR affects the radiosensitivity of cells and the radiocurability of treated tumours. The concept of PLDR was first reported by Phillips and Tolmach [17] through observation of repair of irradiated HeLa S3 cells in the plateau phase *in vitro*.

Plateau phase *in vitro* cultures possess certain characteristics of tumours *in vivo* in which a large proportion of the tumour is in G1 or G0 phase [18–20]. Clonogenic assay has been employed to study PLDR of various types of cell lines irradiated with X-rays, but limited studies have been carried out on GBM [3, 18–20, 24, 25]. Other methods such as kinetics and fidelity of chromosome rejoining and gene expression have also been used to demonstrate PLDR [26, 27].

The ability of cells to repair PLD with high-LET radiation have been investigated using neutrons [14, 28, 29], α-particles [30], ^{12}C [31] and ^{4}He ions [32, 33]. Differences in the results following neutron irradiation have been reported. No PLDR was detected by Hall and Kraljevic, who irradiated Chinese hamster cells [14], or by Shipley *et al.* [28], irradiating Lewis lung carcinoma cells *in situ* with fast neutrons. Conversely, Rasey *et al.* [29] reported substantial PLDR in plateau-phase EMT-6 tumour cells. No PLDR was detected by Raju *et al.* after exposure of Chinese hamster cells to α-particles produced from plutonium ^{238}Pu [30]. Guichard *et al.* [32], using 645 MeV ^{4}He ions in the middle of the spread-out Bragg peak (SOBP), reported comparable PLDR with γ-irradiation on EMT6 cells *in vivo* and *in vitro*. In addition, with extended-Bragg-peak ^{4}He ions, compared with γ-rays Ward *et al.* noted less PLDR in embryonic survival Sprague-Dawley rats when irradiated on the fifth and sixth days of gestation [33]. Furthermore, Wheeler *et al.* [31] observed that the extent of PLDR in 9 L tumour cells irradiated *in situ* with SOBP ^{12}C ions was virtually identical to that observed after X-ray irradiation. In this study, we aimed to ascertain the PLDR of a number of different GBM strains following irradiation with various ion species and levels of LET.

MATERIALS AND METHODS

Cell lines and cell maintenance

Three human Grade IV glioblastoma cell lines (T98G, U87 and LN18) were used in this study. The T98G cells were a gift from Mick Woodcock, Gray Institute for Radiation Oncology and Biology, Oxford, UK; the U87 cells were obtained from the Health Protection Agency Culture Collections (HPACC, Wiltshire, UK), and the LN18 cells were obtained from the American Type Culture Collection (ATCC, Middlesex, UK). All the cell lines were confirmed *Mycoplasma* free using Lonza MycoAlert® *Mycoplasma* Detection Assay.

The cell lines were individually maintained in 75 cm^2 plastic flasks (T75 BD FalconTM 353 084) in Eagle's Minimum Essential Medium (MEM: Nissui Pharmaceutical Co. Ltd, Tokyo) supplemented with 10% fetal bovine serum (FBS: Hyclone, Thermo Scientific, USA) in a humidified 95% air/5% CO_2 incubator at 37°C. Cells were subcultured from a T75 plastic flask by rinsing in calcium- and magnesium-free phosphate-buffered saline (PBS) and exposed to 0.2% trypsin solution containing 0.5 mM EDTA. Cell numbers were determined by Coulter Counter. For all experiments, 3×10^5 cells for each cell line were inoculated into a 25 cm^2 plastic flask (T25 BD Falcon 353014) for each dose point, 3 days before irradiation of the cells (~85–90% in the confluent stage). The medium was changed on the day of radiation. At least two independent tests were performed using X-rays, each ion species and each LET.

Irradiations

Cells were irradiated with ^{12}C (135 MeV/n, LET 100 keV/μm) and ^{28}Si (490 MeV/n, LET 55 and 200 keV/μm) monochromatic beams accelerated at the HIMAC/NIRS, Chiba, Japan. Although the carbon ion is the main heavy ion used for medical treatment, previous studies using neon, helium and silicon ions have been carried out, and silicon ions are considered to be a potential ion [34, 35] for radioresistant hypoxic tumours, of which GBM is one. Silicon ions were once deemed to be of potential significance in the treatment for brain cancer (hypoxic tumours), not only for biological reasons (RBE 3–4 depending on location of peak, LET and dose fraction size) and dose localization advantages, but also for its oxygen enhancement ratio (OER). It has been shown that the magnitude of the OER decreases progressively as the atomic number and LET of the accelerated ion species increase [34, 35]. However, with silicon ions it has not yet been possible to prove or disprove their virtues in clinical therapy for hypoxic tumours, and indeed few institutes

could provide such beams compared with the number able to use carbon ion beam therapy. As silicon is a heavy ion, fragmentations should be taken into consideration and reduced as far as is practically possible to emphasize its depth–dose effectiveness and oxygen gain factor [35]. However, given silicon radiotherapy has been proposed and is under study as a potential ion, it has been included in this work. Clinically, SOBPs are used for treatment based on the tumour size and shape. However, for *in vitro* cells, mono-energetic beams are more appropriate for studies as they are mono-layered and the dose average is homogenous. The LETs chosen for silicon ion were based on the entrance (plateau) and peak positions of the ion Bragg peak, two points on the depth–dose curve of clinical relevance. For carbon ions, 100 keV/μm was deemed to be highly cytotoxic for GBM cells. Further, the particles were chosen for both their potential as clinically feasible ions and also their beam time availabilities, which could be assumed to be approximately equivalent to any particles with the same LET [although there are some differences based on the atomic number of the particle and its radial energy distributions around the trajectory of the heavy charged particle (track-structure effects in cell killing) and its microdosimetry].

To change the energy of the beams, Lucite absorbers of different thicknesses were used. A range of average absorbed dose was used, depending on the LET employed. The details of the HIMAC beam delivery system, physical characteristics, biological irradiation procedures and dosimetry have been described by Kanai *et al.* and Torikoshi *et al.* [36, 37]. For comparison with photons, a 200 kVp X-ray (20 mA) beam filtered with 0.5 mm Cu and 0.5 mm Al (TITAN 320 irradiator; Ge Inspection Technologies Shimadzu, Japan) was used, delivering doses at a dose-rate of 1.00 Gy/min ± 0.02. All the irradiations were carried out at room temperature. The dose-rate of all ion beams was ~3 Gy/min.

For the ions, fragmentations were taken into consideration and applied to convert particle fluence (Φ) to absorbed dose as described in [38, 39]:

$$\text{Absorbed Dose (Gy)} = 1.6021 \times 10^{-9} \times \text{LET (keV/μm)} \times \Phi\ (1/\text{cm}^2) \quad (1)$$

Cell survival assay

The surviving fraction (SF) was measured using the colony formation assay to assess reproductive death. Two identical sets of plateau phase GBM cells in T25 flasks (Set A and B) were irradiated at the same time. Immediately after irradiation, the Set A T25 flasks were placed in the incubator in a humidified 95% air/5% CO_2 incubator at 37°C for 24 h. For Set B T25 flasks, immediately post irradiation, the cells were removed from the T25 flasks and inoculated into triplicate 60 mm plastic dishes (Falcon 353002) to produce 60–70 colonies per dish. The cells were counted using a Coulter Counter (Coulter Electronics Ltd, Japan, Tokyo). Set A T25 flasks were delayed in plating out for 24 h in triplicate 60 mm plastic dishes to allow for repair of the potential lethal damages induced. The plating efficiency (PE) for T98G was 50–60%, for U87 it was 10–15% and for LN18 it was 85–95%. Although U87 had a low PE, ~40 colonies survived post ≤4 Gy dose with LET of 100–200 keV/μm. After 14 days' incubation, colonies were fixed with 20% methanol and stained with 0.2% crystal violet. Triplicate dishes of each dose point colony consisting of more than 50 cells were counted under a stereomicroscope. The SF at each dose point was determined as the ratio of live colonies in the treated dish relative to the the number in the untreated/control. The mean values and standard deviations of triplicate samples were counted with error propagation.

The plateau phase of the cells was based on the 85–90% confluency of the cells in the T25 flasks. Flow cytometry of 10 000 cells was performed on both immediate plating (IP) and delayed plating (DP) cells to determine their stage in the cell cycle. For example, IP cells for LN18 using 100 keV/μm were 74–78% at plateau phase and DP (post 24 h) cells were 74 to 68% for control, 0.2 Gy and 0.4 Gy and approximately 59–52% for the other dose points (data are not shown).

Data analysis

The SF data were obtained from the mean of at least two independent experiments and fitted by a least squares Linear Quadratic (LQ) Model equation:

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

where S is the SF and D is the absorbed dose in gray. The α parameter describes the linear component of the curve, and the β component describes the quadratic portion of the curve. Doses were calculated from particle fluence and the dose-averaged LET values by Equation (1), and the results are presented in Table 1. The α and β values are determined by minimizing the sum of squares calculated by Equation (2). The α/β ratio is the point at which linear cell kill is equivalent to quadratic cell kill.

RBE is defined as the ratio of a photon dose (D_γ) to a corresponding ion dose (D_I) yielding the same biological effect:

$$\text{RBE} = D_\gamma / D_I \quad (3)$$

Ratio of potential lethal damage repair

PLDR time is defined as the interval between irradiation and subculture [20]. The PLDR ratio is the SF of DP (R) divided by the SF of IP (R0) at a single dose. [3, 40]. The PLDR ratio reveals the capability of the cells for repairing PLD.

$$\text{PLDR ratio} = \frac{\text{SF (R)}}{\text{SF (R}_0\text{)}} \quad (4)$$

The extent of PLDR was divided into four levels; definite (PLDR ratio of ≥ 2.0), some evidence (PLDR ratio of ≥ 1.3), minimal (1.0 < PLDR < 1.3) and no evidence (PLDR ≤ 1.0). RBE_{10} is the ratio of absorbed dose required to reduce the SF to 10% for the ion beam irradiations relative to X-rays. $RBE_{2\,Gy}$ is the SF plotted at $RBE_{2\,Gy}$ for ion beam irradiations as compared with $RBE_{2\,Gy}$ for X-rays. The SF graphs, α and β parameters and RBE were plotted/obtained using KaleidaGraph by Synergy software (version 3.5).

Table 1. IP and DP values of α, β, α/β ratio, D_{10}, and RBE_{10} and $RBE_{2\,Gy}$ of T98G, U87 and LN18 for irradiations with LET of 1.7, 55, 100 and 200 keV/μm

T98G Ions	LET	α (Gy^{-1})	β (Gy^{-2})	D_{10} (Gy)	D_{10} RBE	D_{10} α/β (Gy)	2 Gy RBE
200kVp-IP	1.7 ± 0.02	0.049 ± 0.001	0.109 ± 0.009	4.57 ± 0.023	1.00 ± 0.005	0.45	1.00 ± 0.001
200kVp-DP	1.7 ± 0.02	0.226 ± 0001	0.039 ± 0.001	5.33 ± 0.001	0.86 ± 0.001	5.80	1.06 ± 0.001
^{28}Si 490-IP	55.0 ± 0.06	0.451 ± 0.058	0.213 ± 0.009	2.43 ± 0.027	1.88 ± 0.020	2.12	2.37 ± 0.012
^{28}Si 490-DP	55.0 ± 0.06	0.327 ± 0.004	0.202 ± 0.001	2.65 ± 0.002	1.72 ± 0.001	1.62	1.81 ± 0.001
^{12}C 135-IP	100 ± 1.77	0.935 ± 0.010	0.025 ± 0.004	2.32 ± 0.003	1.97 ± 0.002	37.4	3.55 ± 0.010
^{12}C 135-DP	100 ± 1.77	0.821 ± 0.006	0.062 ± 0.001	2.37 ± 0.016	1.93 ± 0.013	13.2	2.85 ± 0.009
^{28}Si 490-IP	200 ± 3.12	0.791 ± 0.004	0.095 ± 0.001	2.25 ± 0.045	2.03 ± 0.040	8.33	3.17 ± 0.026
^{28}Si 490-DP	200 ± 3.12	1.026 ± 0.007	0.055 ± 0.001	2.07 ± 0.035	2.22 ± 0.038	18.7	3.49 ± 0.021
U87 Ions	LET	α (Gy^{-1})	β (Gy^{-2})	D_{10} (Gy)	D_{10} RBE	D_{10} α/β	2 Gy RBE
200kVp-IP	1.7 ± 0.02	0.157 ± 0.006	0.068 ± 0.004	4.81 ± 0.078	1.00 ± 0.016	2.31	1.00 ± 0.001
200kVp-DP	1.7 ± 0.02	0.248 ± 0.004	0.034 ± 0.001	5.35 ± 0.009	0.90 ± 0.001	7.29	1.03 ± 0.001
^{28}Si 490-IP	55.0 ± 0.06	0.515 ± 0.001	0.069 ± 0.001	3.15 ± 0.004	1.53 ± 0.002	7.46	2.00 ± 0.004
^{28}Si 490-DP	55.0 ± 0.06	0.497 ± 0.002	0.034 ± 0.003	3.70 ± 0.005	1.30 ± 0.002	14.6	1.70 ± 0.003
^{12}C 135-IP	100 ± 1.77	1.102 ± 0.101	0.039 ± 0.038	2.27 ± 0.034	2.12 ± 0.032	28.3	3.84 ± 0.011
^{12}C 135-DP	100 ± 1.77	1.039 ± 0.004	0.033 ± 0.002	2.40 ± 0.002	2.00 ± 0.002	31.5	3.36 ± 0.008
^{28}Si 490-IP	200 ± 3.12	1.079 ± 0.015	0.014 ± 0.003	2.05 ± 0.047	2.35 ± 0.054	77.1	3.72 ± 0.021
^{28}Si 490-DP	200 ± 3.12	1.138 ± 0.049	0.025 ± 0.019	1.98 ± 0.020	2.43 ± 0.025	45.5	3.64 ± 0.006
LN18 Ions	LET	α (Gy^{-1})	β (Gy^{-2})	D_{10} (Gy)	D_{10} RBE	D_{10} α/β	2 Gy RBE
200kVp-IP	1.7 ± 0.02	0.316 ± 0.001	0.076 ± 0.001	3.81 ± 0.010	1.00 ± 0.004	4.16	1.00 ± 0.001
200kVp-DP	1.7 ± 0.02	0.355 ± 0.003	0.045 ± 0.001	4.22 ± 0.020	0.90 ± 0.005	7.89	0.98 ± 0.001
^{28}Si 490-IP	55.0 ± 0.06	0.642 ± 0.001	0.105 ± 0.001	2.54 ± 0.001	1.50 ± 0.001	6.11	1.64 ± 0.004
^{28}Si 490-DP	55.0 ± 0.06	0.592 ± 0.002	0.056 ± 0.001	3.02 ± 0.010	1.26 ± 0.004	10.6	1.50 ± 0.004
^{12}C 135-IP	100 ± 1.77	1.079 ± 0.002	0.052 ± 0.001	1.95 ± 0.001	1.95 ± 0.002	20.8	2.40 ± 0.002
^{12}C 135-DP	100 ± 1.77	0.941 ± 0.010	0.079 ± 0.003	2.09 ± 0.001	1.82 ± 0.001	11.9	2.27 ± 0.001
^{28}Si 490-IP	200 ± 3.12	1.126 ± 0.016	0.011 ± 0.010	2.00 ± 0.016	1.91 ± 0.017	102	2.42 ± 0.001
^{28}Si 490-DP	200 ± 3.12	1.205 ± 0.002	0.006 ± 0.001	1.89 ± 0.001	2.00 ± 0.001	201	2.72 ± 0.002

LET error = SD, and for other parameters = SEM; IP = immediate plating; DP = 24 h delayed plating.

RESULTS

The results presented are the means of at least two independent experiments, and the error bars in the survival curves (SCs) represent the standard deviations (SDs). Figures 1 to 4 (Panels A–C) show SC versus dose for T98G, U87 and LN18 from irradiations with LET of 1.7, 55, 100 and 200 keV/μm. Table 1 summarizes the PLDR effects of the three GBM cell lines α, β, α/β ratio, D_{10}, and RBE at 10% and 2 Gy [with SD for LET and standard error of means (SEM) for the other parameters]. Recovery PLDR ratios of the three GBM strains, the doses and the LETs are summarized in Table 2.

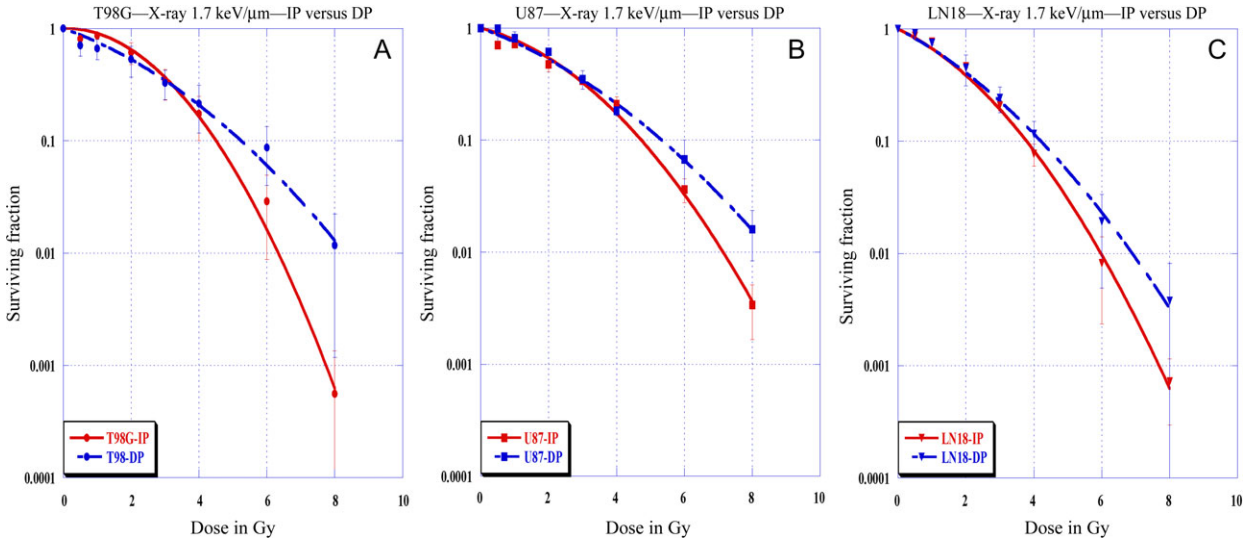

Fig. 1. Cell survival curves of T98G (A), U87 (B) and LN18 (C), IP versus DP when irradiated with X-rays of LET 1.7 keV/µm. IP = immediate plating, DP = 24 h delayed plating. Error bars indicate SD.

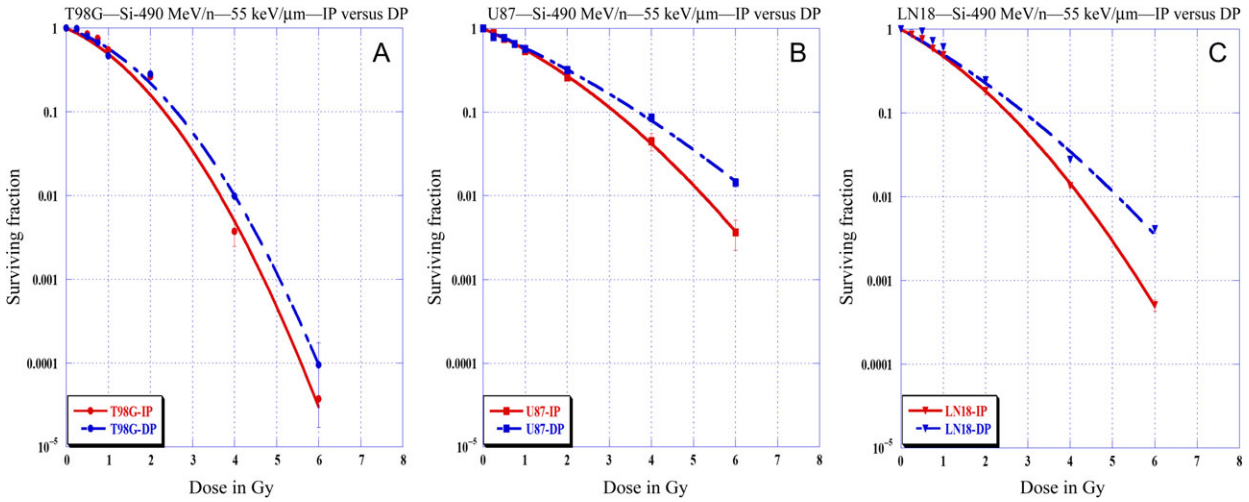

Fig. 2. Cell survival curves of T98G (A), U87 (B) and LN18 (C), IP versus DP when irradiated with ^{28}Si (55 keV/µm). IP = immediate plating, DP = 24 h delayed plating. Error bars indicate SD.

Effects of potential lethal damage repair on survival curves

Our results demonstrated that the PLDR effects showed a minimal change in the shoulder of the SF curve with X-rays, but a significant increase in the gradient of the slope from 4 Gy onwards (Fig. 1). For irradiations with LET of 55 keV/µm, T98G, U87 and LN18 cells showed a change in the slope after 2 Gy (Fig. 2). There was little change in the slope of PLDR for irradiations with LET of 100 keV/µm (Fig. 3). The differences observed between IP and DP survival with 100 keV/µm were statistically significant for both U87 and LN18 but not T98G. This could be due to the low-dose hypersensitive of T98G. The Wilcoxon Signed Ranks Test—exact sig. (2-tailed) with a value of $P < 0.05$ was employed for this statistical test. Figure 4 shows there was no PLDR with high-LET radiation of 200 keV/µm, and the slope was reduced as most cells were inactivated. There is an indication that the change in the slope of the SF curve was dose dependent for repair processes for low-LET radiation.

Note the observed trend for 200 keV/µm, that IP survival was higher than DP, infers that there was no PLDR. The radiation-induced damaged cells, when given the opportunity to repair before dividing, were unable to do so. Hence, the survival of DP decreased,

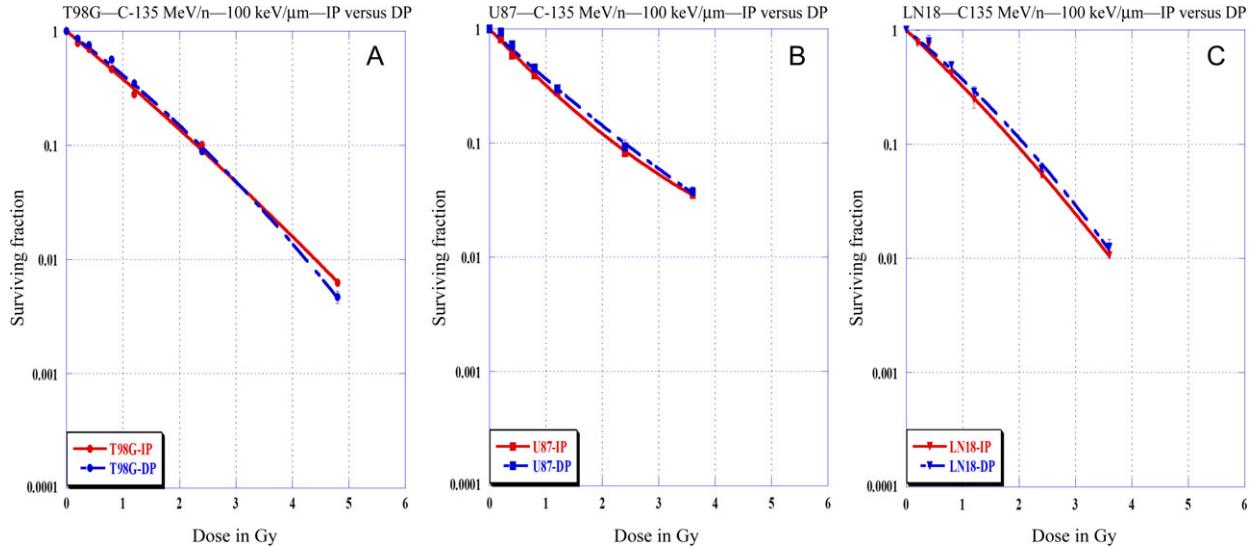

Fig. 3. Cell survival curves of T98G (A), U87 (B) and LN18 (C), IP versus DP when irradiated with ^{12}C (100 keV/µm). IP = immediate plating, DP = 24 h delayed plating. Error bars indicate SD.

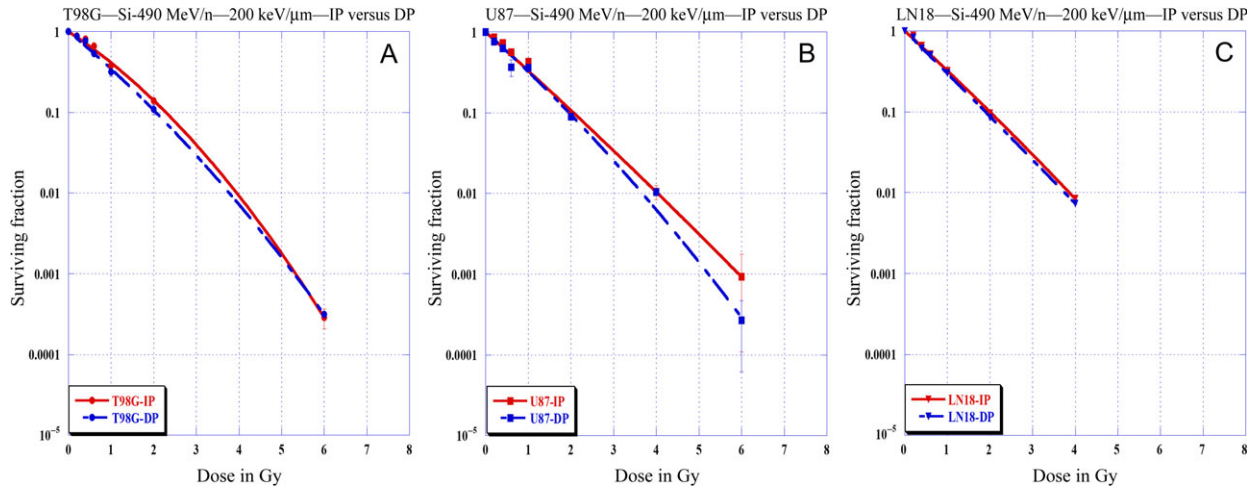

Fig. 4. Cell survival curves of T98G (A), U87 (B) and LN18 (C), IP versus DP when irradiated with ^{28}Si (200 keV/µm). IP = immediate plating, DP = 24 h delayed plating. Error bars indicate SD.

indicating that the damaged cells were not able to repair and unable to proliferate and were inactivated. None of the three GBM cell lines could repair the complex damages induced by 200 keV/µm radiation. When 24 h were allowed for the cells to repair, they were unable to repair, and they died due to the severe radiation-induced damage complexity. Hence, the surviving fractions of DP were reduced.

X-ray potential lethal damage repair

Our results clearly displayed post radiation effects. IP cells were more radiosensitive than the 24 h DP cells irradiated with X-rays of LET 1.7 keV/µm. X-ray PLDR increased for T98G and U87 at ≥ 4 Gy, but for LN18 PLDR from ≥3 Gy onwards (Fig. 1 Panels A–C).

Charged ion potential lethal damage repair

There was apparent PLDR with LET of 55 keV/µm, minimal PLDR for LET of 100 keV/µm and no PLDR for LET of 200 keV/µm. For LET of 55 keV/µm, T98G and U87 PLDR started at >1 Gy onwards. For LN18 cells, PLDR shows a consistent rise with increase in dose (Fig. 2 Panels A–C). LET of 100 keV/µm showed minimal PLDR for all three GBMs (Fig. 3 Panels A–C). There was no PLDR detected in any GBM cell line with the high LET of 200 keV/µm (Fig. 4 Panels A–C).

DISCUSSION

The aim of this study was to quantify the ability of GBM cell lines to repair PLD following charged ion irradiations with different levels

Table 2. T98G, U87 and LN18—LET of 1.7, 55, 100 and 200 keV/μm dose point PLDR ratio[a]

LET	1.7 keV/μm				55 keV/μm			100 keV/μm					200 keV/μm			
GBM	T98G	U87	LN18		T98G	U87	LN18		T98G	U87		LN18		T98G	U87	LN18
Dose (Gy)				Dose (Gy)				Dose (Gy)			Dose (Gy)		Dose (Gy)			
0.00	1.00	1.00	1.00	**0.00**	1.00	1.00	1.00	**0.00**	1.00	1.00	**0.00**	1.00	**0.00**	1.00	1.00	1.00
0.50	0.88	1.40	1.05	**0.25**	1.03	0.90	1.19	**0.20**	1.10	1.15	**0.20**	1.36	**0.20**	0.99	0.87	0.95
1.00	0.77	1.13	0.98	**0.50**	0.95	1.02	1.24	**0.40**	1.08	1.24	**0.40**	1.08	**0.40**	0.95	0.86	0.93
2.00	0.87	1.30	0.96	**0.75**	0.89	1.00	1.23	**0.80**	1.22	1.15	**0.80**	1.18	**0.60**	0.71	0.65	0.97
3.00	0.99	1.04	1.16	**1.00**	0.85	1.06	1.24	**1.20**	1.25	1.07	**1.20**	1.17	**1.00**	0.83	0.84	0.93
4.00	1.23	0.87	1.51	**2.00**	1.07	1.22	1.33	**2.40**	0.88	1.13	**2.40**	1.13	**2.00**	0.79	0.97	0.88
6.00	3.01	1.85	2.38	**4.00**	2.64	1.91	2.05	**4.80**	0.75	1.06	**3.60**	1.18	**4.00**	1.00	0.99	0.87
8.00	21.00	4.72	5.21	**6.00**	2.56	3.95	8.10						**6.00**	1.00	0.29	0.68

[a]PLDR ratio = surviving fraction of delayed plating/surviving fraction of immediate plating (DP/IP). Ratio of ≤1 indicates no PLDR.

of LET in comparison with X-rays. GBM is known clinically to be radioresistant and, *in vitro*, GBM cell lines have revealed large PLDR with photon irradiation [41]. In addition, 9L gliosarcoma have shown PLDR following irradiation with ^{12}C ions [31].

Growth conditions for potential lethal damage repair study

Plateau phase has been used as it has similar characteristics to tumours *in vivo*, in which a large proportion of the tumour is in G1 or G0 phase [18–21, 30, 40, 42–44]. Furthermore, exponential phase (log phase) culture has been shown to display lower PLDR [24, 30]. Hahn *et al.* lists some of the post-irradiation conditions that allow PLDR after X-ray irradiation: reduction in temperature; growth under suboptimal conditions such as in plateau-phase cultures or *in situ* hypoxia; and inhibition of protein synthesis [21]. Analogous to solid tumours, where hypoxic regions are present, a lack of blood vessels, a low level of nutrients and an acidic extracellular environment with high concentration of cellular waste products after the first irradiation may enable cells to repair instead of proliferate (and thus promote PLDR) [21]. GBM is a known hypoxic tumour. In this study, GBM cells were allowed to repair radiation damage for 24 h instead of proliferating, by neither removing them nor changing the medium. Twenty four hours delay was chosen to ensure the PLDR had been completed, as has been researched by Yashiro *et al.* [27], who found that at 18 h post irradiation, PLDR was thought to be stable. Moreover, this also simulated a typical clinical setting as radiotherapy is generally given on a daily basis. Other studies have examined a limited number of dose points [20, 31]. In this study, a wide-range of dose points (0.2, 0.25, 0.4, 0.5, 0.6, 0.75 and 0.8 Gy) were employed to determine low-dose hypersensitivity (HRS), up to doses of 8 Gy. Moreover, Marchese *et al.* report that fitting DP results to SCs increases the accuracy of the recovery ratio as compared with using individual single doses [24]. Doses of >5 Gy with ^{12}C (135 MeV/n, LET 100 keV/μm) were not chosen for T98G as it was estimated to be highly toxic. Similarly, doses of >4 Gy for U87 and LN18 were not used for fear of 'overkill'. More than 8 Gy for high-LET radiation was not practical as the large number of cells necessary to be used may have given rise to statistical uncertainties (due to very low number of surviving colonies versus number of cells inoculated).

Charged ion potential lethal damage repair and potential lethal damage repair ratio

The majority of PLDR studies have been carried out using photons (X- or γ-rays) [16–18, 20, 21, 43, 45] and other types of cell line. We believe that this study of the PLDR relationship to the type of ion species and the level of LET of the irradiation is the first to be performed on three different GBM strains. For charged particle irradiation, Guichard *et al.* [32] reported that PLDR in EMT-6 (mouse mammary carcinoma) tumours irradiated with SOBP ^{4}He ions was similar to that observed with γ-irradiation. In addition, Wheeler *et al.* [31] observed recovery of PLDR in 9L gliosarcoma cells (*in situ*) that were stereotactically implanted into the left cerebral hemisphere of male Fisher 344 rats irradiated with a dose of 13.5 Gy at a dose rate of 10 Gy/min (using 400 MeV/nucleon ^{12}C ions (SOBP) with a median LET of ~40 keV/μm) in comparison with X-rays at a dose rate of 2 Gy/min; the extent of recovery was almost identical to that observed with X-rays. We concur with Wheeler *et al.* as our irradiations with a LET of 55 keV/μm showed evidence of PLDR. Moreover, the PLDR ratio demonstrated that, with a LET of 100 keV/μm, there was minimal PLDR for the three GBM cell lines. This suggests that with a LET of 100 keV/μm, low doses were not sufficient to produce irreparable damages, even though it has been accepted that it is the optimal LET for producing a biological effect [46]. (At this density of ionization, the average separation between ionizing events approximately coincides

with the diameter of a DNA double helix, i.e. 2 nm in width, and has the highest probability of a single charged particle causing a double-strand break). In contrast, irradiation with a LET of 200 keV/μm showed no PLDR for any of the three GBM cell lines. Our results showed that a high LET of 200 keV/μm induced mostly lethal damage in the GBM cells (such as local multiple damage sites that are complicated and complex, and which, according to Ward, are difficult to repair to the original status or not reparable at all [47]).

X-ray potential lethal damage repair and potential lethal damage repair ratio

From Table 2, it is intriguing to note that the X-ray PLDR ratio for T98G increased by a factor of 1.2 with 4 Gy, 3.0 with 6 Gy and 21.0 with 8 Gy. This reveals the potential ineffectiveness of high doses of irradiation for T98G types of tumour. It could be inferred that escalating doses may not benefit strains of GBM that exhibit low-dose HRS. Our results showed that with T98G the PLDR ratio increased with increasing dosage, but its known low-dose HRS GBM cell lines [48] demonstrated no PLDR at doses below 2 Gy (Table 2 and Fig. 1 Panel A). While GBMs are known radioresistant tumours, our results concurred with Short *et al.* in that T98G and U87 displayed low-dose HRS to X-ray radiation. However, U87 cells only exhibited low-dose HRS to doses of 0.25 Gy for LET 55 keV/μm [48, 49]. Although, Short *et al.* report that low-dose HRS usually occurs at doses <1 Gy with X-rays, our results showed PLDR up to 2 Gy, which could be due to the dose rate of 1 Gy/min (compared with Short *et al.*, who employed 0.2–0.4 Gy/min); the methods and medium employed were also different. Low-dose HRS is common in radioresistant glioma and is more marked in more radioresistant cell lines [49]. Therefore, treating GBM with high doses for both low- (X-rays) and high-LET radiation may result in an increase in radioresistance, although the clinical outcome will obviously be different for both, assuming the high-LET radiation hits the GBM cells.

The X-ray PLDR ratios at 6 Gy were similar to the results of Weichselbaum *et al.* [3, 20], who reported a PLDR ratio of 2.8 at 7 Gy (based on a single dose point) for GBM; our results showed a similar but varied range of 1.85 to 3.01 at 6 Gy (Table 2—LET 1.7 keV/μm, X-ray column).

Potential lethal damage repair RBE_{10} and $RBE_{2\,Gy}$, α, β and α/β

From Table 1, comparing RBE_{D10} and $RBE_{D2\ Gy}$, a distinct difference was found with X-ray RBE_{10}. While DP RBE_{10} decreased, DP $RBE_{2\,Gy}$ increased for T98G and U87, and this indicates that T98G and U87 exhibited the presence of low-dose HRS to X-rays [48, 49]. $RBE_{2\,Gy}$ could be used as a good indicator for intrinsic tumour cell radiosensitivity, especially with high-LET radiation as it can demonstrate the effect for typical clinical 2 Gy daily treatments.

There was a distinct difference in PLDR α values between X-rays and ion radiations; X-ray PLDR α values showed a definite increase in all three GBM strains but decrease with increasing LET < 200 keV/μm. This differs from the findings of Malaise *et al.* [50], who reported that PLDR led to a decrease in α value in their study of published data that were comprised of both fibroblast and tumour-derived cells but did not include GBM cell lines. Their report suggested that a link may exist between the repair capacity and the intrinsic radiosensitivity [50]. The X-ray β-value was higher at IP, which concurs with Malaise *et al.* [50]. Generally, the trend for PLDR β-value decreased as LET increased. From Table 1, T98G and LN18 DP α/β ratios increased significantly with X-rays (LET 1.7 keV/μm) and ^{28}Si-ions (LET 200 keV/μm). For U87 DP, the α/β ratio increase was observed for X-rays (LET 1.7 keV/μm) and for ions of up to ~100 keV/μm LET, but not 200 keV/μm. LN18 was the only GBM cell line that had an α/β ratio in the hundreds with LET 200 keV/μm and decreased to the order of tens at LET of <200 keV/μm. Both T98G and U87 showed peak α/β ratios at LET 100 and 200 keV/μm, respectively. For LN18, the α/β ratio increased as the LET increased. The increase in the α/β ratio for high-LET radiation may imply that fractionation effects are not crucial, but this needs to be confirmed with *in vivo* experiments. This observation supports the increasing use of hypofractionationed regimens for the treatment of tumours with high-LET radiation therapy. However, further in-depth studies are required.

Our results concurred with Weichselbaum *et al.* [3]; they indicated that the capacity for PLDR is a cellular repair characteristic that may differ between cell types, and also that the more radioresistant the tumour, the higher the PLDR. This inherent cellular radioresistance in GBM may play an important factor in clinical radiocurability. PLDR in GBM may be responsible for failure in radiotherapy especially with X-ray treatment [19, 49]. Even with a high LET of 100 keV/μm, these slow-growing cells are able to induce PLDR. Our results show that at low LET, GBM PLDR is dose dependent [43], except for in T98G cells, which demonstrate HRS [49]. Conversely, Marchese *et al.* [24] describe no correlation between PLDR and *in vitro* radiosensitivity or clinical radiosensitivity of the tumour type.

These results demonstrated that GBM PLDR depended on LET and on the HRS of the cells. Even though charged ion therapy may be a prospective candidate treatment option for improving and progressing the treatment of GBM, our results showed that GBM cells were able to repair damage induced by high-LET charged ion radiation, even up to 100 keV/μm. In conclusion, our results indicated that PLDR of GBM is dependent on LET, dose, and cell strain. The intrinsic radiosensitivity of GBMs based on their genetic alterations will need to be examined in future work.

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