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First Human Cell Experiments With FLASH Carbon Ions

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Abstract. *Background/Aim: This study aimed to establish a setup for ultra-high-dose-rate (FLASH) carbon-ion irradiation, and to conduct the first human cell experiments using FLASH carbon ions. Materials and Methods: A system for FLASH carbon-ion irradiation (1-3 Gy at 13 or 50 keV/μm) was developed. The growth and senescence of HFL1 lung fibroblasts were assessed by crystal violet staining assays and senescence-associated β-galactosidase staining, respectively. Survival of HSGc-C5 cancer cells was assessed by clonogenic assays. Results: The dose rates of carbon ions ranged from 96-195 Gy/s, meeting the definition of FLASH. With both 13 and 50 keV/μm beams, no FLASH sparing effect was observed on the growth suppression and senescence of HFL1 cells, nor on the survival of HSGc-C5 cells. Conclusion: We successfully conducted the first human cell experiments with FLASH carbon ions. No FLASH effect was observed under the conditions examined.*

Radiotherapy is the most widely-used and effective anti-tumor treatment (1). The key to successful radiotherapy relies on expanding the therapeutic window by increasing the dose delivered to tumors while sparing normal tissues. On the basis of this concept, photon radiotherapy technologies have advanced towards modalities providing greater dose conformality, such as intensity-modulated radiotherapy and stereotactic body radiotherapy (1). Particle therapy using protons or carbon ions has also been pursued because protons and carbon ions show greater dose conformality than photons. In addition, carbon ions show a 2-3 fold greater anti-tumor effect than photons (2). However, even with these advanced radiotherapy modalities, some tumors remain incurable because of insufficient dose delivery (*e.g.*, highly radioresistant tumors or tumors surrounded by radiosensitive organs), highlighting the need to establish methods that further expand the therapeutic window of radiotherapy.

In recent years, ultra-high-dose-rate (FLASH) irradiation has attracted great interest because of its potential for lower toxicity to normal tissue (1, 3). FLASH is defined as a single irradiation with a dose rate equal to or greater than 40 Gy/s (1). To date, the biological effect of FLASH irradiation has been investigated using mainly photons and electrons (1, 3). The results of previous animal studies are broadly consistent in terms of showing a sparing effect of FLASH irradiation on normal tissues of mice, including brain (4-9), lung (10-12), intestine (13, 14), skin (15, 16), and other organs (17, 18); as well as some organs of rats (19), mini-pigs (20), and zebrafish (21), although a few studies have reported contradicting outcomes (22, 23). Previous animal studies also agree on the similar anti-tumor effects of FLASH and non-FLASH irradiation in both orthotopic tumor models (4, 11, 19) and xenograft models (7, 11, 12, 24). Furthermore, in 2018, FLASH electrons (15 Gy in 90 ms) were used to treat a human patient with disseminated cutaneous lymphoma (25). The treatment resulted in a rapid tumor response with mild epithelitis and transient edema, and is awaiting verification in a clinical trial. In contrast to photons and electrons, the effects of FLASH irradiation on particle therapy have not been fully investigated. Although there have been several biological studies on FLASH protons, the results are largely inconsistent in terms of normal tissue sparing (26-31), whereas anti-tumor effects may be comparable between FLASH and non-FLASH settings (26, 30, 32-36). However, biological data on FLASH carbon-ion irradiation are lacking.

When conducting FLASH irradiation with carbon ions, it is important to clarify the dose rate for the entire irradiation target. In the scanning irradiation utilized in the clinic, a given target is irradiated spot-by-spot over a period of time. Thus, the average dose rate for the entire target is lower than the dose rate for a given spot, which can be quite high at the moment the pencil beam irradiation is delivered. Recently,

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we established a finely-tuned technique for irradiating high linear energy transfer (LET) carbon ions, which we named 'Carbon Knife'; this achieved a steep dose concentration to a small target (*i.e.*, 1-10 mm in width) (37, 38). In a previous study, we estimated the dose distributions of 1 mm-sized fine carbon-ion beams, resulting in an average dose rate of ~90 Gy/s (which corresponds to ~200 Gy/s during the beam extraction from the synchrotron) at the center of the fine beam near the Bragg peak depth (38). These data led us to speculate that FLASH carbon ions could be achieved by removing the collimators from the Carbon Knife device. This study aimed to establish the technique of FLASH carbon-ion irradiation at a single spot, and to conduct the first human cell experiments using FLASH carbon ions.

Materials and Methods

FLASH carbon-ion irradiation systems. In this study, two beam settings with different dose rates, namely, FLASH and non-FLASH, were established. To assure the homogeneity of the dose rate over the irradiation time, both settings were adjusted so that the irradiation expired within one spill. Carbon-ion irradiation was performed using a scanning vertical beam port at Gunma University Heavy Ion Medical Center (GHMC) (39). The beam energy and intensity were 290 MeV/u and ~1×10^9 particles per second, respectively, similar to values used in the clinic for cancer treatment (40). In the 2.67 s operating cycle of the synchrotron, the flat-top duration was usually ~1 s, and the extraction duration was reduced by approximately half to increase the beam intensity. For the non-FLASH irradiation, the chopper opening duration and the attenuators of the accelerator injector were adjusted to reduce the beam intensity by ~2%. The delivered beam profile at the isocenter (*i.e.*, sample position) was measured by radiochromic film. The standard deviation of the beam profile, approximated by a Gaussian distribution, was ~2.4 mm. Nitrogen gas was flowed to the dose monitor, which was of a parallel plate ionization chamber type. To examine the dose monitor response at a high dose rate, dose measurements were performed at the isocenter position while changing the dose monitor voltage applied. An Advanced Markus ionization chamber (PTW 34045, PTW, Freiburg, Germany) was used for the dose and dose-rate measurements. In addition, the ionization chamber voltage dependence was measured to confirm the response of the dosimeter near to the Bragg peak depth of high-dose-rate beams.

FLASH carbon-ion irradiation to biological samples. In reality, the spill structure of the beam extracted from the synchrotron is not absolutely constant at the beginning, with an abrupt increase generally seen. Therefore, especially at high-dose-rate irradiation, the beam intensity can change rapidly over a short moment, representing irradiation with deposition of a dose of several Gy. It takes approximately 10 ms to irradiate a dose of several Gy with high-dose-rate irradiation. To avoid using the first rising part of the spill and to obtain a constant dose rate at the sample position, the beam was irradiated to a spot sufficiently off the sample position for approximately the first 100 ms of the spill, and was then directed to irradiate the central target. The preset of the dose monitor was calibrated using the ionization chamber for each irradiation condition, to deal with possible changes in monitor response due to dose rate, and the dose rate was obtained from the reading of the dosimeter and the time of the monitor output signal.

The LETs were set to 13 and 50 keV/μm, corresponding to the plateau region and vicinity of the Bragg peak of the carbon-ion beams, respectively. To adjust the LETs, the beam range was shifted by 124.5 mm in the latter case with a variable-water-thickness phantom including totally 20-mm-thick acrylic windows. The beam sizes were ~2.6 and ~3.7 mm in standard deviations for LETs of 13 and 50 keV/μm, respectively. The doses to the cell samples were set at 1, 2, and 3 Gy. The well bottom diameter of the cell sample was 6.35 mm, and its dose and dose rate were evaluated with an Advanced Markus chamber with a sensitive area of 5 mm. Due to single spot irradiations with specific beam sizes, there were variations in the doses in the ionization chamber and sample wells. Therefore, in the following, the measured values, *i.e.*, the average values, in the ionization chamber were used as nominal values to indicate the dose and dose rate.

X-ray irradiation. To verify the robustness of the experimental systems, X-ray irradiation was performed using an MX-160Labo irradiator (160 kVp, 1.06 Gy/min; mediXtec, Matsudo, Japan) (41, 42).

Cell lines and cell culture. The human lung fibroblast HFL1 and human salivary gland tumor line HSGc-C5 were used in this study. HFL1 was selected to represent normal cells considering the fact that the normal tissue protective effect of FLASH irradiation was first discovered with lung fibrosis as the endpoint (12). HSGc-C5 was selected as being representative of cancer cells because this line demonstrates a typical tumor response to ionizing radiation, and has been used as a reference in the beam design of clinical carbon-ion radiotherapy equipment (43, 44). HFL1 was obtained from Riken Bioresource Center (Tsukuba, Japan), and HSGc-C5 was obtained from JCRB Cell Bank (Ibaraki, Japan). Cells were cultured in RPMI-1640 (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (Life Technologies, Carlsbad, CA, USA) at 37˚C with 5% CO_2.

Crystal violet staining assays for cell proliferation. The radiosensitivity of HFL1 cells, which lack a colony-forming ability, was assessed using crystal violet staining assays, as described previously (45, 46). Briefly, cells were seeded on 96-well plates and incubated at 37˚C with 5% CO_2 for 24 hours. Cells were then irradiated and incubated for an additional 5 days, after which they were fixed with 25% methanol (FUJIFILM Wako Chemicals, Osaka, Japan), stained with 0.1% crystal violet (Sigma-Aldrich), and solubilized in 200 μl of 10% acetic acid (FUJIFILM). The absorbance of the solution at 570 nm was measured using a Multiskan FC microplate photometer (Thermo Fisher Scientific, Waltham, MA, USA). The absorbance for a given dose was normalized to that of the non-irradiated control.

Senescence-associated β-galactosidase staining. Cellular senescence in HFL1 cells was assessed by senescence-associated β-galactosidase (SA-β-gal) activity using a senescence β-galactosidase staining kit (Cell Signaling Technology, Danvers, MA, USA) (45, 47, 48). Cells were seeded on 96-well plates and incubated at 37˚C with 5% CO_2 for 24 hours. Cells were then irradiated and incubated for an additional 5 days, before being subjected to SA-β-gal staining following the manufacturer's protocol. Blue-stained cells observed on light microscopy were considered positive for the staining. Using

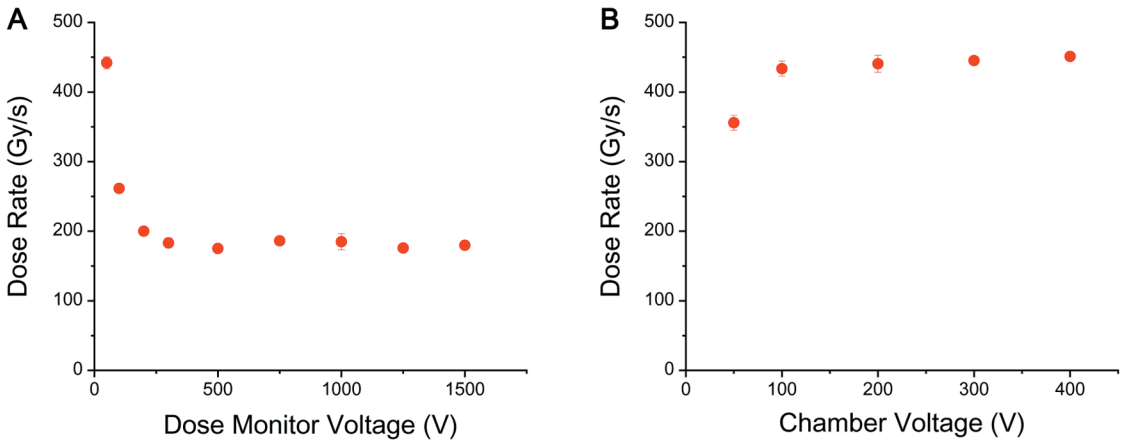

Figure 1. *(A) Dose rate reading at the surface plateau versus dose monitor voltage. The voltage used normally is 1500 V. (B) Dose rate reading at the Bragg peak versus chamber voltage measured by the Advanced Markus ionization chamber. The voltage used normally is 300 V.*

a 10× objective lens, the number of positive-stained cells in a randomly selected field was counted. The count was performed in triplicate for each experimental setting and three independent experiments were performed.

Clonogenic assays. The radiosensitivity of HSGc-C5 cells was assessed using clonogenic assays, as described previously (49). Briefly, cells were seeded on culture plates and incubated at 37˚C with 5% CO_2 for 12 h. Cells were then irradiated and incubated for an additional 7 days, before being fixed with 25% methanol and stained with 0.1% crystal violet. Colonies consisting of 50 or more cells were counted using an inverted microscope. The surviving fraction for a given dose was calculated by dividing the number of colonies for the dose by the number of seeded cells for the dose, which was further divided by the plating efficiency calculated from unirradiated controls. The surviving fractions were fitted to a linear quadratic model (50), from which α, β, and D50 (*i.e.*, the dose reducing cell survival to 50%) were calculated (43). Each experiment was performed in quadruplicate, and three independent experiments were performed.

In this study, clonogenic assays were performed using 96-well plates to ensure the robustness of the FLASH carbon-ion dosimetry. Although clonogenic assays are more commonly performed using 6-well plates or culture flasks with a greater area, the robustness of clonogenic assays using 96-well plates has been demonstrated in multiple studies over several decades (51-53). Furthermore, to verify the robustness of the 96-well plate-based clonogenic assays, the consistency of the experimental outcomes of these assays were compared with those of 6-well plate-based assays (which are routinely performed in-house) (47, 48, 50, 54, 55) using HSGc-C5 cells. The results showed that the X-ray sensitivity of HSGc-C5 cells was highly consistent between 96-well and 6-well plate-based clonogenic assays (Supplementary Figure 1), suggesting the technical robustness of the 96-well plate-based clonogenic assays performed in this study.

Statistical analysis. Differences between two groups were examined using the Mann–Whitney *U*-test and were considered statistically significant when the *p*-value was below 0.05 after Bonferroni correction for multiple comparisons. All statistical analyses were performed using GraphPad Prism 8 (GraphPad Software Inc., San Diego, CA, USA).

Results

The applied voltage dependences of the dose monitor and ionization chamber are shown in Figure 1. The responses of the dose monitor and ionization chamber at the voltages normally used were almost constant, thereby inferring that the recombination effect was almost negligible.

We then examined the dose rate and dose for the high-dose-rate carbon ions under the settings used for the cell experiments (*i.e.*, 1, 2, and 3 Gy at 13 and 50 keV/μm; Table I). Five repeated measurements resulted in dose rates of 96-195 Gy/s with a standard deviation of 13-22%, meeting the definition of FLASH. The standard deviation of the dose for FLASH carbon ions was 3-6%. For use as a control for the cell experiments, a non-FLASH setting (carbon ions exerted in a single spill, as in the FLASH setting) was established, which gave dose rates for non-FLASH carbon ions of 8-13 Gy/s with standard deviation of 4-9%. The standard deviation of the dose for non-FLASH carbon ions was 0.1-0.4%.

Having established the irradiation setting for FLASH carbon ions, we performed *in vitro* experiments using human cell lines with two different LETs, *i.e.*, 13 keV/μm and 50 keV/μm, representing the entrance and high-LET region of clinical carbon-ion beams, respectively. In lung fibroblast HFL1 cells, there was no significant difference in post-irradiation growth suppression between FLASH and non-FLASH carbon ions for both 13 keV/μm and 50 keV/μm beams (Figure 2A). There was also no significant difference in the induction of senescence [the predominant mode of post-irradiation cell death in fibroblasts (45)] between

Table I. *Measured dose and dose rate under each condition for cell irradiation.*

Setting	LET (keV/μm)	Nominal dose (Gy)	Dose (Gy)	Dose rate (Gy/s)
FLASH	13	1	0.945±0.056	96.5±13.4
		2	2.036±0.073	153.4±27.7
		3	3.023±0.118	145.8±18.8
	50	1	0.976±0.039	177.7±39.0
		2	2.073±0.119	179.5±34.3
		3	3.006±0.087	195.1±27.3
Non-FLASH	13	1	0.980±0.004	7.7±0.5
		2	1.999±0.004	9.3±0.3
		3	2.994±0.008	8.4±0.8
	50	1	1.006±0.001	7.9±0.5
		2	2.008±0.002	12.7±0.9
		3	3.007±0.004	12.3±1.1

For each setting, mean and standard deviation of five measurements are shown. FLASH, Ultra-high dose rate; LET, linear energy transfer.

FLASH and non-FLASH carbon ions for both 13 keV/μm and 50 keV/μm beams (Figure 2B, 2C). In the HSGc-C5 cancer cell line, there was no significant difference in post-irradiation clonogenic survival between FLASH and non-FLASH carbon ions for both 13 keV/μm and 50 keV/μm beams (Figure 3, Table II).

Discussion

In this study, we established a system for investigating the FLASH effects of carbon-ion beams and irradiated cell samples while adjusting the dose, dose rate and LET. This is the first report to present the response of human cells to carbon-ion irradiation by changing all of these parameters.

In scanning irradiation, each spot distributed within a target is irradiated with a pencil beam to deliver a prescribed dose to the entire target, so that the dose rate at each point in the target changes with time. It is important to specify the irradiation conditions such as the dose rate to discuss the FLASH effect of carbon-ion beams. This study provides the advantage that the dose rate, as well as the dose, can be specified by completing single spot irradiation to a sample with just one spill. However, due to the beam's lateral profile, the dose and dose rate within the irradiated sample size are not constant. Nevertheless, the dose rate variation is in the range that satisfies the FLASH or non-FLASH condition, and the comparative studies on biological responses are considered possible between both conditions because the beam size is almost the same under each condition.

The biological mechanism by which FLASH irradiation spares normal tissue remains unclear; however, several hypotheses have been proposed (1, 3, 21). One hypothesis is that FLASH irradiation depletes oxygen from the irradiated tissues, which functions to decrease the indirect effects of DNA damage caused by ionizing radiation (3, 21). This hypothesis rationalizes a greater sparing effect in normal tissues than in tumors because the physiological oxygen concentration is generally higher in normal tissues. Importantly, the oxygen effect of carbon ions is LET-dependent, and is generally smaller than that of photons. Thus, the FLASH sparing effect of carbon ions should be investigated with a specific focus on LET. The irradiation system presented in the present study will be of great value for this purpose because it enables carbon ions to be irradiated at any point within the clinically relevant spread-out Bragg peak. The present study showed no FLASH sparing effect under normoxic conditions, irrespective of the LET (13 and 50 keV/μm), warranting a deeper investigation using anoxic, hypoxic and physoxic conditions. Another hypothesis is the so-called immune hypothesis, which proposes the sparing of lymphocytes circulating within the irradiation field (3). For example, Rama *et al*. reported that FLASH irradiation increased intratumoral recruitment of T lymphocytes from peripheral regions in an orthotopic mouse lung tumor model (32). Despite accumulating pre-clinical data, an important caveat in investigations into FLASH irradiation is the lack of consistency between experimental parameters such as dose rate, total dose, and pulse rate, making it difficult to interpret experimental outcomes in comparison with each other. Furthermore, it may be possible that the mechanism by which the FLASH sparing effect is exerted varies between tissues and tumors, underscoring the need for further mechanistic research.

During the preparation of this manuscript, another group independently released an online report of FLASH carbon-ion experiments using Chinese hamster ovary cells (56). In their study, the authors evaluated 13 keV/μm FLASH carbon-ion irradiation for an area of 10×10 mm, which is greater than that achieved in the present study. Nevertheless, it is likely that the dose rate at each point in the irradiated

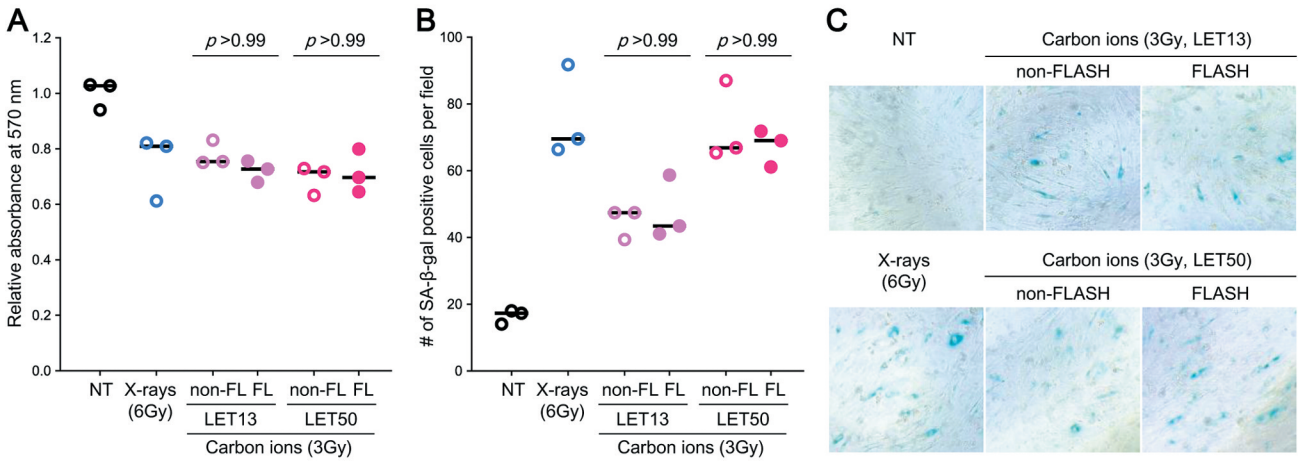

Figure 2. *Sensitivity of human fibroblast HFL1 to FLASH or non-FLASH carbon ions. (A) Growth suppression at 5 days post-irradiation assessed by crystal violet staining assays using absorbance at 570 nm (n=3). Bars indicate median. (B) Induction of senescence at 5 days post-irradiation assessed by senescence-associated β-galactosidase (SA-β-gal) staining (n=3). Bars indicate median. Data are shown after normalizing to the absorbance at 570 nm obtained from crystal violet staining assays for the corresponding experimental settings. (C) Representative micrographs of SA-β-gal staining samples. To confirm the robustness of the experimental systems, the data from cells treated with 6 Gy of X-rays are also shown, on the basis of a previous study reporting that the relative biological effectiveness of carbon ions over X-rays (as assessed by crystal violet staining assays) was approximately 2 (45). FL, FLASH; NT, no treatment; #, number. p-Values assessed by Mann–Whitney U-test are shown after Bonferroni correction.*

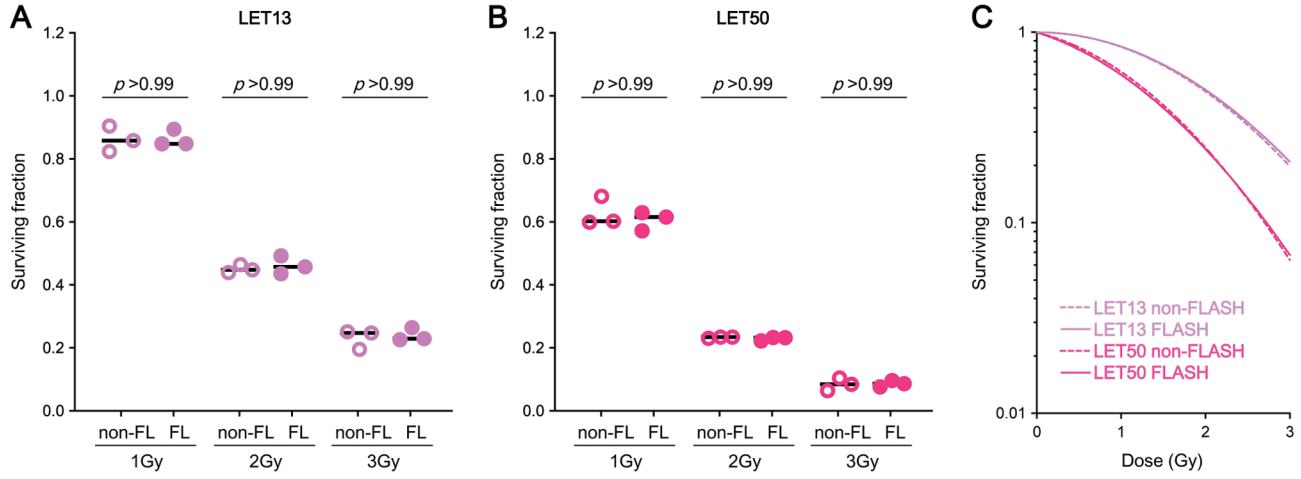

Figure 3. *Sensitivity of human cancer cell line HSGc-C5 to FLASH or non-FLASH carbon ions, as assessed by clonogenic assays (n=3). (A) Clonogenic survival at 13 keV/μm. (B) Clonogenic survival at 50 keV/μm. (C) Survival curves generated by fitting the data shown in A and B to a linear quadratic model. FL, FLASH. p-Values assessed by Mann–Whitney U-test are shown after Bonferroni correction.*

area may differ from the nominal value considering the scanning nature of the irradiation. In the FLASH carbon-ion irradiation technique established in the present study, the dose rate can be specified because a single spot is irradiated within one spill duration; however, the dose and dose rate have considerable variations due to the beam size. In any case, it is important to make the irradiation conditions used for cell experiments (including dose rate) quantifiable. From a biological standpoint, the authors showed the results of two independent clonogenic assays using different oxygen concentrations and observed the FLASH sparing effect in two experiments at 0.5% O_2, and one experiment at 4% O_2, but not at 0% and 21% O_2, warranting future study.

On the basis of the work presented here, we suggest pursuing the following points to investigate the clinical applicability and biological mechanism of FLASH carbon

Table II. *Clonogenic assay parameters for HSG cells treated with FLASH or non-FLASH carbon-ions.*

LET (keV/μm)	Parameters	Non-FLASH	FLASH	*p*-Value
13	α	0.08±0.037	0.08±0.041	>0.99
	β	0.13±0.022	0.13±0.017	>0.99
	D_{50}	1.95±0.082	1.98±0.066	>0.99
50	α	0.39±0.060	0.45±0.018	0.60
	β	0.14±0.043	0.12±0.017	>0.99
	D_{50}	1.20±0.025	1.16±0.006	0.60

α, β, D_{50} values calculated using the linear quadratic model are shown. *p*-Values assessed by Mann-Whitney *U*-test are shown after Bonferroni correction. FLASH, Ultra-high dose rate; LET, linear energy transfer; D_{50}, the dose that reduces survival to 50%.

ions. Considerable fluctuations in dose and dose rate are observed, especially at high-dose-rate conditions, as shown in Table I. We consider that each spill extracted from the accelerator is unstable, and the spill itself may include spike-like time structures. Therefore, the response on the dose monitor can change instantaneously, although it seems to be almost stable in the measurement of the entire spill, as shown in Figure 1A. To obtain further stable dose rates and doses, it would be necessary to improve the spill structure by adjusting the accelerator. In addition, the system requires development so that it can form a larger, more uniform field size and a spread-out Bragg peak to realize irradiations under FLASH conditions closer to clinical situations. In such cases, it will be important to define the irradiation conditions, such as the average and instantaneous dose rates at the spot position, to accumulate data, and to develop an understanding of the dose-rate dependence of the biological response. From a biological standpoint, the effect of FLASH carbon ions needs to be evaluated in relation to LET and oxygen concentration *in vitro*, which should then be followed by *in vivo* experiments.

In conclusion, we established the technique of FLASH carbon-ion irradiation with a specific dose rate at a single spot. We also established spill-matched non-FLASH carbon-ion irradiation for use as a control in biological experiments. Using these techniques, we present the first human cell experiments using FLASH carbon ions. Our results indicated no FLASH sparing effect in normal and cancer cells under normoxia. This is the first study to report the response of cells to FLASH carbon ions with varying dose, dose rate and LET.

Supplementary Material

Supplementary material is available at: https://www.researchgate.net/publication/358808249_Supplementary_Figure_1

Conflicts of Interest

Tatsuya Ohno received honorarium from Hitachi, Ltd. (Tokyo, Japan). All the other Authors declare no conflicts of interest.

Authors' Contributions

M.T. contributed to dosimetry and wrote the manuscript. Y.Y. contributed to the biology experiment setup and wrote the manuscript. T. Oike performed the biology experiment and wrote the manuscript. M.N. and K.Y. contributed to dosimetry. Y.H. performed the biology experiment. T. Ohno supervised the project and acquired funding. All Authors have read and agreed to the publication of the manuscript.

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