Unusual prolongation of radiation-induced G2 arrest in tumor xenografts derived from HeLa cells

Atsushi Kaida and Masahiko Miura

Section of Oral Radiation Oncology, Department of Oral Health Science, Graduate School of Medical and Dental Sciences, Tokyo Medical and Dental University, Tokyo, Japan

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Correspondence
Masahiko Miura, Section of Oral Radiation Oncology, Department of Oral Health Science, Graduate School of Medical and Dental Sciences, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8549, Japan. Tel/Fax: +81-3-5803-5897; E-mail: masa.mdth@tmd.ac.jp

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Solid tumors have microenvironments characterized by hypoxia, lack of nutrients, and low pH. Tumor cells often acquire therapeutic resistance in tumor microenvironments. Formulating effective therapeutic strategies requires understanding the cell cycle kinetics that follow DNA damage in solid tumors where such environments are present; however, it has been difficult to obtain information on these kinetics due to limitations of techniques, such as flow cytometric analysis, which do not retain spatial information. Sakaue-Sawano et al. developed a novel system, the fluorescent ubiquitination-based cell cycle indicator (Fucci), to visualize each cell cycle phase in vivo. This technique uses two proteins that originate from corals, monomeric Azami Green (mAG) and monomeric Kusabira Orange 2 (mKO2), which fluoresce in vivo during the S/G2/M and G1/G0 phases, respectively. Using this system, Yano et al. reported that G1/G0-phase cells survived after treatment with ionizing radiation or cisplatin in spheroids and tumors with wild-type p53 because only growing cells were killed by the agents, but the kinetics of cell cycle arrest were not described in detail.

Ionizing radiation causes a variety of types of DNA damage in tumor cells, with double-strand breaks (DSB) being the most lethal. It is well-known that many kinds of tumor cells lack functional p53, which accumulate in G2 phase due to DSB, leading to G2 arrest. We previously visualized radiation-induced G2 arrest in live monolayer-cultured HeLa cells expressing Fucci (HeLa-Fucci cells). Further described radiation-induced cell cycle kinetics in multi-cellular spheroids consisting of HeLa-Fucci cells as a tumor-like model. Interestingly, the duration of radiation-induced G2 arrest in spheroids was significantly prolonged, and furthermore, cell cycle kinetics after irradiation differed between the outer and the inner spheroid layers. In this study, for the first time, we visualized unusual cell cycle kinetics after irradiation of tumor xenografts derived from HeLa-Fucci cells that were generated by tumor microenvironments. We further suggest that the unusual kinetics may provide a potential radioresistance mechanism.

Materials and methods
Cell line and culture conditions. HeLa-Fucci cells were provided by the RIKEN BRC through the National Bio-Resource Project of MEXT, Japan. Cells were maintained in DMEM (Sigma-Aldrich, St. Louis, MO, USA) with 100 units/mL penicillin and 100 μg/mL streptomycin, supplemented with 10% fetal bovine serum, at 37°C in a 5% CO2 humidified atmosphere.

Xenograft establishment. All animal experiments were approved by the Institutional Animal Care and Use Committee of Tokyo Medical and Dental University. HeLa-Fucci cells (1 × 10⁶ cells in 50 μL PBS) were implanted subcutaneously into the hind legs of male KSN nude mice (6 weeks old). Tumor xenografts took about a month to grow to the appropriate size (length of about 1 cm), at which point the mice
underwent the experiments described below and then were properly sacrificed. Tumor volume (V) was calculated using the following formula: \( V = \text{length} \times \text{width}^2 / 2 \).

**Irradiation.** Each xenograft tumor in non-anesthetized mice was irradiated with an HS-225 X-ray therapeutic machine (225 kVp, 15 mA, 1.0 mm Cu filtration; Shimadzu, Kyoto, Japan) at a dose rate of 0.9 Gy/min while shielding the body with lead.

**Optical imaging of mAG and mKO2 in tumor xenografts of HeLa-Fucci cells.** Before the imaging, mice were anesthetized with 40 mg/kg pentobarbital sodium in physiological saline. Optical imaging was performed with a Photon Imager (BIOSPACE LAB, Paris, France) to detect both mAG and mKO2. Acquired images were analyzed using M3Vision™ software (BIOSPACE LAB).

**Immunohistochemistry.** At each time point after irradiation, tumors were excised and fixed in 4% paraformaldehyde in PBS, then immersed in 20% sucrose in PBS until tumors sank to the bottom of the tube. Fixed tumors were embedded in OCT compound (Sakura, Tokyo, Japan) and stored at −80°C. For the detection of blood perfusion, 16 mg/kg Hoechst 33342 was injected intravenously 1 min before sacrifice. As a hypoxia marker, 60 μg/mL pimonidazole-HCl (Hypoxyprobe-1: Hypoxyprobe, Burlington, MA, USA) was injected intraperitoneally 30 min before sacrifice. Frozen and fixed tumors were cut by a cryostat into 10-μm sections. For detections of cyclin B1 and CENPF as G2 markers, tumor sections were boiled in Histostool One solution (Nakalai Tesque, Kyoto, Japan) for at least 20 min at 90°C to retrieve the antigen. For the detection of pimonidazole, Target Retrieval Solution (Dako, Glostrup, Denmark) was applied and tumor sections were boiled for at least 20 min at 70°C. After the antigen retrieval treatment, non-specific antigens were blocked in Protein Block, Serum Free (Dako) for 10 min. Sections were incubated with anti-cyclin B1 antibody (clone: GNS1: Santa Cruz Biotechnology, Dallas, TX, USA), anti-CENPF (ab5: Abcam, Cambridge, UK), or anti-pimonidazole (Hypoxyprobe) for 60 min at room temperature; thereafter, Alexa Fluor 555 Goat Anti-Mouse IgG (H+L), Alexa Fluor 488 Goat Anti-Rabbit IgG (H+L), Alexa Fluor 647 Goat Anti-Mouse IgG (H+L), or Alexa Fluor 488 Goat Anti-Mouse IgG (H+L) (Molecular Probes, Eugene, OR, USA) was applied for 30 min at room temperature as the secondary antibody, respectively. After the immunoreaction, sections were mounted with ProLong Gold Antifade Reagent with DAPI (Molecular Probes). For detection of mAG/mKO2 and perfusion, neither the retrieval treatment nor the immunoreaction was performed, and sections were mounted only. Fluorescence images were obtained using a BIORIVO BX-9000 fluorescence microscope (KEYENCE, Osaka, Japan). For the quantitative analysis, at least three regions in each section were randomly chosen and fluorescent areas were measured using ImageJ 1.44 software (available at http://rsweb.nih.gov/ij/).

**Clonogenic survival assay.** To assess the effect of prolonged in vivo G2 arrest on cell survival, tumors were excised immediately after sacrifice at specific times after 10-Gy irradiation. Excised tumors were minced using scalpels and disaggregated by collagenase and trypsin for preparation of single cell suspensions. Equal number of isolated single cells were plated on dishes and incubated for about 10 days. Colonies were fixed and stained with crystal violet. Colonies consisting of more than 50 cells were counted and surviving fractions (SF) were calculated as follows:

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SF = \frac{\text{number of colonies/number of irradiated cells plated}}{\text{number of colonies/number of non-irradiated cells plated}}.
\]

**Statistical analysis.** Mean values were statistically compared using the two-tailed t-test. P-values < 0.05 were considered statistically significant.

**Results**

**Characterization of tumor xenografts derived from HeLa-Fucci cells.** We established tumor xenografts derived from HeLa-Fucci cells and analyzed the expression of mAG and mKO2 in tumor sections. There was no specific localization of mAG or mKO2 in peripheral or perinecrotic regions; however, expression of mKO2 was higher than that of mAG throughout the tumor sections (Fig. 1a). By injecting Hoechst 33342 immediately before sacrifice, perfused blood vessels were visualized in tumor sections (Fig. 1b), where expression of mKO2 was also higher than that of mAG. Interestingly, perinecrotic regions contained areas in which Fucci fluorescence was negative and only DAPI staining was positive (designated DAPI (+) areas) (Fig. 1c). To examine the relationship between DAPI (+) areas and hypoxic fractions, pimonidazole was administered as a hypoxic marker.\(^{(13)}\) DAPI (+) areas were not stained with pimonidazole. Hypoxic fractions were observed outside of DAPI (+) areas, where mKO2 was more highly expressed (Fig. 1d). It was speculated that DAPI (+) areas consisted of death-committed cells that had lost most biochemical activity. Taken together, mKO2 expression was predominant in tumor xenografts that had not been irradiated. Perinecrotic regions were hypoxic, but there was no characteristic difference in fluorescence distribution between oxic and hypoxic areas.

**Fluorescence kinetics in tumor xenografts of HeLa-Fucci cells after irradiation.** To characterize cell cycle kinetics following irradiation of tumor xenografts, we analyzed fluorescence distributions in tumor sections at varying times after 10-Gy irradiation (Fig. 2a). Interestingly, a remarkable change was observed on Day 1 after irradiation compared to Day 0: peripheral areas were green, whereas perinecrotic ones were red (Fig. 2a: Day 1). Furthermore, most cells in all areas, including perinecrotic regions, became green 2 days after irradiation. This green-dominant state persisted for at least 5 days (Fig. 2a,b). As a reference, the fluorescence kinetics in monolayer cultures are shown in Supplementary Figure S1.

The fluorescence intensities of both mAG and mKO2 could be quantitated in live mice (Suppl. Fig. S2a). The fluorescence intensity ratio of mAG to mKO2 (mAG/mKO2) was similar to that in tumor sections (Suppl. Fig. S2b,c). Next, we analyzed fluorescence kinetics after irradiation in live nude mice bearing tumor xenografts (Fig. 2c). In non-irradiated tumor xenografts (0 Gy), the fluorescence of both mAG and mKO2 was always detectable and the mAG/mKO2 fluorescence ratio was <1 (Fig. 2d: 0 Gy). Following 10-Gy irradiation, the ratio increased dramatically and peaked around 2 days after irradiation. The predominance of green fluorescence persisted for more than 5 days in live mice bearing tumor xenografts as well as in tumor sections. Fluorescence kinetics showed a similar tendency in independent experiments albeit with slight differences in the peak time (Suppl. Fig. S3). The reason why the values in Figure 2(d) are enhanced compared to those in Figure 2(b) is due to Fucci properties; the promoter activity in...
Fucci probes is constitutively active, and therefore, intensity of green fluorescence remarkably increases when cells stop at G2 phase for a long time.

**Verification of G2 arrest in tumor xenografts after irradiation.** Previous studies on cell cycle kinetics in monolayer cultures\(^{12,13}\) strongly suggested that the green-dominant state in irradiated tumor xenografts represented prolongation of radiation-induced G2 arrest. To verify this, we examined the expression of cyclin B1 and CENPF as G2 phase markers.\(^{16,17}\) In tumor sections 5 days after irradiation, most green cells expressed cyclin B1 in the cytoplasm and CENPF in the nucleus (Fig. 3a,c). Furthermore, quantitative analysis of both proteins supported the above hypothesis (Fig. 3b,d).

**Potential contribution of prolonged G2 arrest to radioresistance in xenografted tumors.** We previously reported that the release from radiation-induced G2 arrest was accelerated by attaching spheroids to the bottom of the culture dish.\(^{14}\) G2-arrested cells isolated from a tumor xenograft one day or 5 days after irradiation were more rapidly released from G2 arrest when attached to a culture dish (Fig. 4a, Suppl. Fig. S4) than cells in an *in vivo* condition (Fig. 2). This rapid release from prolonged G2 arrest prompted us to consider the possibility of induction of radiosensitization. We therefore examined cell survival in tumor cells isolated from tumor xenografts at various times after irradiation (Fig. 4b). The surviving fraction significantly increased in isolated tumor cells 5 days after irradiation, compared to those isolated immediately or one day after irradiation (Fig. 4c). The trypan blue exclusion test showed that cell viability of isolated tumor cells was similar between non-irradiated tumors and tumors one day or five days after irradiation, and there were no significant changes (Control: 63.67% ± 12.4%; Day 1: 52.9% ± 5.4%; Day 5: 55.1% ± 15.7%). Also, the mean tumor volume on Day 6 decreased to 90.5% ± 10.5% of that before irradiation, but

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**Fig. 1.** Characterization of tumor xenografts originating from HeLa-Fucci cells. (a) Fluorescence images of tumor xenograft sections. Bar: 500 μm. (b and c) Fluorescence images of tumor xenograft sections. Low magnification images of mAG and mKO2 are merged with images of perfused vessels and nuclei, respectively. Bar: 500 μm. High magnification images are composed of each staining and a merged one. Bar: 200 μm. (d) Localization of a hypoxic maker (Hypoxyprobe-1: Pimo) in sections of tumor xenografts. Bar: 200 μm. Data are representative of four independent experiments.
this difference was not significant. Thus, we speculate that the increase in the surviving fraction on Day 5 was not an artifact of cell loss at the early time. Taken together, we reasoned that the prolonged G2 arrest served \textit{in vivo} after irradiation is likely to contribute to a potential radioresistance mechanism.

Discussion

In this study, we demonstrated the following novel findings regarding tumor xenografts derived from HeLa-Fucci cells after irradiation: (i) remarkable prolongation, up to 5 days, of the green phase, representing radiation-induced G2 arrest; (ii) two distinct red (in the perinecrotic region) and green (in the peripheral region) fluorescent regions observed 24 h after irradiation; (iii) transition from red to green fluorescence two days after irradiation; and (iv) enhanced cell survival during the elongated G2 arrest.

The fluorescence kinetics are reminiscent of those we previously observed in spheroids,\(^{(14)}\) although there was more significant prolongation of G2 arrest in solid tumors. In 500-μm-diameter spheroids consisting of HeLa-Fucci cells, there was no significant difference in the distribution of either red or green.
fluorescence, though red was somewhat predominant between the outer and inner layers. The outer layer, with a thickness of approximately 100 μm, became green 16 h after irradiation, while the inner region remained red. Interestingly, following irradiation the green phase persisted in the outer layer of spheroids for more than 48 h longer than in monolayer cultures. The inner region began turning green 24 h after irradiation and remained green for as long as 48 h, suggesting recruitment from the quiescent to the growing phase. Judging from the structural homology between spheroids and solid tumors, the outer and inner regions correspond to the peripheral and perinecrotic areas of solid tumors, respectively. However, the elongation of G2 arrest was somehow remarkably enhanced in solid tumors (Fig. 2), which may have resulted both from complicated structures and the degree of tumor microenvironments. The spheroids used in the study had diameters of approximately 500 μm with no internal necrosis while the solid tumors used in this study had diameters of approximately 1 cm and were characterized by internal necrosis and vasculature. It is well-established that the tumor microenvironment is acidic. Park et al. reported that DSB repair is affected by other tumor microenvironmental factors, such as acidic conditions and extracellular matrix expression. Further study is needed to elucidate the detailed mechanism.

We next considered how the unusually prolonged G2 arrest observed in this study influenced radiosensitivity. Although a slow DSB repair rate is generally thought to be a disadvantage in cell survival, cells remaining in G2 phase for a long time demonstrated a higher surviving fraction than those isolated immediately after irradiation (Fig. 4c). Thus, prolongation of G2 arrest is likely to be advantageous in tumor-cell survival. As in this study, previous reports performed colony formation assays on cells isolated from xenografted tumors at various times after irradiation. The surviving fractions of tumor cells isolated several hours after irradiation were larger than those of cells isolated immediately after irradiation, which is a well-known result of potentially lethal damage repair (PLDR). This phenomenon is mainly associated with the non-homologous end-joining (NHEJ) pathway due to DSB repair of quiescent solid tumor cells at the G0 phase. Our findings are inconsistent with previous studies in that we observed recovery of cell survival during G2 arrest, not G1/G0 phase. A recent study demonstrated PLDR in G2 phase, where the HR pathway...
plays a critical role. It is still unclear whether the same mechanism also occurs in the very slow PLDR during the prolonged G2 arrest in vivo. Nevertheless, two plausible radiosensitizing strategies could be used in the context of such a radiosensitivity mechanism. One is fractionated stereotactic radiotherapy using more than 10 Gy per fraction and total treatment duration within 1 week. In this regimen, prolonged G2 arrest provides quite an efficient redistribution of the cell cycle toward the radiosensitive G2/M boundary. Another approach might be to release cells from elongated G2 arrest using Chk1 or Wee1 inhibitors, which demonstrate G2/M checkpoint inhibition, resulting in enhanced radiosensitization.

In this study, we demonstrated that radiation-induced G2 arrest was remarkably prolonged in tumor xenografts derived from HeLa-Fucci cells, which seemed to be attributed to enhanced cell survival. Because HeLa-Fucci cells do not show G1 arrest after irradiation due to p53 deficiency, further studies will be needed in order to verify whether this phenomenon occurs only in p53-deficient cells or not. Understanding radiation-induced cell cycle kinetics in solid tumors is important to formulate the effective therapeutic strategies. This study will provide supportive information as a valuable piece of evidence that tumor microenvironments affects cell cycle kinetics and cell survival in irradiated solid tumors.

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Disclosure Statement

The authors do not have any conflicts of interest.

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Supporting Information

Additional supporting information may be found in the online version of this article:

Data S1. Supporting Materials and Methods

Fig. S1. Fluorescence kinetics after 10-Gy irradiation of monolayer cultures.

Fig. S2. Detection of mAG and mKO2 in live nude mice bearing tumor xenografts.

Fig. S3. Values of mAG/mKO2 over time in other live mice bearing tumor xenografts after 10-Gy irradiation.

Fig. S4. Fluorescence kinetics of HeLa-Fucci cells isolated from a tumor xenograft following irradiation.

Fig. S5. Nuclear formation of γH2A.X foci in tumor sections before and after 10-Gy irradiation.