and MAPK signaling, known as a major intracellular signaling of transformation into cancer cells. Although morphological change of nuclear envelope has been known in cancer cells, the molecular mechanism of the processes is not well understood. Recent studies revealed molecular signaling of laminar formation is altered during carcinogenesis. Our study revealed that PARylation is involved in these molecular events. Oocytes are capable of being isolated without other cell types by adding hyarulonidase, which is far easier compared with the difficulty in homogenous isolation of purified cancer cells. Our data suggest oocytes provide with a unique biological window for elucidating the mechanism of PARylation in carcinogenesis. Further comparative analysis between cancer cells and oocytes may highlight the uncovered mechanisms underlying the carcinogenesis processes.

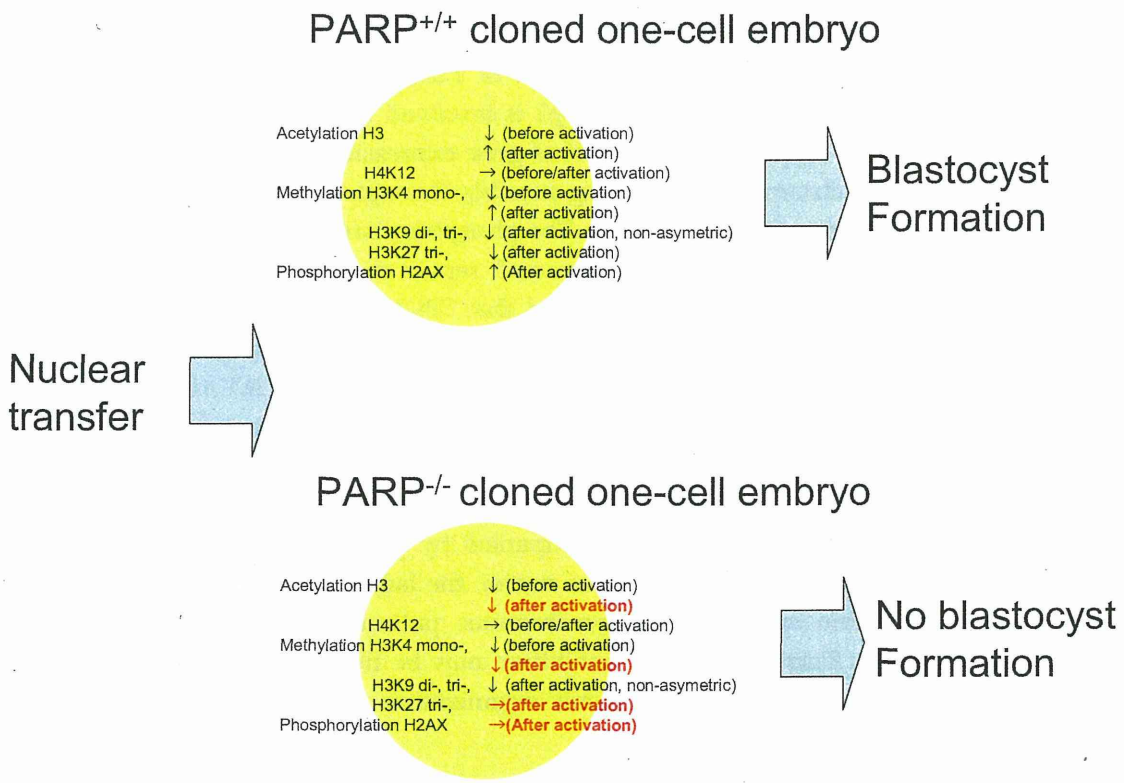

Figure 3. The scheme of histone modification and the effects of *Parp1* deficiency during the first cleavage of NT embryogenesis. Histone modifications of the one-cell reconstructed oocytes were indicated in *Parp*$^{+/+}$, and those which *Parp1* deficiency influences were indicated in *Parp*$^{-/-}$ in red.

We also described here that PARylation is a novel target of anti-conception. Inhibition of PARylation may easy to be handled, because exposure of an inhibitor to oocytes is effective enough to stop fertilization. Further extensive analysis should be carried out regarding the optimized dose of inhibitors and non-toxic dose on other tissues. Biological phenomena, in which PARylation is involved, are variable and its inhibitors could serve as possible pharmaceutical targets including inflammation or brain injury as well as carcinogenesis and reproduction as discussed in this chapter. Further basic investigation of the roles of PARylation in the cells will broaden the view for the understanding the embryogenesis and proof of mechanism of human diseases for drug discovery.

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Radiosensitization effect of poly(ADP-ribose) polymerase inhibition in cells exposed to low and high liner energy transfer radiation

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Poly(ADP-ribose) polymerase (PARP)-1 promotes base excision repair and DNA strand break repair. Inhibitors of PARP enhance the cytotoxic effects of γ-irradiation and X-irradiation. We investigated the impact of PARP inhibition on the responses to γ-irradiation (low liner energy transfer [LET] radiation) and carbon-ion irradiation (high LET radiation) in the human pancreatic cancer cell line MIA PaCa-2. Cell survival was assessed by colony formation assay after combination treatment with the PARP inhibitor AZD2281 and single fraction γ-irradiation and carbon-ion irradiation (13 and 70 keV/μm [LET 13 and LET 70]). The DNA damage response (DDR) was assessed by pulse field gel electrophoresis, western blotting and flow cytometry. Treatment with a PARP inhibitor enhanced the cytotoxic effect of γ-irradiation and LET 13 and LET 70 carbon-ion irradiation. Moreover, the radiosensitization effect was greater for LET 70 than for LET 13 irradiation. Prolonged and increased levels of γ-H2AX were observed both after γ-irradiation and carbon-ion irradiation in the presence of the PARP inhibitor. Enhanced level of phosphorylated-p53 (Ser-15) was observed after γ-irradiation but not after carbon-ion irradiation. PARP inhibitor treatment induced S phase arrest and enhanced subsequent G2/M arrest both after γ-irradiation and carbon-ion irradiation. These results suggest that the induction of S phase arrest through an enhanced DDR and a local delay in DNA double strand break processing by PARP inhibition caused sensitization to γ-irradiation and carbon-ion irradiation. Taken together, PARP inhibitors might be applicable to a wide therapeutic range of LET radiation through their effects on the DDR. (*Cancer Sci*, doi: 10.1111/j.1349-7006.2012.02268.x, 2012)

A definite cell-killing effect with minimal adverse events during the lifetime of patients is among the main goals of radiotherapy for cancer treatment. To achieve this goal, both the improvement of dose distribution and the development of efficient radiosensitizers are important.

In addition to conventional photons, such as X-rays and γ-rays, other types of radiation, such as high liner energy transfer (LET) charged particles and protons, are being used in cancer therapy with good clinical outcomes.[1] Carbon-ion radiation has significant biological advantages compared with photon beams,[2] and radiosensitizers should result in further improvement of the effectiveness of carbon-ion radiation therapy. However, effective radiosensitizers for high LET radiation are not currently available.

In the search for chemotherapeutic agents, recent interest has focused on DNA repair pathways as potential targets for novel cancer treatments.[3] The poly(ADP-ribose) polymerase (PARP) superfamily consists of 17 members, which are multifunctional enzymes, and PARP-1 is the most abundant. PARP-1 detects the presence of DNA single and double strand breaks (SSB and DSB) and binds to the sites of damage, promoting DNA repair by modifying key proteins.[4] PARP-1 is upregulated in various cancers, presumably to compensate for genomic instability,[5] making this enzyme a target of cancer therapy. PARP inhibitors cause synthetic lethality in cells with mutations in *BRCA1* or *BRCA2*, which encode important proteins for homologous recombination (HR)[6] or in HR-deficient cancer cells. In fact, clinical studies suggest that PARP inhibitors are effective as mono-therapy against *BRCA*-mutated cancers, showing few adverse effects compared with conventional chemotherapy,[7] and PARP inhibitor treatment in combination with conventional chemotherapy improves survival of cancer patients without increasing toxic effects.[8,9] PARP inhibitors also enhance the cytotoxicity of ionizing radiation in various cancer cells and animal models.[10–12] Because PARP-1 is an important enzyme for base excision repair (BER)[13] and radiation-induced SSB are mainly repaired by BER, the radiosensitization effect of PARP inhibitors is thought to occur through a block in the BER pathway, leading to an increase of collapsed replication forks generating persistent DSB, which are potentially lethal lesions.[14] Dungey *et al.*[10] demonstrate that the radiosensitizing effects of PARP inhibitors on photon beams are S phase-dependent.

Because biological enhancement of high LET radiation might contribute to the development of more effective cancer therapies, we investigated the effect of PARP inhibition on the responses to γ-irradiation (low LET radiation) as well as carbon-ion irradiation (high LET radiation).

Materials and Methods

Chemicals and antibodies. AZD2281 (Olaparib) was obtained from Selleck Chemicals (Houston, TX, USA) and dissolved in DMSO. Anti-γ-H2AX (Ser-139) antibody was purchased from Millipore (Billerica, MA, USA). Anti-phosphorylated p53 (Ser-15), and anti-histone H3 antibodies were obtained from Cell Signaling Technology (Danvers, MA, USA). Anti-phosphorylated histone H3 (Ser-10) antibody was purchased from Abcam (Cambridge, UK). Anti-β-actin was purchased from Sigma-Aldrich (St. Louis, MO, USA).

Cell culture. The human pancreatic cancer cell line MIA PaCa-2 was obtained from the American Type Culture Collection (Rockville, MD, USA) and maintained in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin at 37°C in a humidified atmosphere containing 5% CO_2 and 95% air.

Irradiation. Exponentially growing cells were irradiated with γ-rays and carbon-ion beams. For γ-irradiation, ^{60}Co γ-irradiator (Gammacell 220, Nordion, Canada) was used at 0.29 Gy/sec at the National Cancer Center Research Institute. Carbon-ion

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beams (290 MeV/n, 13 and 70 keV/μm) were generated by a heavy ion medical accelerator at the National Institute of Radiological Sciences (NIRS). LET 13 and LET 70 carbon-ion monobeams were used because they are the representative LET at the entrance region to which normal tissues adjacent to tumors are exposed and near at the Bragg peak to target tumor volume, respectively. Cells in flasks were placed in a specially designed rack for correct positioning. Irradiation was conducted using horizontal carbon-ion beams with a dose rate of approximately 1.2 Gy/min. The energy at the irradiation site was obtained by comparing the calculated and measured depth–dose distribution. The LET values (dose averaged LET) at the sample position were compensated with Lucite absorbers.

Clonogenic survival assays. Cells were seeded in triplicate in six-well tissue culture dishes with 3 mL of culture medium and in 25-cm^2 flasks with 5 mL of culture medium 10–14 h before γ-irradiation and carbon-ion beam (LET 13 and 70 keV/μm) irradiation, respectively. This 4-h interval was the time needed for seeding and irradiation. AZD2281 at 1 μM, 5 μM or 0.1% of DMSO (solvent control) were added to the medium 2–3 h before irradiation. Following irradiation, the cells were incubated at 37°C in a CO_2 incubator. After 5–6 days, surviving colonies were fixed with 4% formalin solution and stained with 0.02% crystal violet solution. In the literature, for colony formation assay, approximately 5 Gy is the usual maximum dose for carbon-ion irradiation.[15,16] Therefore, the cell survival and the sensitization effect of the chemicals were evaluated with 1–5 Gy of carbon-ion irradiation. Colonies composed of more than approximately 50 cells were counted. Cell survival was calculated by dividing the number of colonies of irradiated cells by the number of inoculated cells and plating efficiencies of the control cells in each condition. The result of γ-ray irradiation was calculated from the average of at least three independent experiments performed in triplicate. The average of nine data points was used to generate a survival curve. Standard deviation was calculated and statistical analysis was carried out. For carbon-ion irradiation, experiments were performed three times, and the representative result is shown. The plating efficiency of MIA PaCa-2 was 0.55 ± 0.15. To evaluate the radio-sensitizing effects of AZD2281, the ratio of radiation doses to give 10% cell survival for radiation alone and radiation plus AZD2281 was calculated.

Pulse field gel electrophoresis. Cells were trypsinized and 1.5×10^5 cells were embedded in 1% agarose (Bio-Rad, Hercules, CA, USA) and digested in a proteinase K buffer (0.5 M EDTA; 1% sodium N-lauroylsarcosine, 0.5 mg/mL proteinase K) at 50°C for 20 h, followed by washing in 50 mM EDTA (pH 8.0). Electrophoresis was performed for 20 h at 14°C in 1% pulse field-certified agarose (Bio-Rad) gels containing 0.5 × Tris-borate/EDTA buffer and adapted to a CHEF Mapper Pulse Field Electrophoresis System (Bio-Rad). Markers of λ phage DNA digested with Hind III were loaded. Gels were stained with ethidium bromide and analyzed with LAS 3000 (Fuji Film, Tokyo, Japan).

A modification of a Southern blot hybridization method that uses total human genomic DNA as a probe was used. Briefly, total human genomic DNA was labeled with [α-^{32}P] dCTP using the Megaprime DNA labeling System (GE Healthcare, Waukesha, WI, USA) and hybridization was carried out as described elsewhere[17] and analyzed by BAS 2500 (Fuji Film).

Western blot analysis. MIA.PaCa-2 cells were extracted with Laemmli buffer, sonicated as previously described, electrophoresed on SDS-polyacrylamide gels, and transferred to Sequi-Blot PVDF membranes (Bio-Rad). Western blotting was performed with anti-γ H2AX (Ser-139), anti-p53, anti-phosphorylated p53 (Ser-15), anti-histone H3 and anti-phosphorylated histone H3 (Ser-10), and anti-β-actin at the indicated dilutions. Blots were incubated in a horseradish peroxidase-linked secondary antibody and the immune complex was detected using an enhanced chemiluminescence reaction kit (Millipore).

Flow cytometry. MIA PaCa-2 cells irradiated in the presence or absence of AZD2281 were trypsinized, fixed with 70% ethanol, treated with RNase A, stained with propidium iodide (PI) and analyzed by FACS Calibur (Beckton and Dickinson, Franklin Lakes, NJ, USA).

Statistical analysis. Statistical analysis was conducted using PASW Statistics 18 software (SPSS, Chicago, IL, USA). Levine's test was used to check the equality of variance. If the significance based on Levine's test was 0.05 or below, then probability was automatically calculated with Welch's *t*-test. Otherwise, the probability was calculated by Student's *t*-test. When the *P*-value was <0.05, the difference was considered statistically significant.

Results

Sensitization effect on γ-irradiation and carbon-ion irradiation. Figure 1 shows the dose-response curves of MIA PaCa-2 cells irradiated with γ-ray and carbon-ion beams with two different LET values (13 and 70 keV/μm) in the presence of the PARP inhibitor AZD2281. AZD2281-treated cells showed decreased survival both after γ-irradiation and carbon-ion irradiation compared to the controls. The enhancement ratios of AZD2281 at 1 and 5 μM are shown in Table 1. Isoeffective doses of γ-ray, LET13 carbon-ion and LET 70 carbon-ion irradiation that resulted in 10% cell survival were 5, 3.5 and 2.6 Gy, respectively. Therefore, cells were irradiated with 5 and 3 Gy doses of γ-ray and carbon-ion (LET 13 and 70) irradiation, respectively, for western blot analysis and flow cytometry.

Effect on double strand break levels. To analyze the effect of the PARP inhibitor on DSB, we used pulse field gel electrophoresis (PFGE) under neutral conditions. A 2-h exposure to AZD2281 induced an increase of DSB when the gel was analyzed after Southern blot hybridization. These induced DSB were diminished 12 h after incubation (Fig. 2A). Increase of DSB after 5 Gy of γ-irradiation was not detected in the absence or presence of AZD2281 between 5 and 24 h (data not shown).

Figure 2(B,C) shows the results of PFGE with ethidium bromide staining of DNA from cells at 18 h after carbon-ion irradiation. As described later, western blot analysis of cells irradiated with LET 13 and LET 70 carbon-ions showed a prolonged increase in the level of γ-H2AX, a DSB marker, when the cells were incubated for 10–24 h in the presence of PARP inhibitor. In the control cells, the repair occurred earlier, at 10 h. Therefore, we chose to analyze DSB in cells at 18 h by PFGE to demonstrate the increase in the level of persistent DSB caused by delayed repair in the presence of PARP inhibitor. LET 13 carbon-ion irradiation did not cause DSB with increasing radiation doses 18 h after irradiation in the presence of AZD2281 (Fig. 2B). By contrast, LET 70 carbon-ion irradiation increased the DSB level in a dose-dependent manner only in the presence of AZD2281 (Fig. 2C). A similar increase of DSB in the presence of PARP inhibitor was observed 24 h after LET 70 carbon-ion irradiation (data not shown).

Effect on DNA damage response. To further analyze the effect of PARP inhibition on DSB processing, the level of phosphorylated H2AX (γ-H2AX), which is a marker for DSB, was examined by western blot analysis. As shown in Figure 3(A,B) prolonged and increased levels of γ-H2AX were observed both after γ-irradiation and carbon-ion irradiation in the presence of the PARP inhibitor. The increase in the levels of γ-H2AX peaked 10 h after LET 13 irradiation alone. By contrast, the peak in γ-H2AX levels was shortened to 1-h post-irradiation in the presence of the PARP inhibitor. The persistent presence of γ-H2AX (arrow) and the 25-kDa form of γ-H2AX (marked with an asterisk), which is a mono-ubiquitinated form, was

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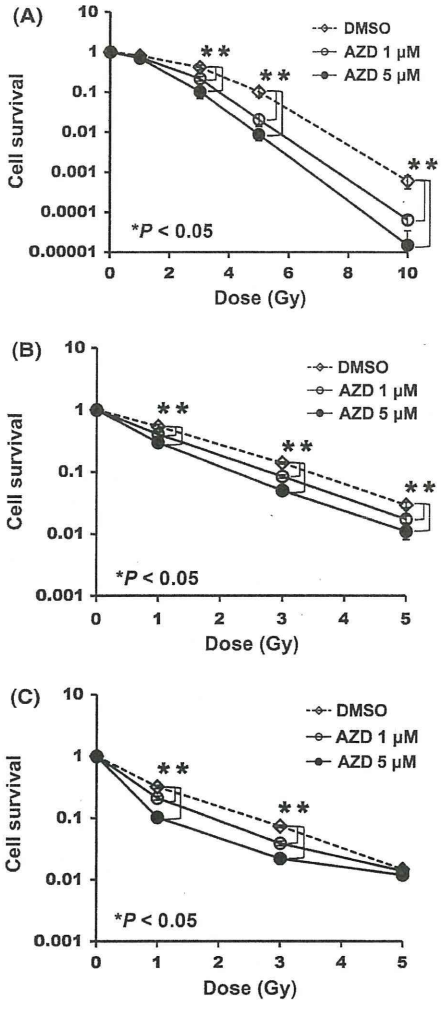

Fig. 1. (A) Clonogenic survival of MIA PaCa-2 cells treated with γ-irradiation alone and in combination with AZD2281. Surviving colonies (defined as > 50 cells) were counted after 5–6 days. The error bars indicate standard deviation calculated from three independent experiments. Asterisks and brackets indicate significant differences in response to γ-irradiation alone at $P < 0.05$. (A,B) Clonogenic survival of MIA PaCa-2 cells treated with liner energy transfer (LET) 13 keV/μm (B) and LET 70 keV/μm (C) carbon-ion irradiation alone and in combination with AZD2281. The cells were exposed to the indicated concentrations of AZD2281 for 2 h and irradiation was then performed. Experiments were carried out three times, and a representative result is shown. The error bars indicate standard deviation. Asterisks and brackets indicate significant differences in response to carbon-ion (LET 13 and 70 keV/μm) irradiation alone at $P < 0.05$.

Table 1. Enhancement ratios of radiosensitivity by AZD2281 at 10% survival

	γ-ray	Carbon-ion beam	
		LET 13 keV/μm	LET 70 keV/μm
AZD2281 1 μM	1.4	1.2	1.4
AZD2281 5 μM	1.7	1.5	2.5

LET, liner energy transfer.

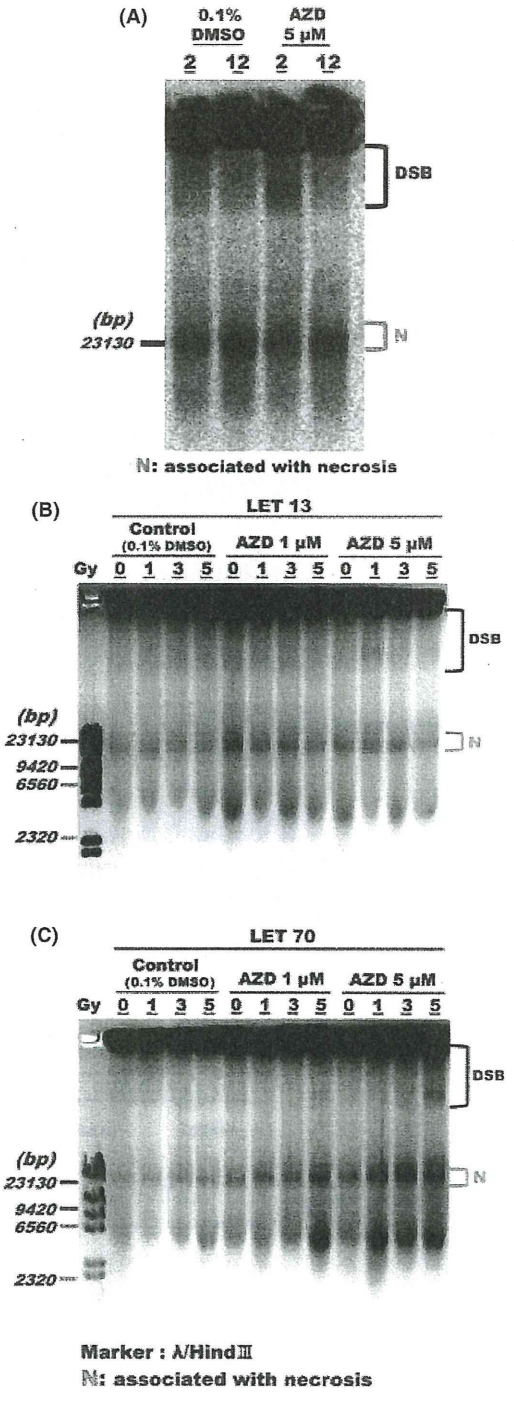

Fig. 2. The effect of double strand breaks (DSB) analyzed by neutral pulse field gel electrophoresis (PFGE). (A) The result of Southern blot analysis 2 and 12 h after addition of either AZD2281 or DMSO (control). (B,C) Ethidium bromide staining result of neutral PFGE 18 h after carbon-ion (liner energy transfer (LET) 13 keV/μm (B) and 70 keV/μm (C)) irradiation in the presence or absence of AZD2281. The positions of DSB and the bands associated with necrosis (N) are shown in brackets.

observed after LET 70 irradiation in the presence of AZD2281. Enhanced levels of p-p53 (Ser-15) were detected after γ-irradiation, but not after carbon-ion irradiation (Fig. 3B). In addition, decreased levels of phosphorylated histone H3, which is a G2/M marker, were observed after γ-irradiation and carbon-ion irradiation in the presence of AZD2281 compared

to its absence (Fig. 3A,B). Analysis of cell cycle progression using flow cytometry (Fig. 4) revealed that PARP inhibition induced S phase arrest and enhanced subsequent G2/M arrest both after γ-irradiation and LET 13 and 70 carbon-ion irradiation.

Discussion

In the present study, we have demonstrated that PARP inhibition is an effective radiosensitizer for carbon-ion irradiation. The underlying mechanism of radiosensitization by PARP inhibitors to both γ-irradiation and carbon-ion irradiation is suggested to be caused by a delay in DDR and DSB processing, which leads to increased S phase arrest and a subsequent arrest at the G2/M phase (Figs 3,4). Western blot analysis showed that this effect occurred independently of p53 phosphorylation status after carbon-ion irradiation. We speculate that these delays in DDR might be due to both the increase in persisting DSB generated by collapsed replication forks[10] and the effect of PARP inhibition on DSB repair pathways. Because MIA PaCa-2 cells did not show a subG1 apoptotic population either in the presence or absence of the PARP inhibitor (Fig. 4), but showed enhanced G2/M arrest, cell death through mitotic catastrophe or necrosis could be the plausible cell death pathways enhanced by the PARP inhibitor.[18]

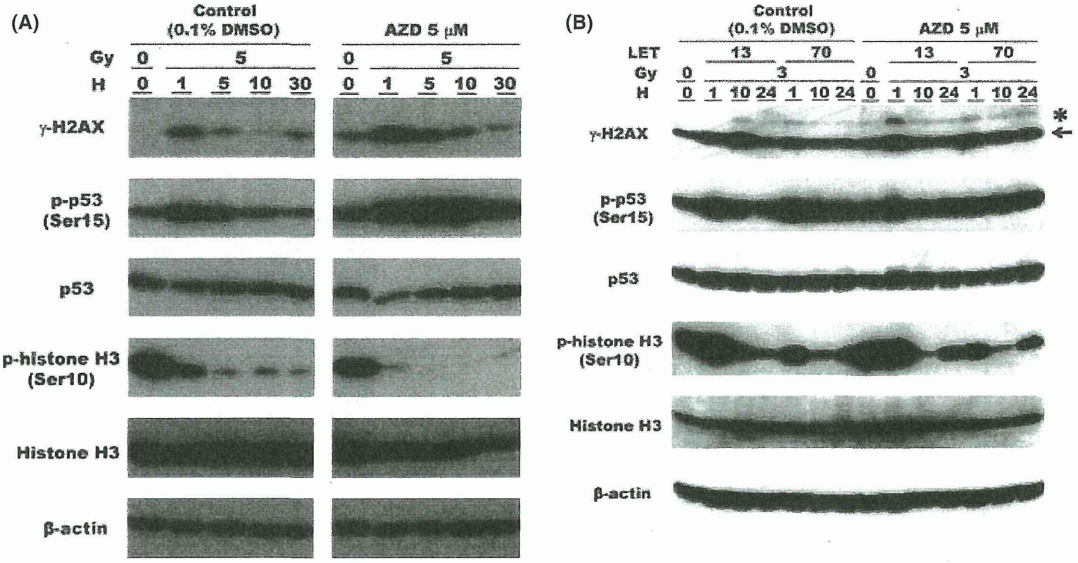

Fig. 3. The level of γ-H2AX, phosphorylated p53, p53, phosphorylated histone H3, histone H3 and β-actin, analyzed by western blotting after γ-irradiation at 5 Gy (A) or carbon-ion (liner energy transfer [LET] 13 and 70) irradiation at 3 Gy (B) in the presence or absence of AZD2281 at 5 μ. The arrow indicates γ-H2AX and an asterisk indicates mono-ubiquitinated γ-H2AX.

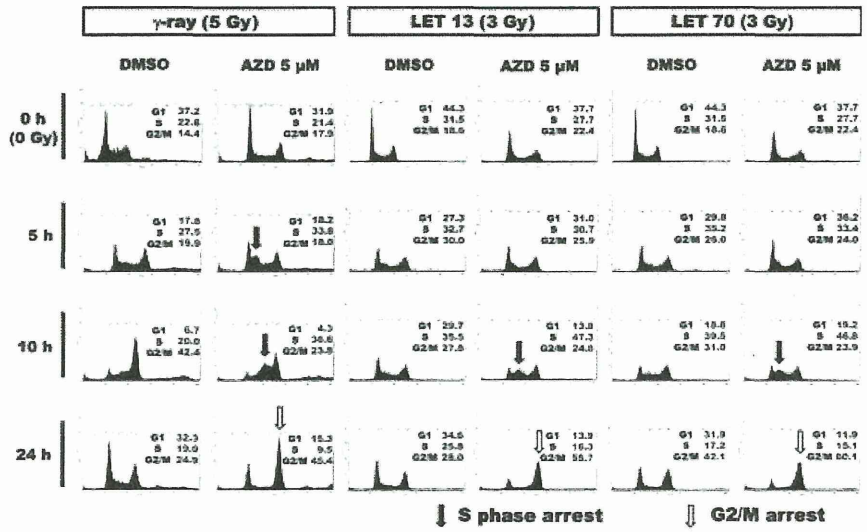

Fig. 4. Flow cytometry analysis with propidium iodide staining after γ-irradiation and carbon-ion irradiation. Percent distributions of cells in different phases of cell cycle are shown in each histogram. The black arrows indicate S phase arrest and the outlined arrows indicate G2/M arrest. The poly(ADP-ribose) polymerase (PARP) inhibitor AZD2281 induced S phase arrest and subsequent G2/M arrest both after γ-irradiation and carbon-ion irradiation.

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DNA damage induced by high LET heavy ion radiation is more complex than that caused by photons, and complex clustered DNA damage can be categorized into two major groups; namely, DSB and non-DSB oxidative clustered DNA lesions (OCDL).[19,20] OCDL are defined as two or more closely associated DNA lesions existing on both strands, usually within one or two helical turns.[19] Yields of clustered damage increase depending on the LET value.[21] OCDL include oxidized bases, apurinic–apyrimidinic sites and SSB, and these lesions are repaired mainly by BER, in which PARP plays a significant role.

We speculate that the sensitization effect of PARP inhibitors on carbon-ion irradiation may be mediated by the conversion of sub-lethal OCDL to lethal damage caused by blocking the BER pathway. Consistent with these premises, the survival curve parameters and the effects of the PARP inhibitor were quite different dependent on what type of irradiation was used; γ-ray resulted in a distinct shoulder on the survival curve, which was not present with LET 13, and was small with LET 70 at the higher dose range close to log-linear. This difference in the survival curve parameters could result in differences in the enhancement ratios, as shown in Table 1. In the presence of 5 μM PARP inhibitor, the enhancement ratio at 10% survival was higher for LET 70 carbon-ion irradiation than for γ-ray and LET 13 (ER_{10}:1.7 for γ-ray, 1.5 for LET 13 and 2.5 for LET 70).

At the lower doses that gave more than 10% survival, PARP inhibitor could affect cell death on OCDL induced by LET 70 carbon-ion irradiation. At the higher dose that gave lower than 10% survival, namely, at 5 Gy of LET 70 carbon-ion irradiation, the amount of clustered lethal DSB may increase and be a main cause of cell death. Thus, the effects of AZD on OCDL or SSB may not contribute much to cell death. This could be the reason why we could not detect the sensitization effect of the PARP inhibitor in the present study at 5 Gy of LET 70 carbon-ion irradiation. In contrast, for γ-irradiation and LET 13 carbon-ion irradiation, the ratio of clustered lethal DSB is low and the amount of SSB and OCDL, which are repaired by the BER pathway, increases dose-dependently so that AZD2281 could sensitize more at higher doses of irradiation. Therefore, the PARP inhibitor enhanced the effect of LET 70 carbon-ion irradiation at a lower dose than 5 Gy and enhanced the effects of γ-irradiation and LET 13 carbon-ion irradiation at all doses.

Conversion of OCDL to lethal DSB by binding of the PARP inhibitor to PARP at strand break ends may be more effective with OCDL produced by LET 70 carbon-ion irradiation compared to those produced by γ-irradiation and LET 13 carbon-ion irradiation. This could be the reason why we observed a higher enhancement ratio by the PARP inhibitor for LET 70 carbon-ion irradiation at low doses.

Therefore, the effect of PARP inhibition on clustered damage should be analyzed further. However, our results suggest that inhibition of PARP sensitizes cells to various forms of radiation by affecting the repair of OCDL, which leads to a delay of the DDR. The effect of PARP inhibition on DSB repair pathways is also thought to be an important mechanism for sensitization to carbon-ion irradiation. These results imply that the PARP inhibitor might sensitize cells at low doses (i.e. 3 Gy or less) of carbon-ion irradiation at the Bragg peak more than at the entrance region. Therefore, doses lower than 5 Gy of LET 70 carbon-ion irradiation could be the appropriate range of radiosensitization with a blockade of DNA repair by PARP inhibitor.

As the sensitization effect of PARP inhibitors combined with photon beams is well characterized *in vitro* and *in vivo*, it is important to compare the sensitizing effect of PARP inhibitors for proton and other types of radiation with clinical applications. Furthermore, radiosensitizers for charged particle radiation therapy evaluated using animal models should show a lower cell-killing effect on normal cells at the entrance region and a pronounced definite effect on cancer cells at spread-out Bragg peaks.[22]

Few factors are known to induce sensitization to charged particle radiation, and we have demonstrated that PARP inhibition is a radiosensitizer for carbon-ion irradiation. The present results show that the inhibition of PARP enhances radiosensitivity to γ-ray and carbon-ion irradiation by disturbing DDR, possibly by increasing the conversion of non-DSB lesions to lethal DNA damage, and suggest that functional inhibition of PARP should be useful for sensitizing to both low and high LET radiation therapies.

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Disclosure Statement

The authors have no conflict of interest.

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