Effects of carbon ion irradiation and X-ray irradiation on the ubiquitylated protein accumulation

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Abstract. C-ion radiotherapy is associated with improved local control and survival in several types of tumors. Although C-ion irradiation is widely reported to effectively induce DNA damage in tumor cells, the effects of irradiation on proteins, such as protein stability or degradation in response to radiation stress, remain unknown. We aimed to compare the effects of C-ion and X-ray irradiation focusing on the cellular accumulation of ubiquitylated proteins. Cells from two human colorectal cancer cell lines, SW620 and SW480, were subjected to C-ion or X-ray irradiation and determination of ubiquitylated protein levels. High levels of ubiquitylated protein accumulation were observed in the C-ion-irradiated SW620 with a peak at 3 Gy; the accumulation was significantly lower in the X-ray-irradiated SW620 at all doses. Enhanced levels of ubiquitylated proteins were also detected in C-ion or X-ray-irradiated SW480, however, those levels were significantly lower than the peak detected in the C-ion-irradiated SW620. The levels of irradiation-induced ubiquitylated proteins decreased in a time-dependent manner, suggesting that the proteins were eliminated after irradiation. The treatment of C-ion-irradiated SW620 with a proteasome inhibitor (epoxomicin) enhanced the cell killing activity. The accumulated ubiquitylated proteins were co-localized with γ-H2AX, and with TP53BP1, in C-ion-irradiated SW620, indicating C-ion-induced ubiquitylated proteins may have some functions in the DNA repair system. Overall, we showed C-ion irradiation strongly induces the accumulation of ubiquitylated proteins in SW620. These characteristics may play a role in improving the therapeutic ratio of C-ion beams; blocking the clearance of ubiquitylated proteins may enhance sensitivity to C-ion radiation.

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Abbreviations: C-ion, carbon ion; LET, linear energy transfer; RBE, relative biological effectiveness; DSB, DNA double-strand break; γ-H2AX, phosphorylation of the histone variant H2AX; DMEM, Dulbecco's modified Eagle's medium; HIMAC, heavy-ion medical accelerator in Chiba; NIRS, National Institute of Radiological Sciences; DAPI, 4',6-diamidino-2-phenyllindole; Tritc, tetramethylrhodamine isothiocyanate; LPS, lipopolysaccharide

Key words: carbon ion radiotherapy, colorectal cancer, ubiquitin, proteasome inhibitors, radiosensitizing agents

Introduction

Colorectal cancer is currently the most common gastrointestinal malignancy and remains the third most common form of cancer and second most common cause of cancer-related death in developed countries (1). Although surgical resection is the first choice of treatment for colorectal cancer, radiation therapy and chemotherapy are also essential interventions. In addition, many patients with local recurrence are not eligible for surgical resection and are frequently referred for radiotherapy. However, the results of conventional photon radiotherapy remain far from satisfactory, with many studies in the literature reporting 1- and 3-year survival rates of 50 and 10%, respectively (2,3). Several reports have revealed that C-ion irradiation offers advantages over conventional photon irradiation, such as accurate dose distribution, and enhanced biological effects due to higher LET (4,5). Thus, C-ion radiotherapy is expected to be promising alternative to surgery for colorectal cancer.

The RBE of C-ion irradiation with respect to reference photon radiation sources, such as X-ray- or γ-ray-irradiation, as assessed by biological endpoints such as cell death, DNA damage, and chromosomal aberrations, is known to be ~2-3-fold (5-7). However, it is not known how the effects of C-ion irradiation on cellular proteins, such as protein stability or degradation compare to the effects of photon irradiation. Protein ubiquitylation has crucial role in protein function through the modulation of its stability or its activity (8-10). Proteins destined for degradation are labeled with poly-ubiquitin chains by the sequential activity of a multi-enzymatic system, and the poly-ubiquitin chains then serve as a recognition signal for protein degradation via proteasomes (11).

It is known that proteasomes are located in the cell cytosol, endoplasmic reticulum, and nucleus, and are thought to have a significant role in degrading the majority of endogeneous cellular proteins, which can have a marked effect on cell behavior (12). The role of the ubiquitin proteasome pathway on the classical effects of photon irradiation, such as DNA repair, chromosome instability, cell cycle arrest, and cell death, have been studied (12,13). During DSB repair, a series of phosphorylation events such as γ-H2AX are initiated, which leads to the ubiquitylation of histon H2A and other unknown proteins which elicits the chromatin association of BRCA1 as well as TP53BP1 (14,15).

Interestingly, we have previously reported that C-ion irradiation at a dose of 2 Gy induced a greater amount of ubiquitylated proteins than X-ray irradiation at a dose of 4 Gy in a human pancreatic cancer cell line (MIAPaCa-2) (16). The RBE of C-ion irradiation with respect to the X-ray irradiation of MIAPaCa-2 was 2.0, as assessed by cell death, thus C-ion irradiation at 2 Gy and X-ray irradiation at 4 Gy could have a similar cell killing effect. However, an increase in the formation of ubiquitylated proteins was observed in C-ion-irradiated MIAPaCa-2 cells. It would be intriguing to study whether this accumulation of ubiquitylated proteins represents one of the unique effects of C-ion radiation on cells. Thus far, however, no studies have focused on the accumulation of ubiquitylated proteins to examine the characteristics of C-ion irradiation in comparison to photon irradiation. In this study, we used two human colon cancer cell lines, SW620 and SW480, and examined the effects of C-ion and X-ray irradiation on the accumulation of ubiquitylated proteins.

Materials and methods

Cell culture and reagents. The two human colon cancer cell lines, SW620 and SW480, were purchased from ATCC (Manassas, VA, USA) and cultured in DMEM (Nissui, Tokyo, Japan) supplemented with 10% FBS (Hyclone, UT, USA), 1% L-glutamine (Gibco, CA, USA), and 1% penicillin/streptomycin (Gibco). RAW264.7, the mouse macrophage cell line, was purchased from ATCC. The cells were maintained in DMEM. The cells were incubated with or without LPS (100 ng/ml) for 24 h.

Irradiation. Cells were subjected to C-ion (1, 2, 3 or 4) or X-ray irradiation (2, 4, 6 or 8 Gy). The C-ions were accelerated by HIMAC, and X-rays were produced by a PANTAK HF-320S generator (Shimadzu, Kyoto, Japan) at NIRS, Japan, as described previously (17).

Immunofluorescence labeling and image acquisition. Immunofluorescence labeling and image acquisition was performed as described previously (18). The primary antibodies against multi-ubiquitin, which recognizes K29-, K48-, K63-linked poly-ubiquitylated and mono-ubiquitylated protein purified from hybridoma, clone FK2 (MBL, Nagoya, Japan), γ-H2AX (Cell Signaling Technology, MA, USA), and TP53BP1 (Cell Signaling Technology), were suspended with Can Get Signal solution 1 (Toyobo, Tokyo, Japan) at 1:250 and used for the assay. Cells were then treated with Alexa Flour 555- or 488-labeled anti-mouse IgG or anti-rabbit IgG secondary antibodies (Invitrogen, Carlsbad, CA, USA). The slides were mounted with ProLong Gold Antifade Reagent containing the nuclear counterstain DAPI (Invitrogen).

Cellular ubiquitylated proteins were visualized and photographed with a BZ-9000 fluorescence microscope (Keyence, Osaka, Japan) using a 20X Plan fluorescence lens (N.A 0.45) with BZ filters for Tritc and DAPI.

To examine the co-localization of proteins, a DSU-IX70 fluorescence microscope (Olympus, Tokyo, Japan) with MetaMorph's 3D Deconvolution module (Molecular Devices, CA, USA) was used (19). Representative images were uniformly processed in Adobe Photoshop using the brightness and contrast tools.

The measurement of ubiquitylated protein accumulation levels in each of the cell types. To compare the accumulation levels of ubiquitylated proteins within non-irradiated, X-ray-irradiated or C-ion-irradiated cells, we used immunofluorescence-labeled images stained with anti-multi-ubiquitin antibody and DAPI, and analyzed the images with the ImageJ software program (Fig. 1A). We first used DAPI images and counted the number of cell nuclei per image, which represents the total number of cells per image. Next, the anti-multi-ubiquitin antibody-stained images were converted into 8 bit grayscale images. The thresholding tool of the ImageJ software program, which can separate the pixels that fall within a desired range of intensity values from those which do not, was used to separate the cells with low, intermediate or high accumulation of ubiquitylated proteins. The criteria for the cells containing low, intermediate or high ubiquitylated protein accumulation were stated as follows: the cells in which the distribution of pixel intensity was <25 was classified as Low-Ub, the cells with the pixel intensity ≥40 was classified High-Ub, and the cells with the pixel intensity between 25 and 39 and the High-Ub cells were subtracted was classified as the Intermediate-Ub cells. The numbers of Low-Ub, Intermediate-Ub, and High-Ub cells were counted for each image, and divided by the total number of cells per image to evaluate the percentage of Low-Ub, Intermediate-Ub or High-Ub cells within the total cell population. Three images per group were used for the analysis. The number of cells ranged from 18 to 78 per group. The cell numbers tended to be lower at 5 days after irradiation because cell death continued in the period after irradiation.

Cell viability assays. The cells were grown to ~60% confluence in a 12-well plate and used for the irradiation. The conditioning medium was replaced with fresh medium, and the cells were incubated for 51 h in a CO_2 incubator. The cells were then fixed and stained with Diff-Quick (Sysmex, Kobe, Japan). Six random fields were photographed and cell numbers were counted. For the proteasome inhibitor treatment, 5, 10, 15 or 20 nM epoxomicin was added to the conditioning medium at 3 h after irradiation, and the cells were fixed and stained at 51 h after the irradiation, and used for the count.

Western blotting. Primary antibodies against LC3 (MBL) or GAPDH (Trevigen, MD, USA) were suspended with Can Get Signal solution 1 at 1:2,500 or 1:10,000, respectively. The membranes were then washed and incubated with horseradish peroxidase-conjugated anti-mouse or -rabbit IgG (Amersham Biosciences, Buckinghamshire, UK) suspended with Can Get

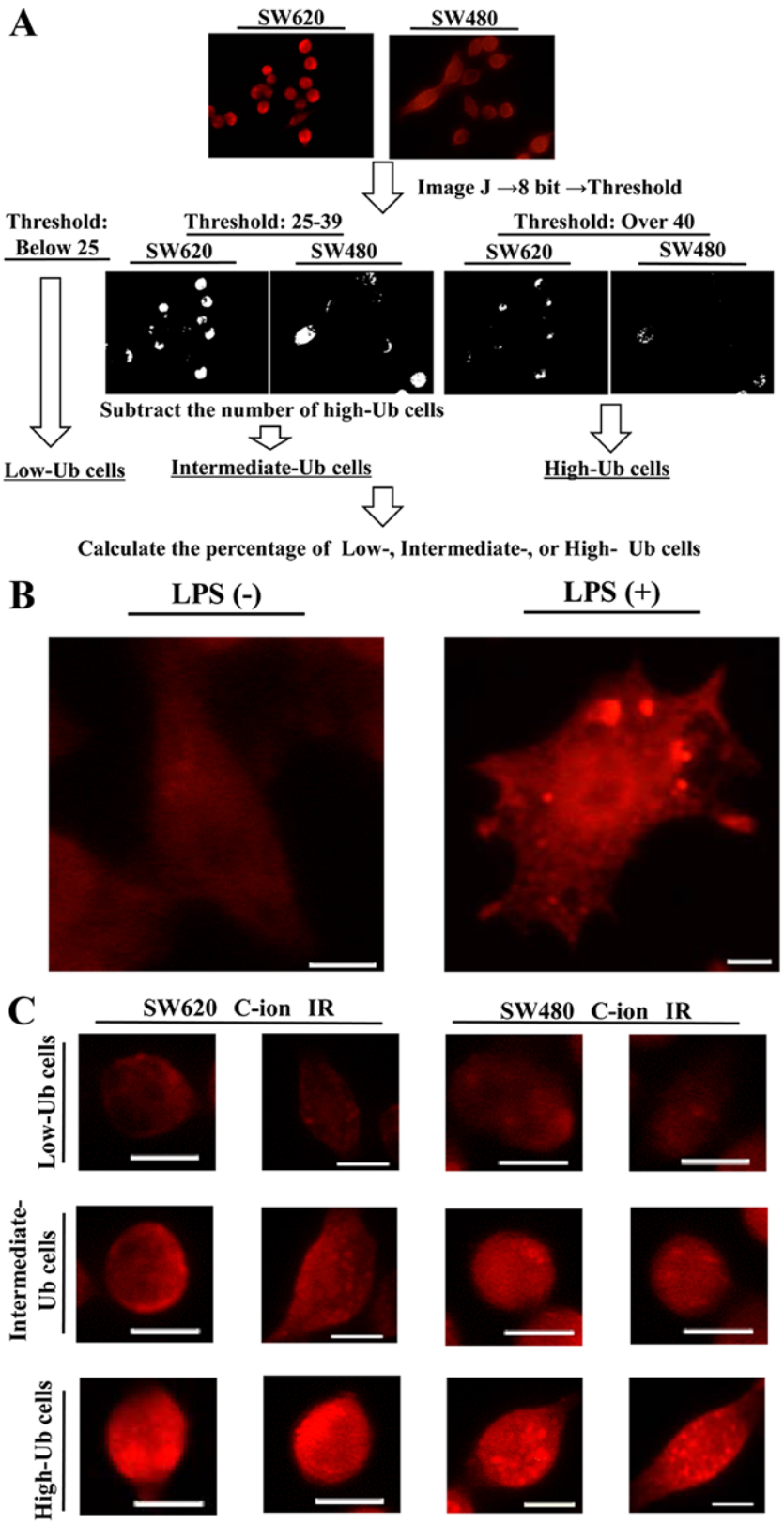

Figure 1. The criteria for Low-Ub, Intermediate-Ub and High-Ub cells. (A) A summary of the criteria for Low-Ub, Intermediate-Ub and High-Ub cells are shown. (B) Accumulation of ubiquitylated protein in RAW264.7 cells treated with or without 100 ng/ml LPS for 24 h, and stained the cells with anti-multi-ubiquitin antibody. Red, ubiquitylated proteins. Scale bar, 10 μm. (C) Representative images of Low-Ub, Intermediate-Ub and High-Ub cells are shown. Scale bar, 10 μm.

Signal solution 2 (Toyobo) at 1:10,000. Protein bands were detected by enhanced chemiluminescence and imaged with an LAS 4000 Lumino image analyzer (Fujifilm, Tokyo, Japan). siRNA targeting Atg5 and negative control siRNA were purchased from Cell Signaling Technology (Danvers, MS, USA). Cells were grown to ~60% confluence in a 6-well plate

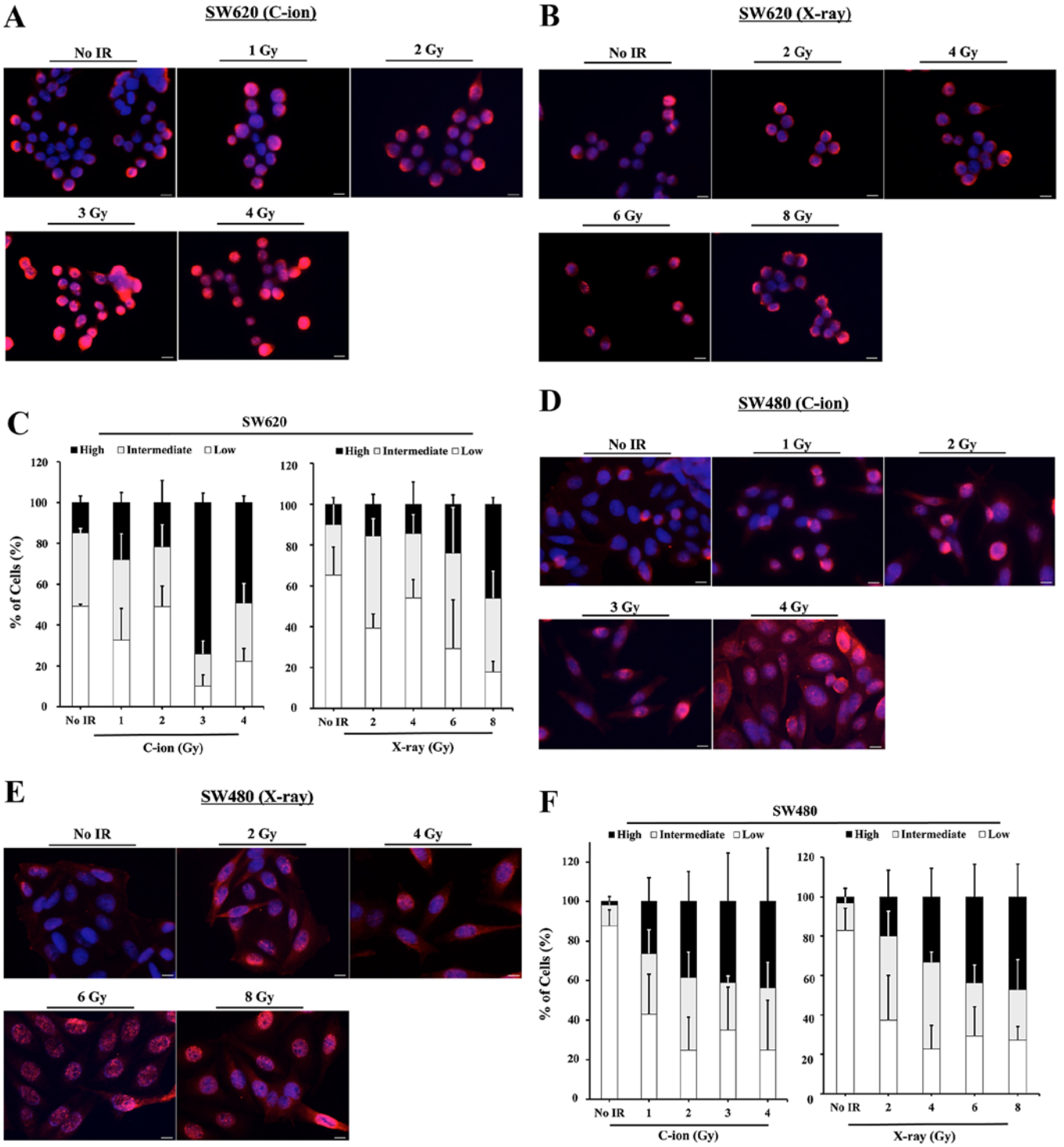

Figure 2. The dose-dependency of ubiquitylated protein accumulation in C-ion- or X-ray-irradiated SW620 and SW480 cells. The cells were irradiated and fixed at 6 h after irradiation, then used for immunofluorescence staining with anti-multi-ubiquitin antibody. The representative images of the cellular accumulation of ubiquitylated protein in SW620 and SW480 cells after C-ion irradiation (1, 2, 3 or 4 Gy) (A and D), and X-ray irradiation (2, 4, 6 and 8 Gy) (B and E) are shown. Red, ubiquitylated proteins. Blue, DAPI. Scale bar, 10 μm. The numbers of Low-Ub, Intermediate-Ub, and High-Ub cells were counted using the ImageJ software program. The percentages of Low-Ub, Intermediate-Ub, and High-Ub cells are shown in the graph (C and F). The data represent the mean ± SD, n=3 images, 28-71 cells per image.

and then incubated with a transfection mixture containing LipoTrust Ex Oligo (Hokkaido System Science Co., Ltd., Hokkaido, Japan) and 50 nmol siRNA for 40 h. Primary antibodies against Atg5 (Cell Signaling Technology), and GAPDH were suspended with Can Get Signal solution 1 at 1:2,500 and 1:10,000, respectively.

Statistical analysis. The statistical analyses were performed using an unpaired Student's t-test, and the differences between groups were assessed with a two-tailed test. P-values of <0.05 were considered to indicate statistical significance. Each experiment was performed in triplicate and independently repeated at least twice on different days.

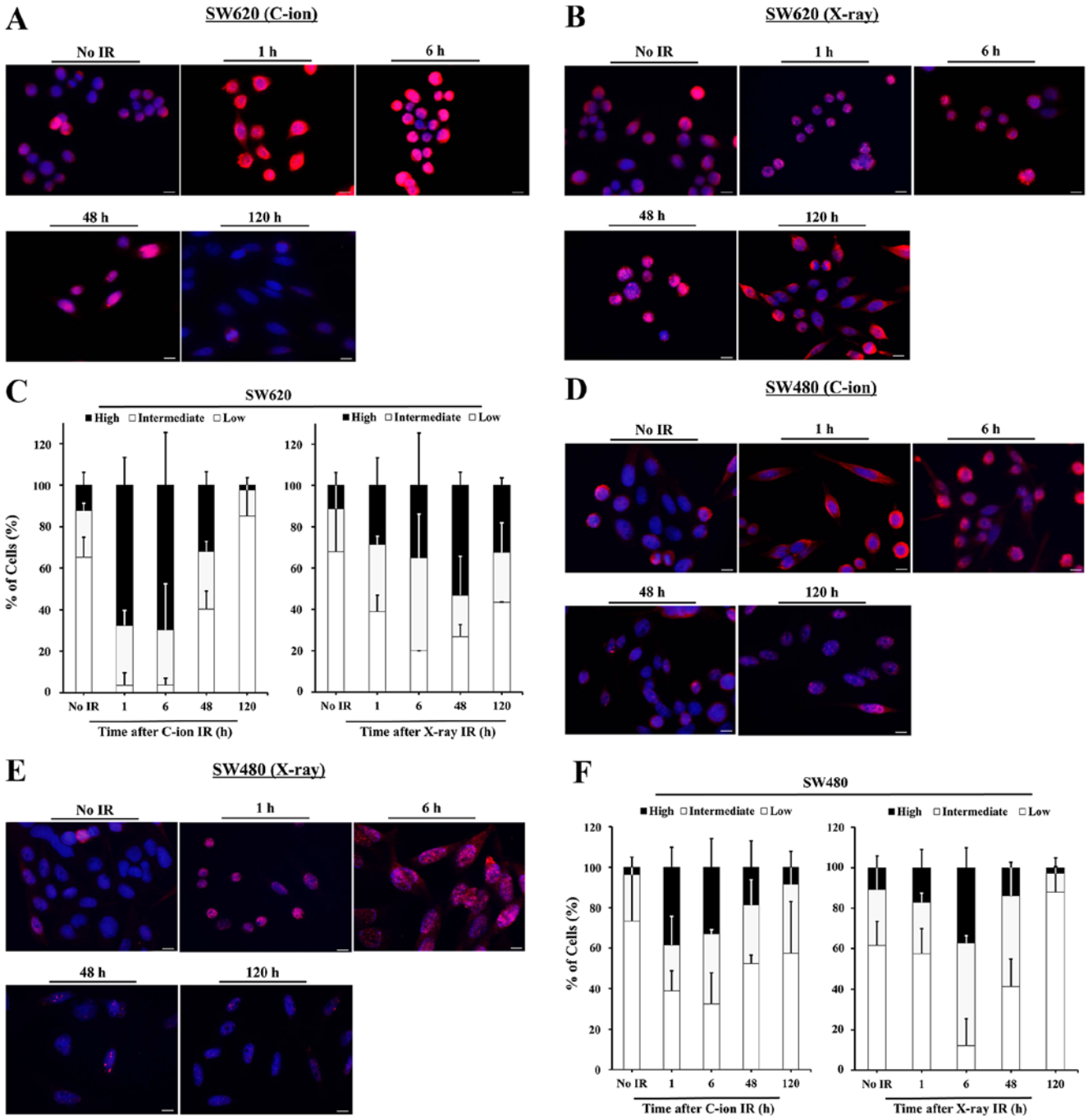

Figure 3. The time-dependency of ubiquitylated protein accumulation in C-ion- or X-ray-irradiated SW620 and SW480 cells. The cells were irradiated and fixed at 1, 6, 48 or 120 h after irradiation, then used for immunofluorescence staining with anti-multi ubiquitin antibody. Representative images of the changes of cellular ubiquitylated proteins over time in C-ion-irradiated (A and D) or X-ray-irradiated SW620 and SW480 cells (B and E) are shown. Red, ubiquitylated proteins. Blue, DAPI. Scale bar, 10 μm. The number of Low-Ub, Intermediate-Ub, and High-Ub cells was gained by ImageJ software program. The percentages of Low-Ub, Intermediate-Ub, and High-Ub cells are shown in the graph (C and F). The data represent the mean ± SD, n=3 images, 18-78 cells per image.

Results

The high accumulation of ubiquitylated proteins was observed in C-ion-irradiated SW620 cells. First, we used RAW264.7 treated with 100 ng/ml LPS for 24 h, and confirmed the immunofluorescence staining of ubiquitylated protein accumulation as reported in a previous report (Fig. 1B) (20). Next, we examined the effects of C-ion and X-ray irradiation on the accumulation of ubiquitylated protein. A summary of the criteria for the Low-Ub, Intermediate-Ub, and High-Ub cells, is shown in Fig. 1A, representative images of the cells are show in Fig. 1C.

The percentages of Low-Ub, Intermediate-Ub or High-Ub cells were determined in SW620 and SW480 cells at 6 h after C-ion irradiation (1, 2, 3 or 4 Gy) or X-ray irradiation (2, 4, 6 or 8 Gy). Increased percentages of High-Ub cells were observed in the C-ion-irradiated-SW620 with a peak at 3 Gy, 73% within total cells, whereas, the numbers of High-Ub cells in the X-ray-irradiated SW620 were lower, even at higher doses (Fig. 2A-C). The peak number of High-Ub cells in the

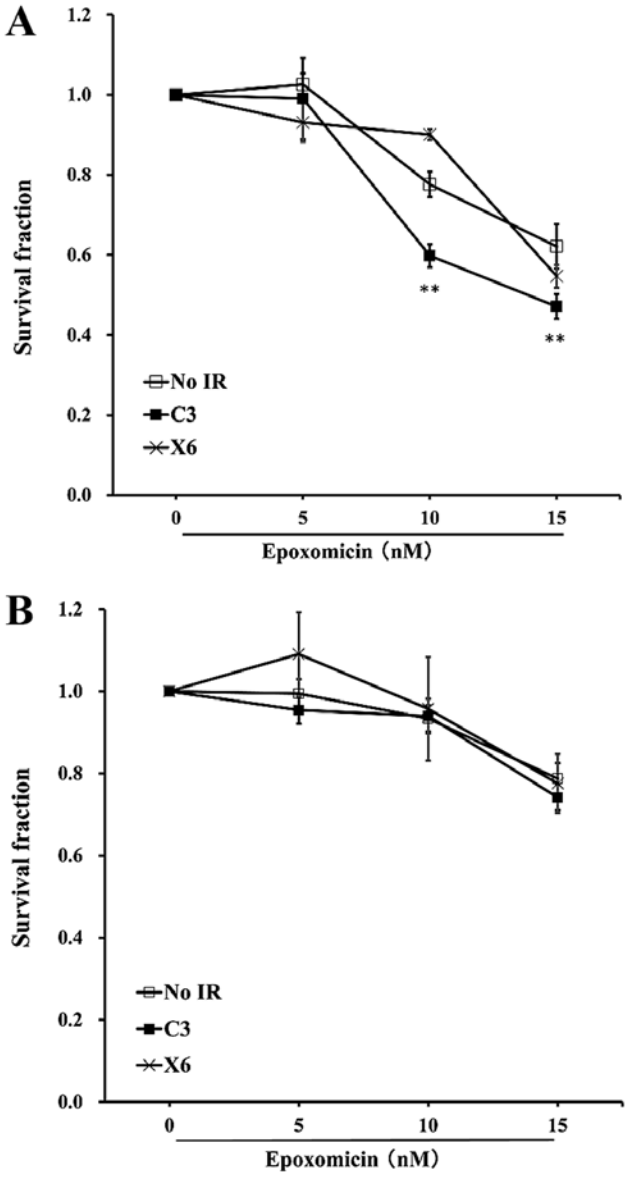

Figure 4. The treatment of SW620 cells with a proteasome inhibitor enhanced their sensitivity to C-ion irradiation. SW620 or SW480 cells were irradiated with 3 Gy C-ions or 6 Gy X-rays, and 10 nM epoxomicin was added 3 h after irradiation. The cells were stained 51 h after irradiation and the surviving fraction was analyzed by counting the cell numbers. The surviving fractions of the SW620 and SW480 cells are summarized in (A) and (B), respectively. The data represent the mean ± SD; *P<0.05 and **P<0.01 vs. control.

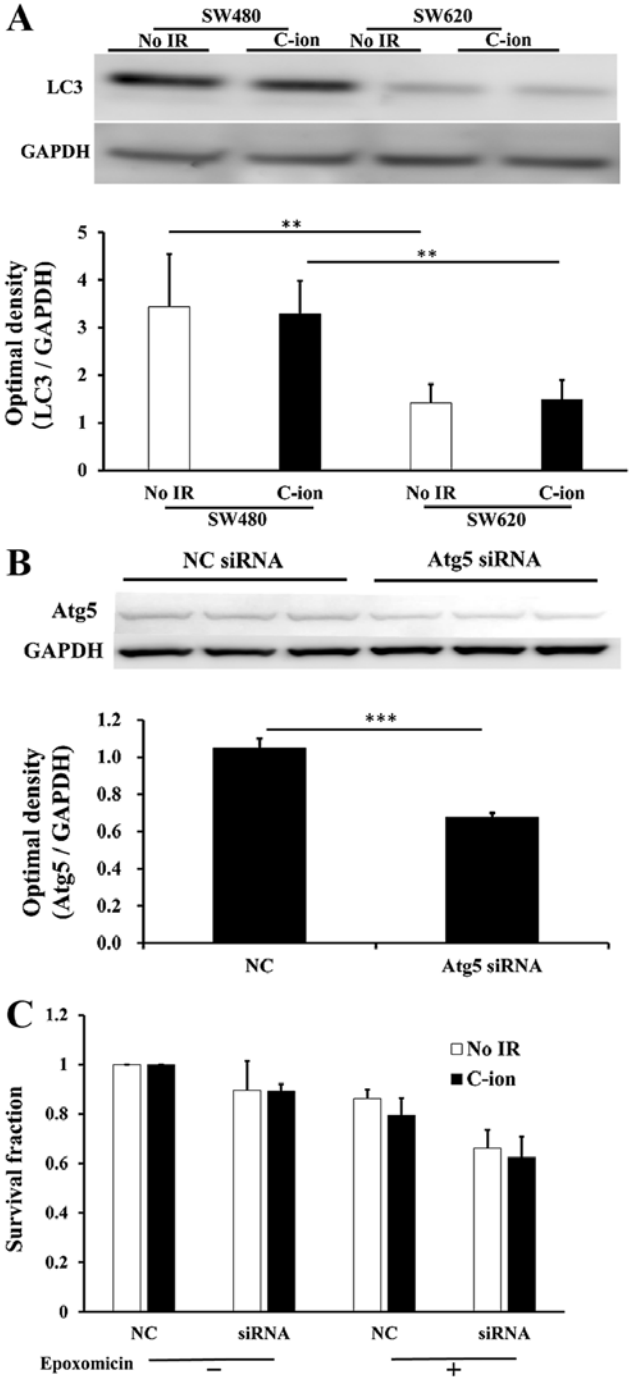

Figure 5. Surviving fraction of C-ion irradiated SW480 was unchanged with Atg5 siRNA treatment. (A) Protein levels of LC3 in No IR or 3 Gy C-ion irradiated SW620 and SW480. Protein levels of LC3 and GAPDH in no irradiated (No IR) or 3 Gy C-ion irradiated SW620 or SW480 were analyzed by western blotting. The data represent the mean ± SD; **P<0.01 vs. control (n=3). (B) Reduction of Atg5 protein expression in Atg5 siRNA treated SW480. The data represent the mean ± SD; ***P<0.001 vs. control (n=3). NC, negative control siRNA. (C) Surviving fraction of C-ion-irradiated SW480 after Atg5 siRNA treatment with or without epoxomicin siRNA targeting Atg5 or negative control siRNA (NC) were transfected to the No IR or 3 Gy C-ion irradiated SW480. Cells were treated with or without epoxomicin (10 nM), and survival fractions were analyzed. The data represent the mean ± SD; n=3.

C-ion-irradiated SW620 was also higher than that detected in the C-ion or X-ray-irradiated SW480, which was 49% in 4 Gy C-ion-irradiated SW480 or 46% in 8 Gy X-ray irradiated SW480, respectively (Fig. 2D-F).

We further examined the time-dependency of ubiquitylated protein accumulation in 3 Gy C-ion-irradiated cells and 6 Gy X-ray-irradiated cells. An increased number of High-Ub cells was already observed at 1 h after C-ion or X-ray irradiation. In SW620, the number of High-Ub cells peaked at 6 h after C-ion irradiation, 69% within total cells, at which point the number of High-Ub cells was higher than that in the X-ray irradiated cells at any time (Fig. 3A-C). In SW480, the number of High-Ub cells peaked at 6 h in both C-ion or X-ray irradiated cells, at 41 or 43%, which were both lower than the peak value observed in the C-ion-irradiated SW620 (Fig. 3D-F). The difference in the peak values of the High-Ub cells that were detected in C-ion-irradiated cells versus X-ray-irradiated cells was greater

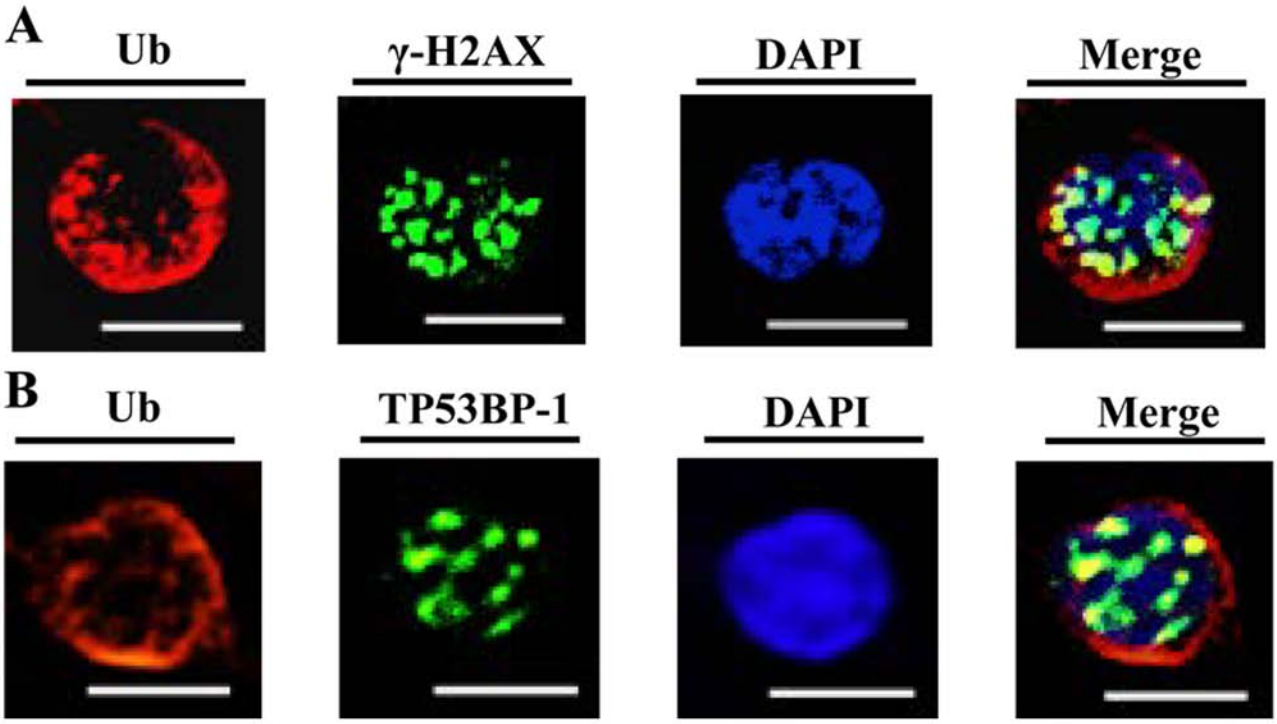

Figure 6. Ubiquitylated proteins were co-localized with γ-H2AX (A) and TP53BP1 (B) in C-ion-irradiated SW620 cells. The 3 Gy C-ion-irradiated SW620 cells were fixed at 6 h after irradiation, and used for immunofluorescence staining with anti-ubiquitin antibody and anti-γ-H2AX antibody or anti-TP53BP1 antibody. The co-localization of ubiquitylated proteins and γ-H2AX or ubiquitylated proteins and TP53BP1 was analyzed with a DSU-IX70 fluorescence microscope with MetaMorph 3D Deconvolution module. Representative images are shown. Red, ubiquitylated proteins. Green, γ-H2AX or TP53BP1. Blue, DAPI. Scale bar, 10 μm.

in the SW620 cells than it was in the SW480 cells. In addition, ubiquitylated protein accumulation was decreased at 5 days after irradiation, indicating that ubiquitylated proteins may be degraded over time after radiation treatment.

The treatment of SW620 cells with a proteasome inhibitor enhances cellular sensitivity to C-ion irradiation. It is well known that damaged proteins are toxic to cells (21), the proteins were therefore ubiquitylated and degraded through proteasomes or autophagy (22,23). It would be intriguing to see whether blocking the clearance of ubiquitylated proteins enhances the radiosensitivity to radiation. To clarify this, we irradiated SW620 and SW480 cells with C-ion at 3 Gy or X-ray at 6 Gy, and treated the cells with epoxomicin at 3 h after irradiation and the cells were incubated for another 48 h. The treatment of SW620 with 10 or 15 nM epoxomicin significantly reduced the numbers of surviving cells and increased the radiosensitivity to C-ion radiation. A reduction in the number of surviving cells was not observed in X-ray-irradiated SW620 cells; 10 nM epoxomicin even enhanced the cell survival after X-ray irradiation (Fig. 4A). In the case of SW480 cells, none of the doses of epoxomicin enhanced radiosensitivity to C-ion or X-ray irradiation (Fig. 4B).

The protein levels of LC3, a component of autophagy, were much higher in SW480 cells than in SW620 cells (Fig. 5A). We therefore hypothesized that ubiquitylated proteins might be degraded by autophagy rather than by proteasomes in SW480 cells, and the blocking of autophagy or the blocking of both autophagy and proteasomes may increase the radiosensitivity to radiation. Thus, we next treated SW480 cells with siRNA specific for Atg5, which is the functional component of autophagy, with or without treatment with 10 nM epoxomicin, in order to examine cellular survival in C-ion-irradiated cells. The reduction of Atg5 protein expression was confirmed in SW480 cells that were treated with Atg5 siRNA (Fig. 5B). However, Atg5 siRNA treatment (with or without epoxomicin) did not enhance the radiosensitivity of C-ion-irradiated SW480 cells (Fig. 5C).

Ubiquitylated proteins were co-localized with γ-H2AX and TP53BP1 in C-ion-irradiated SW620 cells. C-ion irradiation is known to induce greater amount of DNA damage such as DSBs (5). During DSBs repair, DNA damage-induced ubiquitylation cascade was fundamental for eliciting the chromatin association of TP53BP1 (14,15). To examine the relation of ubiquitylated proteins and DNA repair system after the C-ion irradiation, we used 3 Gy C-ion-irradiated SW620 cells, which were fixed at 6 h after irradiation, and stained the cells with anti-multi-ubiquitylated chain antibody with anti-γ-H2AX antibody, the marker of DSBs or anti-TP53BP1 antibody. We found that some of the ubiquitylated proteins were partially co-localized with γ-H2AX (Fig. 6A) or TP53BP1 (Fig. 6B) in C-ion-irradiated SW620 cells, indicated that C-ion induced ubiquitylated proteins may have some function in DNA repair system.

Discussion

In this study, we compared the effects of irradiation with C-ions and X-rays, focusing on the cellular accumulation of

ubiquitylated protein. We found the greatest accumulation of ubiquitylated proteins, occurred in C-ion-irradiated SW620. Higher levels of ubiquitylated proteins were also reported in C-ion-irradiated pancreatic cancer cell line, MIAPaCa-2 in comparison to those detected in non-irradiated or X-ray-irradiated MIAPaCa-2 (16). Thus, the induction of a great amount of ubiquitylated proteins in cells may be a unique property of C-ion radiation in several cell lines. The treatment of SW620 with a proteasome inhibitor enhanced the cell killing of C-ion-irradiated SW620 cells. Thus, blocking the clearance of ubiquitylated proteins may be promising candidate treatment for enhancing the radiosensitivity of tumor cells to C-ion radiation.

Since heavy ions, such as C-ions, have a higher ionization density in the track of individual particles, they can induce greater DNA damage and cytotoxicity in tumor cells (24-26). Thus, it can be hypothesized that C-ion irradiation may also induce greater effects on cellular proteins. Radiation-induced ubiquitylated proteins may represent the sum of the effects of irradiation on cellular proteins, such as the damage of proteins via radiation stress, which leads to their elimination (27), and functional proteins, which are involved such as in the DNA repair system (14,15). There have been a number of studies showing ubiquitin accumulation at DSB sites (28,29); ubiquitylated proteins were detected as foci in the nucleus of irradiated cells, which were colocalized with Rad51 (29). In this study, we only examined the co-localization of C-ion-induced ubiquitylated proteins involved in DNA repair system, γ-H2AX and TP53BP1. However, it would be intriguing to examine the relationship between ubiquitylated proteins and other proteins that are involved in the cellular stress responses, since some of the ubiquitylated proteins were detected in the periphery of the nuclear or in the cytoplasm.

Classically, it has been reported that proteasome-mediated protein degradation requires the ubiquitylation of proteins, which is then recognized by 26S proteasomes, whereas autophagy is considered to be a random cytoplasmic degradation system. However, several studies have suggested that ubiquitylated proteins are also degraded by autophagy, and there is crosstalk between the proteasome- and autophagy-mediated protein degradation (22,30,31). In this study, blocking the clearance of ubiquitylated proteins by treatment with a proteasome inhibitor enhanced the cell killing of C-ion-irradiated SW620 cells. However, the proteasome inhibitor treatment did not enhance the radiosensitivity of SW480 cells to C-ions. It may be because of less accumulation of ubiquitylated proteins in C-ion irradiated SW480, however, we also detected the protein levels of LC3, an autophagy marker, were much higher in SW480 cells than in SW620 cells. We therefore hypothesized that SW480 cells may use autophagy-mediated protein degradation more dominantly than proteasome-mediated degradation or use both systems to degrade ubiquitylated proteins. However, the treatment of siRNA specific for Atg5, a functional component of autophagy or Atg5 siRNA with a proteasome inhibitor did not increase cell killing in C-ion-irradiated SW480 cells. Thus, another system seemed to be involved in reducing the ubiquitylated proteins in SW480 cells. Further studies will be required to clarify the mechanism by which the clearance of ubiquitylated proteins is blocked in this type of cells.

Blocking the clearance system of aggregated proteins with the use of a proteasome inhibitor has already been used in clinical studies, and it has also been reported in combination with photon radiotherapy (32-35). The potential of proteasome inhibitor, bortezomib, in combination with radiotherapy has been shown in several types of tumors, however, increase of side effects was also reported (33-35). C-ion radiotherapy has the advantage over photon radiation with accurate dose distribution to the target tumor (4-7), thus the accumulation of ubiquitylated proteins may largely occur in tumor cells, while smaller amounts accumulate in normal tissues. It is still challenging to use radiation on colorectal cancer treatment, because the intestines are a highly radiosensitive organ. Thus, blocking the clearance of ubiquitylated proteins is expected to greatly impact on the increase of therapeutic ratio of C-ion radiation for colorectal cancer, which can induce cell death, especially in tumors without side effects in normal tissues.

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