Fra-1 enhances the radioresistance of colon cancer cells to X-ray or C-ion radiation

SATOSHI ENDO[1], MAYUMI FUJITA[2], SHIGERU YAMADA[3], KAORI IMADOME[2], FUMIAKI NAKAYAMA[2], TETSURO ISOZAKI[1], TAKESHI YASUDA[2], TAKASHI IMAI[4] and HISAHIRO MATSUBARA[1]

[1]Department of Frontier Surgery, Chiba University Graduate School of Medicine, Chiba 260-8670; [2]Department of Basic Medical Sciences for Radiation Damage; [3]Hospital of The National Institute of Radiological Sciences; [4]National Institute of Radiological Sciences, National Institutes for Quantum and Radiological Science and Technology, Japan

Abstract. Fos-related antigen 1 (Fra-1) has roles in a variety of cell functions, including cell proliferation, differentiation, transformation, and invasiveness, and it is upregulated in various cancers. We investigated the role of Fra-1 in cellular radioresistance using cells of two human colorectal cancer cell lines, SW620 and SW480. We found that SW620 cells are more sensitive than SW480 cells at doses greater than 6 Gy for X-ray or 3 Gy for carbon-ion (C-ion) radiation. Fra-1 expression tended to be decreased by the radiation in a dose-dependent manner in both cell lines; of note, a greater reduction of Fra-1 expression was observed in SW620 cells, especially at 6 Gy of X-ray or 3 Gy of C-ion irradiation, than in SW480 cells, indicating a possible association between Fra-1 downregulation and cellular radiosensitivity. Knockdown of Fra-1 in SW480 cells significantly increased the radiosensitivity to X-ray or C-ion radiation. On the other hand, overexpression of Fra-1 in SW620 cells significantly enhanced the radioresistance to C-ion radiation, suggesting a role of Fra-1 in radioresistance. Furthermore, we found that downregulation of Fra-1 protein in irradiated SW620 cells was regulated via protein degradation through a proteasome-dependent pathway. Overall, our results indicate a role of Fra-1 in radioresistance to both X-ray and C-ion radiation for colorectal cancer cell lines.

Introduction

Fos-related antigen 1 (Fra-1) protein forms activator protein-1 complexes in association with members of the JUN family,

Correspondence to: Dr Satoshi Endo, Department of Frontier Surgery, Chiba University Graduate School of Medicine, 1-8-1 Inohana, Chuo-ku, Chiba-shi, Chiba 260-8670, Japan
E-mail: satoshi.endo0808@gmail.com

which drives the expression of genes involved in various biological processes, including cell proliferation, differentiation, transformation, and invasiveness, in several cancer cell lines (1-3). Fra-1 is usually absent in normal epithelial cells but is upregulated in various cancers, such as lung, breast, colon, prostate, brain, head and neck, esophagus, ovary, and nasopharynx cancers (3-13). Increased Fra-1 expression has been shown to be correlated with tumor stage in esophageal squamous cell carcinoma (8), and high levels of Fra-1 expression are associated with severe malignancy in breast cancer progression (13); thus, Fra-1 is recognized as a prognostic factor for certain cancers (2,8).

Colorectal cancer is currently the most common gastrointestinal malignancy, and it remains the third most common cancer and second leading cause of cancer-related death in developed countries (14). Although surgical resection is the first choice of treatment for colorectal cancer, radiation therapy and chemotherapy are also essential interventions in colorectal cancer treatment. In addition, many patients with local recurrences are not eligible for surgical resection, and they are frequently referred for radiotherapy. However, the results of conventional photon radiotherapy are still far from satisfactory, with many studies in the literature reporting a 50% 1-year survival rate and a 10% 3-year survival rate (15). Thus, the role of photon radiotherapy is often described as mere pain control (16). Carbon-ion (C-ion) beam therapy is well known for its high linear energy transfer (LET), and it has some unique advantages over photon irradiation, including more accurate dose distribution (17-19), a high rate of double-strand breaks of the DNA chain (20,21), and high relative biologic effectiveness of tumor cell killing (22-24). Thus, C-ion radiotherapy is expected to become a promising alternative to surgery for colorectal cancer treatment. Previous research has shown that C-ion radiotherapy may be a safe and effective treatment option for locally recurrent rectal cancer and may serve as an alternative to surgery (25-31).

The radiation dose required for tumor control varies widely among human tumors and depends on a range of factors, such as inherent cellular radiosensitivity, repair and repopulation phenomena, and tumor hypoxia (32-34). Since resistance to radiation is one of the reasons for treatment failure, the identification of key factors involved in cancer radioresistance

is important for developing an effective method of chemoradiotherapy. A previous study reported that downregulation of Fra-1 reduced the radioresistance of a prostate cancer cell line, PC-3, after treatment with 4-Gy photon beam irradiation (35). However, there are no published studies of the role of Fra-1 in radioresistance to X-ray or C-ion radiation for colorectal cancer cells.

Herein, we used two human colon cancer cell lines, SW620 and SW480, and demonstrated that Fra-1 has a role in the radioresistance to both X-ray and C-ion radiation.

Materials and methods

Cell culture and reagents. Cells of the human colon cancer cell lines SW620 and SW480 were purchased from ATCC (Manassas, VA, USA) and cultured in Dulbecco's modified Eagle's medium (DMEM; Nissui, Tokyo, Japan) supplemented with 10% fetal bovine serum (FBS; HyClone, Logan, UT, USA), and penicillin/streptomycin (Gibco, Carlsbad, CA, USA).

Irradiation. Cells were irradiated with X-rays or C-ions at room temperature. X-rays were produced by a PANTAK HF-320S generator (Shimadzu Corp., Kyoto, Japan), at 200 kVp and 20 mA, and filtered with 0.5 mm Al and 0.5 mm Cu (36). C-ions were accelerated by the Heavy-Ion Medical Accelerator in Chiba at the National Institute of Radiological Sciences, Chiba, Japan (37). The initial energy of the C-ion beams was 290 MeV/nucleon, and the LET value was 80 keV/μm with a monoenergetic beam (20). An outline of the experimental procedures after irradiation is shown in Fig. 1.

siRNA transfection. The cells were transiently transfected with siRNA specific for Fra-1 using Lipofectamine 2000 Reagent (Invitrogen, Carlsbad, CA, USA), as previously described (8). The sequences of the Fra-1 siRNA were as follows: Sense, agaaaucugggcugcagcgagagau, and antisense, aucucucgcug cagcccagauuucu. Fra-1 protein expression was evaluated by western blotting, and Fra-1 downregulation was confirmed at 48 h after siRNA transfection by comparison with the Fra-1 expression level of the cells transfected with scrambled negative control siRNA (Invitrogen).

Lentivirus production and transduction. The coding sequence of the human FRA1 gene was amplified from cDNA derived from SW480 cells by PCR using a gene-specific primer set: Sense, ggggacaagtttgtacaaaaaagcaggcttcaccatgttccgagactt cggggaacccggccccg, and antisense, ggggaccactttgtacaagaaagc tgggtctcacaaagcgaggagggttggagagccaag. The PCR fragment was introduced into a pDONR221 vector for cloning of the gene, in accordance with the instructions for Gateway Cloning Technology (Invitrogen), and confirmed by sequencing. Then, this gene was transferred by LR recombination from its entry clone into a pLenti7.3V5-DEST vector containing Emerald Green Fluorescent Protein (EmGFP). pLenti7.3/V5-GW/lacZ was the construct for the negative control. Lentiviral stocks were produced in 293FT cells in accordance with a modification of the manufacturer's protocol (Invitrogen). Briefly, 18 μl of FuGENE 6 was diluted in 0.6 ml of Opti-MEM I medium, and then 1.5 μg of plasmid DNA and 4.5 μg of packaging mix (Applied Biological Materials Inc., Richmond, BC, Canada)
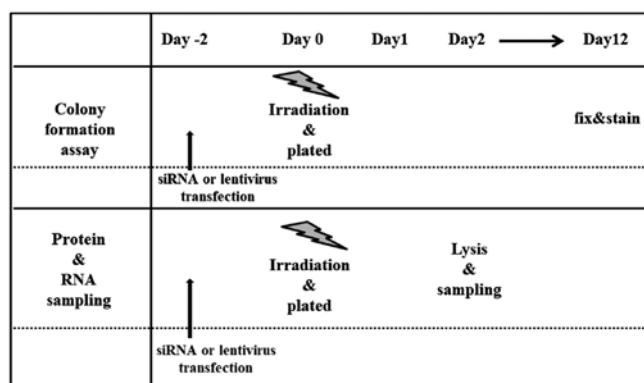

Figure 1. An outline of the experimental procedures after irradiation.

were added to this medium. These transfection complexes were incubated at room temperature and added to 5 ml of Opti-MEM I containing 6x10^6 cells. After incubation at 37˚C for 8 h, the culture medium was replaced with 5 ml of DMEM supplemented with 10% heat-inactivated FBS. Virus-containing supernatants were harvested 48 h after transfection and then centrifuged at 3,000 rpm at 4˚C for 15 min and passed through a 0.45-μm Millex-HV filter to remove debris. The virus was precipitated at 4˚C overnight by adding 3.3 ml of cold 40% PEG6000, to concentrate the virus, and then suspended in 100 μl of phosphate-buffered saline (PBS). Then, 1x10^5 cells were transduced by 10 μl of the virus preparation in the presence of 6 μg/ml hexadimethrine bromide (Polybrene) for 48 h. Fra-1 protein expression was evaluated by western blotting, and upregulation of Fra-1 was confirmed at 48 h after lentivirus transfection by comparison with the Fra-1 expression level of the cells transfected with the negative control.

Colony formation assay. Cell survival curves were determined by a colony formation assay as previously described, with some modifications (38). Briefly, cell cultures at 70% confluence were rinsed with PBS and detached with 0.1% trypsin/PBS. Cell numbers were determined with a hemocytometer. Cells were plated in triplicate onto 60-mm diameter plastic dishes and incubated for 12 days, whereupon the colonies were fixed and stained with 1% methylene blue in 30% methanol. Colonies consisting of more than 50 cells were scored as surviving colonies.

For the radiosensitivity analysis, non-irradiated cells or cells irradiated with X-rays at 1, 2, 4, 6, or 8 Gy or C-ions at 0.5, 1, 2, 3, or 4 Gy were used. The cells were trypsinized and counted immediately after irradiation. Eighty cells for non-irradiated cells or 150, 300, 1,500, or 3,000 cells for X-ray irradiation at 1, 2, 4, 6, or 8 Gy or C-ion irradiation at 0.5, 1, 2, 3, or 4 Gy were plated onto 60-mm diameter dishes, respectively. The surviving fraction was normalized to that of the non-irradiated control.

To assess the clonogenicity of the Fra-1 siRNA-transfected or lentivirus-transfected cells, cells were treated with siRNA or lentivirus vector for 48 h before irradiation. Immediately after irradiation, the cells were trypsinized, and the same numbers of cells as that used in the radiosensitivity analysis were plated onto dishes containing fresh media; colony-forming assays were then performed.
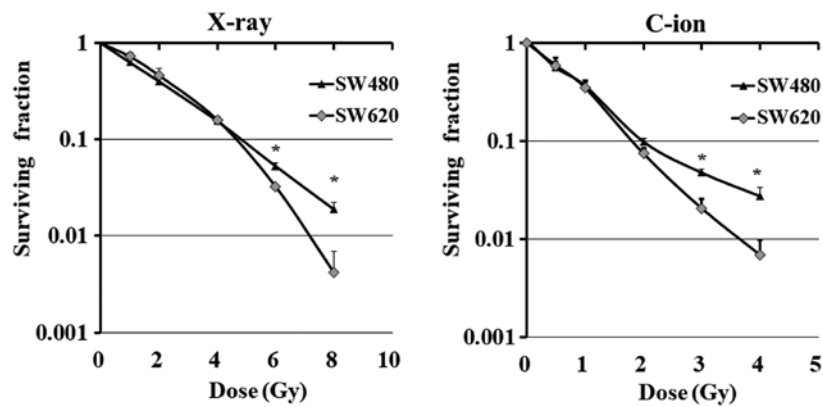
Figure 2. Radiosensitivity of SW620 and SW480 cells to X-ray or C-ion irradiation. The clonogenic survival curves of SW620 and SW480 cells after X-ray or C-ion irradiation were determined. Data are presented as the means ± standard deviations of triplicate samples. *P<0.05 vs. SW480.
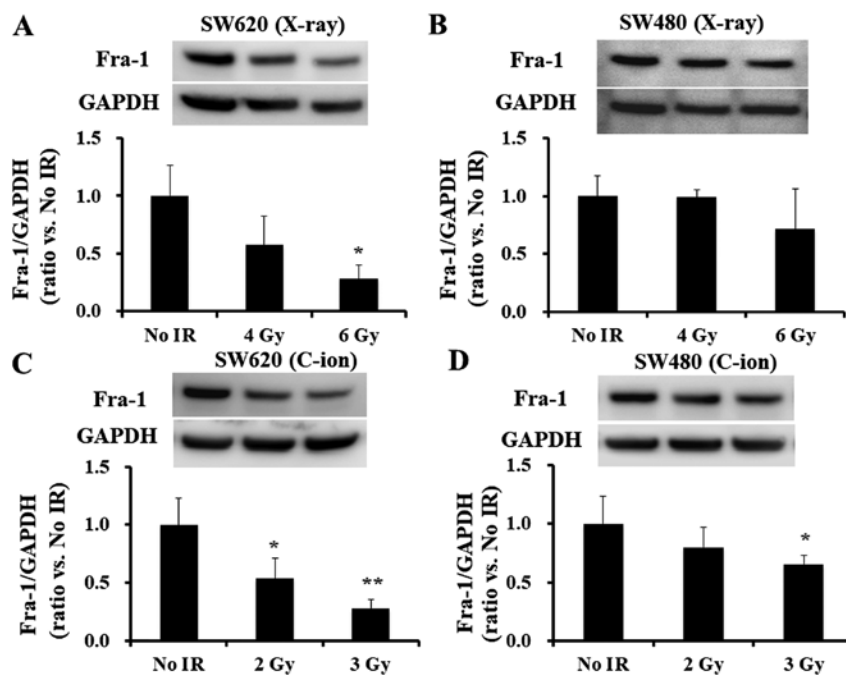

Figure 3. Fra-1 expression of SW620 and SW480 cells. Fra-1 expression of non-irradiated cells (No IR) vs. 4 or 6-Gy X-ray-irradiated SW620 (A) or SW480 (B) cells and 2 or 3-Gy C-ion-irradiated SW620 (C) or SW480 (D) cells were determined, respectively. Bands and quantitative densitometric results for Fra-1 protein are shown. n=3, *P<0.05, **P<0.01 vs. No IR.

Protein sampling and western blotting. Non-irradiated or irradiated cells were trypsinized and counted immediately after irradiation, and the same number of non-irradiated and irradiated cells was plated onto dishes containing fresh media. Two days after irradiation, cells were lysed with RIPA lysis buffer containing PMSF and sodium orthovanadate (Santa Cruz Biotechnology, Dalla, TX, USA) and then used for the western blotting.

For the proteasome inhibitor treatment, two patterns of schedule were used: 1) 10 nM of epoxomicin (proteasome inhibitor; Peptide Institute Inc., Osaka, Japan) was added to the culture media immediately before cell irradiation, and the cells were cultured for 48 h in a 5% CO_2 incubator at 37˚C and then lysed, or 2) 10 nM of epoxomicin was added to the culture media 48 h after irradiation, and the cells were cultured in a 5% CO_2 incubator at 37˚C for 24 h. The cells were then lysed with RIPA lysis buffer and used for the western blotting.

Immunoblotting was performed as previously described (37). Primary antibodies for human Fra-1 (Santa Cruz Biotechnology) and GAPDH (Trevigen, Bristol, UK) with horseradish peroxidase-conjugated anti-mouse IgG or anti-rabbit IgG (Amersham Biosciences, Buckinghamshire, UK) were used for this study. Protein bands were detected by enhanced chemiluminescence and imaged using a Lumino image analyzer (LAS4000; Fujifilm, Tokyo, Japan).

Quantitative real-time PCR. Quantitative real-time PCR (qRT-PCR) was performed on a LightCycler 480 with Probes Master (Roche Diagnostics, Basel, Switzerland) as previously described (38). The Universal Probe Library (UPL; Roche Diagnostics) probes and primer sequences for the
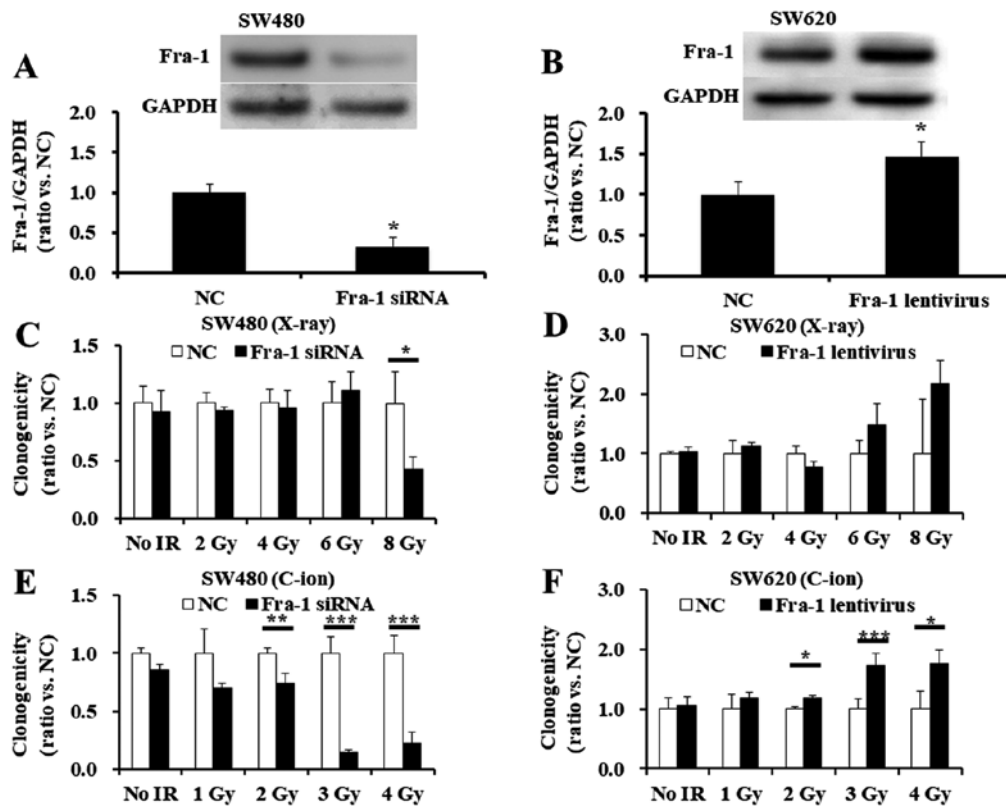
Figure 4. (A) Fra-1 expression of Fra-1 siRNA vs. scrambled negative control (NC)-transfected SW480 cells. The reduction of Fra-1 levels with siRNA transfection was determined by western blotting. Band and quantitative densitometric results for Fra-1 protein are shown. n=3, *P<0.05 vs. scrambled NC. (B) Fra-1 expression of Fra-1 lentivirus vector vs. NC vector-transfected SW620 cells. The over-expression of Fra-1 levels with lentivirus transfection was determined by western blotting. Band and quantitative densitometric results for Fra-1 protein are shown. n=3, *P<0.05 vs. NC. Role of Fra-1 in the clonogenicity of SW480 and SW620 cells in (C-F). The clonogenicity of Fra-1 siRNA- vs. scrambled negative control (NC)-transfected SW480 cells after X-ray irradiation (C) or C-ion irradiation (E) is shown. The clonogenicity of Fra-1 lentivirus vector- vs. negative control vector (NC)-transfected SW620 cells after X-ray irradiation (D) or C-ion irradiation (F) is shown, n=3. Data are presented as the means ± standard deviations of triplicate samples. *P<0.05, **P<0.01, ***P<0.001 vs. NC.

Fra-1 (*FOSL1*) and *GAPDH* genes were as follows: *FOSL1* (UPL probe: 26) sense, aggaactgaccgacttcctg, and antisense, cagctctaggcgctccttc; *GAPDH* (UPL probe: 60) sense, agccac atcgctcagaca, and antisense, gcccaatacgaccaaatcc.

Statistical analysis. Statistical analyses were performed using unpaired Student's t-tests or Mann-Whitney U-tests. P-values of <0.05 was considered to indicate a statistically significant difference.

Results

Role of Fra-1 in radioresistance. Surviving fractions of SW620 and SW480 cells were determined after X-ray or C-ion irradiation. Sensitivity to X-ray or C-ion irradiation differed between the cell lines; SW620 showed lower surviving fractions than SW480 at doses greater than 6 Gy for X-ray or 3 Gy for C-ion radiation (Fig. 2). Of note, SW620 cells showed a greater decrease in Fra-1 after 6 Gy for X-ray or 3 Gy for C-ion irradiation than SW480 cells (Fig. 3A and B for X-ray and Fig. 3C and D for C-ion irradiation, respectively).

To investigate a possible association between Fra-1 down-regulation and cellular radiosensitivity, we first treated SW480 cells with Fra-1 siRNA. The effectiveness of Fra-1 reduction with siRNA transfection is shown in Fig. 4A. Downregulation of Fra-1 in siRNA-treated SW480 cells showed increased

radiosensitivity to 8-Gy X-ray radiation (Fig. 4C) and to 2-, 3-, or 4-Gy C-ion radiation (Fig. 4E), compared with that of the scrambled negative control-treated SW480 cells.

To further clarify the significance of Fra-1 in radioresistance, we next overexpressed Fra-1 in SW620 cells via transfection with a lentivirus vector. Fra-1 induction with lentivirus transfection is shown in Fig. 4B. Further, overexpression of Fra-1 in lentivirus-transfected SW620 cells tended to increase the resistance to X-ray radiation (Fig. 4D) and significantly enhanced the resistance to C-ion radiation at doses greater than 2 Gy (Fig. 4F). Overall, the results indicate that Fra-1 has some role in radioresistance to X-ray or C-ion radiation for SW480 and SW620 cells.

Fra-1 levels in irradiated SW620 cells were downregulated by protein degradation through a proteasome pathway. To identify the molecular mechanisms modulating Fra-1 levels after irradiation, we first compared the changes of Fra-1 protein and the corresponding FOSL1 transcript levels in SW620 and SW480 cells after X-ray or C-ion irradiation. Although Fra-1 protein levels significantly decreased after irradiation with 6-Gy X-ray or 3-Gy C-ion radiation (Fig. 5A-D), no alteration in the Fra-1 transcript, FOSL1, levels was found for either the SW620 (Fig. 5A and B) or the SW480 cell lines (Fig. 5C and D), which indicates discrepancies between the reduction of Fra-1 protein and transcript levels in the irradiated cells.
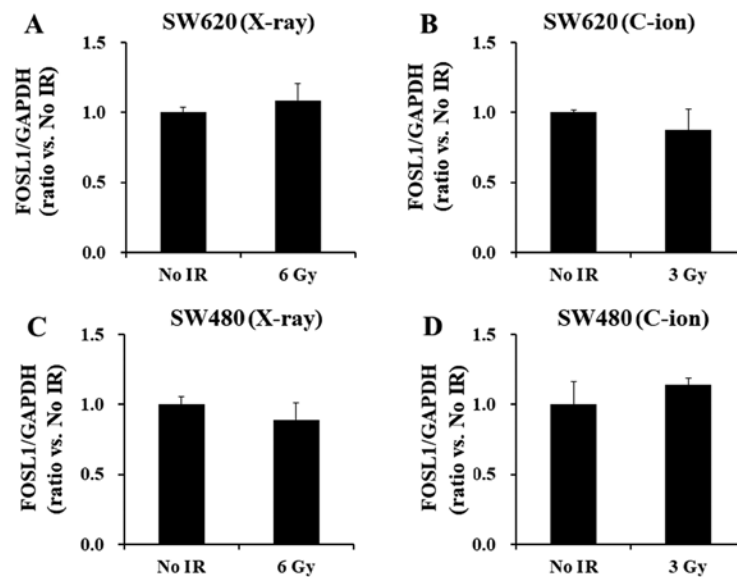
Figure 5. Expression of Fra-1 transcripts in irradiated SW620 or SW480. Expression of Fra-1 transcripts (FOSL1) of X-ray 6 Gy irradiated, or C-ion 3 Gy irradiated, SW620 (A and B) or SW480 (C and D) are shown, n=3, respectively. Data represent the means ± SD values of triplicate samples.
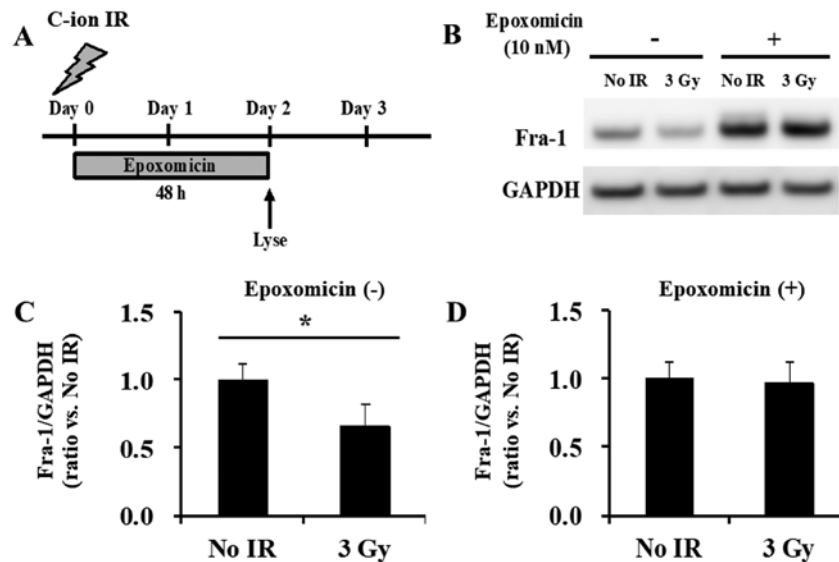

Figure 6. Role of proteasome in Fra-1 degradation in first 48 h post C-ion irradiation. Time course of the epoxomicin treatment of SW620 cells is shown in (A). Fra-1 expression levels in non-irradiated cells (No IR) vs. C-ion 3 Gy irradiated SW620 with or without the epoxomicin treatment were determined by western blotting (B). Quantitative densitometric results for Fra-1 protein without epoxomicin treatment (C) or with epoxomicin treatment (D) are shown, respectively. n=3, *P<0.05 vs. No IR.

The expression of Fra-1 protein is known to be highly regulated by proteasomal degradation (39). To clarify whether proteasomal degradation was involved in the reduction of the Fra-1 protein levels observed in the irradiated cells, C-ion-irradiated SW620 cells were further studied, because a clear discrepancy between the Fra-1 protein and transcript levels was observed in these cells (Figs. 3C and 5B). Pre-treatment of SW620 cells with the proteasome inhibitor epoxomicin and continued treatment for another 48 h after irradiation (Fig. 6A) blocked the Fra-1 degradation of these irradiated cells compared with that of the non-epoxomicin-treated SW620 cells (Fig. 6B-D). Of note, epoxomicin treatment from 48 h after C-ion irradiation (Fig. 7A) failed to block the reduction of Fra-1 (Fig. 7B-D), which indicates that the degradation

of Fra-1 via the proteasome occurred at some time during the first 48 h after irradiation.

Discussion

An understanding of the molecular mechanisms involved in radioresistance is necessary for improving the clinical outcomes of cancer radiotherapy. In this study, we demonstrated that Fra-1 has a significant role in the radioresistance of two colorectal cancer cell lines, SW620 and SW480. It is well known that irradiation, especially high LET radiation such as C-ion irradiation, induces cell cycle delay at the G2 phase, arresting the cells at the G2 checkpoint for DNA repair and/or committing them to undergo apoptosis (40). Cyclin A,
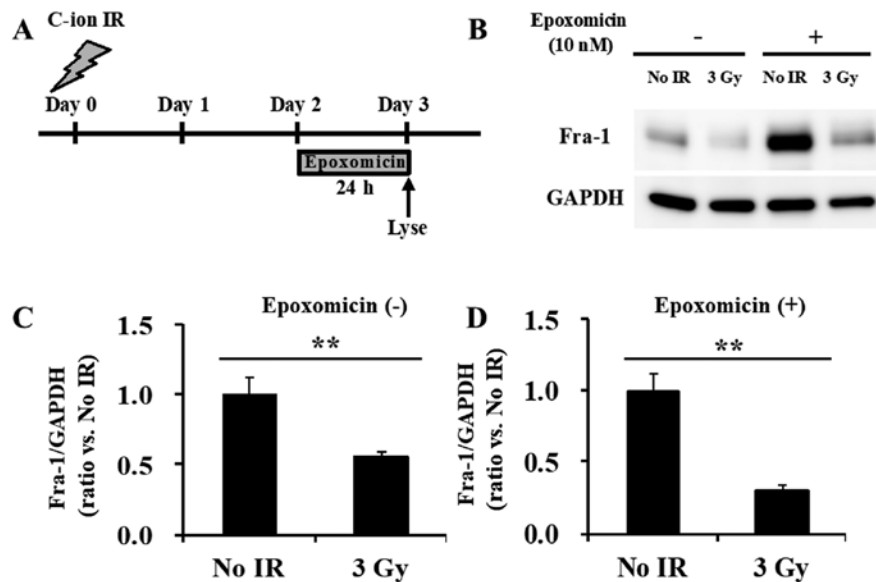
Figure 7. Role of proteasome in Fra-1 degradation from 48 to 72 h post C-ion irradiation. Time course of the epoxomicin treatment of SW620 cells was shown in (A). Fra-1 expression levels in non-irradiated cells (No IR) vs. C-ion 3 Gy irradiated SW620 with or without the epoxomicin treatment were determined by western blotting (B). Quantitative densitometric results for Fra-1 protein without epoxomicin treatment (C) or with epoxomicin treatment (D) are shown, respectively. n=3, **P<0.01 vs. No IR.

an important factor for the initiation of DNA replication, is known as a transcriptional target of Fra-1 (41). Thus, greater reduction of Fra-1 may interrupt DNA repair, which could induce cell death.

Fra-1 is known to be involved in various biological processes (1,2). In order to avoid the potential pathological effects of Fra-1 overexpression, the stability of the Fra-1 protein is highly regulated by phosphorylation-dependent proteasomal degradation (39). Thus, Fra-1 is usually absent in normal epithelial cells, but it is upregulated in several cancers (2). Several studies have suggested that the stability of the Fra-1 protein is regulated by phosphorylation upon ERK-MAPK pathway activation (39,42-44). The upstream signaling effectors, such as proteins encoded by oncogenic KRAS, found in colon carcinoma cell lines have been shown to result in constitutive ERK activation, followed by Fra-1 accumulation (38). To clarify whether Fra-1 phosphorylation status is involved in the Fra-1 degradation of irradiated cells, we also determined whether irradiation reduced the phospho-Fra-1 (p-Fra-1) levels in SW620 cells, as Fra-1 de-phosphorylation causes Fra-1 to become unstable and be degraded via the proteasome. Treatment of irradiated SW620 cells with epoxomicin for 48 h upon irradiation clearly blocked the downregulation of Fra-1 (Fig. 6); therefore, we hypothesized that the remaining undegraded Fra-1 contained many of the dephosphorylated Fra-1 proteins, which were destined to be degraded via the proteasome but remained because we had blocked this proteasome function. However, the levels of p-Fra-1 in the epoxomicin-treated SW620 cells were unchanged even after irradiation; the remaining undegraded Fra-1 following epoxomicin treatment did not contain dephosphorylated Fra-1 proteins, and most of them were phosphorylated Fra-1 (data not shown). These results indicate that the dephosphorylation of Fra-1 is not the trigger of proteasomal degradation upon irradiation. We also intended to check the levels of ubiquitinated Fra-1, but we could

not detect the ubiquitination of Fra-1 protein (data not shown). Thus far, we have not yet discovered how irradiation leads to Fra-1 degradation via the proteasome, without the involvement of dephosphorylation or ubiquitination of Fra-1, and further studies are required to solve this question.

In conclusion, we found that Fra-1 has a role in the radioresistance to X-ray or C-ion irradiation. To our knowledge, this is the first study indicating the role of Fra-1 in the radioresistance of colorectal cancer cells. In addition, we observed Fra-1 degradation within 48 h after irradiation. It would be of interest to further study whether Fra-1 level could be a candidate as an early response marker to reflect the effectiveness of radiotherapy.

Acknowledgements

Competing interests

The authors declare that they have no competing interests.

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