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Biology Contribution

Carbon-Ion Irradiation Suppresses Migration and Invasiveness of Human Pancreatic Carcinoma Cells MIAPaCa-2 via Rac1 and RhoA Degradation

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Summary

Carbon (C)-ion irradiation effectively reduces tumor cell migration and invasion; however, the underlying mechanisms are not well understood. We provide evidence that C-ion treatment suppresses the activities of Rac1 and RhoA, 2 major regulators of cell motility, via ubiquitin-mediated proteasomal degradation in human pancreatic carcinoma cells. This is the first report describing the inhibition of cancer cell migration and invasion via suppression of

Purpose: To investigate the mechanisms underlying the inhibition of cancer cell migration and invasion by carbon (C)-ion irradiation.

Methods and Materials: Human pancreatic cancer cells MIAPaCa-2, AsPC-1, and BxPC-3 were treated by x-ray (4 Gy) or C-ion (0.5, 1, 2, or 4 Gy) irradiation, and their migration and invasion were assessed 2 days later. The levels of guanosine triphosphate (GTP)-bound Rac1 and RhoA were determined by the active GTPase pull-down assay with or without a proteasome inhibitor, and the binding of E3 ubiquitin ligase to GTP-bound Rac1 was examined by immunoprecipitation.

Results: Carbon-ion irradiation reduced the levels of GTP-bound Rac1 and RhoA, 2 major regulators of cell motility, in MIAPaCa-2 cells and GTP-bound Rac1 in AsPC-1 and BxPC-3 cells. Proteasome inhibition reversed the effect, indicating that C-ion irradiation induced Rac1 and RhoA degradation via the ubiquitin (Ub)-proteasome pathway. E3 Ub ligase X-linked inhibitor of apoptosis protein (XIAP), which directly targets Rac1, was selectively induced in C-ion–irradiated MIAPaCa-2 cells and co-precipitated with GTP-bound Rac1 in C-ion–irradiated cells, which was associated with Rac1 ubiquitination. Cell migration and invasion reduced by C-ion radiation were restored by short interfering RNA–mediated XIAP knockdown, indicating that XIAP is involved in C-ion–induced inhibition of cell motility.

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Rho GTPases by C-ion radiation.

Conclusion: In contrast to x-ray irradiation, C-ion treatment inhibited the activity of Rac1 and RhoA in MIAPaCa-2 cells and Rac1 in AsPC-1 and BxPC-3 cells via Ub-mediated proteasomal degradation, thereby blocking the motility of these pancreatic cancer cells.

Introduction

In cancer, carbon (C)-ion irradiation has advantages over conventional photon therapy, such as accurate dose distribution and enhanced biological effects due to higher linear energy transfer (LET) translated into 2- to 3-fold higher cytotoxicity, as evidenced by the analysis of biological endpoints, including cell death, DNA damage, and chromosomal aberrations (1, 2).

It has been previously reported that C-ion and photon radiation produced different effects on the migration and invasiveness of tumor cells (3-5). X-ray irradiation enhanced the invasiveness of MIAPaCa-2 and PANC-1 pancreatic cancer cells (3, 4), whereas C-ions suppressed invasion and migration of MIAPaCa-2 as well as BxPC-3 and AsPC-1 pancreatic adenocarcinoma cells (4), but enhanced PANC-1 cell invasion (4). Cell movement requires dynamic remodeling of cellular architecture, including actin filament rearrangement and actomyosin contraction, and Rho guanosine triphosphate (GTP)ases Rac1 and RhoA are key components of signaling networks regulating cell migration (6). We have previously demonstrated that nitric oxide—induced RhoA activation enhanced the invasiveness of C-ion—irradiated PANC-1 cells (4). However, the role of Rac1 and RhoA in the response of cancer cells to C-ion irradiation has not been explored.

The activity of Rac1 and RhoA is tightly regulated by guanine nucleotide exchange factors (GEFs) that activate Rho GTPases by stimulating the exchange of guanosine diphosphate (GDP) to GTP, and by GTPase-activating proteins (GAPs) that inactivate Rho GTPases by enhancing GTP hydrolysis (6). The cytosolic localization of inactive Rho GTPases is regulated by Rho-specific guanine nucleotide dissociation inhibitors (GDIs) that maintain Rho GTPases in an inactive GDP-bound state (7). Recent studies have shown that the activity of Rac1 and RhoA is regulated by their degradation via the ubiquitin (Ub)-proteasome system, which modulates cell migration (8, 9).

The Ub-proteasome system executes selective degradation of target proteins labeled with poly-Ub chains by the sequential activities of E1, E2, and E3 Ub ligases; the latter transfers Ub from E2 to the target protein (10). Thus, E3 ligase plays a major role in regulating protein turnover, which maintains specific cellular phenotypes (11).

In this study we demonstrate that C-ion irradiation inhibits the motility of pancreatic cancer cells by suppressing Rac1 and RhoA activation via the Ub-proteasome degradation pathway.

Methods and Materials

Cell culture and reagents

Human pancreatic cancer cell lines MIAPaCa-2, AsPC-1, BxPC-3, and PANC-1 were purchased from the American Type Culture Collection (Manassas, VA). Cells were cultured in Dulbecco's modified eagle medium (DMEM) (MIAPaCa-2 and PANC-1) and Roswell Park Memorial Institute medium 1640 (AsPC-1, BxPC-3) purchased from Nissui (Tokyo, Japan) and supplemented with 10% fetal bovine serum (HyClone, Logan, UT). A proteasome inhibitor, epoxomicin, was purchased from Peptide Institute (Ibaraki, Japan).

Irradiation

Cells were treated with x-ray or C-ion radiation as previously described (4). The initial energy of the C-ion beam was 290 MeV per nucleon and the LET value was 80 keV/μm, corresponding to a monoenergetic beam with narrow Bragg peak at a depth of 10 cm. Cells were irradiated at doses of 4 Gy (X rays) and 0.5, 1, 2, or 4 Gy (C-ions) at the rate of 1 Gy/min. An outline of the experimental procedure is presented in Figure 1.

Migration and invasion assay

Cell migration and invasion were examined as previously described, with some modifications (5). Briefly, 2 days after irradiation, cells were trypsinized and stained with trypan blue to determine cell viability. Cells were then resuspended in serum-free DMEM containing 0.35% bovine serum albumin and seeded at a concentration of 1.5×10^5 viable cells per well in transwell chambers containing 6.5-mm filters with 8-μm pores (Corning, Corning, NY); cell migration and invasion were evaluated after 24 hours (Fig. 1). Cells that migrated through the transwell membrane were fixed and stained with Diff Quick (Sysmex, Kobe, Japan).

Short interfering RNA transfection

Short interfering RNA (siRNA) targeting X-linked inhibitor of apoptosis protein (XIAP) and negative control

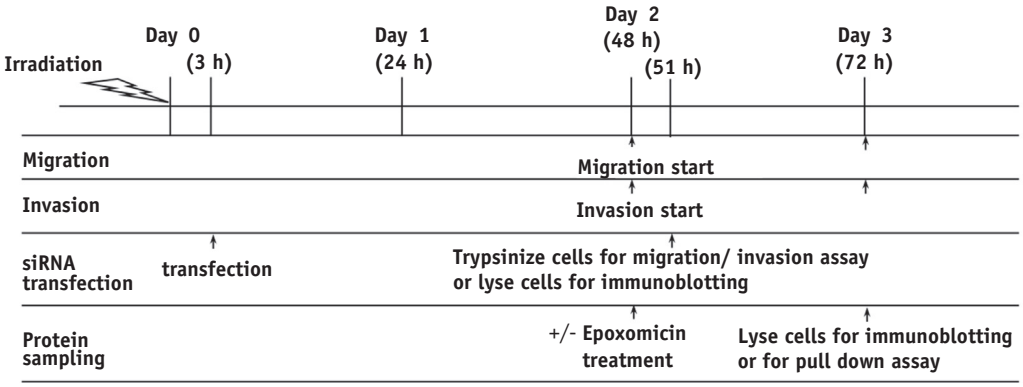

Fig. 1. An outline of the experimental procedures. siRNA = short interfering RNA.

siRNA (Santa Cruz Biotechnology, Santa Cruz, CA) were used as previously described (12). Cells grown to approximately 60% confluence in 6-well plates were transfected with 50 pmol siRNA mixed with LipoTrust Ex Oligo reagent (Hokkaido System Science Co, Hokkaido, Japan) for 48 hours in serum-free medium. Cells were then trypsinized and used for migration and invasion assays, or analyzed for protein expression by immunoblotting.

Immunoblotting

The time course of protein expression analysis is shown in Figure 1. Cells were lysed in radioimmunoprecipitation buffer containing 200 mM phenylmethylsulfonyl fluoride and protease inhibitor cocktail (Santa Cruz Biotechnology) as previously described (5). Total cell proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, transferred to nitrocellulose membranes, and incubated with primary antibodies for 1 hour at room temperature. The antibodies against the following proteins were used: matrix metalloproteinase-2 (1:2500; Daiichi Fine Chemical, Toyama, Japan), XIAP, IAP1, HECT domain and ankyrin repeat-containing E3 ubiquitin ligase (HACE)1 (1:1000; all from Cell Signaling Technology, Danvers, MA), protein Ub chain (1:2000; MBL, Nagoya, Japan), Rac1 and RhoA (1:2500; the Rac1 and RhoA activity assay kit, Cell Biolabs, San Diego, CA), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (1:10,000; Trevigen, Gaithersburg, MD). Can Get Signal Solution 1 (Toyobo, Tokyo, Japan) was used as the dilution buffer. The membranes were washed and incubated for 1 hour at room temperature with horseradish peroxidase–conjugated anti-mouse or anti-rabbit IgG (Amersham Biosciences, Buckinghamshire, United Kingdom) diluted 1:10,000 with Can Get Signal Solution 2 (Toyobo). Protein bands were detected by enhanced chemiluminescence using an LAS 4000 Lumino image analyzer (Fujifilm, Tokyo, Japan).

For proteasome inhibition, 2 days after irradiation cells were treated with 10 or 20 nM epoxomicin for 24 hours, lysed as described, and analyzed for protein expression or Rac1 and RhoA activity.

Glutathione S-transferase pull-down of polyubiquitinated proteins

Polyubiquitinated proteins were isolated using the glutathione S-transferase (GST)-tagged Tandem Ubiquitin Binding Entity (GST-TUBE) (Nacalai Tesque, Kyoto, Japan), according to the manufacturer's instructions. Briefly, cells were lysed as described earlier with or without GST-TUBE. The GST-TUBE–containing samples were collected in ice-cold tubes, incubated for 15 minutes on ice, centrifuged at 14,000 × *g* for 10 minutes at 4°C, and the supernatant was incubated with Glutathione Sepharose 4B beads (GE Healthcare, Buckinghamshire, United Kingdom) for 2 hours at 4°C with rotation; immunoprecipitated proteins were eluted and analyzed by immunoblotting. Cell lysates without GST-TUBE were used to measure protein concentration by the Bicinchoninic acid (BCA) protein assay (Pierce Biotechnology, Rockford, IL).

Rac1 and RhoA activity assay

Small GTPase activity was measured using the active Rho GTPase pull-down assay and the Rac1 and RhoA activation assay kit (Cell Biolabs) according to the manufacturer's instructions. Briefly, cells were lysed on ice, centrifuged at 14,000 × *g* for 10 minutes at 4°C, and the supernatant was incubated with agarose beads containing immobilized protein-activated kinase 1 or Rhotekin Rho-binding domain (Cell Biolabs) for 1 hour at 4°C with rotation. The precipitated protein was eluted and analyzed by immunoblotting.

Statistical analysis

Statistical analyses were performed using an unpaired Student *t* test, and the differences between groups were assessed with a 2-tailed test; $P<.05$ was considered

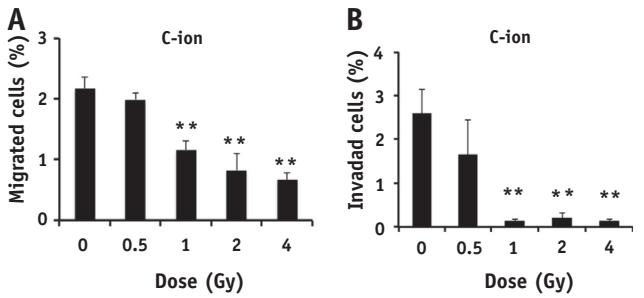

Fig. 2. Carbon-ion (C-ion) irradiation suppresses MIAPaCa-2 cell migration and invasiveness in a dose-dependent manner. (A) Migration and (B) invasion of MIAPaCa-2 cells. $^{**}P<.01$ versus control (n = 3).

significant. Each experiment was performed in triplicate and independently repeated at least twice on different days.

Results

Carbon-ion radiation suppresses MIAPaCa-2 cell migration and invasion in a dose-dependent manner

We have previously demonstrated that irradiation of MIAPaCa-2 cells with 2 Gy C-ions suppressed their migration and invasion (4). Here, we found that C-ion irradiation at 0.5, 1, 2, and 4 Gy dose-dependently inhibited the migration and invasion of MIAPaCa-2 cells (Fig. 2). Consistent with reduced invasiveness, the expression of activated matrix metalloproteinase-2, a critical protease involved in MIAPaCa-2 cell invasion (3), was decreased in C-ion–irradiated cells (Supplemental Figure E1; available online at www.redjournal.org). Although MIAPaCa-2 cell migration and invasion were markedly inhibited by C-ions at doses >1 Gy (Fig. 2), they were induced by x-ray irradiation at 4 Gy (3). The relative biological effectiveness of x-ray versus C-ion radiation, as represented by the D_{10} value, was 2.0 (Supplemental Figure E2; available online at www.redjournal.org); therefore, in the subsequent experiments, X rays and C-ions were used at the doses of 4 and 2 Gy, respectively.

Carbon-ion radiation inhibits Rac1 and RhoA activities via proteasomal degradation

Carbon-ion treatment suppressed MIAPaCa-2 cell migration and invasion, indicating that cell motility was affected. Therefore, we examined the effect of C-ion radiation on the activity of Rac1 and RhoA, 2 major regulators of cell motility (6). Although total levels of Rac1 and RhoA were unchanged, the expression of the GTP-bound active Rac1 was increased in X ray–irradiated cells (Fig. 3A). In contrast, C-ion radiation markedly reduced the levels of GTP-bound Rac1 and RhoA (Fig. 3A, B), indicating the inhibition of Rac1 and RhoA activation in MIAPaCa-2 cells. However, in C-ion–irradiated PANC-1 cells, the level of GTP-bound Rac1 was not changed (Fig. 3C). We have previously found that in PANC-1 cells, C-ion irradiation enhanced invasiveness and increased GTP-bound RhoA without affecting total RhoA (5).

The molecular switch between active GTP-bound and inactive GDP-bound forms of Rac1 and RhoA is regulated by GEFs and GAPs (6); however, their messenger RNA (mRNA) expression was not altered in irradiated MIAPaCa-2 cells (Supplemental Figure E3; available

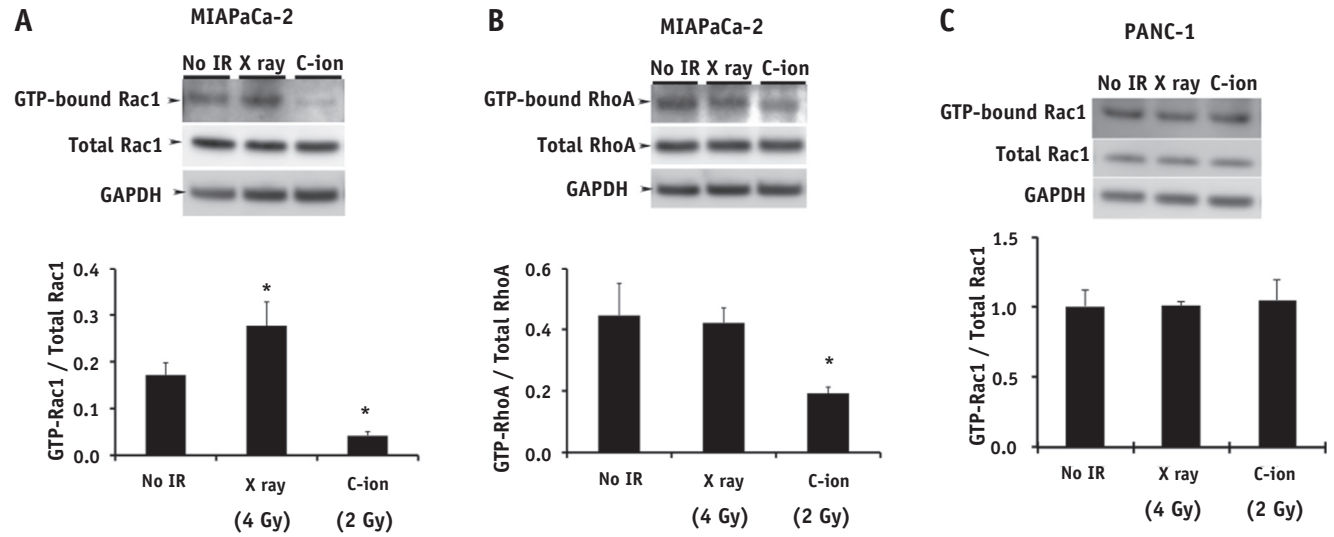

Fig. 3. Carbon-ion (C-ion) irradiation (IR) reduces the levels of guanosine triphosphate (GTP)-bound Rac1 and RhoA in MIAPaCa-2 cells, but not in PANC-1 cells. Expression of GTP-bound and total Rac1 (A) and GTP-bound and total RhoA (B) in MIAPaCa-2, and GTP-bound and total Rac1 in PANC-1 cells (C) irradiated with X rays or C-ions was analyzed by Western blotting; glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as loading control. $^{*}P<.05$ versus control (n = 3).

online at www.redjournal.org). In addition, the protein level of Rho-specific GDI, which sequesters GDP-bound GTPases in the cytosol, as well as the subcellular localization of Rac1 and RhoA, were also not affected by irradiation (Supplemental Figure E4; available online at www.redjournal.org). Interestingly, the treatment of MIAPaCa-2 cells with a proteasome inhibitor restored GTP-bound Rac1 and RhoA levels (Fig. 4A, B). Furthermore, we found that in AsPC-1 and BxPC-3 cells, C-ion irradiation also significantly decreased the levels of GTP-bound Rac1 without affecting total Rac1 expression (Fig. 4C, D), which correlated with the inhibition of cell migration (4). The decrease in GTP-bound Rac1 was rescued by the treatment with epoxomicin (Fig. 4C, D), suggesting that C-ion irradiation reduced BxPC-3 and AsPC-1 cell migration by down-regulating Rac1 activity via proteasomal degradation of GTP-bound Rac1, which is similar to the effect observed in MIAPaCa-2 cells. Together, these data indicate that the response of PANC-1 cells to C-ion treatment differs from that of the other tested pancreatic cancer cells.

Carbon-ion radiation induced stronger polyubiquitination than x-ray radiation

To examine the effect of C-ion irradiation on Ub-mediated proteasomal degradation of Rac1 and RhoA, we analyzed the level of polyubiquitinated proteins in irradiated cells. In C-ion–treated cells, proteasome inhibition caused higher accumulation of polyubiquitinated proteins compared with X ray–treated cells (Fig. 5A), indicating that the Ub-proteasome system was activated by C-ions. Accordingly, E3 Ub ligase mRNA was upregulated by >50% after C-ion irradiation (Supplemental Table E1; available online at www.redjournal.org).

E3 Ub ligase XIAP mediates C-ion–induced degradation of GTP-bound Rac1

Two IAPs, XIAP and cIAP1, and HACE1 have been identified as E3 Ub ligases specific for Rac1 (9, 13). The

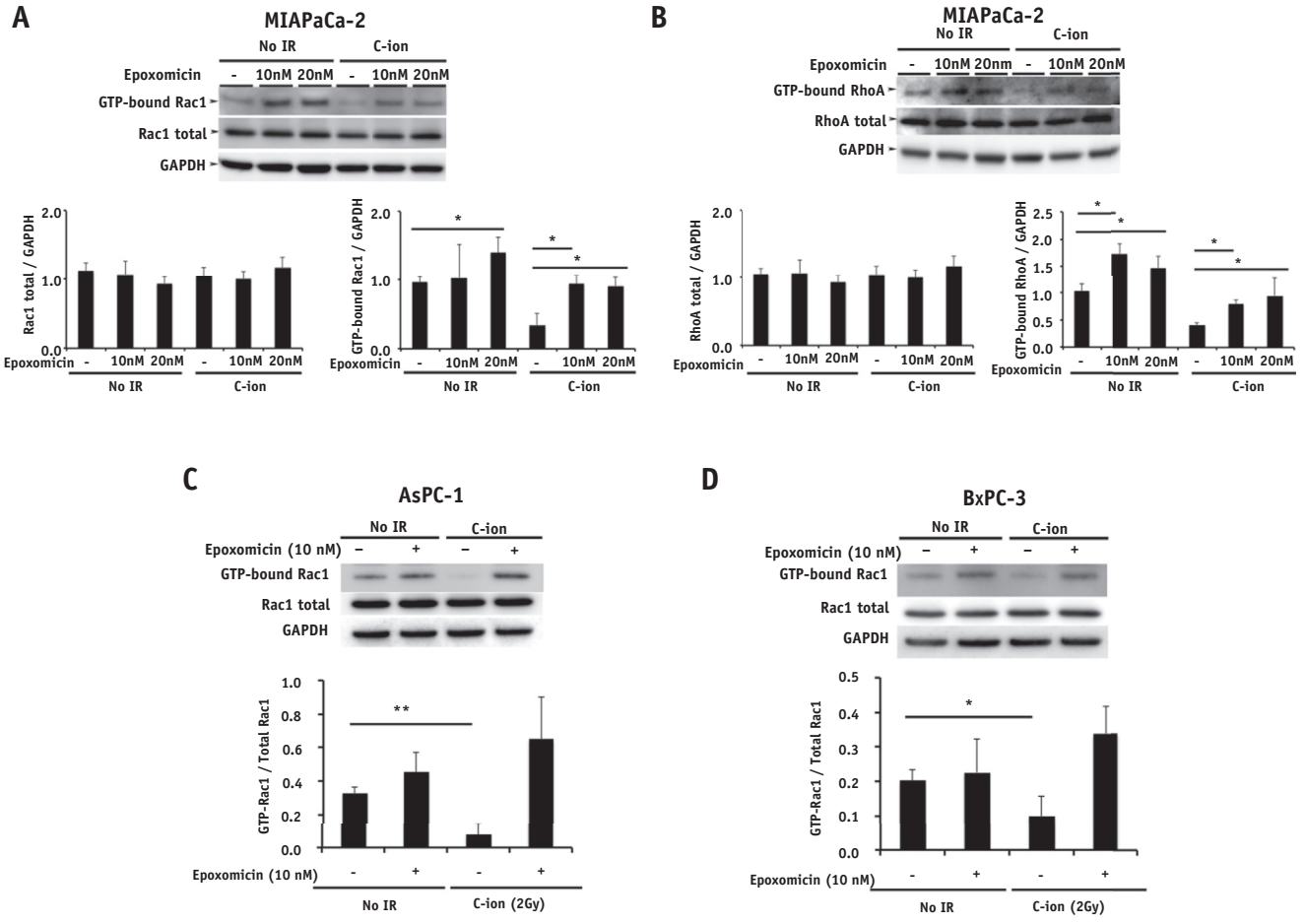

Fig. 4. Carbon-ion (C-ion) irradiation (IR) reduces the guanosine triphosphate (GTP)-bound Rac1 and RhoA in pancreatic cancer cells via proteasomal degradation. Nonirradiated or C-ion–irradiated MIAPaCa-2, AsPC-1, and BsPC-3 cells were treated or not with epoxomicin, and the expression of GTP-bound and total Rac1 (A) and RhoA (B) in MIAPaCa-2 cells, and GTP-bound and total Rac1 in AsPC-1 (C) and BxPC-3 (D) cells was determined by Western blotting; glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as loading control. *$P<.05$ versus control (n = 3).

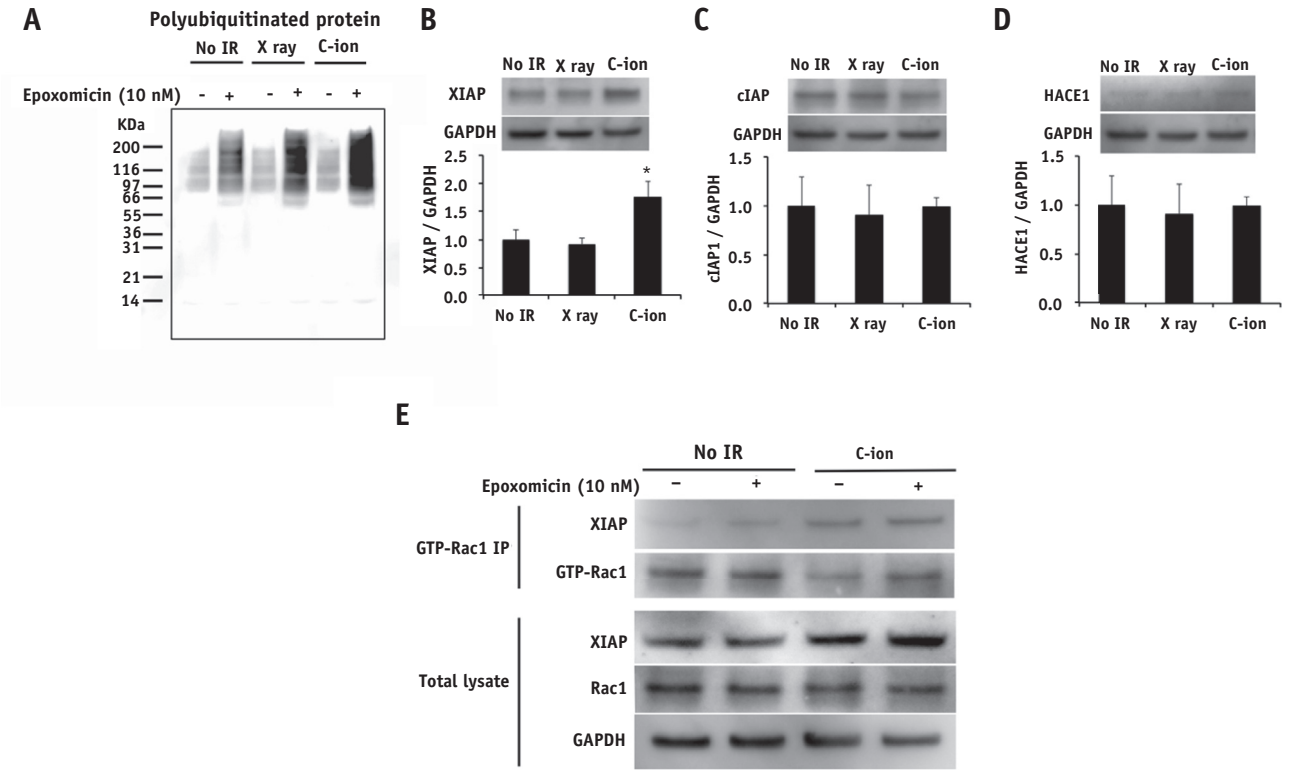

Fig. 5. Role of X-linked inhibitor of apoptosis protein (XIAP) in carbon ion (C-ion)—induced degradation of guanosine triphosphate (GTP)-Rac1. MIAPaCa-2 cells were irradiated with X rays or C-ions and analyzed by Western blotting for the expression of polyubiquitinated proteins (A), XIAP (B), cIAP1 (C), and HACE1 (D). The data represent the mean ± SD; n = 3 for (B-D); $*P<.05$ versus control. (E) GTP-bound Rac1 was immunoprecipitated from the lysates of control and irradiated MIAPaCa-2 cells treated or not with epoxomicin using PAC1-agarose beads and analyzed by Western blotting with the anti-XIAP antibody. GAPDH = glyceraldehyde-3-phosphate dehydrogenase.

XIAP transcript (Supplemental Table E1; available online at www.redjournal.org) and protein (Fig. 5B) were increased after C-ion irradiation, whereas the expression of cIAP1 and HACE1 was unchanged (Fig. 5C, D). X-linked inhibitor of apoptosis protein also coprecipitated with GTP-bound Rac1 in the lysates of C-ion—irradiated cells (Fig. 5E), suggesting that XIAP binds to and Ub-labels the GTP-bound Rac1 to target it for degradation; indeed, Rac1 was ubiquitinated in C-ion—irradiated cells (Supplemental Figure E5; available online at www.redjournal.org). RhoA was also ubiquitinated under these conditions (Supplemental Figure E5; available online at www.redjournal.org); however, none of E3 Ub ligases involved in RhoA degradation was upregulated by C-ion radiation (Supplemental Table E1; available online at www.redjournal.org).

XIAP mediates the inhibitory effects of C-ion radiation on cell migration and invasion

To determine XIAP's role in C-ion—induced inhibition of cell migration and invasion, XIAP expression was blocked by specific siRNA (Fig. 6A). Cell migration and invasion suppressed by C-ion radiation were restored by XIAP knockdown (Fig. 6B, C), indicating that XIAP is the E3 ligase responsible for Rac1 degradation and suppression of migration and invasion of C-ion—irradiated pancreatic cancer cells.

Discussion

Radiation therapy is a standard method of cancer treatment, and the therapeutic efficacy of C-ion radiation in pancreatic cancer has been recently analyzed (14). We have previously found that C-ion irradiation suppressed the motility of AsPC-1, BxPC-3, and MIAPaCa-2 pancreatic cancer cells but increased the invasion of PANC-1 cells (4). We have also observed that C-ions reduced the invasiveness of several other tumor cell lines; only SF126 human glioblastoma cells exhibited increase (5). The response of cancer cells to radiation may vary depending on genetic background; indeed, we have identified mutations in the genes encoding the components of the NO-PI3K-AKT2 pathway (data not shown), which is a critical signaling mechanism underlying irradiation-enhanced invasion of PANC-1 cells (5). Given that C-ion irradiation activated

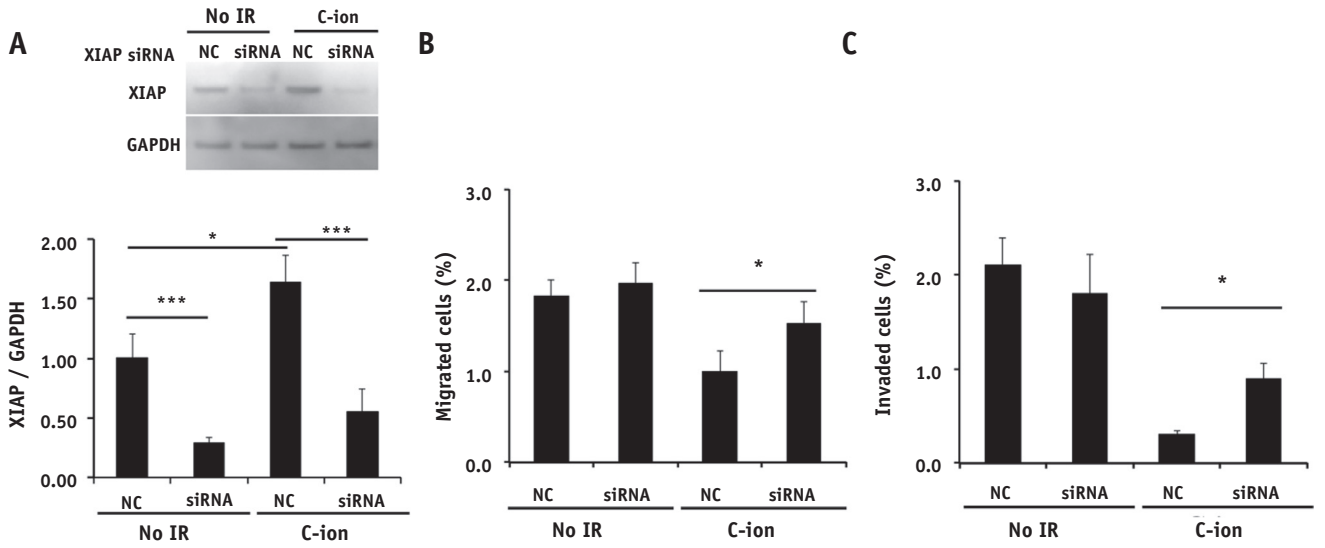

Fig. 6. Role of X-linked inhibitor of apoptosis protein (XIAP) in the suppression of cell migration and invasion by carbon ion (C-ion) irradiation (IR). MIAPaCa-2 cells expressing XIAP-specific short interfering RNA (siRNA) were C-ion–irradiated and analyzed for (A) XIAP expression and cell migration (B) and invasion (C). The data represent the mean ± SD; *$P<.05$ and **$P<.01$ versus control (n = 3). NC = negative control siRNA.

RhoA in PANC-1 cells (5) but exerted the opposite effects in other pancreatic cancer cells (ie, inhibiting RhoA and Rac1 in MIAPaCa-2 cells and Rac1 in AsPC-1 and BxPC-3 cells, via proteasomal degradation), the involvement of cell-specific mutations in Rac1 and RhoA stability should be further investigated.

Small GTPase activity is regulated by multiple factors, including GEFs and GAPs which, serve as molecular switches between GTP- and GDP-bound forms of Rac1 and RhoA (6), and GDIs, which sequester GDP-bound Rac1 and RhoA in the cytosol, preventing their membrane localization (7, 15). Patients with pancreatic adenocarcinomas positive for VAV1, a GEF regulating Rac1 activation, have lower survival rates than those with VAV1-negative tumors (16), suggesting that GEFs or GAPs may be potential therapeutic targets (17). Surprisingly, we did not observe any changes in the expression of GEF and GAP mRNA and GDI protein, or subcellular localization of Rac1 and RhoA in irradiated MIAPaCa-2 cells. Radiation reduces intracellular GTP pool via oxidation of GTP to 8-oxo-dGTP, which is further hydrolyzed by MutT homolog 1 and released from the cells (18, 19); however, GTP level was unchanged in irradiated MIAPaCa-2 cells (data not shown).

In MIAPaCa-2 cells, XIAP levels were upregulated by C-ions but not by x-ray radiation. Transcription of XIAP is regulated by nuclear factor κB (20), which is more strongly activated by particle than by photon radiation via IκB phosphorylation and degradation (21-23), a process modulated by tumor necrosis factor receptor–associated factor (TRAF)2 (24, 25). Deoxyribonucleic acid microarray analysis revealed the increase in TRAF2 levels in C-ion- but not in X ray–irradiated MIAPaCa-2 cells (data not shown). Thus, TRAF2 may play a role in the up-regulation of XIAP expression by C-ion radiation; however, additional studies are needed to elucidate the mechanism of GTP-bound RhoA degradation induced by C-ions.

In this study we observed a unique effect of C-ion radiation on the protein degradation system in MIAPaCa-2, AsPC-1, and BxPC-3 cells. Protein polyubiquitination after C-ion irradiation was dramatically induced compared with x-ray treatment in MIAPaCa-2. Heavy ions, including C-ion, have high ionization density in each track of individual particles and can induce significant DNA damage and cytotoxicity in tumor cells (26, 27). Radiation also triggers cellular oxidative stress via generation of reactive oxygen species such as hydroxyl radicals (•OH) and reactive nitrogen oxide species (28), leading to structural and functional alterations of cellular proteins (29, 30) and their degradation through the ubiquitin-proteasome system (31). Interestingly, Matsumoto et al (32) have reported that the generation of dense •OH radicals was enhanced with increasing LET and that different types of dense •OH may be produced by x-ray and C-ion irradiation. Therefore, C-ions may have more severe effects on protein stability than photon radiation. Protein ubiquitination-mediated degradation can affect cell behavior (33), as evidenced by the change in MIAPaCa-2 cell motility induced by C-ion irradiation. E3 Ub ligase regulates the turnover of specific proteins responsible for particular cellular phenotypes (10, 11); therefore, E3 Ub ligases and their substrates are likely to be involved in cellular responses to C-ion radiation (11, 34).

In conclusion, we show, for the first time, that Ub-proteasome–mediated degradation of Rac1 and RhoA is a mechanism underlying the suppression of pancreatic cancer cell motility by C-ion radiation. Further investigation of

genetic mechanisms regulating the activity of Rac1 and RhoA in tumors may provide clues to effective inhibition of cancer invasiveness by C-ion radiation.

References

1. Kamada T, Tsujii H, Blakely EA, et al. Carbon ion radiotherapy in Japan: An assessment of 20 years of clinical experience. *Lancet Oncol* 2015;16:e93-e100.
2. Fokas E, Kraft G, An H, et al. Ion beam radiobiology and cancer: Time to update ourselves. *Biochim Biophys Acta* 2009;1796:216-229.
3. Fujita M, Otsuka Y, Yamada S, et al. X-ray irradiation and Rho-kinase inhibitor additively induce invasiveness of the cells of the pancreatic cancer line, MIAPaCa-2, which exhibits mesenchymal and amoeboid motility. *Cancer Sci* 2011;102:792-798.
4. Fujita M, Otsuka Y, Imadome K, et al. Carbon-ion radiation enhances migration ability and invasiveness of the pancreatic cancer cell, PANC-1, in vitro. *Cancer Sci* 2012;103:677-683.
5. Fujita M, Imadome K, Endo S, et al. Nitric oxide increases the invasion of pancreatic cancer cells via activation of the PI3K-AKT and RhoA pathways after carbon ion irradiation. *FEBS Lett* 2014;588:3240-3250.
6. Sadok A, Marshall CJ. Rho GTPases, masters of cell migration. *Small GTPases* 2014;5:e29710-e29711.
7. Garcia-Mata R, Boulter E, Burridge K. The 'invisible hand': Regulation of RHO GTPases by RHOGDIs. *Nat Rev Mol Cell Biol* 2011;12:493-504.
8. Nethe M, Hordijk PL. The role of ubiquitylation and degradation in RhoGTPase signaling. *J Cell Sci* 2010;123:4011-4018.
9. Oberoi TK, Dogan T, Hocking JC, et al. IAPs regulate the plasticity of cell migration by directly targeting Rac1 for degradation. *EMBO J* 2012;31:14-28.
10. Micel LN, Tentler JJ, Smith PG, et al. Role of ubiquitin ligases and the proteasome in oncogenesis: Novel targets for anticancer therapies. *J Clin Oncol* 2013;31:1231-1238.
11. Deshaies RJ, Joazeiro CA. RING domain E3 ubiquitin ligases. *Ann Rev Biochem* 2009;78:399-434.
12. Yin S, Wang R, Zhou F, et al. Bcl-xL is a dominant antiapoptotic protein that inhibits homoharringtonine-induced apoptosis in leukemia cells. *Mol Pharmacol* 2011;79:1072-1083.
13. Oberoi-Khanuja TK, Murali A, Rajalingam K, et al. IAPs on the move: Role of inhibitors of apoptosis proteins in cell migration. *Cell Death Dis* 2013;4:e784.
14. Shinoto M, Yamada S, Yasuda S, et al. Phase 1 trial of preoperative, short-course carbon-ion radiotherapy for patients with resectable pancreatic cancer. *Cancer* 2013;119:45-51.
15. Dransart E, Olofsson B, Cherfils J. RhoGDIs revisited: Novel roles in Rho regulation. *Traffic* 2005;6:957-966.
16. Chari ST, Urrutia R, Billadeau DD. Ectopic expression of VAV1 reveals an unexpected role in pancreatic cancer tumorigenesis. *Cancer Cell* 2005;7:39-49.
17. Denicola G, Tuveson DA. VAV1: A new target in pancreatic cancer? *Cancer Biol Ther* 2005;4:509-511.
18. Sangsuwan T, Haghdoost S. The nucleotide pool, a target for low-dose gamma-ray-induced oxidative stress. *Radiat Res* 2008;170:776-783.
19. Fratini E, Licursi V, Artibani M, et al. Dose-dependent onset of regenerative program in neutron irradiated mouse skin. *PLoS One* 2011;6:e19242.
20. Stehlik C, Martin DR, Kumabashiri I, et al. Nuclear factor (NF)-kappaB-regulated X-chromosome-linked iap gene expression protects endothelial cells from tumor necrosis factor alpha-induced apoptosis. *J Exp Med* 1998;188:211-216.
21. Mitra AK, Sarma A, Krishna M, et al. Expression of NF-kappaB and ERK following heavy ion irradiation. *J Environ Pathol Toxicol Oncol* 2004;23:53-59.
22. Mitra AK, Bhat N, Sarma A, et al. Alteration in the expression of signaling parameters following carbon ion irradiation. *Mol Cell Biochem* 2005;276:169-173.
23. Hellweg CE, Baumstark-Khan C, Schmitz C, et al. Activation of the nuclear factor κB pathway by heavy ion beams of different linear energy transfer. *Int J Radiat Biol* 2011;87:954-963.
24. Shishodia S, Aggarwal BB. Guggulsterone inhibits NF-kappaB and IkappaBalpha kinase activation, suppresses expression of anti-apoptotic gene products, and enhances apoptosis. *J Biol Chem* 2004;279:47148-47158.
25. Cabal-Hierro L, Rodríguez M, Artime N, et al. TRAF-mediated modulation of NF-kB AND JNK Activation by TNFR2. *Cell Signal* 2014;26:2658-2666.
26. Nikjoo H, Uehara S, Wilson WE, et al. Track structure in radiation biology: Theory and applications. *Int J Radiat Biol* 1994;65:7-17.
27. Goodhead DT. Initial events in the cellular effects of ionizing radiations: Clustered damage in DNA. *Int J Radiat Biol* 1998;73:355-364.
28. Mikkelsen RB, Wardman P. Biological chemistry of reactive oxygen and nitrogen and radiation-induced signal transduction mechanisms. *Oncogene* 2003;22:5734-5754.
29. Grimsrud PA, Xie H, Griffin TJ, et al. Oxidative stress and covalent modification of protein with bioactive aldehydes. *J Biol Chem* 2008;283:21837-21841.
30. Switzer CH, Glynn SA, Cheng RY, et al. S-nitrosylation of EGFR and Src activates an oncogenic signaling network in human basal-like breast cancer. *Mol Cancer Res* 2012;10:1203-1215.
31. Kastle M, Grune T. Protein oxidative modification in the aging organism and the role of the ubiquitin proteasomal system. *Curr Pharm Des* 2011;17:4007-4022.
32. Matsumoto K, Ueno M, Nakanishi I, et al. Density of hydroxyl radicals generated in an aqueous solution by irradiating carbon-ion beam. *Chem Pharm Bull* 2015;63:195-199.
33. Powers ET, Balch WE. Diversity in the origins of proteostasis networks—a driver for protein function in evolution. *Nat Rev Mol Cell Biol* 2013;14:237-248.
34. Yen HC, Elledge SJ. Identification of SCF ubiquitin ligase substrates by global protein stability profiling. *Science* 2009;322:923-929.