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Silencing of mitochondrial NADP$^+$-dependent isocitrate dehydrogenase gene enhances glioma radiosensitivity

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ABSTRACT

Reactive oxygen species (ROS) levels are elevated in organisms that have been exposed to ionizing radiation and are protagonists in the induction of cell death. Recently, we demonstrated that the control of mitochondrial redox balance and the cellular defense against oxidative damage are primary functions of mitochondrial NADP$^+$-dependent isocitrate dehydrogenase (IDPm) via the supply of NADPH for antioxidant systems. In the present study, we report an autophagic response to ionizing radiation in A172 glioma cells transfected with small interfering RNA (siRNA) targeting the IDPm gene. Autophagy in A172 transfectant cells was associated with enhanced autophagolysosome formation and GFP–LC3 punctuation/aggregation. Furthermore, we found that the inhibition of autophagy by chloroquine augmented apoptotic cell death of irradiated A172 cells transfected with IDPm siRNA. Taken together, our data suggest that autophagy functions as a survival mechanism in A172 cells against ionizing radiation-induced apoptosis and the sensitizing effect of IDPm siRNA and autophagy inhibitor on the ionizing radiation-induced apoptotic cell death of glioma cells offers a novel redox-active therapeutic strategy for the treatment of cancer.

1. Introduction

Gliomas are the most common type of primary brain tumors and are classified from grade I to grade IV based on histopathological and clinical criteria established by the World Health Organization (WHO) [1]. Radiotherapy is a standard adjuvant treatment for patients with malignant glioma, but the resistance of glial tumor cells to ionizing radiation limits the success of this treatment, to the extent that the survival benefits of radiotherapy remain minimal [2]. Therefore, much research interest has been shown in augmenting the effects of radiation on tumors using targeted therapeutic agents [3].

Ionizing radiation generates reactive oxygen species (ROS) in many cell types [4]. In addition to the generating hydroxyl radicals, the hydrated electrons formed can reduce O_2 to O_2^-, which can dismutate to H_2O_2 and generate more $^{\cdot}OH$ via the metal-catalyzed Fenton reaction [5]. These ROS have the potential to damage critical cellular components and to modulate signaling pathways, and thus, have the potential to cause cell death or generate neoplastic transformation [6]. In order to maintain redox balance, biological systems have evolved an elaborated network of antioxidant defense systems that allow cells to cope with lethal oxidative environments. Recently, we showed that the mitochondrial isoenzyme of NADP$^+$-dependent isocitrate dehydrogenase (IDPm) is critical for maintaining the cellular redox environment via its ability to generate NADPH by catalyzing the oxidative decarboxylation of isocitrate to α-ketoglutarate [7]. NADPH is essential for the regeneration of reduced glutathione (GSH) by glutathione reductase and for the activity of the NADPH-dependent thioredoxin system [8,9], which are both important for the protection of cells from oxidative damage. In this respect, reduced IDPm activity could disrupt redox homeostasis and it may induce cell death or increase tumor cell sensitivity to other anticancer agents.

In the present study, we investigated the role of IDPm in ionizing radiation-induced cell death by transfecting the A172 glioma cell line with the small interfering RNA (siRNA) of IDPm. The knockdown of IDPm in A172 cells by siRNA transfection was found to enhance ionizing radiation-induced autophagy. Furthermore, we found that the inhibition of autophagy increased the apoptosis of irradiated IDPm siRNA transfected A172 cells. The sensitizing effect of IDPm siRNA and of an autophagy inhibitor on the ionizing radiation-induced apoptosis of glioma cells offers a novel redox-active therapeutic strategy for the treatment of cancer.

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2. Materials and methods

2.1. Materials

β-$NADP^+$, isocitrate, chloroquine (CQ), acridine orange, *N*-acetylcysteine (NAC), PEG-SOD, propidium iodide (PI), Trichostatin A (TSA), and 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) were obtained from Sigma Chemical Co. (St. Louis, MO). 2′,7′-Dichlorofluoroscin diacetate (DCFH-DA) was purchased from Molecular Probes (Eugene, OR). Antibodies were purchased from Santa Cruz (Santa Cruz, CA) and Cell Signaling (Beverly, MA). A peptide representing the N-terminal 16 amino acids of mouse IDPm (ADKRIKVAKPVVEMPG) was used to prepare polyclonal anti-IDPm antibodies [7].

2.2. Knock-down of IDPm by siRNA

IDPm siRNA and scrambled siRNA were purchased from ST Pharm. (Seoul, Korea). The sequences of the dsRNAs of IDPm and control used in the current experiments were as follows. For IDPm, sense and antisense siRNAs were 5′-AGACCGACUUCGACAAGAA dTdT-3′ and 5′-UUCUUGUCGAAGUCGGUCUdTdT-3′, respectively, and for the scrambled control, sense and antisense siRNAs were 5’-CUGAUGACCUGAGUGAAUGdTdT-3′ and 5′-CAUUCACUCAGGU CAUCAGdTdT-3′, respectively. A172 cells were transfected with 20 nM of oligonucleotide by using Lipofectamine (Invitrogen) in serum-free conditions, according to the manufacturer's instructions. After incubation for 2 days, the cells were washed and supplemented with fresh medium containing 10% FBS.

2.3. Reverse transcription-polymerase chain reaction (RT-PCR) analysis

RNA was isolated using the RNeasy kit (Qiagen, Hilden, Germany), and total RNA (1 μg) was reverse-transcribed into cDNA using a first-strand cDNA synthesis kit (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. The cDNA template was then amplified by quantitative reverse transcription-polymerase chain reaction (RT-PCR) performed using the Perkin-Elmer GeneAmp PCR System 2400 (Perkin-Elmer Cetus, Emeryville, CA), according to the manufacturer's instructions. The primer sequences used were as follows: *β-actin* forward 5′-TCTACAATGAGCTGCGTGTG-3′, reverse 5′-ATCTCCTTCTGCATCCTGTC-3′; and *IDPm* forward 5′-ATCAAGGAGAAGCTCATCCTGC-3′, reverse 5′-TCTGTGGCCTTGTACTGGTCG-3′. β-Actin was used as an internal control. Amplified DNA products were resolved on 1% agarose gels, which were subsequently stained with ethidium bromide.

2.4. Cell culture and cytotoxicity

The A172 human glioblastoma cell line was purchased from the American Type Culture Collection (ATCC, Manassas, VA). Cells were grown in RPMI 1640 medium containing 10% (v/v) FBS, penicillin (50 units/ml), and 50 μg/ml streptomycin at 37 °C in an atmosphere of 5% CO_2/95% air atmosphere. Cells were irradiated at room temperature using a ^{137}Cs source at a dose rate of 1 Gy/min. Cell viability following exposure to ionizing radiation was assessed using a trypan blue dye exclusion assay.

2.5. Enzyme assays

IDPm activities were measured by determining NADPH levels at 340 nm and 25 °C, as described previously [7]. For caspase-3 activity assays, cells were collected with a cell scraper, washed with PBS, mixed with lysis buffer (50 mM HEPES, pH 7.4, 100 mM NaCl, 0.1% CHAPS, 1 mM DTT, and 0.1 mM EDTA) for 10 min at 0 °C, and centrifuged at 10,000*g* for 10 min at 4 °C. Supernatant aliquots containing 100 μg of protein were then added to the reaction buffer (100 mM HEPES, pH 7.4, 0.5 mM PMSF, 10 mM dithiothreitol, 1 mM EDTA, and 10% glycerol) containing the chromogenic peptide substrate Ac-DEVD-pNA (Calbiochem, San Diego, CA) and incubated for 2 h at 37 °C. Absorbances were then monitored at 405 nm to determine caspase activities.

2.6. Detection of acidic vesicular organelles and GFP–LC3 translocation

Acidic vesicular organelle (AVO) formation, a morphological characteristic of autophagy, was detected by acridine orange staining. A172 cells were stained with 1 μg/ml of acridine orange for 15 min and samples were observed under a Zeiss Axiovert 200 inverted microscope (excitation, 546 nm; emission, 575/640 nm). Autophagy was also quantified by calculating the percentage of cells showing green fluorescent protein GFP–LC3 accumulation in aggregates or vacuoles [10]. Cells were transfected with 2 μg of GFP–LC3 expression plasmid using Lipofectamine 2000 and the distribution and fluorescence of GFP–LC3 was visualized by fluorescence microscopy. Cells with a mostly diffuse distribution of GFP–LC3 in cytoplasm and nucleus were considered non-autophagic, whereas cells with several intense punctate GFP–LC3 aggregates with no nuclear GFP–LC3 fluorescence were considered autophagic.

2.7. FACS

A172 cells were collected at 2000*g* for 5 min, washed twice with cold PBS, fixed in 70% ethanol for at least 2 h at −20 °C, decanted ethanol by centrifuge, and stained with 1 ml of PI staining solution (50 μg PI, 100 units RNase A, 1.5% Triton X-100) for at least 1 h in the dark at 4 °C. Labeled cells were analyzed by flow cytometry.

2.8. Cellular redox status

NADPH levels was measured using an enzymatic cycling method as previously described [7], and expressed as ratios versus the total NADP pool. Total glutathione concentrations were determined by measuring the rate of formation of 5-thio-2-nitrobenzoic acid at 412 nm ($\varepsilon = 1.36 \times 10^4\ M^{-1}\,cm^{-1}$), as described previously [7], and oxidized glutathione (GSSG) levels was measured using a DTNB–GSSG reductase recycling assay after treating GSH with 2-vinylpyridine [7]. Intracellular ROS production was measured using the oxidant-sensitive fluorescent probe DCFH-DA using a fluorescence-activated cell sorter (FACS) unit.

2.9. Immunoblot analysis

Whole cell lysates were separated by 10–12.5% SDS–PAGE, and proteins were electrotransferred to nitrocellulose. Membranes were incubated with the desired primary antibody and then treated with horseradish peroxidase-labeled anti-rabbit IgG. Immune-reactive bands were visualized using an enhanced chemiluminescence detection kit (Amersham Pharmacia Biotech, Buckinghamshire, UK).

2.10. Statistical analysis

All experiments were performed at least three times. Results are expressed as means ± SD. The Student's *t*-test was used to determine the significances between two mean values, and p values of <0.05 were considered statistically significant.

3. Results and discussion

RNA interference (RNAi) using small RNA duplexes, provides a powerful means of analyzing gene function in mammalian cells [11]. Since ionizing radiation is commonly used in combination with other therapeutic modalities to treat brain cancer patients, we evaluated whether IDPm siRNA enhances the therapeutic potential of γ-radiation using the A172 human glioma cell line. The transient transfection of *in vitro*-transcribed siRNA targeting the human IDPm gene was found to down-regulate IDPm effectively at the protein (Fig. 1A) and mRNA levels (Fig. 1B). The IDPm siRNA-transfected A172 cells exhibited ~75% less IDPm activity than scrambled siRNA-transfected control cells. However, the activity of cytosolic $NADP^+$-dependent isocitrate dehydrogenase (IDPc) was not affected by the transfection of IDPm siRNA. The decreased activity caused by knockdown of IDPm siRNA was also observed in U87MG glioma cell line (Fig. 1C). When cultured A172 cells were treated with 5 Gy γ-radiation, an increase in cell death was observed, but A172 cells transfected with IDPm siRNA were significantly more sensitive than control cells in this respect (Fig. 1D).

In vitro anticancer activity is due to induction of the apoptotic program, G_1 arrest, and reduced DNA synthesis [12]. To determine whether IDPm knockdown induces cell cycle arrest, we analyzed the effect of IDPm siRNA plus ionizing radiation on the cell cycle distribution using PI staining and flow cytometry. When A172 cells transfected with IDPm siRNA were treated with 5 Gy of γ-radiation, the population of cells in the G_1 phase increased significantly (Fig. 1E). Further examination of molecular markers associated with G_1 arrest showed a remarkable up-regulation of p21, a cyclin-dependent kinase inhibitor that mediates cell cycle arrest (Fig. 1F), suggesting that knockdown of IDPm plus ionizing radiation induces G_1 arrest. The PI3K/Akt pathway is a cell survival pathway and is important for normal cell growth and proliferation [13]. This pathway has also been implicated in tumorigenesis [14] and is becoming an important cancer therapy target [15,16]. PI3K/Akt signaling is frequently activated in various types of cancers, and therefore, represents a major cell survival pathway. In this respect, the activation of PI3K/Akt signaling has long been associated with malignant transformation and apoptotic resistance [17,18]. As shown in Fig. 1F, control cells were more resistant to the antiproliferative effects of ionizing radiation than IDPm siRNA transfected cells, and this resistance was correlated with higher phosphorylated Akt levels.

ROS can initiate several cellular signal transduction pathways that may help cells cope with excess oxidative stress resulting from exposure to ionizing radiation or set into motion pathways that lead to the irretrievable cell damage [4,19]. Therefore, several reports have suggested that antioxidant enzymes may prevent the oxidative damage and cell death caused by ionizing radiation [20,21]. Levels of intracellular peroxides in the A172 cells were evaluated by FACS using the oxidant-sensitive probe DCFH-DA. As shown in Fig. 2A, an increase in DCF fluorescence was observed in A172 cells when they were exposed to 5 Gy of γ-radiation, and this increase in fluorescence was significantly greater in IDPm siRNA transfected cells. The ratio of GSSG to GSH plus GSSG (GSHt) is important parameter of GSH metabolism and may reflect the efficiency of GSH turnover [20]. When the cells were exposed to ionizing radiation, the ratio of cellular GSSG/GSHt was increased in IDPm siRNA-transfected cells compared to control (Fig. 2B). NADPH is an essential factor of the cellular defense system against oxidative stress, and we found that the [NADPH]/[$NADP^+$ + NADPH] ratio was significantly lower in A172 cells exposed to ionizing radiation. This decrease in was much more pronounced in IDPm siRNA-transfected cells (Fig. 2C). The link between the prooxidant state

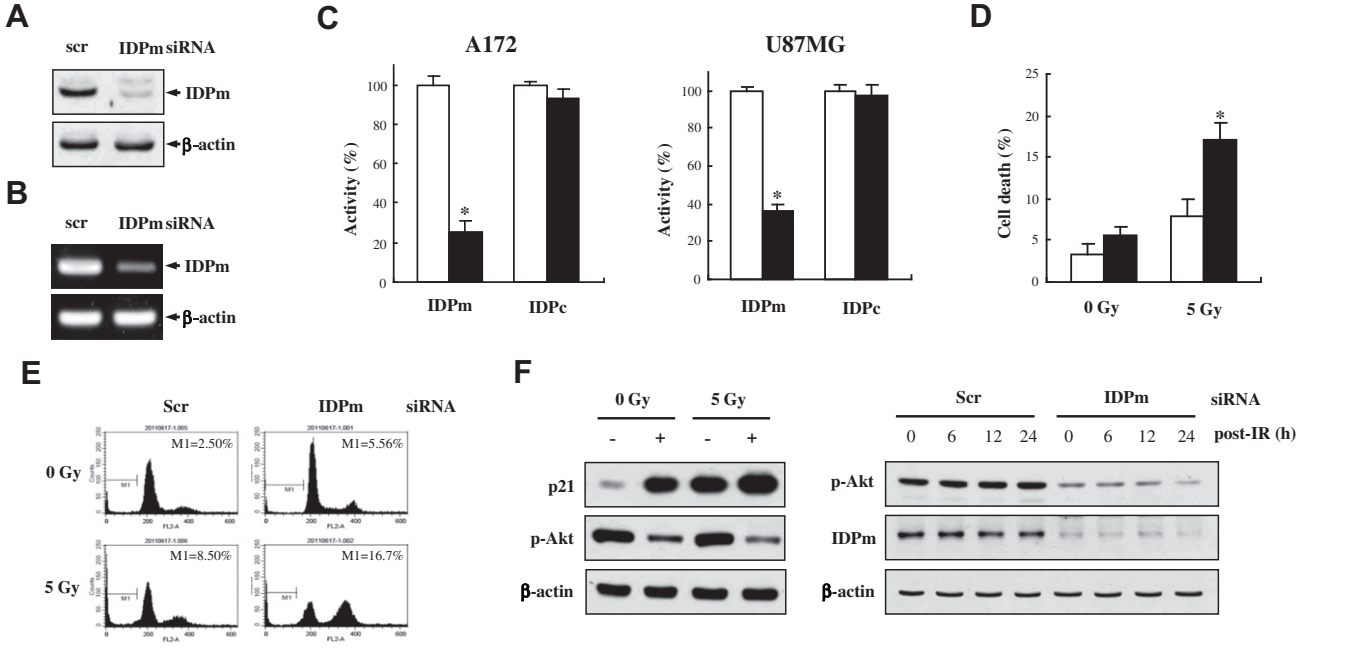

Fig. 1. Knockdown of IDPm by siRNA in A172 cells. (A) A172 cells were transfected with a control scrambled siRNA (scr) or siRNA targeting *idh2* (IDPm), and then IDPm expression was analyzed by immunoblotting using an anti-IDPm IgG. β-Actin was used as a loading control. (B) RT-PCR analysis of IDPm expression in A172 transfectant cells. Total RNA was isolated from the indicated transfected cells and IDPm mRNA levels were determined by RT-PCR followed by agarose gel electrophoresis. β-Actin was used as a control. (C) Activities of IDPm and IDPc in A172 and U87MG cells transfected with scrambled siRNA (*open bars*) or IDPm siRNA (*shaded bars*). Activities are given as percentages of the control values. Data represent the means ± SD of three independent experiments. $^*p < 0.01$ vs. control cells. (D) Forty-eight hours after A172 cells transfected with scrambled siRNA (*open bars*) or IDPm siRNA (*shaded bars*) exposed to 5 Gy of γ-irradiation, cell viabilities were determined using a trypan blue dye exclusion assay. Data represent the means ± SD of three independent experiments. $^*p < 0.01$ vs. irradiated control cells. (E) Knockdown of IDPm plus exposure to ionizing radiation resulted in G_1 arrest in A172 cells. DNA content analysis of A172 transfectant cells exposed to 5 Gy of γ-radiation. (F) Immunoblot analysis of p21 and pAkt. β-Actin was used as an internal control.

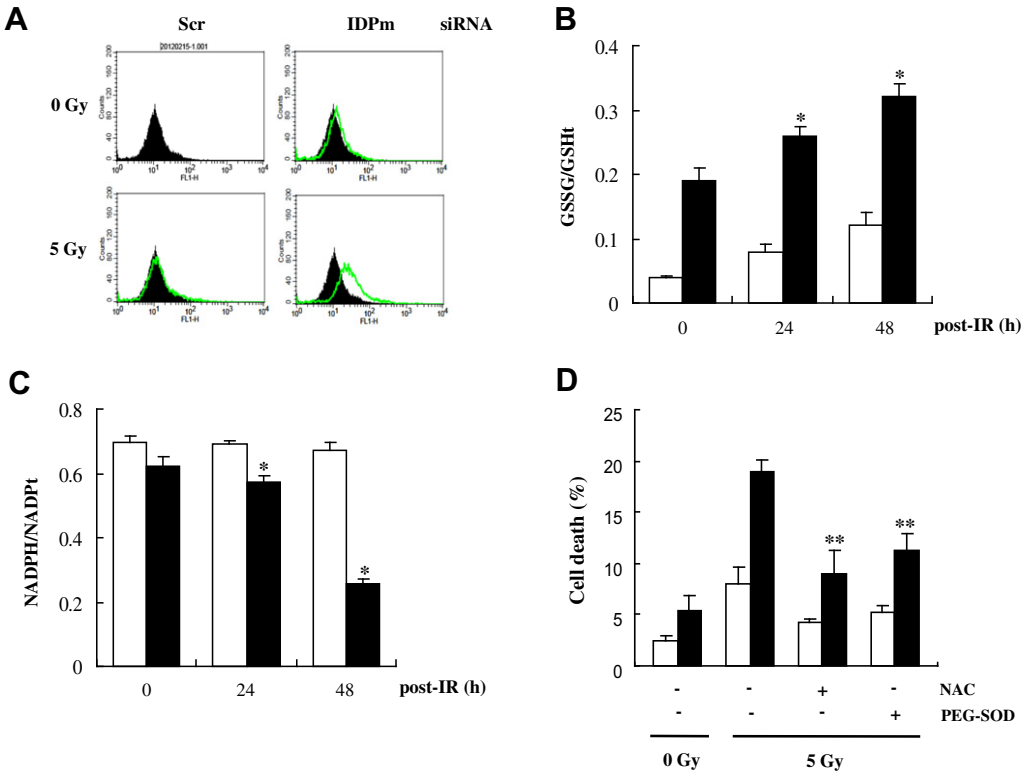

Fig. 2. Effects of IDPm knockdown on cellular redox status. (A) DCF fluorescence was measured in A172 transfectant cells. ROS was detected using DCFH-DA and flow cytometry. (B) GSSG vs. the total GSH pool in A172 transfectant cells. (C) NADPH vs. total NADPH pool in A172 transfectant cells. (D) Viabilities of A172 transfectant cells as determined by trypan blue dye exclusion assay. (B–D) Open and shaded bars represent control and IDPm siRNA-transfected cells, respectively. Data represent the means ± SD of three independent experiments. $^{*}p < 0.01$ vs. scrambled siRNA transfected cells exposed to ionizing radiation. $^{**}p < 0.01$ vs. IDPm siRNA transfected cells exposed to ionizing radiation.

and ionizing radiation-induced cell death in IDPm siRNA-transfected cells was further confirmed by the blocking effect of NAC, a glutathione precursor and scavenger of ROS, and PEG-SOD (Fig. 2D).

Although IDPm knockdown triggered G_1 phase arrest, it did not induce apoptosis during the early stages of ionizing radiation-induced cell death, as determined by immunoblotting for caspase-3 and PARP and by spectrophotometric measurements of caspase-3 activation (Fig. 3A). Recent investigations indicate that chemotherapeutic drugs, or any of several other anticancer stimuli, induce autophagy but not apoptosis in various cancer cells [22]. Furthermore, the cytotoxicities of many anticancer agents are mediated by autophagy activation associated with G_1 arrest [12,23,24]. The process of autophagy starts with autophagosome formation and then progresses to autophagolysosomes via the fusion of acidic lysosomes and autophagosomes [25]. In order to confirm that IDPm knockdown induces the autophagic pathway, acridine orange staining of live cells was employed to visualize acidic autophagolysosomes in ionizing radiation treated IDPm transfectant A172 cells. As shown in Fig. 3B, an increase in acridine orange fluorescence was observed in A172 cells when they were exposed to ionizing radiation, and this increase in fluorescence was markedly elevated in IDPm siRNA transfected cells. The induction of autophagy by treatment with IDPm siRNA plus ionizing radiation was confirmed by monitoring A172 cells transfected with GFP–LC3 expressing vector. Fluorescence microscopy revealed a diffused localization of GFP–LC3 in control cells, whereas IDPm siRNA plus GFP–LC3 transfected cells exposed to 5 Gy of γ-radiation produced puntas of GFP–LC3 fluorescence (Fig. 3C).

To elucidate the involvement of autophagy in ionizing radiation-induced apoptosis, we examined the effects CQ, which inhibits lysosomal acidification and therefore prevents autophagy by blocking autophagosome fusion and degradation [26]. A significant increase in cell death was observed in CQ treated/IDPm siRNA transfected cells as compared with IDPm siRNA transfected cells after exposure to ionizing radiation (Fig. 4A). This CQ-mediated augmentation of ionizing radiation-induced apoptosis was confirmed by PARP cleavage and by the releases of cytochrome c and apoptosis inducing factor (AIF) from mitochondria (Fig. 4B), the latter is a critical event that provokes a cascade of caspases and eventually irreversible cell death [27]. Collectively, these findings suggest that the induction of autophagy has a pro-survival role and delays cell death after ionizing radiation-induced injury to A172 cells. Autophagy may act as a protective mechanism for malignant cells or exhibit opposing effects and promote generation of antineoplastic responses, depending on the cellular context and/or initiating stimulus [28]. Although autophagy has been regarded as a tumor repressive process [12,29], it was recently reported that the inhibition of autophagy enhanced apoptosis, which suggests that autophagy may be a protective response against anti-cancer agents that contribute to tumor progression [29,30–32]. In this context, the autophagic pathway is a novel therapeutic target in cancer [30–32]. Furthermore, autophagy inhibition has been reported to enhance the anticancer effects of arsenic trioxide [33], hyperthermia [33], sulforaphane [34], and of p53 or alkylating drugs [35]. These studies suggest that the induction of autophagy during the initial period of ionizing radiation insult provides an appropriate environment for the maintenance of cellular homeostasis before the threshold of ionizing radiation-induced cell death is reached. Therefore, during the initial stages after ionizing radiation injury, autophagy may function efficiently by eliminating unwanted or damaged organelles and other cytoplasmic macromolecules to establish cellular homeostasis.

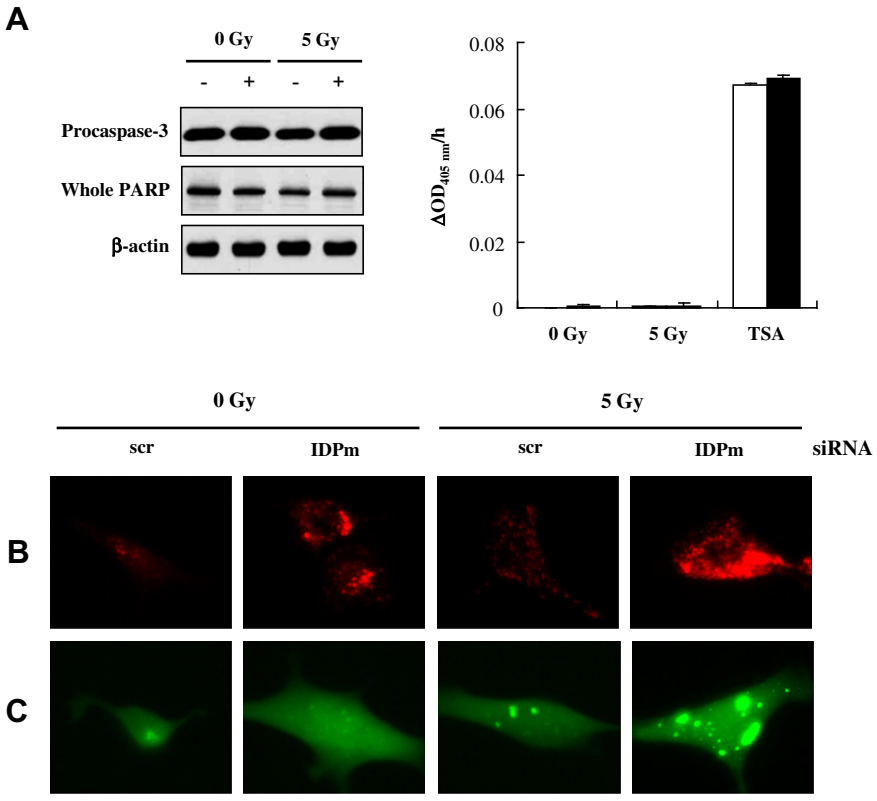

Fig. 3. Ionizing radiation triggered autophagy in A172 transfectant cells. (A) Immunoblot analysis of procaspase-3 and whole PARP. β-Actin was used as an internal control. Caspase-3 protease activation in A172 transfectant cells exposed to ionizing radiation was measured using caspase colorimetric activity. The result of TSA was presented as a positive control of apoptosis. Open and shaded bars represent control and IDPm siRNA-transfected cells, respectively. Data represent the means ± SD of three independent experiments. (B) Ionizing radiation-induced formation of autophagic vacuoles in A172 transfectant cells. Acridine orange staining was used to detect the formation of autophagic vacuoles in A712 cells exposed to 5 Gy of γ-radiation. (C) Analysis of LC3 aggregation in A172 transfectant cells. A172 cells transiently transfected with GFP–LC3 using Lipofectamine 2000 (Invitrogen) were cultured in RPMI with 10% FBS. Accumulations of GFP–LC3 puncta were observed in ionizing radiation exposed transfectants.

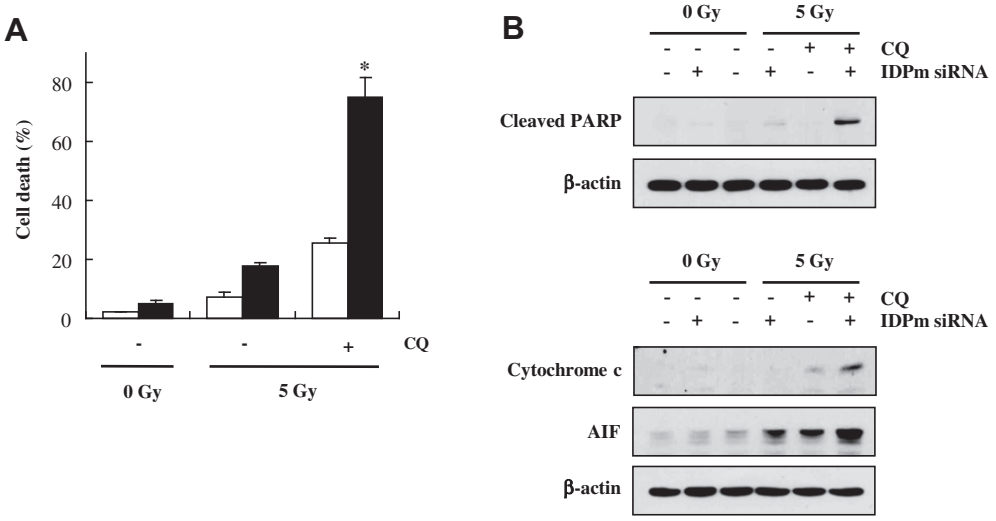

Fig. 4. Autophagy inhibition by CQ (30 μM) enhanced the ionizing radiation-induced apoptosis of A172 transfectant cells. (A) Viabilities of A172 transfectant cells as determined by trypan blue dye exclusion assay. Open and shaded bars represent control and IDPm siRNA-transfected cells, respectively. Data represent the means ± SD of three independent experiments. *$p < 0.01$ vs. IDPm siRNA transfected cells exposed to ionizing radiation. (B) Immunoblot analysis of cleaved PARP, cytochrome c and AIF. β-Actin was used as an internal control.

The present study demonstrates that knockdown of IDPm induces G_1 cell cycle arrest and autophagy during the early stage after exposure to ionizing radiation. Furthermore, the study shows autophagy offers cells a survival mechanism, and that combined treatment with IDPm siRNA and an autophagy inhibitor enhances the therapeutic efficacy of ionizing radiation by promoting apoptosis. Finally, our findings suggest that knockdown of IDPm in the presence of an autophagy inhibitor may provide an effective means of overcoming glioma cell resistance to radiotherapy.

Acknowledgments

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