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The role of autophagy in cell survival from heavy ion irradiation in the plateau region*

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To study cytotoxic effect of heavy ion irradiation in the plateau region, and investigate whether autophagy induced by heavy ion irradiation is cytoprotective, HeLa cells were irradiated with 350 MeV/u carbon ions beams, and the clonogenic survival was analyzed. The results showed that cell survival decreased with increasing doses. It was also found that G2/M-phase cells increased, and the autophagy-related activity was significantly higher than the control. When autophagy was blocked by 3-methyladenine in carbon-ion irradiated cells, G2/M phase arrest and the percentage of apoptosis cells were further elevated, and cell survival decreased significantly, indicating the induction of cytoprotective autophagy by carbon-ion irradiation. Our results demonstrated that autophagy induced by carbon ion irradiation provided a self-protective mechanism in HeLa cells, short-time inhibition of autophagy before carbon-ion irradiation could enhance radiation cytotoxicity in HeLa cells.

Keywords: Carbon ions irradiation, Cell survival, Apoptosis, Autophagy

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I. INTRODUCTION

It is well established that ionizing radiation (IR) can be an effective treatment of many types of cancers. IR induces cell death through evoking a series of events inside the cell, including many important cellular processes, such as DNA damage and repair, apoptosis, cell cycle control, oxidative stress response, etc. [1–3].

Radiotherapy with high LET (linear energy transfer) heavy ion beams is advantageous because of their Bragg peak and increased RBE (relative biological effectiveness) within the peak. Heavy ion beams allow precise delivery of high doses to tumors while minimizing radiation damage to normal tissues, hence a better cancer radiotherapy method than conventional methods of low-LET γ- and X-rays, of which the absorbed dose usually undergoes an exponential attenuation along the penetration depth in patient bodies, causing serious damage to normal tissues around the tumor [4, 5]. The clinical carbon ion radiotherapy has been proved successful, particularly in the deep-seated tumours that cannot be completely eradicated by surgery [6, 7].

Autophagy is an evolutionarily conserved process that lysosomes degraded cytoplasm and cellular organelles to recycle amino acid and energy. Its main function is to maintain intracellular metabolic homeostasis [8]. In some conditions, autophagy is a survival pathway that response to nutrient deprivation and stressful stimuli [9]. However, persistent activation of autophagy can cause depletion of organelles and critical proteins in cells, leading to a specific form of programmed cell death [10].

It was reported that an alternative response to γ-rays irradiation involves the lysosomal compartment, in addition to DNA damage and repair, apoptosis, and cell cycle control [11]. Yao *et al*. [12] found that γ-rays could induce autophagy, regardless of the relative radiosensitivity of the cell line. High-LET radiation has been reported to disrupt the cellular architecture, leading to autophagy induction in mouse skeletal muscles [13]. An understanding of the molecular responses of tumor cells to administration of carbon ions radiation may offer potential new targets for future therapy.

In this study, we irradiated Hela cells with high energy carbon ion beams (350 MeV/u, corresponding LET is 11 keV/μm in water) in the plateau region, and evaluated the responses of HeLa cells to carbon ions radiation; we inhibited autophagic sequestration with 3-methyladenine in irradiated cells, and assessed the effect of blocked autophagy on cell response to radiation by detecting cell survival, cell cycle progression and apoptosis. The results may help to understand the response of tumor cells to heavy ion irradiation.

II. EXPERIMENTAL PROCEDURES

A. Tumor cell lines and reagents

Hela cell line, human cervical carcinoma cell, was preserved by our laboratory. Acridine orange and 3-methyladenine were purchased from Sigma. Cellular DNA flow cytometric analysis kit was purchased from Multisciences (CCS012). Apoptosis detection kit was purchased from Beyotime (C1063-2). Rabbit polyclonal anti-Beclin 1 antibody was purchased from Abcam (ab16998). Fluorescein-labeled affinity purified antibody to rabbit IgG(H+L) produced in goat was purchased from Gaithersburg(02-15-06).

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B. Cell culture, carbon ion irradiation and grouping of the cell samples

HeLa cells were grown under standard conditions in RPMI 1640 medium, supplemented with 10% fetal bovine serum and 100 μg/mL streptomycin, and 100 U/mL penicillin at 37 °C, saturated humidity atmosphere and 5% CO_2/95% air atmosphere.

Exponentially growing HeLa cells in Petri dishes were irradiated in the plateau region using 365 MeV/u (LET=11 keV/μm) carbon ion beams from the Heavy Ion Research Facility in Lanzhou (HIRFL) at Institute of Modern Physics, Chinese Academy of Sciences. The dose rate was 1 Gy/min.

The irradiated cells were incubated for further cultivation and were harvested by trypsinizing with 0.25% trypsin in phosphate-buffered saline (PBS) at 24 h or 48 h after irradiation. For description convenience, a group of irradiated cells is termed with the irradiation dose, for example, the 2 Gy group.

In the autophagy inhibition experiment, 3-methyladenine (3-MA) was used. The cells were pre-treated for 3 hours with 5 mM 3-MA before 2 Gy carbon ion irradiation. After irradiation, the cells were further cultured for 24 h, and harvested by trypsinizing, or cultured for another 24 h in fresh medium without 3-MA. The sample group is termed as 3-MA+2 Gy group. The cells treated with just 3-MA is named as the 3-MA group, while the sample group without any of the two treatments is the control group.

C. Clonogenic survival assay

After 48 h irradiation, the cells treated with various modalities were seeded in Φ60 mm plates, incubated for 10 days. Cells were fixed with ethanol, and stained with 0.25% crystal violet, and colonies that contained $\geq$ 50 cells were counted under microscope [14].

D. Cell cycle analysis

Flow cytometry was performed by propidium iodide staining according to the manufacturer's instructions. Collected cells were washed in phosphate-buffered saline and fixed in ice-cold ethanol. Fixed cells were pelleted and resuspended in 500 μL of phosphate-buffered saline. Then the cells were stained with propidium iodide and analyzed for cell-cycle distribution by flow cytometry (Becton Dickinson). The percentage of each phase was evaluated by Modfit software.

E. Analysis of apoptosis

Apoptosis was evaluated by staining of cells with Annexin V/PI. FITC-Annexin V bound to the apoptotic cells with exposed phosphatidylserine, and PI labeled the cells with membrane damage. The percentage of apoptosis cells was quantized by flow cytometry (Becton Dickinson).

F. Detection of autophagy-related activity

For detection of autophagic cells by staining with acridine orange, the cells were incubated with medium containing 50 μg/mL acridine orange for 15 min. The acridine orange was removed. The cells were washed once with PBS, fresh media was added, and fluorescent micrographs were taken using an Olympus fluorescent microscope. All images presented are at the same magnification. The number of cells with increased acidic vesicular organelles (AVO) was determined by flow cytometry (Becton Dickinson) [8].

Autophagy-related protein expression of Beclin 1 was evaluated by flow cytometry. Briefly, cells were collected, fixed, and incubated with rabbit polyclonal anti-Beclin 1 antibody for 12 h in 4 °C,washed with PBS and labeled with a goat anti-rabbit IgG conjugated with fluorescein isothiocyanate (FITC) for 1h. Finally, the cells were washed in PBS, and the expression rate of Beclin 1 was detected using Flow cytometry (Becton Dickinson).

G. Statistical analysis

The survival data were fitted to the linear-quadratic model: $\ln S = -\alpha D - \beta D^2$, where S is the survival fraction, D is the irradiation dose, and α and β are coefficients of the radiation killing contribution by a single and double hit, respectively.

Statistical significance was analyzed by student t-test. $p \leq 0.05$ was considered statistically significant.

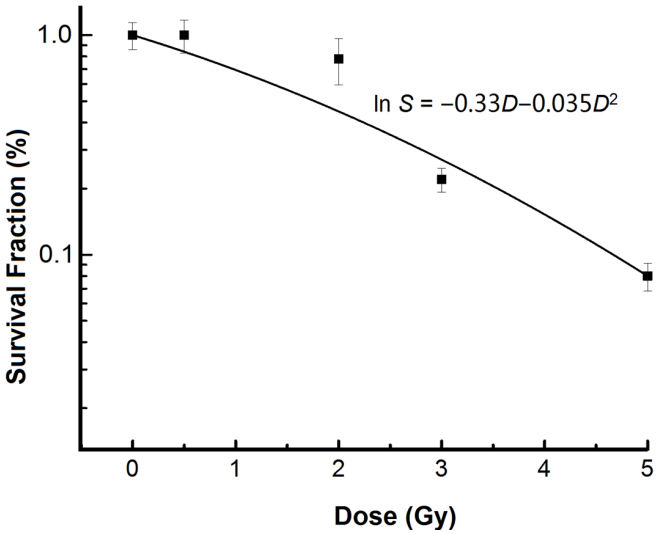

Fig. 1. Effect of carbon-ion irradiation on HeLa cell survival. The irradiated cells were incubated for 10 days to count the colonies. The data (mean ±SD) were averaged from three parallel experiments and fitted to the model of $\ln S = -\alpha D - \beta D^2$

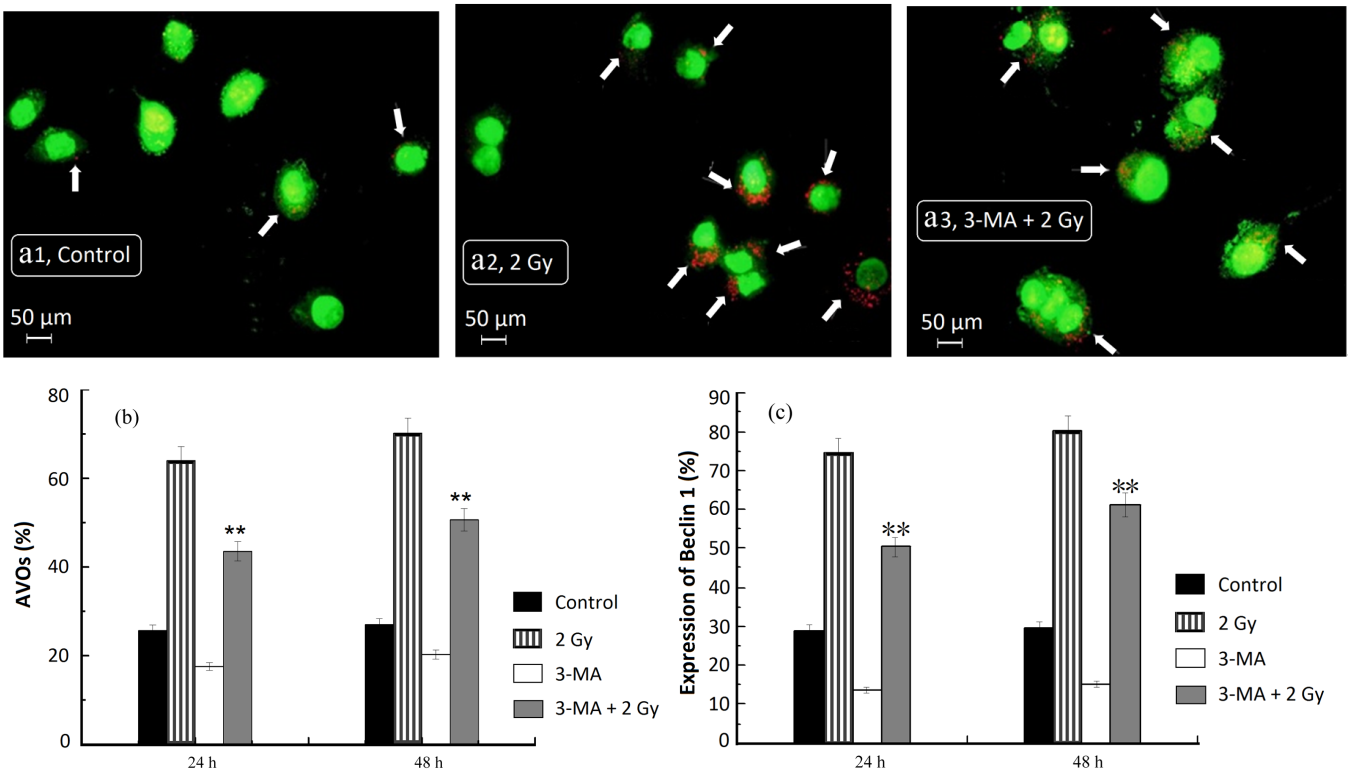

Fig. 2. (Color online) Autophagy (arrows in Fig. 2(a)) in Hela cells treated with 5 mM3-MA inhibition, 2 Gy carbon-ion irradiation, and their combination (3-MA+2 Gy). The cells were harvested 24 h or 48 h after irradiation, and detected with 50 g/mL acridine orange staining, (a) Typical fluorescent micrograph of autophagy taken by an Olympus fluorescent microscope ($\times$400). (b) The acidic vesicular organelles, and (c) expression of Beclin 1 determined by flow cytometry. Data were shown as the means $\pm$SD of three parallel experiments (*p $<$ 0.05; **p $<$ 0.01 *vs*. the 2 Gy group).

III. RESULTS

The cell survival 48 h after carbon-ions irradiation was evaluated with clonogenic survival assay. The results showed that cell survival decreased with increasing dose (Fig. 1), or the cytotoxic of carbon-ion irradiation increased with dose. The survival data were fitted to the linear-quadratic model, and the α and β values of HeLa cell line were 0.33 and 0.035, respectively, with $R^2 = 0.9$.

A. Induction of autophagy in Hela cells by carbon ions, and autophagy inhibition by 3-MA

Acidic vesicular organelle (AVO), a marker of autophagy, can be detected and measured by vital staining of acridine orange. Acridine orange moves freely to cross biological membranes and accumulates in acidic compartment, where it is seen as fluorescence bright red (Fig. 2(a)) [11, 15, 16]. To determine autophagy in Hela cells of the 2 Gy and 3-MA + 2 Gy groups , vital staining with acridine orange was performed. The results showed that 2 Gy carbon ion irradiation increased the proportion of AVOs-positive cells, and the appearance of AVOs occurred in carbon ion irradiated Hela cells was partly inhibited by 3-MA (Fig. 2(b)).

B. The cytotoxic of carbon-ions irradiation

To obtain further support, Beclin 1 (an essential mediator of autophagy) expression was examined. Flow cytometry assay showed that the level of Beclin 1 increased rapidly in HeLa cells 24 h and 48 h after irradiation, while with the supplement of 3-MA, the expressions of Beclin 1 decreased (Fig. 2(c)).

C. Effect of autophagy inhibition on cytotoxic of carbon-ion irradiation

The autophagy inhibition on cytotoxic of carbon ion irradiation was performed by treating HeLa cells with carbon-ion irradiation alone or 3-MA inhibition before carbon ion irradiation. Clonogenic survival assay was done with the treated cells. In Fig. 3, for the 3-MA + 2 Gy group, the cell survival fraction decreased dramatically. The results indicated that the presence of AVOs may protect the cell against carbon ion irradiation-induced damage, and autophagy inhibition might be an approach to enhance the cytotoxic of carbon-ion irradiation.

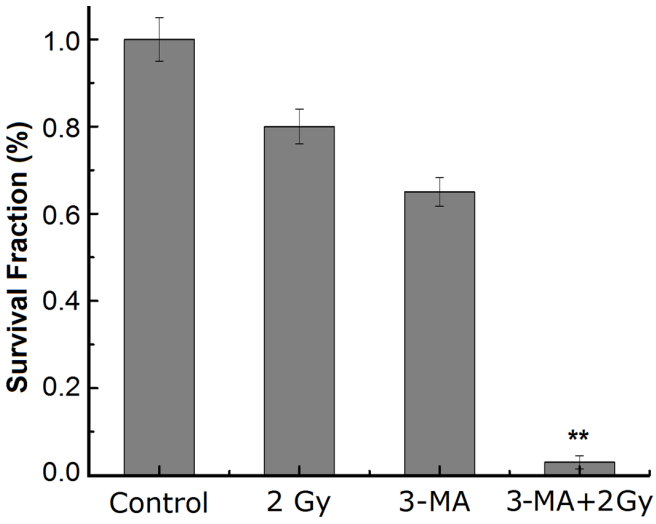

Fig. 3. Survival fractionof Hela cells treated with 5 mM 3-MA inhibition, 2 Gy carbon-ion irradiation, and their combination (3-MA+2 Gy). At 48 h after irradiation, the cells were incubated for 10 days and the colonies were counted. Data (mean $\pm$SD) were averaged from three parallel experiments (*p $<$ 0.05 **p $<$ 0.01 *vs.* the 2 Gy group).

D. Strengthened G2/M cell-cycle arrest and cell apoptosis

The analysis of FCM showed changes in cell cycle phase distribution of HeLa cells (Fig. 4(a)). THe G2/M phase arrest was observed in the 2 Gy group, while the G2/M phase arrest was further elevated in the 3-MA + 2 Gy group, after the 24 h 3-MA treatment (Fig. 4(b)). Even at 24 hours after removal of 3-MA (Fig. 4(c)), the level of G2/M phase arrest was the same.

Cell apoptosis was analyzed by staining of cells with Annexin V/PI. FITC-Annexin V bound to the apoptotic cells with exposed phosphatidylserine (green under fluorescence microscope, namely early apoptosis), PI labeled the cells with membrane damage (red or green and red under fluorescence microscope, namely necrosis or late apoptosis). The percentage of apoptosis cells was quantized by flow cytometry (Becton Dickinson). As shown in Fig. 5, the number of apoptotic cells of the 2 Gy group was lightly higher (no significance) than that of the control, while in 3-MA + 2 Gy group, the number of apoptotic cells increased after 24 hours of the 3-MA treatment, and it continued to increase 24 hours after removal of 3-MA.

IV. DISCUSSION

An understanding of molecular responses of cells to heavy ion irradiation may open a way for future therapy. The responses of Hela cells to carbon-ion irradiation in the plateau region (350 MeV/u) have been assessed.

The cell survival from the carbon-ion irradiation decreases with increasing doses in a linear-quadratic manner, which is consistent with previous studies [18–20]. This indicates that the cytotoxic of carbon-ions irradiation increases with dose (Fig. 1). For the cell samples exposed to 2 Gy carbon-ion irradiation, the number of apoptotic cells differ little from the control (Fig. 5), but the autophagy activity is significantly higher than the control (Fig. 2).

Autophagy, activated by environmental factors, is a pathway characterized by the formation of membrane-bound vacuoles in the cytoplasm [21]. The autophagic vacuoles sequester cytosolic components have been implicated in cellular defense mechanisms such as protection against infectious agents, drug resistance [22], and survival under starvation conditions [9].

The induction of autophagy by γ-irradiation was reported by Paglin *et al.* [11] and Yao *et al.* [12]. It was also reported that, in human breast adenocarcinoma, human colon adenocarcinoma and human prostate carcinoma cell lines, the progenies of γ-irradiated cells contain an increased level of AVOs [11]. Our findings indicated that the carbon ion irradiation effectively induce accumulation of AVOs and elevated level of autophagy-related protein expression in HeLa cell line (Fig. 2).

Studies have shown that autophagy represents a mechanism of resistance to radiation-mediated cell death; the inhibition of autophagy process could lead to enhanced cytotoxicity of radiotherapy in cancer cells and increase the radiosensitivity [23–26]. Autophagic vacuoles protect the cells against radiation damage by preventing cytoplasmic acidification, by providing catabolites required for repair processes, and by removing dysfunctional mitochondria and endoplasmic reticulum. Our results indicate that autophagy in HeLa cells induced by carbon-ion irradiation could be reduced by pretreatment of 5 mM 3-MA (Fig. 2), and cytoprotective effect has limited synergistic damage to the cells (Fig. 3). The cell cycle was significantly blocked at G2/M phase (Fig. 4), which was conducive to radiation-induced cell killing; and cell apoptosis was more serious (Fig. 5). Maybe, cell death occurred through apoptosis under conditions of nutrient depletion if autophagy is inhibited [10]. Our results suggested that autophagy might be a pro-survival pathway in 2 Gy carbon-ion irradiated HeLa cells, and sufficient autophagy block with adequate time duration could cause an enhanced radiosensitizing effect. However, more studies are needed to support the conclusion.

Apoptosis was thought to be a major type of cell death. Now, autophagic cell death or "Type II programmed cell death", is described as an alternative response to cellular damage [22, 27]. Also, autophagy is a mechanism of radiation-induced cell death. It was found that, after irradiation, human glioblastoma cells were authophagic rather than apoptotic [12], and greater autophagic death was induced when apoptosis pathway was blocked [28].

Peng *et al.* [29] reported that autophagic cell death, instead of apoptosis, was the main cell death pattern in the cell-killing effect on A549 cells with combined treatment of berberine and irradiation. Perhaps, continuous accretion of AVOs may negate their protective effect, and lead to eventual autophagocytosis and cell death [11].

The role of autophagy in cancer cells in response to can-

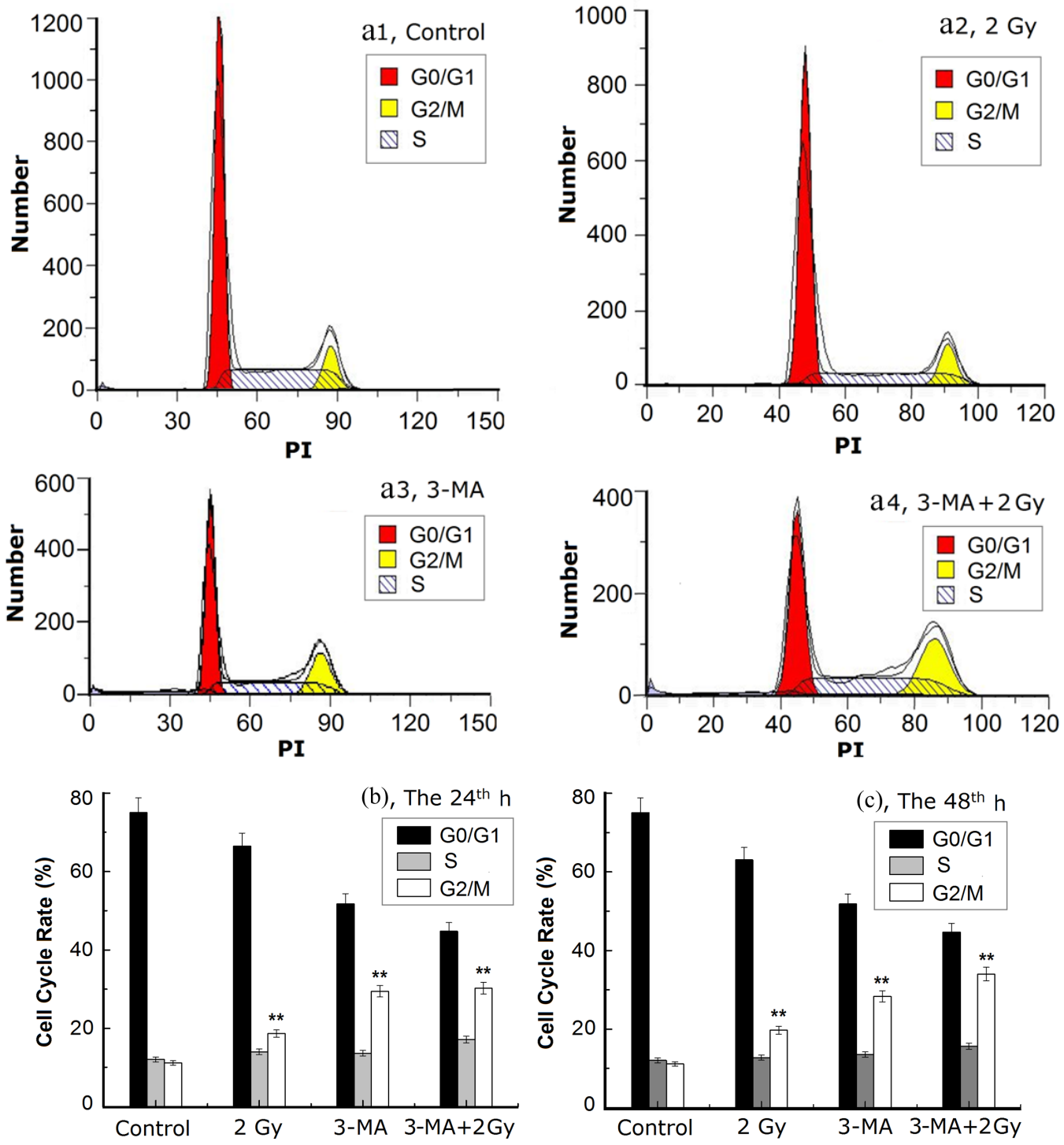

Fig. 4. (Color online) Cell cycle distribution of Hela cells treated with 5 mM 3-MA inhibition, 2 Gy carbon-ion irradiation and their combination (3-MA+2 Gy), analyzed by flow cytomtery 24 h or 48 h after irradiation. (a) histograms of flow cytometry analysis for cell population with counts. (b) and (c), the cell cycle rate in each phase of the cells at 24 h and 48 h, respectively. Data were shown as the means ±SD of three parallel experiments (*p $<$ 0.05; **p $<$ 0.01 *vs.* the 2 Gy group).

cer therapy is still a controversy [30, 31]. At least, one can regulate the radiation sensitivity, including heavy ion beams, through interference autophagy. In addition to relevance to radiation therapy, the study of biological effects of heavy-ion irradiation is also significant to radiation protection issues.

Probably the most obvious effect of irradiation on proliferating cells is induction of cell cycle arrest [32]. In this study, the cells irradiated to 2 Gy showed a G2/M cell-cycle arrest,

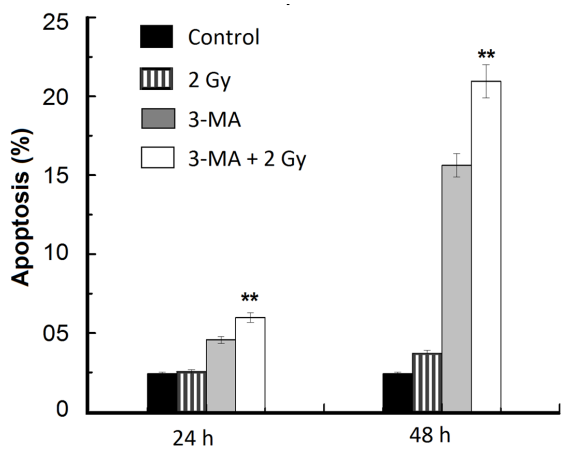

Fig. 5. Apoptosis in Hela cells treated with 3-MA inhibition, 2 Gy carbon-ion irradiation and their combination (3-MA+2 Gy). The number of cells with apoptosis was determined by flow cytometry. Data were shown as the means ±SD of three parallel experiments (*p < 0.05; **p < 0.01 *vs*. the 2 Gy group).

which was further elevated by the 3-MA treatment, and 24 h after removal of 3-MA, the level of G2/M phase arrest was the same (Fig. 5).

In conclusion, short-time inhibition of autophagy could enhance cytotoxicity of carbon-ion irradiation in HeLa cells, indicating that radiation-induced autophagy may provide a self-protective mechanism in HeLa cells against carbon ion radiation-induced damage. Autophagy inhibition enhances cytotoxicity of carbon-ions irradiation by strengthened G2/M cell-cycle arrest and cell apoptosis.

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[1] Amundson S A, Bittner M, Fornace A J Jr. Oncogene, 2003, **22**: 5828–5833.
[2] Anscher M S and Vujaskovic Z. Semin Oncol, 2005, **32**: 86–91.
[3] Feinendegen L E, Pollycove M, Neumann R D. Exp Hematol, 2007, **35**: 37–46.
[4] Nobuyuki H. J Radiat Res, 2009, **50**: 1–9.
[5] Kato S, Ohno T, Tsujii H, *et al*. Int J Radiat Oncol, 2006, **65**: 388–397.
[6] Tsujii H, Mizoe J, Kamada T, *et al*. J Radiat Res, 2007, **48**: A1–A13.
[7] Takahashi S, Kawase T, Yoshida K, *et al*. Acta Neurochir, 2009, **151**: 759–769.
[8] Tseng H, Liu W, Tyan Y, *et al*. Chemico-Bio Interact, 2011, **192**: 201–208.
[9] Mizushima N, Noda T, Yoshimori T, *et al*. Nature, 1998, **395**: 395–398.
[10] Boya P, Gonzalez-Polo R A, Casares N, *et al*. Mol Cell Biol, 2005, **25**: 1025–40.
[11] Paglin S, Hollister T, Delohery T, *et al*. Cancer Res, 2001, **61**: 439–444.
[12] Yao K C, Komata T, Kondo Y, *et al*. J Neurosurg, 2003, **98**: 378–384.
[13] Hino M, Wada S, Tajika Y, *et al*. Cell Struct Funct, 2007, **32**: 51–56.
[14] Wang Z Z, Li W J, Zhang H, *et al*. World J Gastroentero, 2006, **12**: 2601–2605.
[15] Traganos F and Darzynkiewicz Z. Methods Cell Biol, 1994, **41**: 185–194.
[16] Stankiewicz M, Jonas W, Hadas E, *et al*. Int J Parasitol, 1996, **26**: 445–446.
[17] Apel A, Herr I, Schwarz H, *et al*. Cancer Res, 2008, **68**: 1485–1494.
[18] Yang J S, Jing X G, Wang Z Z. Cent Eur J Biol, 2006, **3**: 451–462.
[19] Friedrich T, Scholz U, Els*ddot*asser T, *et al*. J Radiat Res, 2013, **54**: 494–514.
[20] Suzuki M, Kase Y, Yamaguchi H, *et al*. Int J Radiat Oncol, 2000, **48**: 241–50
[21] Bursch W. Cell Death Differ, 2001, **8**: 569–581.
[22] Altan N, Chen Y, Schindler M, *et al*. J Exp Med, 1998, **187**: 1583–1598.
[23] Apel A, Herr I, Schwarz H, *et al*. Cancer Res, 2008, **68**: 1485–1494.
[24] Benzina S, Altmeyer A, Malek F, *et al*. Cancer Lett, 2008, **264**: 63–70.
[25] Brech A, Ahlquist T, Lothe R A, *et al*. Mol Oncol, 2009, **3**: 366–375.
[26] Lomonaco S L, Finniss S, Xiang C, *et al*. Int J Cancer, 2009, **125**: 717–722.
[27] Bursch W, Ellinger A, Kienzl H, *et al*. Carcinogenesis, 1996, **17**: 1595–1607.
[28] Demasters G, Di X, Newsham I, *et al*. Mol Cancer Ther, 2006, **5**: 2786–2797.
[29] Peng P L, Kuo W H, Tseng H C, *et al*. Int J Radiat Oncol, 2008, **70**: 529–542.
[30] Eisenberg-Lerner A and Kimchi A. Apoptosis, 2009, **14**: 376–391.
[31] Tsuchihara K, Fujii S, Esumi H. Cancer Lett, 2009, 2**78**: 130–138.
[32] Bernhard E J, Maity A, Muschel R J, *et al*. Radiat Environ Bioph, 1995, **34**: 79–83.