Contribution of caspase-independent pathway to apoptosis in malignant glioma induced by carbon ion beams

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Abstract. High linear energy transfer (LET) carbon ion beam (CIB) is becoming the best tool for external radiotherapy of inoperable tumors because of its greater cell killing than conventional low LET gamma or X-rays. In the present study, whether the caspase-independent pathway exerts the important contribution in CIB-induced cell apoptosis was explored. Herein we showed, despite the absence of caspase activity using a pan caspase inhibitor Z-VAD-FMK, that apoptosis induced by high LET CIB were clearly observed in the glioma cells. Simultaneously, the increased 8-OHdG level, PARP-1 activity and AIF translocation occurred in response to CIB irradiation. Moreover, it was distinctly higher in the nuclear translocation frequency along with PARP-1 activation when the caspase protease cascade was suppressed in the irradiated glioma cells. Nuclear colocalization between PARP-1 and AIF as well as a positive association of the PARP-1 mRNA expression with AIF translocation frequency indicated that PARP-1 activation controlled the translocation of AIF to the nucleus. Our findings strongly demonstrated that caspase-independent cell apoptosis provided a prominent compensation in the glioma cell death involving the PARP-1/AIF signaling pathway at 24 h after CIB exposure, and likely triggered by oxidative damage to DNA. The knowledge on the molecular mechanism of AIF-mediated cell death may be very useful for the improvement of the therapeutic efficacy of malignant gliomas with heavy charged particles.

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Abbreviations: AIF, apoptosis inducing factor; PARP-1, poly(ADP-ribose) polymerase-1; CIB, carbon ion beam; LET, linear energy transfer; DAPI, 4', 6-diamidino-2-phenylindole; FITC, fluorescein isothiocyanate; PI, propidium iodide

Key words: apoptosis, CIB, caspase-independent pathway, apoptosis inducing factor, PARP-1, glioma

Introduction

Radiation therapy has remained the mainstay of treatment for glioblastoma, but these tumors are often associated with radioresistance (1). Heavy ions represent the best tool for various tumors, especially for radioresistant tumors mediated by hypoxia, due to several advantages over conventional radiotherapy (gamma and X-rays) such as an inverted depth-dose distribution, a higher relative biological effectiveness, a reduction in oxygen enhancement ratio, less variation in cell cycle related sensitivity and cells having decreased ability to repair (2).

Cell apoptosis is a key mechanism through which ionizing radiation kills tumor cells via two principal signaling pathways, the extrinsic death pathway involving the ligation of death receptors and the intrinsic death pathway initiated at the mitochondrion (3). Among them, intrinsic apoptosis seems to play a key part in the regulation of susceptibility of tumor cells to radiation (4). Caspases, a family of cysteine proteases, have been recognized as important mediators of apoptosis in the intrinsic apoptotic cascade. There is accumulating evidence indicating that heavy ion radiation can effectively trigger apoptosis as described in previous findings (5,6) which can be through activation of caspase-3 and caspase-9 (7); however, so far little information is available on the caspase-independent form of apoptosis.

Apoptosis-inducing factor (AIF), a mitochondrion-localized flavoprotein initially discovered as a caspase-independent death effector, is released in response to death stimuli, and subsequently results in chromatin condensation and large-scale fragmentation of DNA (8-10). There is definite evidence demonstrating that AIF has been shown to associate well with induction of cell death after ischemia/reperfusion (11), hypoglycemia (12), brain trauma (13) and neurodegenerative diseases (14).

Poly(ADP-ribose) polymerase 1 (PARP-1), the major isoform of the poly(ADP-ribose) polymerase family, is a constitutive nuclear and mitochondrial protein with well-recognized roles in base excision repair and DNA strand break repair (15). Over activation of PARP-1 is required for the translocation of AIF from the mitochondria to nucleus as an important activator of caspase-independent cell death (16-18). PARP-1 activity was reported to affect the radiation sensitivity of tumor cells by manipulation of the DNA repair processes (19), cell cycle (20) and autophagy (21) following exposure to ionizing radiation.

Among all high-LET heavy ion radiations, CIB is becoming popular and excellent in treatment of malignant tumors. In the current study we present preliminary data which could help to clarify the role of caspase-independent manner in CIB-induced glioma cell death by means of caspase inhibitor Z-VAD-FMK. Furthermore, PARP-1/AIF pathway might contribute to the development of more effective cancer radiotherapy. This study provides the supplement to the apoptosis mechanisms caused by the heavy ion radiation and demonstrates a potential benefit of therapeutic strategies for high-LET particle therapy.

Materials and methods

Cell culture and irradiation. Human glioblastoma multiform (GBM) U251 cell line was obtained from China Center for Type Culture Collection (CCTCC), Wuhan, China. Cells were maintained in DMEM medium supplemented with 10% FBS, 100 U/ml penicillin and 100 U/ml streptomycin in a humid atmosphere of 5% CO_2 and 95% air at 37˚C. U251 cells were irradiated with 2 Gy $^{12}C6^{+}$ ions at room temperature. A carbon ion beam of 350 MeV/u was supplied by the Heavy Ion Research Facility in Lanzhou (HIRFL) at the Institute of Modern Physics, Chinese Academy of Sciences (Lanzhou, China). The dose rate was adjusted to be approximately 0.2 Gy/min.

Apoptosis assay. Hoechst 33258 staining was performed with a few modifications. Briefly, after exposure to radiation, confluent cells were incubated for 24 h at 37˚C in 6-well plates. Then these cells were fixed with 4% paraformaldehyde for 30 min at RT and washed once with PBS. Fixed cells were stained with Hoechst 33258 of 50 ng/ml and incubated for 30 min at RT and washed with PBS. Apoptotic cells were identified by condensation and fragmentation of nuclei examined by fluorescence microscopy.

Quantification of apoptotic cells was obtained using the Annexin V-FITC detection kit (Invitrogen, Eugen, Oregon, USA) according to the manufacturer's protocol. Following controls were used to set up compensation and quadrants: unstained cells and cells stained with FITC-Annexin V or with PI alone. The apoptotic/necrotic cell population was analyzed with a FACS Calibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA).

Flowsight data acquisition and analysis. Acquisition speed was set up to low speed and the highest resolution, an automated condition provided in Flowsight (Amnis/Merck Millipore, Darmstadt, Germany). Approximately 1000-5000 cells were acquired. Channel 5 was used to acquire DRAQ5 and channel 2 was used to detect Alexa Fluor 488. Data were analyzed in IDEAS software after compensation of single color control samples using a compensation matrix. The frequency of AIF translocation to the nuclei was analyzed using the nuclear translocation application wizard in IDEAS software.

Gene expression analysis. Total RNA was extracted from glioma cells at 24 h after CIB irradiation using the TRIzol Reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. The cDNA was synthesized from 1 mg total RNA using RT reagent kit with gDNA Eraser (Takara, Tokyo, Japan) following the manufacturer's protocols. Real-time polymerase chain reaction (quantitative PCR) was carried out the SYBR Premix EX Taq II kit (Takara, Dalian, China) on an FTC-3000+ instrument (Funglyn Biotech Inc., Toronto, ON, Canada). Gene expression was detected using qPCR primers for PARP-1 and β-actin which was used to normalize the mRNA and cDNA quantity and quality. Sequences of the primers are as follows: PARP-1 sense, 5'-TAGGCATGATTGACCGCTGG-3', and antisense, 5'-ACCATGCCATCAGCTACTCG-3'; β-actin sense, 5'-TGA GCGCAAGTACTCTGTGTGGAT-3', and antisense, 5'-TAGA AGCATTTGCGGTGCACGATG-3'. The PCR program was denatured at 95˚C for 30 sec, followed by 40 cycles of 95˚C for 5 sec, 60˚C for 30 sec. The fold changes of gene expression of the treatment groups were calculated by the $2^{-\Delta\Delta Ct}$ method.

Immunofluorescence. GBM U251 cells were fixed in 4% paraformaldehyde for 10 min and permeabilized with 0.2% Triton X-100 for 10 min. Cells were blocked for 1 h with TBST containing 0.1% BSA followed by a 1 h incubation with rabbit polyclonal or mouse polyclonal antibody, PARP-1 (1:400, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) and AIF (1:400, CapitalBio, Beijing, China). The primary antibody was detected with Alexa Fluor-555 donkey anti-rabbi secondary antibody (1:1000, Invitrogen, Carlsbad, CA, USA) or Alexa Fluor-488 goat anti-mouse (1:1000, Invitrogen). The cells were counterstained with DAPI (Vector Laboratories, Burlingame, CA, USA). Confocal images were acquired using a Zeiss LSM-700 confocal microscope. Fluorescence intensity was quantificationally analyzed using ZEN 2010 software (Carl Zeiss).

Statistical analysis. Statistical analysis for each of the studied parameters was performed on the means of the data obtained from at least 3 independent experiments. Data are presented as means ± SD. Student's t-test program in Microsoft Excel was used to detect statistical significance. A P-value <0.05 was selected as a criterion for a statistically significant difference.

Results

Cell apoptosis. Apoptotic cells were determined by Hoechst 33258 and Annexin V/PI staining 24 h after CIB irradiation. As illustrated in Fig. 1, it shows radiation-induced augmentation in the proportion of apoptotic cells which displayed the typical morphological features of cell death such as cell shrinkage, nuclear condensation and formation of pyknotic bodies of condensed chromatin. Z-VAD-FMK

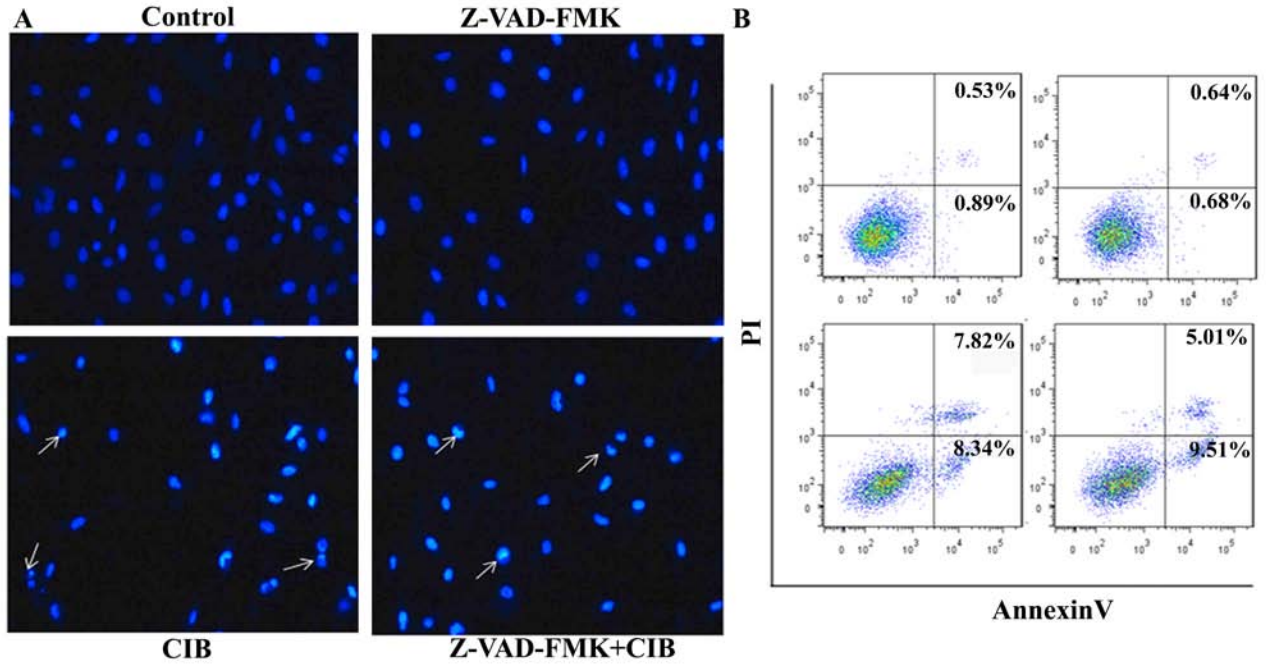

Figure 1. Effect of Z-VAD-FMK and CIB on the apoptosis of U251 cells. Cells were incubated with 50 μM Z-VAD-FMK before 2 Gy CIB irradiation. (A) Annexin V-FITC/PI staining and flow cytometric determination of apoptosis in cells. Percentage denotes proportion of apoptotic cells in right lower quadrant and right upper quadrant. (B) The nuclear morphological changes by Hoechst staining were assessed under a fluorescence microscope (original magnification, x200).

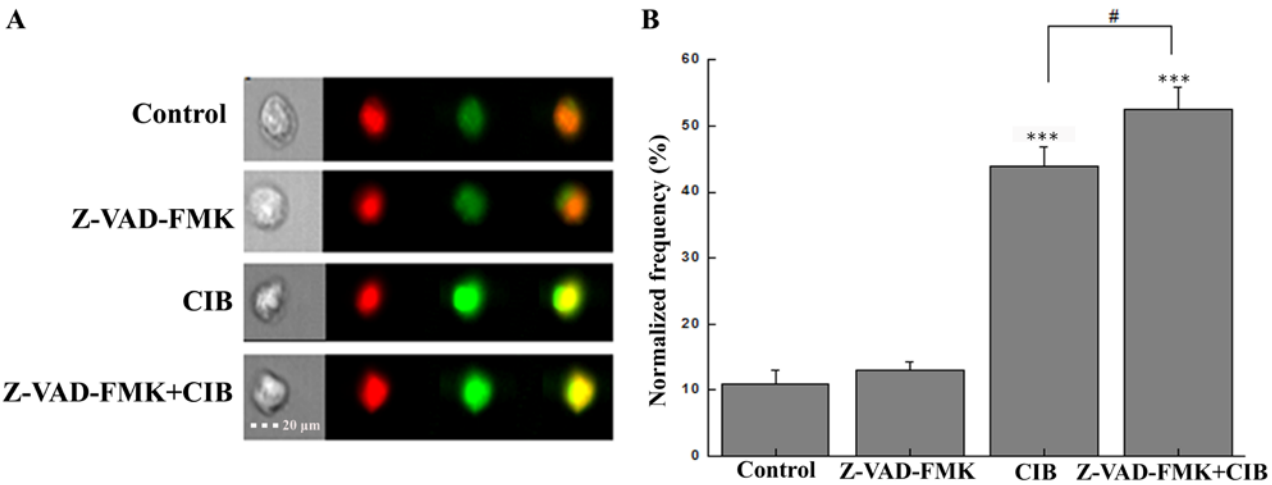

Figure 2. AIF nuclear translocation of U251 cells with/without Z-VAD-FMK treatment (50 μM) before 2 Gy CIB irradiation using a FlowSight. (A) Representative images of AIF translocation into the nucleus. Green color denotes AIF protein and red color indicates nucleus. The merged color regions (orange) represent the AIF translocation into nucleus. (B) The nuclear translocation frequency of AIF protein is presented as means ± SD from three independent experiments. ***P<0.001 compared to the control and $^{\#}$P<0.05 compared to 2 Gy irradiation alone.

(50 μM), pan inhibitor of caspases, blocked caspase-mediated mitochondrial membrane depolarization. Interestingly, no distinct difference was detected in the cellular apoptosis of cells pretreated with Z-VAD-FMK followed by CIB irradiation (14.52%) and cells treated with CIB alone (16.16%), indicating that CIB can trigger apoptosis in U251 cells via an alternative caspase-independent pathway.

AIF translocation. AIF, an important caspase-independent death effector, translocates from mitochondria to the cytosol as well as the nucleus when apoptosis is induced. To explore the detailed mechanism of cell death program elicited by CIB, AIF translocation was investigated by Imaging Flow Cytometry, which is closely linked to the caspase-independent apoptosis. Green color denotes AIF protein and red color denotes nucleus. The merged color regions with high density represent the AIF translocation into nucleus. Herein, CIB radiation promoted the AIF nuclear translocation in glioma cells as observed in Fig. 2A. Amnis data exhibit that the nuclear translocation frequency of AIF protein was increased 3.96- or 4.81-flod in the CIB alone group or in the combination with Z-VAD-FMK group compared with the control

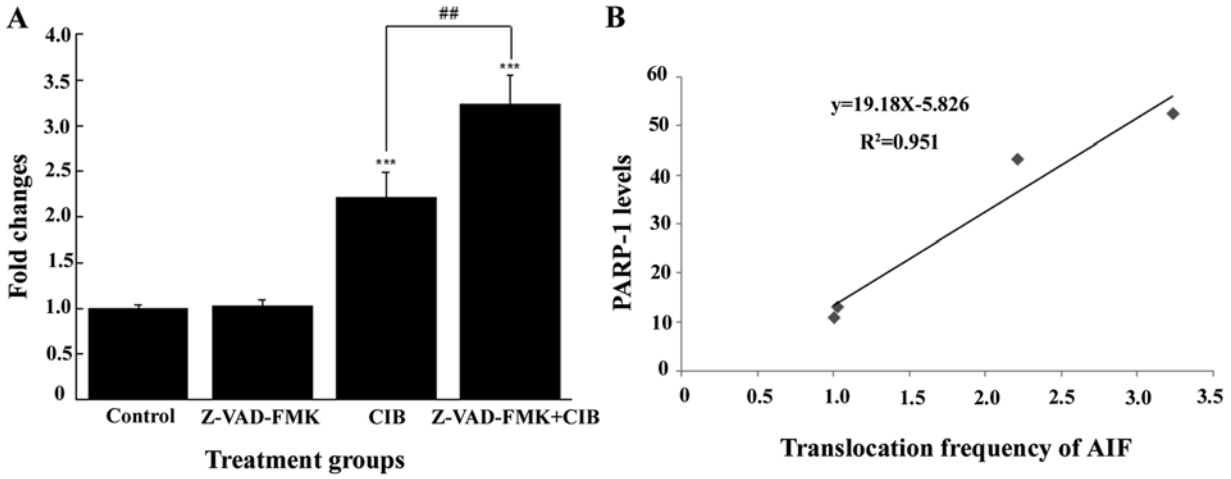

Figure 3. Z-VAD-FMK enhances PARP-1 transcript expression post 24 h irradiation. (A) The relative levels of PARP-1 in the CIB irradiated cells as measured by real-time PCR. (B) The relationship between PARP-1 mRNA levels and translocation frequencies of AIF after Z-VAD-FMK treatment combined with 2 Gy CIB irradiation. Data are presented as means ± SD from three independent experiments. ***P<0.001 compared to the control and $^{\#\#}$P<0.01 compared to 2 Gy irradiation alone.

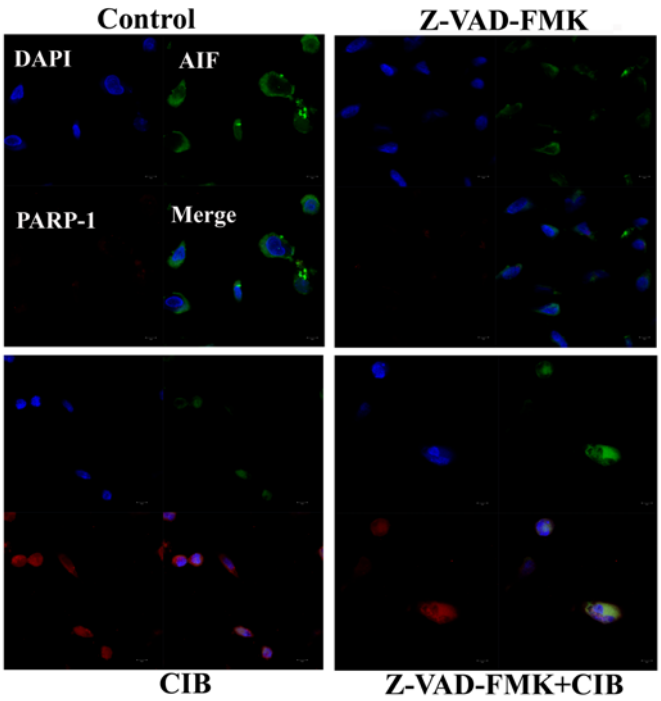

Figure 4. PARP-1 and AIF in U251 cells with/without Z-VAD-FMK treatment (50 μM) before CIB irradiation. Cells were administered with 50 μM Z-VAD-FMK prior to 2 Gy CIB irradiation Confocal microscopy was used to visualize the intracellular colocalization of AIF (green fluorescence) and PARP-1 (red fluorescence).

group, respectively. Furthermore, incubation with or without Z-VAD-FMK had a significant difference on the AIF nuclear translocation (P<0.05, Fig. 2B). These data imply that the inhibition activation of caspase may lead to AIF translocation with compensatory enlargement.

PARP-1 mRNA expression. To ascertain the cause of the AIF translocation in the irradiated glioma cells, we determined the PARP-1 status which is the decisive molecule responsible for the translocation of AIF from the mitochondria to nucleus. As given in Fig. 3, the data from qRT-PCR exhibited that PARP-1 expression level was significantly elevated by irradiation with CIB compared with the control (P<0.001) at 24 h post-irradiation. Remarkably, CIB-induced PARP-1 activation is higher 1.46-fold in the presence of Z-VAD-FMK than in the absence of Z-VAD-FMK, suggesting that caspase inhibition could improve the PARP-1 activation. In addition, a positive correlation between AIF translocation with PARP-1 mRNA level was found (y=19.18x-5.826, R^2=0.951) in Z-VAD-FMK-intervened glioma cells.

Interactions of PARP-1 with AIF. To further characterize the role of PARP-1 activity in AIF translocation of glioma cells following CIB treatment, we confirmed the interactions of AIF and PARP-1 by immunofluorescence analysis. As shown in the Fig. 4, it is evident that the colocalization of PARP-1 and AIF in the nucleus was distinctly potentiated in the cells irradiated with CIB as well as in those supplemented with Z-VAD-FMK prior to CIB irradiation. In addition, immunofluorescence result showed that CIB markedly increased the PARP-1activation, which was possibly in response to the accumulated DNA damage.

Oxidative DNA damage. The percentage of U251 cells possessing oxidative damage to their DNA was assessed using 8-OHdG as the marker. As seen in Fig. 5, CIB radiation enhanced the oxidation of DNA compared to the untreated control. The signal intensity of 8-OHdG-positive cells prominently increased 24 h after irradiation (P<0.01), with most immunoreactivity in the perinuclear region of the cytoplasm (Fig. 5A). The 8-OHdG content was higher in the mitochondrial than the nuclear fraction. Moreover, the supplement of Z-VAD-FMK had no significant effect on glioma cells in terms of the percentage 8-OHdG.

Discussion

Currently, a few studies indicate that inhibition of caspase did not affect apoptosis in malignant glioma cells and various

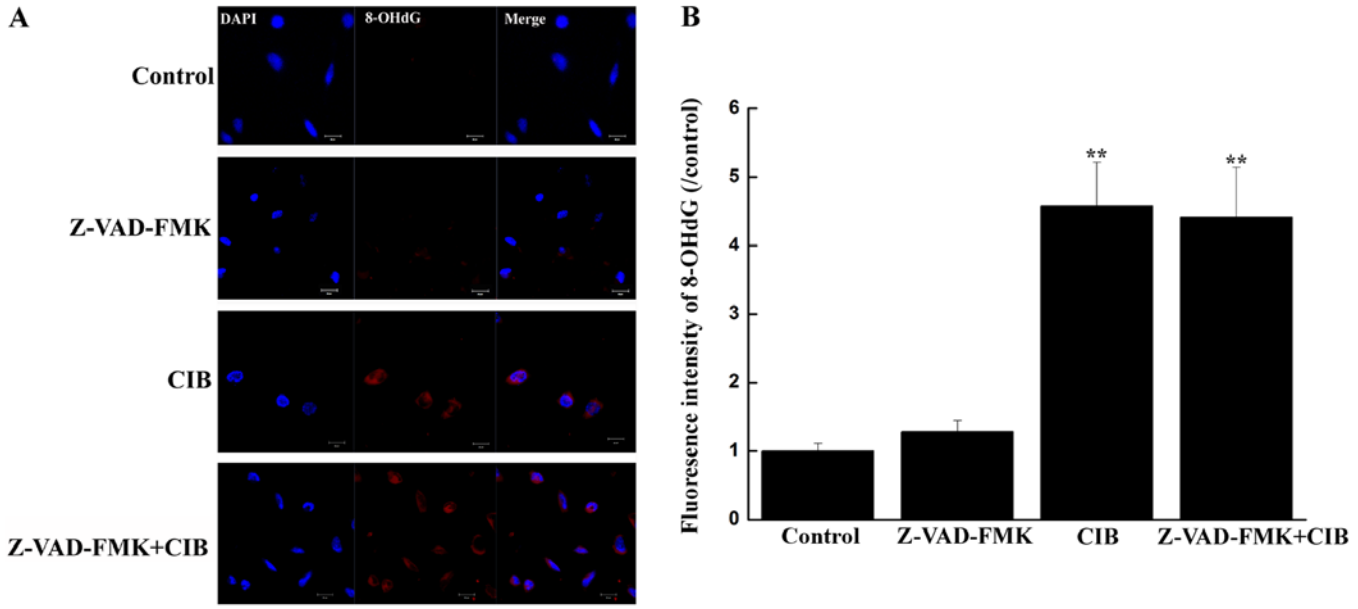

Figure 5. Effect of Z-VAD-FMK and CIB on the oxidative DNA damage. U251 cells were treated with CIB alone, or Z-VAD-FMK alone, or pre-administration with Z-VAD-FMK (50 μM) for 1 h before irradiation. An antibody specific for 8-OHdG lesions was used to visualize DNA oxidative adducts by confocal microscopy. (A) Representative immunofluorescent images of 8-OHdG (red) and DAPI (blue) staining. (B) Relative fluorescence intensity of 8-OHdG is presented as means ± SD from three independent experiments. **P<0.01 compared to the control.

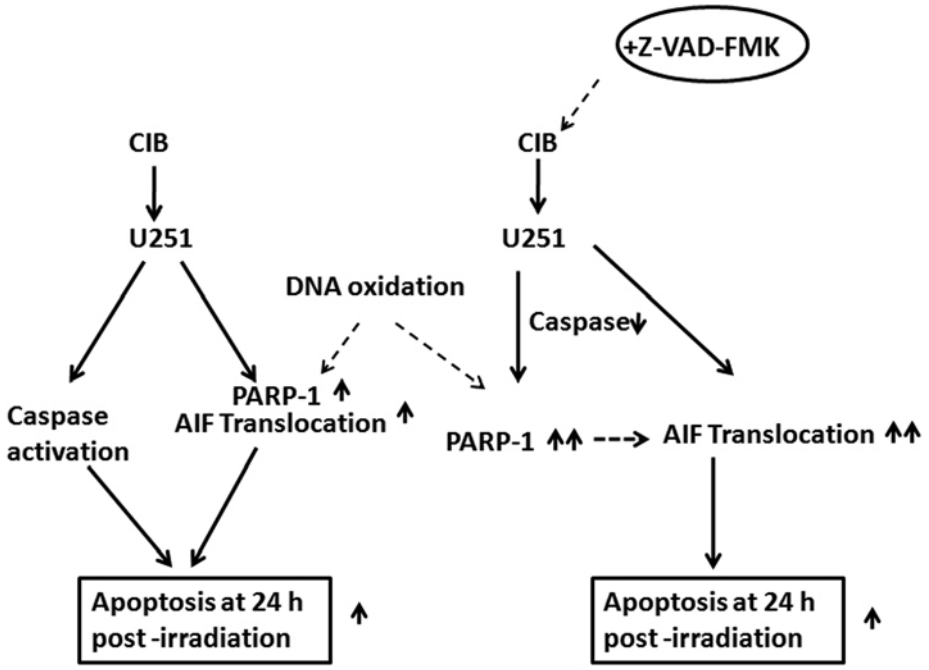

Figure 6. Schematic diagram of the influences of caspase inhibitior Z-VAD-FMK and CIB on the apoptosis pathway in U25 glioma cells. U251 cells were treated with CIB alone, or Z-VAD-FMK alone, or pre-administration with Z-VAD-FMK (50 μM) for 1 h prior to the irradiation. Z-VAD-FMK supplementation suppressed caspase activation and elevated PARP-1 expression. The combination of Z-VAD-FMK with CIB irradiation significantly triggered the AIF nuclear translocation through PARP-1 regulation which was possibly in response to the accumulated oxidative DNA damage. Our findings strongly demonstrated that caspase-independent cell apoptosis provided a prominent compensation in the glioma cell death induced by CIB irradiation.

cell types (22,23). To evaluate whether high-LET CIB irradiation-induced apoptosis is caspase-dependent or not, we immediately applied glioma cells with 50 μM of Z-VAD-FMK, a pan-caspase inhibitor before irradiation with 2 Gy in the present study. The fraction of apoptosis measured at 24 h following CIB irradiation showed that pre-administration with Z-VAD-FMK did not significantly reduce, in particular, the percentage of Annexin V staining cells (early apoptotic indicator, right lower quadrant of Fig. 1B), suggesting that caspase-independent cell death at an early stage could be triggered effectively by high-LET CIB. Similarly, the data from Ghorai *et al* also found the existence of caspase-independent

signaling pathway of apoptosis in cervical cancer HeLa cells caused by CIB irradiation (7).

For further insight into the caspase-independent apoptotic signal evoked by high-LET CIB, we consider that AIF as an important factor involved in the regulation of the glioma cell death. There is strong evidence that AIF translocation occurred in response to neuronal stimuli, including hypoxia, cerebral ischemia and traumatic brain injury (13,24). Our results demonstrate that CIB prominently promoted the mitochondrial release and translocation of AIF to the nucleus visualized using a FlowSight, suggesting that CIB could activate AIF-associated caspase-independent apoptosis, and contribute to the execution of cell death. In particular, the nuclear translocation of AIF was facilitated when caspase activation was suppressed. It is likely that the early activation of caspase caused the inactivation of the process responsible for AIF nuclear translocation (25). Commonly, activation of PARP-1, calpain, cytochrome c, Bax has been reported to affect the mitochondrio-nuclear translocation of AIF (11,26).

However, the molecular mechanism of mitochondrial AIF release to the nucleus induced by high-LET CIB remains obscure. PARP-1 activation is proposed to require translocation of AIF from the mitochondria to the nucleus and that AIF is necessary for PARP-1-dependent cell death (parthanatos) (18,27). In the present study, the levels of PARP-1 mRNA and protein were significantly upregulated by CIB irradiation as seen in the Figs. 3 and 4. Furthermore, an obvious difference of PARP-1 activity raised by CIB irradiation was observed in glioma cells cultured in the presence and absence of Z-VAD-FMK ($P<0.01$). Prabhakaran *et al* also reported that inhibition of caspase cascade with Z-VAD-FMK led to a marked increase of PARP-1 in cyanide-treated cortical cells (28). Moreover, the administration of Z-VAD-FMK enhanced 11'-deoxyverticillin A-elicited PAR formation (29) which played a pivotal role in PARP-1 induced cell death (30). In addition, our data showed a positive correlation between PARP-1 activity and AIF translocation frequency, suggesting that PARP-1 may be strongly activated in glioma cells where it caused the CIB-induced AIF translocation into nucleus, which is consistent with the HeLa cells after exposure with CIB (24). As shown in Fig. 4, nuclear colocalization of AIF and PARP-1 positive signals provided further evidence that PARP-1 initiated AIF-mediated glioma cell death with apoptotic features. Some literature reported that oxidative damage to DNA specifically can trigger caspase-independent apoptosis (31). Our data clearly showed that CIB elevated the amount of 8-OHdG and pre-administration with Z-VAD-FMK did not alter radiation-induced oxidative stress to DNA (Fig. 5), and consequently PARP-1 may be activated to involve in base excision repair process that repairs 8-OHdG lesions (32).

Taken together, our results strongly suggested that AIF translocation from the mitochondria to the nucleus governed by the activated PARP-1 could be particularly effective in glioma cells irradiated with high-LET CIB when the caspase protease cascade is inhibited. These findings demonstrated that at 24 h after treatment with 2 Gy CIB, caspase-independent glioma cell death provide an outstanding contribution in cell death via PARP-1/AIF pathway, which may be involved in DNA oxidative stress (Fig. 6).

Acknowledgements

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