Original Paper

Protective Effect of CpG-Oligodeoxynucleotides Against Low- and High-LET Irradiation

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Key Words

CpG-ODNs • Irradiation • Carbon beam • High-LET

Abstract

Background/Aims: CpG-oligodeoxynucleotides (ODNs) are synthetic DNA sequences containing unmethylated cytosine-guanine motifs with potent immunomodulatory effects. Previous reports showed a powerful protective effect of CpG-ODN against the damage induced by low-LET γ -rays. In this study, we explored whether CpG-ODN also protects against the damage induced by high-LET irradiation. Parallel experiments were performed with low-LET irradiation. **Methods:** RAW264.7 cells were incubated with 1 μ M of CpG-ODN after γ -ray or carbon-beam irradiation. Cell death was then measured by PI/DAPI double staining, cell survival was assessed by colony-formation assays, DNA damage was evaluated by comet assays, cell cycle was monitored by flow cytometry, and the levels of apoptosis-related proteins were detected by western blots. **Results:** When irradiated cells were treated with the CpG-ODN, cell viability decreased, cell survival increased, DNA damage and G₂/M-phase arrest were ameliorated, and apoptosis was inhibited. **Conclusions:** The CpG-ODN showed protective effects against low-LET γ -ray and high-LET carbon-beam irradiation. These effects might be associated with the repair of DNA damage and inhibition of apoptosis.

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Introduction

Qualitatively high-linear energy transfer (LET) radiation, such as heavy ions, α -particles and neutrons, is densely ionizing. Due to its physical and radiobiological properties, high-LET radiation is of special interest for tumor therapy. Recent and proposed radiation therapy strategies use carbon ions to effectively treat malignant tumors [1-3]. However, the absorbed dose delivered by high-LET heavy ion irradiation undergoes penetration and then higher-energy deposition in the bodies of patients. The irradiation not only destroys tumor tissue but can also lead to serious damage to normal cells surrounding the tumor [4]. It was reported that high-LET heavy ion irradiation is much more damaging to normal cells than low-LET irradiation, which causes immune cell death, immunosuppression, and an increased risk of opportunistic infections during tumor radiotherapy [5]. In addition to the emerging side effects that occur during radiotherapy, high-LET heavy ion irradiation also constitutes a major health risk for crews aboard air- or spacecraft; outside the protective shielding of the earth's atmosphere, crews are exposed to increased levels of high-LET heavy ion radiation from space [6]. It is estimated that during a 3-year space mission, a significant number of cells in the body will be exposed to high-LET heavy ion radiation, and the total radiation dose that crews may receive could reach 3 Gy [7]. Although high-LET heavy ion irradiation can affect many physiological systems, immune dysfunction, with its potentially serious consequences, is a paramount concern. It is undoubtedly important to find methods to protect immune cells from damage induced by high-LET heavy ion irradiation.

CpG-oligodeoxynucleotides (ODNs), which are synthetic analogs of bacterial DNA, can be generated containing specific unmethylated cytosine-guanine motifs [8]. The signaling pathway is triggered when CpG-ODNs interact with Toll-like receptor 9 (TLR9), which then recruits myeloid differentiation factor 88 (MyD88), IL-1R-associated kinase (IRAK), and tumor necrosis factor receptor-associated factor 6 (TRAF6), culminating in the activation of NF- κ B [9-11]. Previous work determined that CpG-ODNs had a protective effect against low-LET irradiation both in vitro and in vivo. Indeed, Sohn et al. demonstrated that CpG-ODNs inhibited γ -rays irradiation-induced immune cell death [12]. Studies from our laboratory suggested that CpG-ODNs ameliorate the damage to hematopoietic cells and intestinal crypt cells after γ -ray irradiation [13-15]. We speculated that CpG-ODNs would also have the protective effect against high-LET irradiation. To the best of our knowledge, the present study is the first to investigate the effect of CpG-ODNs against the damage induced by high-LET carbon beams and to compare this effect with that of low-LET γ -rays.

Materials and Methods

Agents

The CpG-ODN was synthesized at Shanghai Sangon Biological Engineering Technology Services Co. Ltd. (Shanghai, China). The sequence used in this study was 5'-T***CG TCG***TTT T***CG GCG CGC CG***-3' (letters in regular type represent phosphorothioates; bold and italic letters represent phosphodiester) [16]. Endotoxins were removed. The compound was diluted with phosphate-buffered saline (PBS) to a concentration of 100 μ M and stored at 4°C.

Cell culture and treatments

The mouse macrophage cell line RAW264.7 was obtained from the American Type Culture Collection (ATCC, Manassas, USA). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % fetal bovine serum and maintained at 37 °C with 5 % CO₂ and 95 % humidity. All culture reagents were purchased from HyClone (Pennsylvania, USA). Exponentially growing cells were harvested and seeded in 25 cm² plastic flasks (Corning, New York, USA) 8 h before irradiation. Viability, which was assayed using trypan blue dye exclusion, was typically greater than 95 %. After plating, irradiated cells were treated with or without 1 μ M CpG-ODN for 3 h.

Irradiation

γ -ray irradiation was performed at the radiation facility of Second Military Medical University (SMMU, Shanghai, China). Cells were irradiated with ^{60}Co γ -radiation at 1.0 Gy/min. Carbon-beam radiation was supplied by the Heavy Ion Research Facility in Lanzhou (HIRFL, Institute of Modern Physics, Chinese Academy of Sciences, Lanzhou, China). A carbon beam with an initial energy of 290 MeV/n was generated, and modular films were used to achieve a LET value of 100 keV/ μm . Cells were irradiated at room temperature. The control group was treated with the same protocol, except the irradiation was excluded.

MTT assays

The toxicity of the CpG-ODN on RAW264.7 cells was tested by MTT assay. Cells (1.0×10^4 cells/well) were seeded in 96-well plates and incubated with varying final concentrations of CpG-ODN for 24 h. Subsequently, 20 μl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was added to each well and incubated for 2 h. The medium was then replaced with 200 μl of dimethyl sulfoxide (DMSO). The absorbance of each well at 490 nm was measured spectrophotometrically using a 96-well plate reader. The absorbance of empty wells was used as a control. The MTT assay was repeated three times with six replicates per experiment. The data were analyzed using Microsoft Excel 2010. The half maximal inhibitory concentration (IC_{50}) was calculated by fitting a linear-quadratic function.

DAPI/PI double staining

Cell death was measured using propidium iodide (PI) and 4,6-diamidino-2-phenylindole (DAPI) double staining. Cells were stained with PI and DAPI and then manually counted under a fluorescent microscope (Nikon, Tokyo, Japan) 24 h after irradiation. Cellular states were recorded: all cells were stained blue with DAPI under UV fluorescence, and dead cells were stained red with PI under green fluorescence. The analysis was conducted in a blind manner, i.e., the observer had no knowledge of the identity of the samples. The median lethal dose (LD_{50}) and the dose-modifying factor (DMF) were calculated.

Colony-formation assays and dose-survival fractions

Cell survival was assessed by colony-formation assays. Cells were counted using a cell counter analyzer system (CASY), diluted by geometric proportion, and seeded in flasks to provide 50-800 cells/ml. Increasing numbers of cells were exposed to increasing doses of radiation: a 50 cells/ml solution was exposed to 0 Gy; a 100 cells/ml solution was exposed to 0.5 Gy; a 200 cells/ml solution was exposed to 2 Gy; a 400 cells/ml solution was exposed to 4 Gy; and a solution of 800 cells/ml was exposed to 6 Gy. The irradiated cells were then incubated for approximately 14 days, fixed with methanol:acetic acid (9:1 v/v) for 30 min and stained with 5 μM DAPI. The bottom of the flask was divided into 9 areas, and the number of colonies (more than 50 cells) was counted in each area under a fluorescence microscopy (10 $\times$). The survival fraction = $(N1/n1)/(N2/n2)$ $N1$: the number of cells forming colonies after irradiation; $n1$: the number of seeded cells after irradiation; $N2$: the number of cells forming colonies in the control group; $n2$: the number of seeded cells in the control group). A modified single-hit multi-target model was established to analyze the survival fraction. The formula used was $S = ne^{-kd}$, where the survival fraction is expressed in terms of the four parameters D_q , D_0 , D_{37} and n . D_q (the size or width of the shoulder of the curve, $D_q = \ln n/k$) represents the minimum radiation dose that inevitably leads to cell death, D_0 (the slope of the curve, $D_0 = 1/k$) represents the mean lethal dose, D_{37} represents the dose required for 37 % survival, and n is the extrapolation number [17].

Comet assays (single-cell gel electrophoresis)

DNA damage was evaluated using the comet assay. Cells were incubated for 24 h after irradiation with 6 Gy. Conventional microscope slides were covered with a solution of 85 μl of 1 % normal-melting-point agarose (NMP) and allowed to dry on a flat surface at room temperature. Ten microliters of cell suspension (2.5×10^5 cells/ml) was gently mixed with 75 μl of 0.5 % (w/v) low-melting-point agarose (LMP). The suspension was rapidly layered onto the slides precoated with 1 % NMP and covered with a coverslip. After removing the coverslip, cells were immersed into a freshly made lysis solution at 4 $^{\circ}\text{C}$ for 1 h. The slides were placed into the electrophoresis tank. Electrophoresis (30 V, 300 mA) was conducted for 15 min at 4 $^{\circ}\text{C}$, and the slides were stained with PI for 20 min and observed under a fluorescence microscope [18, 19].

Fig. 1. Cytotoxicity effect of CpG-ODN on cell. RAW264.7 cells were incubated with various final dose of CpG-ODN. MTT assay was used to assess viability of cells after 24 h. The IC_{50} values of 61.4 μ M were calculated. (Data are represented by the mean $\pm$ SEM from 3 independent experiments, * p <0.05 for significant differences between normal group and other concentration group).

Cell cycle analysis

The cell cycle was monitored using a flow cytometer. Irradiated cells were incubated for 12 h and then trypsinized, rinsed, resuspended in PBS, and fixed with ice-cold 70 % ethanol for least 3 h. The fixed cells were rinsed again with PBS and stained with 50 μ g/ml PI. A flow cytometer (Beckman, California, USA) was used to analyze the cell cycle phase of the cells.

Western blots

The expression of apoptosis-related proteins was detected by western blotting. Cells were incubated for 12 h after irradiation and then harvested, washed with cold PBS, lysed with mammalian protein extraction reagent (Thermo, Pennsylvania, USA) for 30 min on ice, and centrifuged at 14,000 $\times g$ for 10 min at 4°C. The concentration of cleared protein supernatants was evaluated using the Bradford method. Proteins were resolved by 12 % SDS-polyacrylamide gel electrophoresis and electrotransferred to nitrocellulose membranes. Membranes were blocked with 5 % nonfat milk in TBS containing 0.1 % Tween-20 (TBST) for 1 h at room temperature. Afterwards, the blots were incubated in primary antibody at 4 °C overnight. The membranes were then incubated with horseradish peroxidase-conjugated secondary antibody for 3 h at room temperature. For densitometry analysis, an enhanced chemiluminescence detection system (Gene Company, Hong Kong, China) was used to measure specific protein bands, and band densities were quantified using the Gene Tools software.

Statistical analysis

Data are presented as means $\pm$ standard errors of the means (means $\pm$ SEMs). The significance of differences between groups was determined by Student's *t*-test. The parameter values (including $D_{q'}$, $D_{0'}$, D_{37}) were determined by corresponding regression analysis. The software analysis program SPSS 13.0 (Release 12.0 K; SPSS Inc., Chicago, USA) was used. All *p* values were obtained using two-tailed tests. P <0.05 was considered to be statistically significant.

Results

Cytotoxic effect of the CpG-ODN on RAW 264.7 cells

The cytotoxic effect of the CpG-ODN on RAW264.7 cells was assessed by MTT assay. The result showed that there was no significant change in cell viability in cells treated with a final concentration of 15.0 μ M CpG-ODN compared with the control cells. However, when the final concentration of CpG-ODN was higher than 15.0 μ M, cell viability was significantly decreased in a dose-dependent manner, with an IC_{50} value of 61.4 μ M (Fig. 1). For all further experiments, a final concentration of 1.0 μ M CpG-ODN was used. These results suggested that the CpG-ODN had little cytotoxic effect on RAW264.7 cells.

Effect of CpG-ODN on cell death after irradiation

To examine the protective effect of the CpG-ODN against cellular damage after γ -ray or carbon-beam irradiation, the viability of the cells was measured by PI/DAPI double staining. As shown in Fig. 2A, γ -ray and carbon-beam irradiation resulted in a large amount of cell death. Compared with the viability of cells exposed to γ -ray radiation, a clear increase in cell

Fig. 2. Effect of CpG-ODN on cell death after irradiation. RAW264.7 cells were treated with or without 1 μ M CpG-ODN after 2 Gy, 4 Gy and 6 Gy of γ -ray or carbon-beam irradiation. (A) Death cell was measured by PI/DAPI double staining at 24 h after irradiation and photographed under a fluorescent microscopy; (B) the mortality of cell was quantified by manually counting; (C) The values of LD₅₀ and DMF were calculated by fitting linear-quadratic function. (Data are represented by the mean $\pm$ SEM from 3 independent experiments, * p <0.05 for significant differences between γ -ray plus CpG-ODN group and γ -ray control group; # p <0.05 for significant differences between carbon-beam plus CpG-ODN group and carbon-beam control group).

death was observed in cells exposed to carbon-beam radiation. However, CpG-ODN treatment reduced cell death. For γ -ray radiation, the percentage of dead cells decreased from 11.1 %, 23.8 % and 51.9 % in the control cells to 5.7 %, 13.5 % and 38.3 % in the CpG-ODN-treated cells after exposure to 2 Gy, 4 Gy and 6 Gy of γ -ray radiation, respectively (Fig. 2B). The LD₅₀ increased from 5.91 Gy to 6.83 Gy after treatment, and the DMF value was 1.16. For carbon-beam radiation, the percentage of dead cells decreased from 37.1 %, 68.2 %, and 90.6 % in the control cells to 27.8 %, 48.5 % and 65.0 % in the CpG-ODN-treated cells after exposure to 2 Gy, 4 Gy and 6 Gy of carbon-beam radiation, respectively (Fig. 2B). The LD₅₀ value increased

Fig. 3. Effect of CpG-ODN on cell survival after irradiation. RAW264.7 cells were treated with or without 1 μ M CpG-ODN after 0.5 Gy, 2 Gy, 4 Gy and 6 Gy of γ -ray or carbon-beam irradiation. Survival fraction was established by colony forming assay. Three parameters are listed in Table 1. (Data are represented by the mean $\pm$ SEM from 3 independent experiments, * p <0.05 for significant differences between γ -ray plus CpG-ODN group and γ -ray control group; # p <0.05 for significant differences between carbon-beam plus CpG-ODN group and carbon-beam control group).

Table 1. Parameters and of survival curves obtained with a modified model

	<i>n</i>	<i>D_q</i>	<i>D₀</i>	<i>D₃₇</i>
γ -ray control	1.215	1.030	5.291	6.291
CpG-ODN + γ -ray	1.327	2.080	7.353	9.255
Carbon-beam control	1.024	0.036	1.511	1.538
CpG-ODN + Carbon-beam	1.118	0.255	2.281	2.525

from 3.03 Gy to 4.35 Gy after CpG-ODN treatment, and the DMF value was 1.44. These results suggested that the CpG-ODN reduced cell death after γ -ray and carbon-beam irradiation.

Effect of CpG-ODN on cell survival after irradiation

Next, the effect of the CpG-ODN on cell survival after γ -ray and carbon-beam irradiation was described by colony-formation assays, and the survival fraction ($S=ne^{-kd}$) was established. The parameters obtained are listed in Table 1. As shown in Fig. 3, γ -ray and carbon-beam irradiation resulted in a dose-dependent decrease in the number of surviving cells. Survival curves were characterized by an initial shoulder region at low doses of radiation that was followed by an exponential decrease in survival. The survival curve of cells exposed to γ -rays showed a wider shoulder ($D_q=1.030$ Gy), and the dose required for 37 % survival (D_{37}) was 6.29 Gy. In contrast, the survival curve of cells treated with carbon-beam radiation showed a small shoulder region ($D_q=0.036$ Gy), and the D_{37} value was 1.54 Gy, indicating that the relative biological effectiveness (REB) of carbon-beam radiation was 4.08.

For γ -ray irradiation, CpG-ODN treatment increased the survival fraction. A significant difference in survival was observed after irradiation with either 4 or 6 Gy of γ -ray radiation. However, when the radiation dose was lower than 2 Gy, there was no significant change in the survival fraction in irradiated cells with or without CpG-ODN treatment, suggesting that the CpG-ODN only altered the slope region of the exponential portion of the survival curves (Fig. 3). As shown in Table 1, the D_0 value, which describes the change in the slope region, increased from 5.291 Gy to 7.353 Gy.

The survival fraction was significantly higher in irradiated cells treated with the CpG-ODN than in irradiated control cells after 0.5 to 6 Gy of carbon-beam irradiation. The shoulder region and the slope region of the exponential portion of the curve were significantly changed (Fig. 3). As shown in Table 1, the D_q value increased from 0.036 to 0.255 Gy, and the D_0 value increased from 1.511 to 2.281 Gy. These results suggested that CpG-ODN treatment enhanced the survival of the cells after γ -ray and carbon-beam irradiation.

Effect of CpG-ODN on cellular DNA damage induced by irradiation

The effect of the CpG-ODN on DNA damage caused by γ -ray or carbon-beam irradiation was evaluated by comet assays. As shown in Fig. 4A, the cells' fragmented DNA migrated away from the comet head and formed a long tail similar to that of a comet after γ -ray or

Fig. 4. Effect of CpG-ODN on cell DNA damage after irradiation. RAW264.7 cells were treated with or without 1 μ M CpG-ODN after 6 Gy of γ -ray or carbon-beam irradiation. (A) Degrees of DNA damage were evaluated by comet assay at 24 h after irradiation and photographed under a fluorescent microscopy. (B) The area of comet tail was quantified. (Data are represented by the mean $\pm$ SEM from 3 independent experiments, * p <0.05 for significant differences between γ -ray plus CpG-ODN group and γ -ray control group; # p <0.05 for significant differences between carbon-beam plus CpG-ODN group and carbon-beam control group).

carbon-beam irradiation. However, when irradiated cells were treated with the CpG-ODN, there was a significant difference in the shape of the comet tail. Considerably shorter and narrower comet tails were observed in irradiated cells treated with the CpG-ODN compared with irradiated control cells.

For γ -ray radiation, the comet tail area decreased by 5- to 11-fold after treatment with the CpG-ODN compared with the control. For carbon-beam radiation, the comet tail area was decreased by 10- to 22-fold compared with the control (Fig. 5B). This result suggested that the CpG-ODN alleviated the DNA strand breaks induced by γ -ray or carbon-beam irradiation.

Effect of CpG-ODN on cell-cycle progression after irradiation

Finally, the effect of CpG-ODN treatment on cell-cycle distribution after γ -ray or carbon-beam irradiation was analyzed by flow cytometry to determine whether the protection of CpG-ODN treatment was due to the promotion of cell-cycle progression. As shown in Fig. 5A, irradiated cells exhibited G_2/M phase arrest, as shown by an increased percentage of cells in G_2/M phase compared with the control cells, but CpG-ODN treatment significantly decreased the fraction of cells in G_2/M phase after irradiation. For γ -ray radiation, the percentage was decreased from 63 % to 35 % with CpG-ODN treatment. For carbon-beam radiation, the percentage was decreased from 79 % to 56 % with CpG-ODN treatment. Moreover, compared with the irradiation control group, a significant increase was also observed in the fraction of cells in G_0/G_1 phase in the irradiation group with CpG-ODN treatment. For γ -ray radiation, the percentage was increased from 23 % to 46 % with CpG-ODN treatment. For carbon-beam radiation, the percentage was increased from 11 % to 29 % with CpG-ODN treatment (Fig. 5B). These results revealed that the CpG-ODN promoted cell-cycle progression after γ -ray and carbon-beam irradiation.

Fig. 5. Effect of CpG-ODN on cell-cycle progression after irradiation. RAW264.7 cells were treated with or without CpG-ODN after 6 Gy of γ -ray or carbon-beam irradiation. Cell cycle was analyzed by flow cytometry at 12 h after irradiation. (A) Representative profiles; (B) The percentage of cells in G_0/G_1 , S and G_2/M phases. (Data are represented by the mean $\pm$ SEM from 3 independent experiments, * $p < 0.05$ for significant differences between γ -ray plus CpG-ODN group and γ -ray control group; # $p < 0.05$ for significant differences between carbon-beam plus CpG-ODN group and carbon-beam control group).

Effect of CpG-ODN on the expression of apoptosis-related proteins after irradiation

Based on previous results, we investigated the mechanism behind the reduction in DNA damage of RAW267.4 cells by the CpG-ODN after γ -ray and carbon-beam irradiation. The level of several apoptosis-related proteins, including Bax, Bcl-2, caspase-3 and cleaved caspase-3, was detected by western blot. As shown in Fig. 6A, Bax expression was upregulated, Bcl-2 expression was downregulated, and the ratio of Bax/Bcl-2, which is considered to be one of the major markers of apoptosis, was also increased [20]. Caspase-3 expression was increased, and cleaved caspase-3 protein appeared after γ -ray and carbon-beam irradiation. However,

Fig. 6. Effect of CpG-ODN on apoptosis-related proteins expression after irradiation. RAW264.7 cells were treated with or without CpG-ODN after 6 Gy of γ-ray or carbon-beam irradiation. (A) The expression of apoptosis-related proteins was assessed by western blot assay at 12 h after irradiation. The expression of Bax (B) and Bcl-2 (C) were quantified by the relative intensity of protein bands, and the ratio of Bax/Bcl-2 was calculated (D); the expression of Caspase-3 and cleaved Caspase-3 proteins was quantified by the relative intensity of protein bands. (Data are represented by the mean ± SEM from 3 independent experiments, * $p < 0.05$ for significant differences between γ-ray plus CpG-ODN group and γ-ray control group; # $p < 0.05$ for significant differences between carbon-beam plus CpG-ODN group and carbon-beam control group).

when irradiated cells were treated with the CpG-ODN, Bax expression was significantly downregulated (Fig. 6B), Bcl-2 expression was substantially upregulated (Fig. 6C), the ratio of Bax/Bcl-2 proteins markedly decreased (Fig. 6D), caspase-3 expression was significantly decreased, and the amount of cleaved caspase-3 was reduced to very low levels (Fig. 6E and Fig. 6F). These results suggested that the CpG-ODN regulated the expression of apoptosis-related proteins and inhibited cellular apoptosis.

Discussion

Tumor therapy with high-LET heavy ion irradiation has been widely used in the clinical setting, and residency on the space station has become a reality. However, due to quality and different physical characteristics, high-LET heavy ion radiation exposure poses a greater danger than low-LET radiation to normal tissue. High-LET heavy ion irradiation induces immunosuppression that increases the risk of opportunistic infections during radiotherapy and space exploration. Agents to reduce the damage induced by heavy ion irradiation are required to enhance efficiency during cancer radiotherapy and improve reliable risk estimates for space radiation. The macrophage cell line RAW264.7 represents a primary immune cell line [21]. TLR9 is highly expressed in the intracellular compartments of these cells. The only known ligand and agonist of TLR9 is a CpG-ODN, which may induce macrophage activation and proliferation [22, 23]. In the present study, we used the mouse macrophage cell line RAW 264.7 to assess the protective effect of a CpG-ODN against high-LET carbon-beam irradiation-induced damage, and parallel experiments were performed with low-LET γ -ray irradiation.

First, cell death was measured by PI/DAPI double staining. The CpG-ODN significantly reduced cell death after γ -ray and carbon-beam irradiation. For γ -ray radiation, the LD₅₀ value was increased from 5.91 Gy to 6.83 Gy, and the DMF value was 1.16. For carbon-beam radiation, the LD₅₀ value was increased from 3.03 Gy to 4.35 Gy, and the DMF value was 1.44. Second, the survival fraction was determined by colony-formation assays. The corresponding survival curve showed a shoulder region that is due to sub-lethal damage repair, whereas the change in the exponential region of the curve is due to the direct action of the radiation [1, 17]. Our results showed that when irradiated cells were treated with the CpG-ODN, survival was significantly increased. For γ -ray radiation, the shoulder region and the exponential part of the survival curve were altered by CpG-ODN treatment. For carbon-beam radiation, although the shoulder region was much narrower and the slope of the exponential region was much steeper than for γ -ray radiation, the CpG-ODN altered the shoulder region and the slope of the exponential region of the survival curve. These results suggested that the CpG-ODN enhanced cell survival after γ -ray and carbon-beam irradiation. We speculate that the CpG-ODN likely exerted a protective response against the direct effects induced by radiation to reduce and repair DNA damage rather than scavenging free radicals.

It was well known that DNA damage is the most serious threat to cells because it results in the loss or rearrangement of genetic information, eventually leading to cell death [19]. To further demonstrate the previously mentioned speculation, the degree of DNA damage was evaluated by comet assays. The study results showed that considerably shorter and narrower comet tails were observed in irradiated cells with CpG-ODN treatment compared with irradiated control cells, suggesting that the CpG-ODN alleviated the effect of DNA damage, which might be related to DNA damage repair after γ -ray and carbon-beam irradiation. In addition, the repair of damaged DNA also typically depends on DNA synthesis. G₂/M phase of the cell cycle is a key part of this process and is tightly correlated with the radiosensitivity of the cells [24, 25]. Normal mammalian cells exposed to radiation were heavily damaged and characteristically delayed transit from G₂ to M phase [26]. Although carbon-beam irradiation caused a higher relative G₂/M phase arrest than γ -ray irradiation, the CpG-ODN significantly ameliorated G₂/M phase arrest after both γ -ray and carbon-beam irradiation and correlated with a strong increase in the G₀/G₁ phase cell population. The G₀/G₁ phase is called the 'prophase' of cell division and is a stage in which rRNA, mRNA, tRNA and ribosomes are produced and heparin is synthesized [27]. These results not only confirmed that the CpG-ODN had the potential to enhance DNA repair but also suggested that the CpG-ODN helped irradiated cells proceed with improper cell division after γ -ray and carbon-beam irradiation.

Although the reason is not fully understood, apoptosis is the main mechanism of cell death after irradiation. The Bcl-2 family proteins are involved in the initiation phase of apoptosis, which represents a critical checkpoint, and act upstream of irreversible steps of cellular damage. Of the Bcl-2 family proteins, Bax causes the permeabilization of the

mitochondria and release of apoptotic molecules, relocating cytochrome *c* into the cytosol. Bcl-2 prevents Bax from initiating the permeabilization of the mitochondria. The balance between these two proteins determines how cells respond to apoptotic or survival signals [28]. Thus, the ratio of Bax/Bcl-2 is considered to be one of the major markers of apoptosis. Our results showed that when irradiated cells were treated with a CpG-ODN, Bax expression was upregulated, Bcl-2 expression was downregulated, and the ratio of Bax/Bcl-2 was increased. These results indicated that Bcl-2 family proteins made a significant contribution to the inhibition of apoptosis by the CpG-ODN after γ -ray and carbon-beam irradiation. Furthermore, a decrease in the ratio of Bax/Bcl-2 reduced the release of cytochrome *c* from the mitochondria into the cytosol and inhibited the activation of caspase family proteins [29]. The caspase family is responsible for the execution phase of apoptosis [30], and caspase-3 plays a central role in this process. Once activated, caspase-3 rapidly induces the cleavage of poly ADP-ribose polymerase (PARP) and forms cleaved caspase-3, which in turn leads to the degradation of DNA into nucleosomal fragments [31]. Our results showed that CpG-ODN treatment led to a low level of caspase-3 expression and the disappearance of cleaved caspase-3 after irradiation, suggesting that caspase-3 activation was inhibited. These results confirmed that the CpG-ODN inhibited apoptosis after γ -ray and carbon-beam irradiation.

In conclusion, this study demonstrated that a CpG-ODN had protective effects against RAW264.7 cell damage induced by γ -ray and carbon-beam irradiation. The results showed that the CpG-ODN reduced cell death, enhanced cell survival, alleviated DNA damage, promoted cell-cycle progression and inhibited apoptosis after γ -ray and carbon-beam irradiation. We speculated that CpG-ODNs might be useful as a potential agent to protect cells from high-LET heavy ion irradiation.

Because this is the first time results of this type have been shown, the investigation described here could facilitate the design of strategies to focus on the protective effects of CpG-ODNs on irradiated cells. In further studies, we will assess the effect of CpG-ODNs on carcinogenesis, mutagenesis and chromosomal aberrations after irradiation.

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Disclosure Statement

The authors report no conflicts of interest. The authors are responsible for the content of this manuscript.

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