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Different mitochondrial fragmentation after irradiation with X-rays and carbon ions in HeLa cells and its influence on cellular apoptosis

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ABSTRACT

Although mitochondria are known to play an important role in radiation-induced cellular damage, the mechanisms by which ionizing radiation modulates mitochondrial dynamics are largely unknown. In this study, human cervical carcinoma cell line HeLa was used to demonstrate the different modes of mitochondrial network in response to different quality radiations such as low linear energy transfer (LET) X-rays and high-LET carbon ions. Mitochondria fragmented into punctate and clustered ones upon carbon ion irradiation in a dose- and LET-dependent manner, which was associated with apoptotic cell death. In contrast, low-dose X-ray irradiation promoted mitochondrial fusion while mitochondrial fission was detected until the radiation dose was more than 1 Gy. This fission was driven by ERK1/2-mediated phosphorylation of Drp1 on Serine 616. Inhibition of mitochondrial fragmentation suppressed the radiation-induced apoptosis and thus enhanced the resistance of cells to carbon ions and high-dose X-rays, but not for cells irradiated with X-rays at the low dose. Our results suggest that radiations of different qualities cause diverse changes of mitochondrial dynamics in cancer cells, which play an important role in determining the cell fate.

1. Introduction

Radiotherapy is an essential modality of cancer therapy. It has been estimated that about 60% of all cancer diseases are cured by radiotherapy alone or in combination with surgery [1]. Previously, radiotherapy with photons (X- or γ-rays) may be the only option for tumor patients. However, for last two decades, the number of patients who received treatment with charged particles, such as protons and heavy ions (typically carbon ions), is rapidly increasing (http://www.ptcog.ch). Compared with radiotherapy with X-rays, charged particle therapy shows some unique properties in physics and/or biology [2]. One of the biological advantages of charged heavy particles like carbon ions is their higher relative biological effectiveness (RBE) in contrast with sparse ionizing radiation such as X-rays.

Generally, radiation-induced nuclear DNA damages have been regarded as the main cause of mutation and cell death [3]. However, mitochondrial damage elicited by radiation is also attracting more and more attentions [4]. The multiple functions of mitochondria allow them to sense cellular stress and contribute to cell adaptation to challenging micro-environment conditions, conferring a high degree of plasticity to tumor cells for growth and survival [5]. Mitochondria are highly dynamic organelles that change their morphology in response to cellular signaling and differentiation. Mitochondrial morphology is maintained by the balance between fusion and fission [6]. In mammals, mitochondrial fusion is regulated by two outer membrane (OM) GTPases such as mitofusin 1 (Mfn1) and mitofusin 2 (Mfn2), whereas the inner membrane (IM) GTPase optic atrophy 1 (OPA1) regulates fusion of the

Abbreviations: LET, linear energy transfer; RBE, relative biological effectiveness; Mfn1, mitofusin1; Mfn2, mitofusin 2; OPA1, optic atrophy 1; Drp1, Dynamin-related protein 1; Fis1, mitochondrial fission 1 protein; HIRFL, the Heavy Ion Research Facility in Lanzhou; IMP, Institute of Modern Physics; HIMAC, the Heavy Ion Medical Accelerator in Chiba; NIRS, National Institute of Radiological Sciences; MAPK, mitogen-activated protein kinase.

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IM. The central players of mitochondria fission include the Dynamin-related protein 1 (Drp1), a GTPase, which is localized mainly in the cytoplasm and recruited to mitochondrial fission sites via interaction with OM receptor proteins, such as mitochondrial fission 1 protein (Fis1) [6,7]. A perturbation of this process is associated with mitochondrial dysfunction in various diseases, including aging [8], neurodegenerative diseases [6], diabetes [9] and tumor [10].

Pioneering work on the influence of radiation on mitochondrial dynamics has shown that γ-rays excited accelerated mitochondrial fission, which was coupled with delayed mitochondrial $O^{\cdot -2}$ production in normal human fibroblast-like cells [11]. Cytoplasmic irradiation using a precision microbeam resulted in mitochondrial fragmentation in human small airway epithelial cells [12]. Moreover, mitochondrial fission was also observed in adenocarcinoma cells after laser irradiation [13]. In contrast, live imaging of mitochondria in hippocampal neurons of Sprague-Dawley rats has revealed that mitochondrial fusion occurred 5 days after irradiation with X-rays of 0.2 Gy [14]. How ionizing radiations, especially high linear energy transfer (LET) carbon ions, influence mitochondrial dynamics remains unclear. Recently, our study has indicated that carbon ions could effectively induce mitochondrial fission and the level of mitochondrial fragmentation rendered either mitophagy or apoptosis as the response of mitochondrial damages to high-LET radiation in breast cancer cell lines MCF-7 and MDA-MB-231 [15]. In this study, different mitochondrial morphological characteristics in conjunction with the key factors of mitochondrial fission and fusion in human cervical carcinoma HeLa cell line after exposure to radiations of different qualities (X-rays and carbon ions, even carbon ions of different LET values) were further analyzed and the relationship between the morphological characteristics and mitochondrial damage responses to X-rays and carbon ions was examined.

2. Materials and methods

2.1. Cell culture and reagents

Human cervical carcinoma cell line HeLa was purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). Cells were maintained in RPMI-1640 medium supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin and 10% (v/v) fetal bovine serum and kept at 37 °C, 5% CO_2 in incubators. PD0325901 treatment: cells were treated with MEK inhibitor PD0325901 (200 nM, Selleck, S1036) for 1 h before irradiation. Its treatment was removed during irradiation and then continued for 24 h after irradiation. Toxicity of PD0325901 to cells was examined before experiments (Fig. S1). Mdivi-1 treatment: cells were treated with mitochondrial division inhibitor mdivi-1 (50 μM, Sigma-Aldrich, M0199) for 4 h before irradiation. Its treatment was removed during irradiation and then continued for 4 h after irradiation. Toxicity of mdivi-1 to cells was examined before experiments (Fig. S1).

2.2. Irradiation

X-rays: Cells were irradiated with X-rays, which was generated with an X-ray machine (FAXITRON RX-650, Faxitron Bioptics, LLC, Tucson, AZ, USA) operated at 100 kVp. The dose rate was about 0.5 Gy/min.

Carbon ions: Most of the irradiation experiments were performed with a carbon ion beam of 165 MeV/u in the heavy ion therapy terminal of the Heavy Ion Research Facility in Lanzhou (HIRFL) at the Institute of Modern Physics (IMP), Chinese Academy of Sciences, China. Some experiments were conducted with a carbon ion beam of 290 MeV/u in the Heavy Ion Medical Accelerator in Chiba (HIMAC) at the National Institute of Radiological Sciences (NIRS), Japan. Dose averaged LET of the carbon ion beams on cell samples was adjusted to be 30, 50 or 70 keV/μm according to our experimental requirements.

All the irradiations were carried out at room temperature and the control groups were sham-irradiated.

2.3. Mitochondrial morphology assessment

At the times indicated following irradiation, live cells were stained with 100 nM MTG (Invitrogen, M7514) in PBS for 30 min at 37 °C, 5% CO_2 in an incubator. Digital fluorescent images were acquired. The lengths of mitochondrial were quantified using the Image J software. At least 50 cells were scored.

2.4. qRT-PCR

Total RNA was extracted, cDNA was synthesized and the expression of relevant genes was detected as reported previously [15]. The primer sequences are shown in Table S1.

2.5. Cell fractionation and western blot analysis

Mitochondrion isolation was performed with the Cell Mitochondria Isolation Kit (Beyotime, C3601) according to the manufacture's instruction. Mitochondrial and cytosolic fractions were transferred to PVDF membrane. Blots were incubated with the antibodies indicated below and visualized by the enhanced chemiluminescence (ECL) procedure, the density of band was quantified using the Quantity One software. Primary antibodies such as ERK1/2(16443-1-AP, Proteintech), cytochrome *c* (C4993, Sigma, USA), Drp 1 (sc271583), MFN1 (sc166644) (Santa Cruz), p-DRP1 S616 (3455), p-ERK1/2 (4370), COXIV (11967) and GAPDH (2118) (Cell Signaling Technology) were employed in this study.

2.6. Apoptosis assay

Twenty-four hours after irradiation, the apoptotic cells were quantified by flow cytometric measurements of Annexin V-FITC and PI double staining (Roche, 11988549001) as reported previously [16].

2.7. Clonogenic assay

After irradiation, cell survival was determined by means of the colony formation assay as reported previously [16].

2.8. Statistics

Data are represented as the mean ± standard deviation (SD). Statistical analysis was conducted using the unpaired Student's *t*-test. A difference was considered significant when $p < 0.05$.

3. Results

3.1. Changes of mitochondrial dynamics in response to different quality radiations

To determine whether the radiations of different qualities may change mitochondrial dynamics, HeLa cells were irradiated with X-rays or carbon ions of 70 keV/μm at various doses. In response to 0.2 Gy X-rays, the morphology of mitochondria in HeLa cells became longer/elongated compared to the control 4 h after irradiation, and the mean of the mitochondrial lengths increased from