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Mitochondrial electron transport chain identified as a novel molecular target of SPIO nanoparticles mediated cancer-specific cytotoxicity

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ABSTRACT

Superparamagnetic iron oxide nanoparticles (SPIONs) are highly cytotoxic and target cancer cells with high specificity; however, the mechanism by which SPIONs induce cancer cell-specific cytotoxicity remains unclear. Herein, the molecular mechanism of SPION-induced cancer cell-specific cytotoxicity to cancer cells is clarified through DNA microarray and bioinformatics analyses. SPIONs can interference with the mitochondrial electron transport chain (METC) in cancer cells, which further affects the production of ATP, mitochondrial membrane potential, and microdistribution of calcium, and induces cell apoptosis. Additionally, SPIONs induce the formation of reactive oxygen species in mitochondria; these reactive oxygen species trigger cancer-specific cytotoxicity due to the lower antioxidative capacity of cancer cells. Moreover, the DNA microarray and gene ontology analyses revealed that SPIONs elevate the expression of metallothioneins in both normal and cancer cells but decrease the expression of METC genes in cancer cells. Overall, these results suggest that SPIONs induce cancer cell death by targeting the METC, which is helpful for designing anti-cancer nanotheranostics and evaluating the safety of future nanomedicines.

1. Introduction

Superparamagnetic iron oxide nanoparticles (SPIONs) have been widely exploited for biomedical applications [1–5], including as magnetic resonance imaging (MRI) contrast agents [6], as drug delivery carriers [2], as cell labeling agents for cell tracking [4,7], and to induce hyperthermia [8]. Due to their superparamagnetic properties, high surface area-to-volume ratio, and easy transport to pathological sites by external electromagnetic fields, SPIONs are considered functional tools for cancer diagnosis and therapy that allow for tissue detailing, real-time guidance, and drug delivery monitoring [1]. Because SPIONs are mainly taken up by the liver (80%), SPIONs are approved for and used in MRI diagnoses of hepatic lesion, inflammation, and cancer [9]. Generally, SPIONs have good biocompatibility and safety, and many SPION formulas have been approved for human use by the FDA and EU health authorities for over ten years [5]. Moreover, SPIONs promote the growth of mesenchymal stem cells (MSCs) [10] without cytotoxic effects [7,11,12] and do not influence the differentiation and self-renewal of embryonic stem cells (ESCs) [4]. Furthermore, neither cytotoxic nor pro-inflammatory responses were induced by SPIONs in mouse bone marrow-derived macrophages [13].

Recently, many nanoparticles have been reported to be specifically cytotoxic to cancer cells and not normal cells; for example, gold-coated iron oxide nanoparticles inhibited the proliferation of cancer cells but did not affect normal cells [14]. Gold nanorods had

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a distinct impact on cancer cell viability, causing the deaths of A549 lung carcinoma cells with only minor effects on 16HBE normal human bronchial epithelial cells and MSCs; it was further demonstrated that the nanocomposites selectively targeted and affected the cancer cells' mitochondria [15]. Additionally, graphene oxide nanosheets inhibited CT26 colon cancer cells via the induction of autophagy [16] and hydroxyapatite nanoparticles also inhibited cancer growth *in vivo* and *in vitro*, which was mainly attributed to significant endocytosis and the inhibition of protein synthesis in cancer cells [17]. Furthermore, SPIONs are cytotoxic to A549 cells, rather than normal lung cells (IMR-90 cells), which might be due to SPION-induced elevation of reactive oxygen species (ROS) formation in cancer cells [18]. In the present study, we also observed that SPIONs promoted cancer cell death but did not induce apoptosis in normal cells. Despite these recent reports of nanoparticle-induced cancer-specific cytotoxicity, the underlying mechanism behind SPIONs' anti-cancer cell activity is still unclear.

In the present study, we investigated the molecular mechanism behind the cancer cell-specific cytotoxicity of SPIONs using DNA microarray and bioinformatics analyses. We found that SPIONs affected the mitochondrial electron transport chain (METC), which fuels mitochondrial oxidative phosphorylation and produces the majority of cellular ATP [19], in cancer cells. The SPIONs damaged cancer cell mitochondria, subsequently affecting ATP production, the mitochondrial membrane potential (MMP), and the subcellular localization of calcium, leading to mitochondria-dependent apoptosis. The METC also contributes to the production of mitochondrial ROS (Mito-SOX) in cancer cells [20]; the elimination of Mito-SOX partially inhibited the SPION-induced apoptosis of cancer cells. Collectively, our results suggest that SPIONs preferentially induce cancer cell death by targeting the METC, which provides new insights into the application of SPIONs in future nanomedicine.

2. Materials and methods

2.1. Preparation and characterization of SPIONs

The SPIO nanocrystals were synthesized according as previously reported [4]. Briefly, iron(III) acetylacetonate (2 mmol), oleic acid (6 mmol), 1, 2-hexadecanediol (10 mmol), and oleylamine (6 mmol) were added to 20 mL of benzyl ether and heated to 300 °C under argon gas protection for 1 h. Afterward, the harvested SPIO nanocrystals (15 mg) and 10 mg stearic-polyethyleneimine (600D) were dissolved in 1 mL of chloroform and sonicated for 24 h. Finally, the chloroform was removed by rotary evaporation to obtain water-dispersible SPIONs. The size and morphology of the nanoparticles were characterized using dynamic light scattering (DLS) (Zetasizer Nano system, Malvern) and transmission electron microscopy (TEM) (Tecnai 20, FEI, USA). The hysteresis loop at 300 K was recorded on a Quantum Design MPMS-XL-7 system.

2.2. Cell culture

Cells were cultured in RPMI medium 1640 (Gibco, USA) containing 10% fetal bovine serum, 100 IU/mL penicillin, and 100 μg/mL streptomycin. Some cells were grown on coverslips for immunofluorescence experiments. All cell cultures were maintained at 37 °C in a 5% CO_2 humidified incubator (150i, Thermo, USA).

2.3. Cell viability

Cell viability was measured using a MTS assay (Promega, USA). Approximately 1×10^4 cells of each cell line were seeded on 96-well plates and treated for 12 h with 2.5, 5, 10, 15, or 20 μg/mL of SPIONs. MTS was added and the cells were incubated for 3 h at 37 °C. The absorbance at 490 nm was quantified using a microplate spectrophotometer system (Multiskan, Thermo, USA).

2.4. Perl's Prussian blue staining

Cells were seeded at a density of 3×10^4 cells/well in 24-well plates. After SPION treatment for 12 h, cells were fixed with 4% paraformaldehyde for 0.5 h. Perl's stain A mixture (LEAGENE, China) was added for 0.5 h and washed with phosphate-buffered saline (PBS). Afterward, the cells were stained using Perl's stain B (LEAGENE, China) for 30 s. Finally, the stained cells were imaged using a phase-contrast reverse microscope (Nikon, Japan).

2.5. Iron content using atomic absorption spectroscopy

The iron content in the cells was determined using an atomic absorption spectrophotometer (AA-6300, Shimadzu, Japan) at 248.3 nm. The atomizer was a graphite furnace (GFA-EX7i, Shimadzu, Japan). Before atomic absorption spectrometry (AAS) analysis, the cells were suspended in 0.1 mL PBS and digested overnight using 1 mL of 6 M hydrochloric acid (HCl). The digestion solutions were diluted with ultrapure water to decrease the HCl concentration to less than 1%. The standard curve for the iron was obtained with Fe solutions at concentrations of 10, 20, 30, 40, and 50 ng/L.

2.6. Subcellular localization using TEM

After treatment with 5 μg/mL of SPIONs for 12 h, the cells were collected, fixed with 0.1 M glutaraldehyde buffer, post-fixed with 1% osmium tetroxide, dehydrated with ethanol and propylene oxide, and finally embedded in Epon. Sections (70–80 nm) were sliced and stained with uranyl acetate and lead citrate and examined with a transmission electron microscope (Tecnai 20, FEI, USA) [21].

2.7. MRI for SPION-labeled cells

Cells were incubated with SPIONs (5, 10, or 20 μg/mL) for 12 h. The cells were washed with PBS and re-suspended in an equal volume of culture media containing 2% agarose. Cells were then immediately transferred to 0.2 mL tubes to prepare MRI phantoms; the relaxation was examined using a 9.4 T MR scanner (Bruker 94/20, Germany). Untreated cells were scanned as a control.

2.8. ATP measurement

Approximately 8×10^3 cells were seeded in 96-well plates for 24 h and then treated with SPIONs (5, 10, or 20 μg/mL) for 12 h. ATP was measured using a Promega kit, according to manufacturer instructions (Promega, USA). Briefly, trichloroacetic acid (TCA) was added to "shock" the cells for 3 min to extract ATP. Afterward, Tris-acetate was added to neutralize the TCA. After centrifugation for 10 min at 12,000 rcf, the supernatants (50 μL/well) were removed and mixed with 100 μL recombinant Luciferase/Luciferin (rL/L) Reagent (Promega, USA). The luminescence values were measured at 560 nm using a microplate luminometer (Berthold Detection Systems, Germany).

2.9. Imaging of intracellular Ca^{2+}

The $[Ca^{2+}]_i$ was measured using the Ca^{2+}-sensitive fluorescent dye Fluo-3 acetoxymethyl ester (Fluo-3 AM), which is converted to Fluo-3 within cells; Ca^{2+} binding to Fluo-3 increases green fluorescence (Beyotime, China). After SPION treatment (5, 10, or 20 μg/