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Europium-doped Gd_2O_3 nanotubes cause the necrosis of primary mouse bone marrow stromal cells through lysosome and mitochondrion damage

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ABSTRACT

With the wide applications of europium-doped Gd_2O_3 nanoparticles (Gd_2O_3:Eu^{3+} NPs) in biomedical fields, it will inevitably increase the chance of human exposure. It was reported that Gd_2O_3:Eu^{3+} NPs could accumulate in bone. However, there have been few reports about the potential effect of Gd_2O_3:Eu^{3+} NPs on bone marrow stromal cells (BMSCs). In this study, the Gd_2O_3:Eu^{3+} nanotubes were prepared and characterized by powder X-ray diffraction (XRD), photoluminescence (PL) excitation and emission spectra, scanning electron microscope (SEM), and transmission electron microscopy (TEM). The cytotoxicity of Gd_2O_3:Eu^{3+} nanotubes on BMSCs and the associated mechanisms were further studied. The results indicated that they could be uptaken into BMSCs by an energy-dependent and macropinocytosis-mediated endocytosis process, and primarily localized in lysosome. Gd_2O_3:Eu^{3+} nanotubes effectively inhibited the viability of BMSCs in concentration and time-dependent manners. A significant increase in the percentage of late apoptotic/necrotic cells, lactate dehydrogenase (LDH) leakage and the number of PI-stained cells was found after BMSCs were treated by 10, 20, and 40 μg/mL of Gd_2O_3:Eu^{3+} nanotubes for 12 h. No obvious DNA ladders were detected, but a dispersed band was observed. The above results revealed that Gd_2O_3:Eu^{3+} nanotubes could trigger cell death by necrosis instead of apoptosis. Two mechanisms were involved in Gd_2O_3:Eu^{3+} nanotube-induced BMSCs necrosis: lysosomal rupture and release of cathepsins B; and the overproduction of reactive oxygen species (ROS) injury to the mitochondria and DNA. The study provides novel evidence to elucidate the toxicity mechanisms and may be beneficial to more rational applications of these nanomaterials in the future.

1. Introduction

In recent years, gadolinium oxide (Gd_2O_3) nanoparticles (NPs) are very important as nuclear, electronic, laser, optical, catalyst and phosphor materials [1]. In addition, Gd_2O_3 has also some potential applications in biomedical fields. For example, it might be used in magnetic resonance imaging (MRI) as a multimodal contrast agent because of its superparamagnetism and T1 relaxation [2]. Gd_2O_3 is known to be an excellent host material for doping with rare earth ions [3]. Liu et al. combined the standard solid-state reaction with the laser ablation to fabricate sub-10 nm monoclinic europium-doped Gd_2O_3 (called as Gd_2O_3: Eu^{3+}) NPs, which exhibited bright red fluorescence emission and were applied as fluorescence probe for cell imaging and MRI contrast agent [4]. Nichkova et al. reported that Gd_2O_3: Eu^{3+} NPs could be used in the optical imaging of antibody micropatterns [5]. Gd_2O_3: Eu^{3+} nanotubes might be used for encapsulation of proper drugs and targeted accessing by modification for their inner and outer surfaces [6,7]. Undoubtedly, these extensive applications increase the chance of human exposure to Gd_2O_3 NPs, thus raise deep concern regarding their riskiness.

Until now, although Gd_2O_3 NPs exhibit some positive bioeffects, they also show adverse bioeffects at the same time. For example, the toxicity assessment showed that the Gd_2O_3 NPs were relatively nontoxic for rat LE cells by intracellular reactive oxygen species (ROS) and 3-[4,5-dimetylthiazole-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assays [3]. Hemmer et al. reported that erbium/ytterbium-doped Gd_2O_3 (Gd_2O_3:Er^{3+}, Yb^{3+}) nanostructures had no inhibitory effect on viability of B cell hybridomas, while cytotoxic effect was found for phagocytic macrophages [8]. Other reports showed that monoclinic Gd_2O_3:Eu^{3+} NPs without surface modification had no significant cytotoxic effect in *vitro*, while the in *vivo* immunotoxicity was higher than that of Gd-diethylenetriamine pentaacetic acid (Gd-DTPA). Our group previously studied the biodistribution and toxicity of Gd_2O_3:Eu^{3+} nanotubes in mice after intraperitoneal injection. The results indicated that there was no significant difference of the blood elements between the control group and the experimental groups (1.25, 12.5, and 125 mg/kg). However, coefficients of spleen in middle- and high-dose groups mice were significantly higher than those of the control group. The results

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of biochemical parameters such as aspartate aminotransferase (AST), alanine aminotransferase (ALT), ALT/AST, uric acid (UA), lactate dehydrogenase (LDH), and hydroxybutyrate dehydrogenase (HBDH) demonstrated that the significant lesions of liver, kidney, and heart were induced by high-dose Gd_2O_3:Eu^{3+} nanotubes, but no obvious toxicity was found in low- and middle-dose groups. Moreover, the biodistribution experiment showed that gadolinium could accumulate in the bone tissue [7]. However, to the best our knowledge, the effect of Gd_2O_3:Eu^{3+} nanotubes on bone metabolism has not been reported.

It is well-known that bone marrow stromal cells (BMSCs) are multipotent stromal cells that can differentiate into a variety of cell types, including osteoblasts, adipocytes, and chondrocytes. BMSCs as cell model have been widely used in bone tissue engineering fields. For example, Yi et al. reported that gold NPs (AuNPs) promoted osteogenic differentiation of BMSCs through p38 mitogen-activated protein kinase (MAPK) pathway [9]. A novel nanocomposite based on type I collagen containing of AuNPs could promote the adhesion, proliferation, and migration of BMSCs, and might be used to construct tissue engineering scaffolds for vascular regeneration [10]. Up to now, the effects of Gd_2O_3:Eu^{3+} nanotubes on BMSCs have not been reported. In this study, we prepared Gd_2O_3:Eu^{3+} nanotubes and used primary mouse BMSCs as a cell model to investigate the cytotoxicity of Gd_2O_3:Eu^{3+} nanotubes on BMSCs and the associated mechanisms for the first time.

2. Experimental

2.1. Synthesis and characterization of Gd_2O_3:Eu^{3+} nanotubes

The Gd_2O_3:Eu^{3+} nanotubes were prepared on basis of the previous report with some slight modifications [11]. In a typical procedure, 9.5 mmol of $Gd(NO_3)_3$ and 0.5 mmol $Eu(NO_3)_3$ aqueous solution were added to 250 mL of deionized water. Then, 25 wt.% of ammonia solution was introduced to the stirred solution until pH 10. Subsequently, the mixture was heated to 75 °C for 18 h with vigorous stirring. The resulting white precipitate was collected, centrifuged, and washed with deionized water for several times and dried in air. The as-dried sample was calcined at 650 °C for 2 h in air to obtain the final Gd_2O_3:Eu^{3+} product.

The samples were characterized by powder X-ray diffraction (XRD) performed on a D8 advance diffractometer (Bruker, Germany). The morphology of the sample was inspected using a scanning electron microscope (SEM, JEOL, JSM-7500F, Japan). Transmission electron microscopy (TEM) image was obtained using a FEI Tecnai G2 S-Twin transmission electron microscope. Photoluminescence (PL) excitation and emission spectra were recorded with a Hitachi F-7000 spectrophotometer equipped with a 150 W xenon lamp as the excitation source. All measurements were performed at room temperature.

2.2. Preparation of Gd_2O_3:Eu^{3+} nanotubes suspension

Gd_2O_3:Eu^{3+} nanotubes suspension was prepared by a fresh Dulbecco's modified eagle medium (DMEM) (Gibco). In brief, 1.6 mg of Gd_2O_3:Eu^{3+} nanotube powder was suspended in 1 mL DMEM as stock solution and dispersed by ultrasonic vibration for 30 min. The suspension was stirred on a vortex agitator before every use.

2.3. Isolation and culture of BMSCs

The mouse primary BMSCs were obtained from 4 to 6 week ICR female mice (Si Bei Fu Laboratory Animal Technology Co., Ltd.) [12]. Briefly, six mice were executed by cervical vertebra, femora and tibiae were aseptically harvested, and the whole bone marrow was flushed by medium. Cells were maintained in DMEM supplemented with 10% neonatal bovine serum (NBS) (Sijiqing Biological Engineering Materials Co., Ltd.) and 100 U/mL penicillin–streptomycin (Sigma) in a humidified atmosphere of 5% CO_2 at 37 °C. The medium was replaced every 3 d in all experiments; the nonadherent cells were removed by fresh DMEM.

2.4. Cell viability assay

MTT (Sigma) assay was employed to measure the viability of BMSCs upon treated with Gd_2O_3:Eu^{3+} nanotubes [13]. Briefly, BMSCs (1×10^5 cells per well) were seeded in 96-well culture plates and cultured at 37 °C in a 5% CO_2 humidified incubator for 24 h. Gd_2O_3:Eu^{3+} nanotubes were added to wells at final concentrations of 1.25, 2.5, 5, 10, 20, 40, 80, and 160 μg/mL. Control wells were prepared by addition of DMEM, and blank wells were set by addition of DMEM without cells. After treatment for 24, 48, and 72 h, MTT solution (5.0 mg/mL) was added to wells and incubated for 4 h at 37 °C. Afterwards, the supernatant was removed and 100 μL DMSO (Sigma-Aldrich) was added to dissolve the dark blue crystals. The optical density (OD) value was read at a wavelength of 570 nm by a microplate reader (Molecular Devices, VersaMax, USA). The cell viability (%) was calculated as percentage according to the formula: $[OD_{treated} - OD_{blank}] / [OD_{control} - OD_{blank}] \times 100$.

2.5. Annexin V/propidium iodide (PI) assay

The cell apoptosis was measured by annexin V-fluorescein isothiocyanate (FITC)/PI apoptosis detection kit (Beyotime Institute of Biotechnology) as described by Aubry et al. [14]. In brief, 5×10^5 cells per well were seeded into 6-well plates and treated by 10, 20, and 40 μg/mL Gd_2O_3:Eu^{3+} nanotubes for 12 h. After treatment, the cells were washed with phosphate buffered saline (PBS), harvested and resuspended in binding buffer. Then 100 μL cell suspension was mixed with 5 μL annexin V-FITC and 5 μL PI (Sigma-Aldrich), and further incubated in the dark at room temperature for 15 min. The fluorescence intensity of annexin V-FITC was analyzed at an excitation/emission wavelength of 488/530 nm; PI fluorescence analysis was performed at an excitation/emission wavelength of 488/617 nm by FACScalibur flow cytometer (Becton Dickinson, FACSCalibur™, USA), respectively. The data were analyzed by Modfit software 3.2 software.

2.6. DNA ladder analysis

The cell apoptosis was studied by the DNA ladder pattern of internucleosomal fragmentation. In brief, cells 5×10^6 cells per well were seeded into 6-well plates and treated by 20 μg/mL Gd_2O_3:Eu^{3+} nanotubes for 6, 12, and 24 h. Then cells were extracted using the DNA ladder detection kit (TIANGEN Biotech Co., Ltd.) according to the manufacturer's instructions. The extracted DNA fragments were dissolved in Tris-EDTA (TE) buffer solution and assayed by electrophoresis on a 1.0% agarose gel containing 0.5 μg/mL ethidium bromide. The images of DNA fragments were visualized by transillumination under UV light [15].

2.7. Lactate dehydrogenase (LDH) leakage assay

LDH leakage assay was performed as described by a commercially available kit (Nanjing Jiancheng Bioengineering Institute) to evaluate the membrane integrity of cells. Briefly, BMSCs were seeded into 96-well culture plates at 5×10^5 cells/mL and incubated with different concentrations of Gd_2O_3:Eu^{3+} nanotubes (10, 20, and 40 μg/mL) for 12 h. After treatment, the plates were centrifuged at 300 ×*g* for 5 min, and 60 μL of the supernatant was collected, and incubated with 30 μL LDH substrate solution for 30 min at room temperature in the dark. The absorbance at 490 nm was measured by a microplate reader. The LDH leakage (%) was calculated according to the formula: $[(OD_{sample} - OD_{blank}) / (OD_{Triton\ X\text{-}100} - OD_{blank})] \times 100$ [16].