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Gd^{3+}对原代培养的小鼠骨髓基质细胞成骨分化和成脂分化的影响

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摘要: 本文采用 MTT 法、碱性磷酸酶活性测定、矿化功能的测定以及油红 O 的染色和定量测定等手段研究了 Gd^{3+}对原代培养的小鼠骨髓基质细胞成骨分化和成脂分化的影响。研究结果表明,浓度为 1×10^{-10} 和 1×10^{-8} mol·L^{-1} 的 Gd^{3+}对小鼠骨髓基质细胞的增殖没有影响,其他测试浓度下的 Gd^{3+}则抑制小鼠骨髓基质细胞的增殖。当 Gd^{3+}与小鼠骨髓基质细胞作用 7 d 时,其对小鼠骨髓基质细胞成骨分化的影响与作用浓度有关,当 Gd^{3+}与小鼠骨髓基质细胞作用 14 d 时,在全部测试浓度范围内,抑制小鼠骨髓基质细胞成骨分化。除 1×10^{-8} 和 1×10^{-5} mol·L^{-1} 外,其他测试浓度下的 Gd^{3+}促进小鼠骨髓基质细胞的矿化功能。当 Gd^{3+}与小鼠骨髓基质细胞作用 10 d 时,其抑制小鼠骨髓基质细胞的成脂分化,当 Gd^{3+}与小鼠骨髓基质细胞作用 16 d 时,除 1×10^{-9} mol·L^{-1} 外,其他浓度的 Gd^{3+}也抑制小鼠骨髓基质细胞的成脂分化。实验结果提示,Gd^{3+}可能通过促进骨髓基质细胞的成骨分化、抑制其成脂分化途径起到对骨的保护作用。Gd^{3+}对原代培养的小鼠骨髓基质细胞成骨分化和成脂分化的影响与作用浓度和时间有关,而且,它们是影响 Gd^{3+}对骨是损伤还是保护作用转变的关键因素。

关键词: 稀土离子; 骨髓基质细胞; 成骨分化; 成脂分化; 矿化
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Effects of Gd^{3+} on Osteogenic and Adipogenic Differentiation of Mouse Primary Bone Marrow Stromal Cells

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Abstract: A series of experimental assays including 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) test, alkaline phosphatase (ALP) activity measurement, mineralized function, oil red O stain and measurement were employed to assess the effects of Gd^{3+} on the osteogenic and adipogenic differentiation of mouse primary bone marrow stromal cells (BMSCs). The results indicate that Gd^{3+} has no effect on the proliferation of BMSCs at concentrations of 1×10^{-10} and 1×10^{-8} mol·L^{-1}, but inhibits the proliferation at other concentrations. The effect of Gd^{3+} on the osteogenic differentiation depends on concentrations at the 7th day, but Gd^{3+} inhibits the osteogenic differentiation at any concentration at the 14th day. Gd^{3+} can promote the formation of mineralized matrix nodules except at concentrations of 1×10^{-8} and 1×10^{-5} mol·L^{-1}. Gd^{3+} can inhibit the adipogenic differentiation at any concentration at the 10th day, but inhibit the adipogenic differentiation except at a concentration of 1×10^{-9} mol·L^{-1} at the 16th day. These findings suggest Gd^{3+} may have protective effect on bone

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at appropriate dose by decreasing adipogenic differentiation and promoting osteogenic differentiation of BMSCs. The effects of Gd^{3+} on the osteogenic and adipogenic differentiation of BMSCs depend on the concentration and culture time, moreover, they are pivotal factors for switching the biological effects of Gd^{3+} from damage to protection.

Key words: rare earth ion; bone marrow stromal cells; osteogenic differentiation; adipogenic differentiation; mineralization

0 Introduction

The biological properties of the lanthanides, primarily based on their similarity to calcium, have been the basis for research into potential therapeutic applications of lanthanides since the early part of the twentieth century. A lanthanide texaphyrin complex (motexafin gadolinium) is in Phase Ⅲ clinical trials in combination with whole body irradiation for the treatment of brain metastases in non-small cell lung cancer. In addition, gadolinium complexes have been used as Magnetic Resonance Imaging (MRI) contrast agents. Of the six clinically approved contrast agents, four are gadolinium-based complexes with a polyaminocarboxylate ligand. It was also found that $Gd@C_{82}(OH)_{22}$ nanoparticles had anticancer activity arising from immunomodulatory effects both in vivo and in vitro[1-2].

It was reported that the lanthanides were rapidly cleared from the blood and redistributed to tissues, primarily the liver and bone after administration. The lighter lanthanides initially go to the liver, then rapidly redistribute to the bone with a half-life of approximately 10~20 days. The heavier lanthanides accumulate in the bone where they can reside for considerable period of time with a half-life of several years[1]. Thus it is likely that the lanthanide ions intervene in bone-remodeling process and affect bone cell function. Quarles et al. found that Gd^{3+} stimulated DNA synthesis in MC3T3-E1 osteoblasts (OBs) dose-dependently in vitro[3]. Previously, we reported that the effects of Gd^{3+} on the proliferation, differentiation and function expression of osteoclasts (OCs) and OBs depended on concentrations and incubation time[4-6].

BMSCs are pluripotent cells which have the capacity to become OBs, adipocytes, chondrocytes, myoblasts or fibroblasts[7-8]. Thus, lineage determination between OBs and adipocytes may be a critical component in the regulatory pathways of osteoblastogenesis[9]. Furthermore, there is more and more evidence that suggests a great degree of plasticity exists between OBs and adipocytes and this transdifferentiation is reciprocal[10]. Indeed, it is now hypothesized that an increase in the number of adipocytes occurs at the expense of OBs in osteopenic disorders. It was reported that there was a therapeutic opportunity to either prevent or treat osteopenic disorders by inhibiting marrow adipogenesis with a concomitant increase in osteoblastogenesis[11]. Therefore, we deduced that Gd^{3+} might intervene in bone-remodeling process by affecting the osteogenic and adipogenic differentiation of BMSCs. Until now, the effects of Gd^{3+} on the osteogenic and adipogenic differentiation of BMSCs were not reported. In this paper, the effects of Gd^{3+} on the osteogenic and adipogenic differentiation of BMSCs were studied in order to further elucidate the effect of Gd^{3+} on bone metabolism.

1 Materials and methods

1.1 Materials and reagents

Kunming (KM) mice (4~6 weeks) were obtained from Experimental Animal Center of Hebei Medical University. Dulbecco′s modified Eagle′s medium (DMEM) and fetal bovine serum (FBS) were from Gibco. Benzylpenicillin, streptomycin, MTT, β-glycerophosphate, trypsin, dexamethasone, ascorbic acid, insulin, alizarin red S (ARS), oil red O stain and cetylpyridium chloride were obtained from Sigma. An ALP activity kit was obtained from Nanjing Jiancheng Biological Engineering Institute, and a micro-protein assay kit was from Beyotime Biotechnology. Gadolinium chloride (Purity > 99.9%) was purchased from Beijing Institute of Rare Earth Sci. & Tech. Co., Ltd.

1.2 Methods

1.2.1 Isolation and culture of primary BMSCs

The mouse BMSCs were obtained from adult KM mice by a modification of the method previously reported[12]. Briefly, mice were sacrificed by decapitation. Femora and tibiae were aseptically harvested, and the whole bone marrow was flushed by DMEM in a 1 mL syringe and a 25-gauge needle. The cells were collected and cultured in a culture flask. After 3 d incubating at 37 ℃, in a 5% CO_2 humidified incubator, the nonadherent cells were removed by gentle aspiration and the medium was replaced with fresh DMEM. Then the medium was changed every 3 d in all experiments.

1.2.2 Cell proliferation assay

The protocol described by Mosmann was followed by some modifications[13]. Briefly, BMSCs (3×10^6 cells per well) were plated in 96-well culture plates and cultured overnight at 37 ℃, in a 5% CO_2 humidified incubator. Gd^{3+} was added at final concentrations of 1×10^{-10}, 1×10^{-9}, 1×10^{-8}, 1×10^{-7}, 1×10^{-6}, and 1×10^{-5} mol·L^{-1}. Control wells were prepared by addition of DMEM. Wells containing DMEM without cells were used as blanks. The plates were incubated for 44 h. Upon completion of the incubation, MTT dye solution (20 μL, 5 mg·mL^{-1}) was added. After 4 h incubation, the supernatant was removed and DMSO (100 μL) was added. The optical density (OD) was measured on a microplate spectrophotometer (MD VersaMax, USA) at a wavelength of 570 nm. The proliferation rate (%) was calculated according to the formula: $(OD_{treated}/OD_{control}-1)\times100\%$.

1.2.3 Measurement of ALP activity

BMSCs (3×10^6 cells per well) were plated in 48-well culture plates, after being induced by osteogenic supplement (10^{-7} mol·L^{-1} dexamethasone, 5.0 mmol·L^{-1} β-glycerophosphate, 50 μg·mL^{-1} ascorbic acid) and treated with Gd^{3+} at final concentrations of 1×10^{-10}, 1×10^{-9}, 1×10^{-8}, 1×10^{-7}, 1×10^{-6}, and 1×10^{-5} mol·L^{-1} for 7 d and 14 d respectively. The plates were washed thrice with ice-cold PBS and lysed by two cycles of freezing and thawing. Aliquots of supernatants were subjected to ALP activity and protein measurement by an ALP kit and a micro-protein assay kit respectively. The osteogenic differentiation promotion rate(%) was calculated according to the formula: $(ALP\ activity_{treated}/ALP\ activity_{control}-1)\times100\%$[14].

1.2.4 Mineralized matrix formation assay

BMSCs (2×10^5 cells per well) were plated in 48-well culture plates and cultured overnight at 37 ℃, in a 5% CO_2 humidified incubator. The medium was then changed to differentiation medium containing 10 mmol·L^{-1} β-glycerophosphate and 50 μg·mL^{-1} ascorbic acid in the presence or absence of the concentrations of 1×10^{-10}, 1×10^{-9}, 1×10^{-8}, 1×10^{-7}, 1×10^{-6}, and 1×10^{-5} mol·L^{-1} Gd^{3+} for 21 d. The formation of mineralized matrix nodules was determined by ARS stain. Briefly, the cells were fixed in 70% ethanol for 1 h at room temperature. The fixed cells were washed with PBS and stained with 40 mmol·L^{-1} ARS, pH 4.2, for 30 min at room temperature. Quantitation of ARS staining was performed by elution with 10% (*w*/*v*) cetylpyridium chloride for 10 min at room temperature and the OD was measured at 570 nm[15]. Results were expressed as moles of ARS/milligram of total cellular protein.

1.2.5 Oil red O stain and measurement

BMSCs (3×10^6 cells per well) were plated in 48-well culture plates, after being induced by adipogenic supplement (10 μg·mL^{-1} insulin, 10^{-7} mol·L^{-1} dexthamethone) and treated with Gd^{3+} at final concentrations of 1×10^{-10}, 1×10^{-9}, 1×10^{-8}, 1×10^{-7}, 1×10^{-6}, and 1×10^{-5} mol·L^{-1}, and fat droplets within differentiated adipocytes were stained using the oil red O[16]. Briefly, cell monolayers were fixed in 4% formaldehyde, washed in water and stained with a 0.6% (*w*/*v*) oil red O solution (60% isopropanol, 40% water) for 15 min at room temperature. For quantification, cell monolayer was washed extensively with water, then 1 mL of isopropyl alcohol was added. After 5 min, the OD was measured at 510 nm. The adipogenic differentiation inhibition rate(%) was calculated according to the formula: $(1-OD_{treated}/OD_{control})\times100\%$.

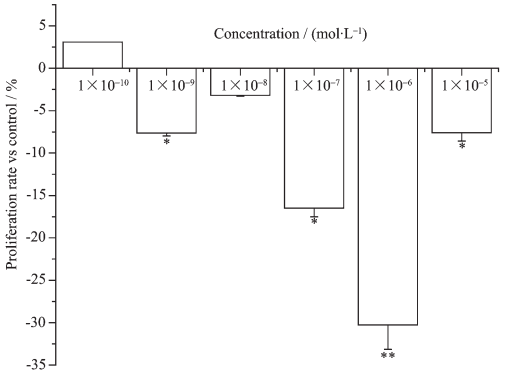
1.2.6 Statistical analysis

Data were collected from at least three separate experiments. The results were expressed as means ± standard deviation (SD). The statistical differences were analyzed using SPSS′ t-test. P values less than 0.05 were considered to indicate statistical differences.

2 Results

2.1 Effect of Gd^{3+} on the BMSC proliferation

As shown in Fig.1, Gd^{3+} can inhibit the BMSC proliferation at concentrations of 1×10^{-9}, 1×10^{-7}, 1×10^{-6}, and 1×10^{-5} mol·L^{-1}, but has no effect at other concentrations.

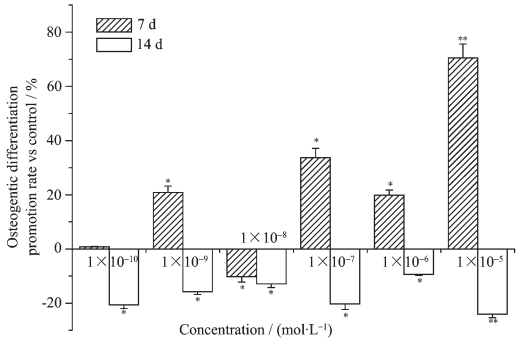


*P<0.05, **P<0.01 vs control group, n=6

Fig.1 Effect of Gd^{3+} on the proliferation of BMSCs

2.2 Effect of Gd^{3+} on the osteogenic differentiation of BMSCs

As shown in Fig.2, Gd^{3+} has no effect on the osteogenic differentiation of BMSCs at a concentration of 1×10^{-10} mol·L^{-1}, inhibits the osteogenic differentiation of BMSCs at a concentration of 1×10^{-8} mol·L^{-1}, but promotes osteogenic differentiation of BMSCs at other concentrations at the 7th day. The osteogenic differentiation of BMSCs is inhibited at any concentration at the 14th day.

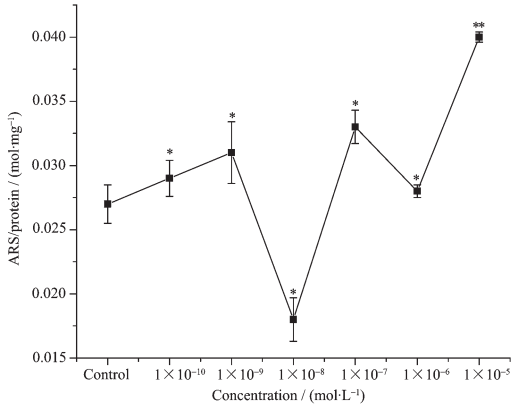


*P<0.05, **P<0.01 vs control group, n=6

Fig.2 Effect of Gd^{3+} on the osteogenic differentiation of BMSCs

2.3 Effect of Gd^{3+} on the formation of mineralized matrix nodules

As shown in Fig.3, Gd^{3+} can inhibit the formation of mineralized matrix nodules of BMSCs at a concentration of 1×10^{-8} mol·L^{-1}, but promote the formation of mineralized matrix nodules at other concentrations.

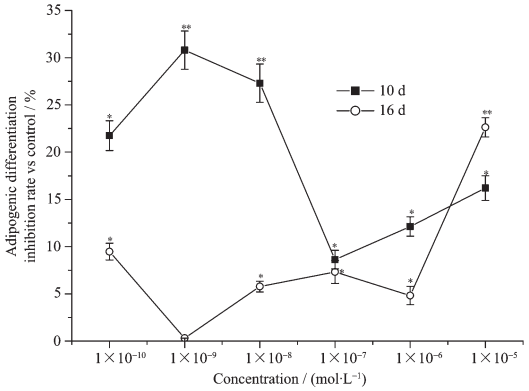


*P<0.05, **P<0.01 vs control group, n=6

Fig.3 Effect of Gd^{3+} on the mineralized fuction of BMSCs

2.4 Effect of Gd^{3+} on the adipogenic differentiation of BMSCs

As shown in Fig.4, Gd^{3+} can inhibit the adipogenic differentiation at any concentration at the 10th day. It has no effect on the adipogenic differentiation at a concentration of 1×10^{-9} mol·L^{-1}, but inhibits the adipogenic differentiation at other concentrations with the maximal inhibition rate at a highest concentration of 1×10^{-5} mol·L^{-1} at the 16th day.



*P<0.05, **P<0.01 vs control group, n=6

Fig.4 Effect of Gd^{3+} on the adipogenic differentiation of BMSCs

3 Discussion

Once BMSCs are committed to the OB lineage, proliferating osteoprogenitors become pre-osteoblasts, cell growth declines, and there is a progressive expression of markers of differentiation by post-mitotic

OBs. The sequence of osteogenic differentiation is characterized by the expression of ALP, followed by the synthesis and deposition of type Ⅰ collagen, bone matrix proteins, and glycosaminoglycans, and an increased expression of osteocalcin and bone sialoprotein at the onset of mineralization[7]. In addition, in vitro studies have shown an inverse relationship between the differentiation of adipocytic and osteogenic cells from BMSCs. Recent data suggested that medullary adipocytes were secretory cells that might influence hematopoiesis and osteogenesis[17]. It was reported that preadipocytes isolated from mouse marrow might regulate the activity and final differentiation of OBs. The condition medium harvested from mouse stromal preadipocytes decreased the ALP activity of a mouse stromal osteoblastic cell line [18]. Benayahu et al. reported that preadipocytes also had the potential to stimulate OC differentiation[19]. Adipocytes synthesized and released a variety of peptide and nonpeptide compounds or secreted cytokines, and the main effect of these cytokines was a stimulation of bone resorption[17].

In this study, we have examined the effects of Gd^{3+} on the osteogenic and adipogenic differentiation of BMSCs in vitro by mouse primary BMSCs. Our results indicate that: (1) Gd^{3+} inhibites the BMSC prolifera-tion at most concentrations; (2) Gd^{3+} promots the osteogenic differentiation of BMSCs at most concentrations at the 7th day, but the osteogenic differentiation is inhibited at any concentration at the 14th day; (3) Gd^{3+} promots the formation of miner-alized matrix nodules except at a concentration of 1×10^{-8} mol·L^{-1}; (4) Gd^{3+} inhibits the adipogenic differen-tiation of BMSCs at any concentrations at the 10th day, but inhibits the adipogenic differentiation except at a concentration of 1×10^{-9} mol·L^{-1} at the 16th day. Liu et al. have studied the effect of La^{3+} on the osteoblastic differentiation by a rat BMSC model. Their results suggest that La^{3+} affects BMSC osteoblastic differentiation depending on differentiation stages. La^{3+} inhibits the osteoblastic differentiation in the early and middle stages by a mitogen-activated protein kinase (MAPK)-dependent mechanism, but does not affect the matrix mineralization in advanced BMSCs[20]. We previously reported that Dy^{3+} had no effect on BMSC proliferation at concentrations of 1×10^{-8} and 1×10^{-5} mol·L^{-1}, but inhibited BMSC proliferation at other concentrations. Dy^{3+} had no effect on the osteogenic differentiation of BMSCs at concentrations of 1×10^{-9} and 1×10^{-7} mol·L^{-1}, and promoted osteogenic differen-tiation at other concentrations at the 7th day. The osteogenic differentiation was inhibited at a concentra-tion of 1×10^{-5} mol·L^{-1}, but promoted osteogenic differe-ntiation at concentrations of 1×10^{-9}, 1×10^{-8}, 1×10^{-7}, and 1×10^{-6} mol·L^{-1} at the 14th day. Dy^{3+} promoted mine-ralized function of BMSCs at any concentration. Dy^{3+} had no effect on adipogenic differentiation at a concen-tration of 1×10^{-7} mol·L^{-1}, but inhibited adipogenic differentiation at other concentrations [21]. This suggests that the different species of rare earth ions might behave differently, this has been recognized in a series of biological effects of lanthanides. These differences might relate to the physico-chemical characteristics of the respective cations depending upon features. In addition, in biological systems, rare earth ions exist as electron-deficient cations and are hence attracted to electron-rich biological molecules. Biological systems themselves provide innumerable examples of designer ligands′ that bind metal ions to perform important biological functions[22]. Thus biological mole-cules in culture medium might bind rare earth ions and form different metal complexes, and further cause their difference of biology behavior. In addition, we previously studied the effect of Gd^{3+} on bone resorbing function of rabbit primary OCs and OBs (UMR106)[4-5]. The results indicated that Gd^{3+} at concentrations of 1.00×10^{-5} and 1.00×10^{-6} mol·L^{-1} inhibited osteoclastic activity as indicated by the dose-dependent reduction in the numbers and surface areas of the lacunae, but the osteoclastic bone resorbing function was significantly enhanced by Gd^{3+} at a concentration of 1.00×10^{-7} mol·L^{-1}. Gd^{3+} promoted the proliferation of OBs at concentrations of 1.00×10^{-9}, 1.00×10^{-8}, 1.00×10^{-7}, 1.00×10^{-6}, and 1.00×10^{-5} mol·L^{-1}, moreover, it significantly improved the differentiation of OBs at concentrations of 1.00×10^{-7} and 1.00×10^{-5} mol·L^{-1}. This also suggests Gd^{3+} might have protective effect on bone at appropriate

dose by decreasing adipogenic differentiation and promoting osteogenic differentiation of BMSCs.

In summary, the effects of Gd^{3+} on the osteogenic and adipogenic differentiation of BMSCs in vitro depend on the concentration and culture time, moreover, they are pivotal factors for switching the biological effects of Gd^{3+} from damage to protection. The mechanism of the effects of Gd^{3+} on the osteogenic and adipogenic differentiation of BMSCs remain to be further studied.

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