Effects of lanthanum and gadolinium on proliferation and differentiation of primary osteoblasts*

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Abstract The effects of La^{3+} and Gd^{3+} on the proliferation, differentiation and adipogenic transdifferentiation of rat calvarial osteoblast-like cells (ROB cells) were evaluated by MTT method, measuring the activity of alkaline phosphatase (ALP) and Oil red O measurement. Both of La^{3+} and Gd^{3+} inhibited the proliferation of ROB cells at all concentrations (1×10^{-5}, 1×10^{-6}, 1×10^{-7}, 1×10^{-8}, 1×10^{-9} mol·L^{-1}). La^{3+} at concentration of 1×10^{-5} mol·L^{-1} significantly increased the alkaline phosphatase activity of ROB cells up to 3 folds ($P<0.01$). However, the effects reversed to inhibit at other concentrations. Gd^{3+} played a negative role in the alkaline phosphatase activity. La^{3+} inhibited the adipogenic transdifferentiation of ROB cells at all concentrations in a dose-dependent way. However, Gd^{3+} promoted the adipogenic transdifferentiation of ROB cells at 1×10^{-8} and 1×10^{-9} mol·L^{-1}. These findings suggested that the effects of rare earth elements on the proliferation, differentiation and adipogenic transdifferentiation of ROB cells were dependent on their concentrations and species.

Keywords: lanthanum, gadolinium, osteoblast-like cells, proliferation, differentiation, transdifferentiation.

Osteoporosis, characterized by low bone mass and microarchitectural deterioration of bone tissue, is a major public health threat in the elderly. Bone is a mineralized tissue which contains two distinct cell types: the osteoblasts and osteoclasts. Osteoblasts synthesize and mineralize the collagenous extracellular matrix of bone; while, osteoclasts are responsible for the normal remodeling and vascularization of bone. Given the variety and the importance of the biological processes in which these two cell types participate in development and in postnatal life, there is great interest in understanding their differentiation and function[1]. Besides, accumulated clinical and experimental studies have revealed the "seesaw effect" between the content of marrow adipocytes and bone loss[2,3]. In addition to supporting lymphohemaetopoiesis[4], preadipocytes or adipocytes support osteoclastogenesis[5,6]. Moreover, the evidence for the reciprocal transdifferentiation between osteoblasts and adipocytes is accumulating[7,8]. Thus, the inhibition of marrow adipogenesis with a concomitant increase in osteogenesis may provide more efficacious prevention or treatment of osteoporosis.

Widely used as fertilizer additives and feedstuff additives[9], rare earth elements have been entering the environment and food chains, including water, soil and food. Are the rare earth elements beneficial or harmful to human? Their potential impacts on human health and the environment will be topics under the serious discussions. As the physical and chemical properties of rare earth ions are mostly similar as Ca^{2+}, they may interfere with bone-reconstruction process[10]. Available precedents suggest that rare earth elements present versatile effects in the process of bone remolding *in vivo* and *in vitro*. Jha et al.[11] found that Pr_6O_{11} and Nd_2O_3 promoted bone-resorption in mice, and Li et al.[12] reported that long-term oral administration of La^{3+} at a low dose in rats caused lanthanum accumulation in the bone tissue, decreased bone density and changed the microstructure of bone. Zhang et al. have investigated the effects of La^{3+} and Gd^{3+} on formation and bone-resorbing activity of osteoclast-like cells and mature osteoclasts[13—15]. In addition, Zhang et al. also studied the effects of La^{3+} and Gd^{3+} on the proliferation, dif-

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ferentiation and function of UMR106 cells[16]. However, the UMR-106 cell line is a clonal derivative of a transplantable rat osteosarcoma, and so far no studies have been carried out to assess the potential effects of La^{3+} and Gd^{3+} on the proliferation and differentiation of rat primary osteoblast-like cells (ROB cells).

1 Materials and methods

1.1 Materials and reagents

New born Kunming (KM) mice were obtained from Guangzhou University of Traditional Chinese Medicine. Minimum essential medium alpha (α-MEM), trypsin and fetal bovine serum were purchased from Gibco. 3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2H tetrazolium bromide (MTT), benzylpenicillin, streptomycin, β-glycerophosphate, dexamethasone, ascorbic acid, collagenase II, insulin, oil red O were from sigma. Alkaline phosphatase (ALP) activity kit and cell alkaline phosphatase (CAKP) stain kit were obtained from Nanjing Jiancheng Biological Engineering Institute (Nanjing, China), and micro-protein assay kit was purchased from Beyotime Biotechnology (Haimen, China). Lanthanum chloride and gadolinium chloride (Purity > 99.9%) were purchased from Beijing Institute of Founder Rare Earth Sci. & Tech. Co., Ltd.

1.2 Methods

1.2.1 Isolation and culture of primary osteoblasts ROB cells were prepared from 3-day-old KM mice calvarias following the sequential enzymatic digestion method described previously with a little modification[17]. Briefly, skull (frontal and perietal bones) were dissected; then endosteum and periosteum were stripped off, and the bone was cut into approximately 1—2 mm^2 pieces and sequentially digested with trypsin (2.5 $mg \cdot ml^{-1}$) for 30 min and collagenase II (1.0 $mg \cdot ml^{-1}$, Sigma) twice for 1 h each time. The cells were collected and cultured in α-MEM with 10% fetal bovine serum, benzylpenicillin (50 $U \cdot ml^{-1}$), and streptomycin (50 $\mu g \cdot ml^{-1}$), for 24 h in a humidified atmosphere of 5% CO_2 in air at 37℃, then old medium were changed. The culture medium was changed every 3 days in all the experiments.

1.2.2 Identification of primary osteoblasts Morphological characteristics of osteoblasts were observed by phase contrast microscopy. Biochemical indices (alkaline phosphatase stain and Alizarin Red stain) were applied to identify the osteoblast phenotype.

ROB cells ($2 \times 10^3/cm^2$) were plated in a 28-cm^2 dish and cultured for 6 days in growth medium as before and 10 days in growth medium supplied with 50 $\mu g \cdot ml^{-1}$ ascorbic acid and 1×10^{-2} $mol \cdot L^{-1}$ β-glycerophosphate respectively. For the experiment of alkaline phosphatase stain, a cell alkaline phosphatase (CAKP) stain kit (Kaplow method) was used according to the protocol by the supplier. For the experiment of alizarin red S stain, cells were fixed in 95% ethanol for 30 min at room temperature, washed with PBS and stained for 20 min with 0.1% alizarin red S (pH 4.2) at room temperature.

1.2.3 Quantitation of cell proliferation ROB cells were seeded in a 96-well plate at the density of 2×10^3/well, and incubated 24 h prior to addition of La^{3+} and Gd^{3+}; then, cultured for another 48 h. Cells were treated with MTT (5 $mg \cdot ml^{-1}$) 4 h prior to the end of the experiment. At the end of this experiment, the supernatant was removed and DMSO was added to dissolve formazan, and absorbance at 570 nm was measured on a microplate spectrophotometer (Bio-rad Model 680, USA). The inhibition rate (%) was evaluated according to the formula: $(OD_{sample} - OD_{blank})/(OD_{control} - OD_{blank}) \times 100\%$.

1.2.4 Measurement of alkaline phosphatase activity ROB cells (2×10^4 cells per well) were seeded in 48-well culture plates and cultured overnight at 37℃ in a 5% CO_2 humidified incubator. La^{3+} and Gd^{3+} were added to culture medium at final concentrations of 1.00×10^{-5}, 1.00×10^{-6}, 1.00×10^{-7}, 1.00×10^{-8}, 1.00×10^{-9} $mol \cdot L^{-1}$ and cultured for further 3 days. The plates were washed twice with ice-cold D-Hank's solution and lysed by two cycles of freezing and thaw. Aliquots of supernatants were subjected to alkaline phosphatase activity (ALP) and protein content measurements by a alkaline phosphatase activity kit and a micro-Bradford assay kit respectively; all results were normalized by protein content. The results were expressed as ALP $activity_{sample}$/ ALP $activity_{control} \times 100\%$.

1.2.5 Oil red O stain and measurement ROB cells (2×10^4 cells per well) were seeded in 48-well culture plates as before, and were cultured for 10

days. The adipogenic supplement ($10\ mg \cdot L^{-1}$ insulin, $1.00 \times 10^{-7}\ mol \cdot L^{-1}$ dexamethasone) and La^{3+} and Gd^{3+} (final concentrations of 1.00×10^{-5}, 1.00×10^{-6}, 1.00×10^{-7}, 1.00×10^{-8}, $1.00 \times 10^{-9}\ mol \cdot L^{-1}$) were added to the culture medium. Fat droplets within transdifferentiated adipocytes from ROB cells were evaluated by oil red O staining method developed by Ichiro et al.[18]. Cell monolayers were washed twice by PBS then stained by 0.6% oil red O solution (60% isoprpanol, 40% water) for 15 min at room temperature. For quantification of oil red O content, the cells were washed with dH_2O three times to remove background staining, and isopropyl alcohol was added to resolve oil red O. Absorbance at 510 nm was measured on a microplate spectrophotometer (Bio-rad Model 680, USA). The adipogenic transdifferentiation rate was evaluated according to the formula: $(OD_{sample} - OD_{control})/(OD_{control} - OD_{blank}) \times 100\%$.

1.2.6 Statistical analysis Data were collected from three separate experiments and expressed as means ± SEM. The statistical differences were analyzed by Student t-test. P values less than 0.05 were considered to be statistically significant.

2 Results

2.1 Identification of osteoblast-like cells

Alkaline phophatase stain combined with mineralization nodes stain identified the osteoblast phenotype. As shown in Fig.1, azo dye precipitated in the cytoplasm of ROB cells, and alizarin red formed an orange-red nest with calcium-containing crystal deposits.

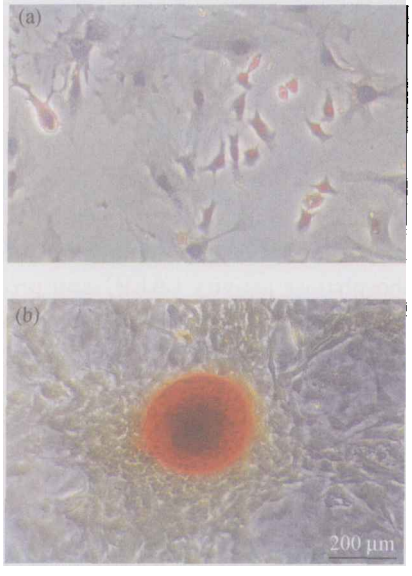

Fig. 1. Identification of the ROB cells. (a) Osteoblasts stained by Kaplow method; (b) osteoblasts stained by alizarin red.

2.2 Effects of La^{3+} and Gd^{3+} on the proliferation of ROB cells

As shown in Fig. 2, La^{3+} and Gd^{3+} at all concentrations (1×10^{-5}, 1×10^{-6}, 1×10^{-7}, 1×10^{-8}, $1 \times 10^{-9}\ mol \cdot L^{-1}$) inhibited the proliferation of ROB cells. La^{3+} at $1 \times 10^{-9}\ mol \cdot L^{-1}$ and Gd^{3+} at $1 \times 10^{-5}\ mol \cdot L^{-1}$ exhibited the most significant effect by 27% and 17% respectively.

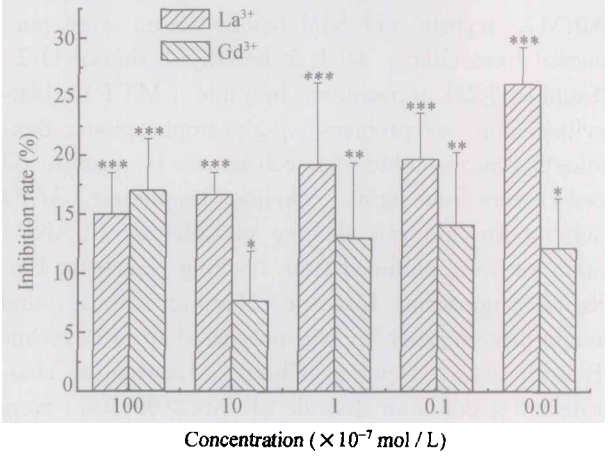

Fig. 2. Effects of La^{3+} and Gd^{3+} on the proliferation of ROB cells. $n = 6$, * $P < 0.05$ vs. control, ** $P < 0.01$ vs. control, *** $P < 0.001$ vs. control.

2.3 Effects of La^{3+} and Gd^{3+} on the alkaline phosphatase activity of ROB cells

As shown in Fig. 3, La^{3+} at concentration of $1 \times 10^{-5}\ mol \cdot L^{-1}$ significantly increased alkaline phos-

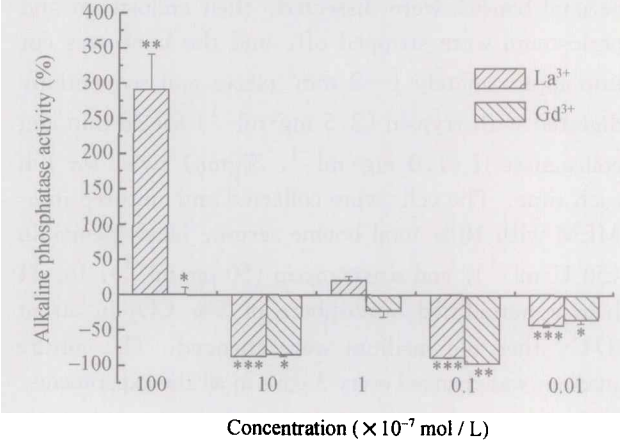

Fig. 3. Effects of La^{3+} and Gd^{3+} on the alkaline phosphatase activity of ROB cells. $n = 6$, * $P < 0.05$ vs. control, ** $P < 0.01$ vs. control, *** $P < 0.001$ vs. control.

phatase activity of ROB cells up to 3 folds ($P < 0.01$). However, there was a sharp decrease in other concentrations and the effects reversed to inhibit. Gd^{3+} exhibited a negative effect on the alkaline phosphatase activity at all concentrations except 1×10^{-5} mol·L^{-1}.

2.4 Effects of La^{3+} and Gd^{3+} on the adipogenic transdifferentiation of ROB cells

As shown in Fig. 4, La^{3+} inhibited the adipogenic transdifferentiation of ROB cells at all concentrations dose-dependently. However, Gd^{3+} promoted the adipogenic transdifferentiation of ROB cells at 1×10^{-8} and 1×10^{-9} mol·L^{-1}. The morphological observation was in accordance with the result (Fig. 5).

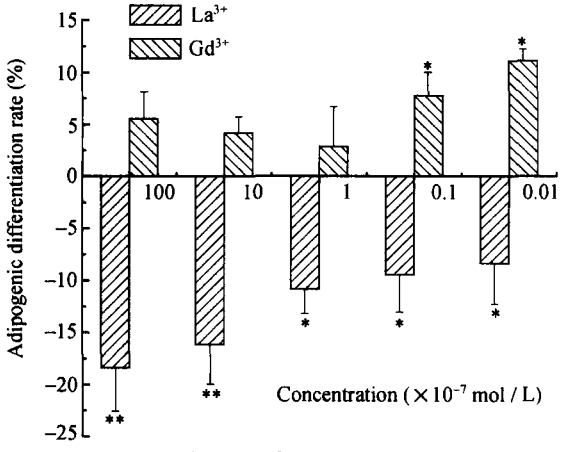

Fig. 4. Effects of La^{3+} and Gd^{3+} on the adipogenic transdifferentiation of ROB cells. $n = 6$, * $P < 0.05$ vs. control, ** $P < 0.01$ vs. control.

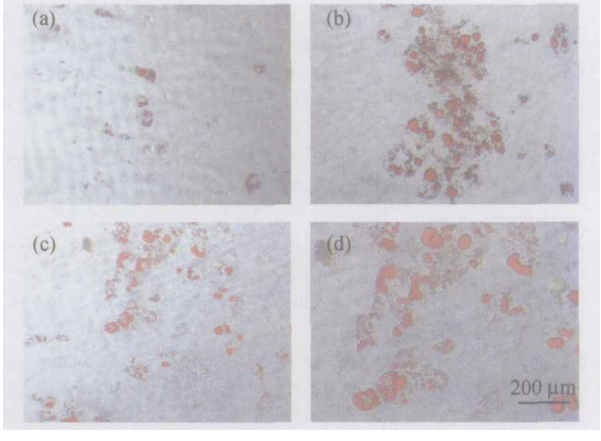

Fig. 5. Effects of La^{3+} and Gd^{3+} on the adipogenic transdifferentiation of ROB cells. (a) Control without adipogenic supplement; (b) adipogenic supplement; (c) adipogenic supplement + 1×10^{-6} mol·L^{-1} La^{3+}; (d) adipogenic supplement + 1×10^{-8} mol·L^{-1} Gd^{3+}.

3 Discussion

Bone remodeling is a very complex process of tightly coordinated action by the bone resorbing osteoclasts and the bone forming osteoblasts. Derived from a mesenchymal cell lineage, osteoblasts are responsible for forming new bone matrix in their differentiated state[19]. It has been postulated that bone loss associated with aging is caused by a defect in the osteoblast cell lineage[20,21]. For instance, the mesenchymal precursor population is either insufficient or has lost the capacity to proliferate and/or differentiate into sufficient numbers of functioning osteoblasts. The progression of osteoblast differentiation has been modeled in cell culture using primary calvarial or bone marrow cells. Bone marrow contains pluripotent stem cells of the adipocytic, osteoblastic, fibroblastic, and hematopoetic cell lineages, thus the effects of agents are more complicate to evaluate than primary osteoblasts. Medullary adipocytes are not only space-filling cells in bone marrow cavity but also secretory cells that may influence hematopoiesis, osteogenesis[22] and osteoclastogenesis[5,6]. Adipocytes express a number of proteins, such as leptin[23] and C3[24], that may contribute to the support of osteoclast-like cells. With ageing, increased marrow adipocytes accompany with decreased trabecular bone volume. Therefore, marrow adipogenesis may be an important complication of osteoporosis. Meanwhile, the evidence for the reciprocal transdifferentiation between osteoblasts and adipocytes is accumulating[7,8]; and results from osteoporotic patients confirm that the increase in the marrow adipogenesis of osteoporotic patients concomits in detriment of marrow osteogenesis[7,26]. Marrow adipocyte may be considered as an important target cell for the therapeutic intervention in osteoporosis. The inhibition of marrow adipogenesis and concomitant enhancement in osteogenesis may serve as a potential approach to increase bone formation and therefore provide more choices and to the prevention or treatment of osteoporosis.

In our present study, the effects of La^{3+} and Gd^{3+} on the proliferation, differentiation, and transdifferentiation of ROB cells *in vitro* were observed. La^{3+} and Gd^{3+} at all concentrations inhibited the proliferation of ROB cells. As to the alkaline phosphatase activity, the results became complicated. La^{3+} dramatically increased the alkaline phosphatase activity of

ROB cells at concentration of 1×10^{-5} mol·L^{-1}, but sharply dropped and reversed to inhibit at other concentrations tested. While, Gd^{3+} inhibited the alkaline phosphatase activity at most concentrations. La^{3+} and Gd^{3+} exhibited their distinctive effects on the adipogenic transdifferentiation of ROB cells. La^{3+} inhibited the adipogenic transdifferentiation of ROB cells at all concentrations; while Gd^{3+} promoted the adipogenic transdifferentiation of ROB cells at 1×10^{-8} and 1×10^{-9} mol·L^{-1}. However, Zhang et al.[16] reported that La^{3+} and Gd^{3+} increased the proliferation (1×10^{-5} to 1×10^{-9} mol·L^{-1}) and alkaline phosphatase activity (1×10^{-5} and 1×10^{-7} mol·L^{-1}) of UMR106 cells at proper concentrations. This discrepancy was due to the different cell phenotype used in these two experiments: UMR-106 cell line is a clonal derivative of a transplantable rat osteosarcoma and primary ROB cells are separated from mice calvarias which is more similar to the osteoblasts *in vivo*. As to adipogenesis, most of the studies were focused on the relation between marrow adipogeniesis and marrow osteogenesis. We found in this study that osteoblasts are readily to transdifferentiate to adipocytes even in the culture medium without adipogenic supplement. Thus, the transdifferentiation between osteoblasts and adipocytes may be very important in the pathogenesis of osteoporosis. As adipocytes express a number of proteins and lipid-derived products contributing to osteoclast-like cells and osteoclast differentiation, considering the relationship between adipogenesis and osteoclastogenesis, the effects of La^{3+} and Gd^{3+} on the adipogenic transdifferentiation may be also reflected in their effects on the osteoclastogenesis. This hypothesis has been confirmed by previous studies that La^{3+} at concentrations of 1×10^{-5}, 1×10^{-6}, and 1×10^{-7} mol·L^{-1} inhibited the formation and bone-resorbing activity of osteoclast-like cells[13] and mature osteoclasts[15]. Gd^{3+} at concentration of 1×10^{-7} mol·L^{-1} promoted the bone-resorbing activity of mature osteoclasts[15]. However, at other concentrations the results may be hard to explain and may be controversal with our results. The mechanism of them remains to be further studied.

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