Effects of the rare earth ions on bone resorbing function of rabbit mature osteoclasts in vitro

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Abstract The effects of rare earth ions on bone resorbing function of osteoclasts were studied by culturing Japanese white rabbit osteoclasts on bone slices. In order to evaluate the activity of osteoclasts, the number and surface areas of lacunae were measured by photomicrography and image analysis, and the calcium concentration in the supernatant was measured by the atomic absorption spectrometry. The lacunae morphology was observed under a scanning electron microscope. The results indicated that La³⁺, Sm³⁺ and Er³⁺ at the concentration of 1.00×10⁻⁶, 1.00×10⁻⁶ and 1.00×10⁻⁸ mol/L and Nd³⁺, Gd³⁺ and Dy³⁺ at the concentration of 1.00×10⁻⁷ and 1.00×10⁻⁶ mol/L inhibited osteoclastic activity as indicated by the dose-dependent reduction in the numbers and surface areas of the lacunae (P<0.01). On the contrary, the number and surface areas of lacunae were increased and osteoclastic bone resorbing function was significantly enhanced by La³⁺, Sm³⁺ and Er³⁺ at the concentration of 1.00×10⁻⁸ mol/L and Nd³⁺, Gd³⁺ and Dy³⁺ at the concentration of 1.00×10⁻⁷ mol/L (P<0.01). Nd³⁺, Gd³⁺ and Dy³⁺ had no effect on osteoclastic bone resorption function at concentrations as low as 1.00×10⁻⁸ mol/L (P>0.05). It is suggested that the effects of rare earth ions on osteoclastic bone resorption are bidirectional, depending on concentrations and species.

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The osteoclast plays initiating and vanguard roles in the course of bone remodeling. The resorption and formation of bone is the basic clues of bone remodeling, which results from the mutual dependent functions of osteoclasts and osteoblasts. When the osteoclasts are activated, the bone resorption function is increased. The excess bone resorption leads to osteoporosis, characterized by formation of many hollows, lacunae and tunnels on the bone surface or in depth[1,2]. The differentiation, proliferation, apoptosis and bone-resorbing activity of osteoclasts depend on the growth media and environmental factors. Among them, the essential trace elements were reported to play important roles in the pathogenesis of osteoporosis. According to the results of laboratory animal and clinical studies, Strause et al. indicated that copper, zinc and manganese were important in maintaining the bone matrix and bone density. The bone density decrease in calcium-supplemented, older postmenopausal women can be further arrested by concomitant increases in trace mineral intake[3]. Strontium can stimulate bone formation, increase osteoid formation and regulate calcium metabolism[4]. It is reported that the short-term oral strontium supplementation reduced the osteoclast activity and long-term treatment with strontium supplementation produced a rapid stimulatory effect on bone formation[5]. Morohashi et al. pointed out that strontium supplementation to ovariectomized rats had no effects on calcium levels in bone and urinary calcium[6]. Brandi suggested it as one of new treatment strategies[7]. It is reported that an appropriate amount of germanium, vanadium and molybdenum is also beneficial for normal development and metabolism of skeletons[8,9]. It is found that the rare earth ions could affect bone formation and dissolution. Jha et al. found that Pr₂O₃ and Nd₂O₃ exhibited a promoting bone-resorbing property in mice[10]. Quarles et al. found that Gd³⁺ could stimulate DNA synthesis in MC3T3-E1 osteoblasts in vitro in a dose-dependent fashion. This was related to Gd³⁺-activated G-proteins in osteoblast membranes[11]. Hartle et al. found that Gd³⁺ inhibited PGE-stimulated cAMP accumulation, but potentiated PTH-stimulated cAMP production[12]. The cAMP level is important in regulating osteoblast function. Li et al. reported that the long-term oral lanthanum supplementation at a low dose to rats caused lanthanum accumulation in the bone tissue, reduced Ca/P ratio, decreased bone density, changed the microstructure of bone and increased bone crystalinity[13]. According to the above results, the rare earth ions affect the bone resorption biphasically. They are able to promote bone resorption and formation. The biochemical behavior of the rare earth ions is basically originated from their similarity to Ca²⁺. Thus it is likely that the lanthanides ions intervene the bone-remodeling process and affect cell function. The effects of rare earth elements on osteoclast activity were an important factor for determining bone resorption. However, the effects of rare earth ions on osteoclastic bone resorption were not reported. In the present work, the experimental results of the study on the effects of rare earth ions on the bone-resorptive capacity of cultured osteoclasts were reported.

1 Materials and methods
(i) Materials and reagents. Newborn Japanese white rabbits were obtained from China Institute of Veterinary Drug Control. Bovine femur were stored at −70°C. Minimum essential medium alpha (α-MEM) was pur-
chased from Gibco. Fetal bovine serum was from Sanli Biological Product Factory. Giemsa stain, Hepes, hematoxylin-eosin (HE) were all from Sigma. Tartrate resistant acid phosphatase (TRAP) kit was from Tianjin Blood Research Institute. The preparation of LnCl₃ solution: Rare earth oxides with 99.99% purity were dissolved in hydrochloric acid (analytical purity). The redundant acid was driven away by heating. The amount of rare earth ions was determined by EDTA titration.

(ii) Isolation of osteoclasts[13,14]. Newborn rabbits were killed by decapitation. Long bones from limbs were dissected and adherent soft tissues and epiphysis on bone surface were removed in D-Hank’s salt solution and cutted with a scalpel blade into α-MEM which was buffered by Hepes (0.025 mol/L) and containing fetal bovine serum (15%), streptomycin (100 μg/mL) and penicillin (100 U/mL) until the marrow cavities turned to pale color. The marrow cavities were rinsed repeatedly by pipetting. Then the bone fragments were discarded and the resulting suspension was collected.

(iii) Preparation of bone slices. Fresh bovine cortical bones were cut by a saw-like microtome and then ground into slices of 10 μm in thickness and 6 × 6 mm in area with a corundum grindstone. The slices were cleaned by repeated ultrasonication in distilled water, then immersed in D-Hank’s salt solution containing penicillin (1000 U/mL) and streptomycin (100 μg/mL). The solution was changed thrice, each 20 min.

(iv) Effects of the rare earth ions on the bone-resorption activity of the osteoclasts cultured on bone slices. One rehydrated bone slice was placed in each well of a 24-well culture plate which contained 1 mL α-MEM solution. The plate was pre-incubated in a CO₂ incubator (Heraeus, BB16/BB5060) for 1 h and then 1 mL of osteoclast suspension was added into each well and the incubation was continued in 5% CO₂ atmosphere at 37°C for 16 h. Afterwards, the slices or the cover glasses were rinsed to remove the nonadherent cells. Then a series of new culture solutions containing 1.00 × 10⁻⁵, 1.00 × 10⁻⁶, 1.00 × 10⁻⁷ and 1.00 × 10⁻⁸ mol/L rare earth ions respectively were added and the incubation was continued for further 7 days. Meanwhile, the control was run in parallel with cover glasses instead of the bone slices and without the addition of rare earth ions.

(v) Phase contrast microscopic observation and the counting of resorption lacunae. After incubation for various time, the morphology and the motion of the cultured osteoclasts were observed under a phase contrast inverted microscope (Olympus, BH-2). At the same time, the cells were stained with hematoxylin-eosin (HE), Giemsa and tartrate resistant acid phosphatase (TRAP) respectively. The formation and the change of bone resorption lacunae on bone surface were observed. The number of lacunae on each slice was counted after 3 d and 7 d culturing. Image analysis was performed by using the LEICA Q550IW system (Germany) to determine the number and surface area of lacunae on each slice.

(vi) Determination of calcium dissolution of bone slices[15]. The supernatant was collected at the end of the experiment and the total concentration of calcium was determined with an atomic absorption spectrometer (FXW-1C, China).

(vii) Scanning electron microscopic observation and chemical analysis of the components of bone. At the end of 1 week culture, the cells were stripped from the bone slices by ultrasonication for 10 min in 0.25 mol/L aqueous ammonia. The slices were then fixed with 3% glutaraldehyde and 1% osmic acid solution, dehydrated, and critically dried using liquid CO₂. The bone resorption lacunae on the specimens were viewed under a scanning electron microscope (S 250 Mk3, Link AN 10000) after sputtering coating with a layer of gold. Four lacunae were scanned respectively at the same time, bone protein and mineral composition in the lacunae were determined by X-ray energy spectroscopy (S 250 Mk3, Link AN 10000), meanwhile the control group was run in parallel with normal bone slices. In addition, the lacunae and normal bone slices were analyzed using IR micro-spectroscopy (Nico-MAGNA-IR 750, Nico-Plan™ IR Microscope) as above.

(viii) Statistical analysis. The relative resorption lacuna number, relative resorption lacuna surface area and bone resorbing index were calculated as the ratio of the average value of resorption lacuna numbers, resorption lacuna surface areas and calcium concentration of experimental group to the control’s. A value higher than 1.00 indicates the enhanced bone resorption. Data were expressed as mean ± SEM. The statistical significance of the difference between the control and the experimental group was determined by SPSS’ t-test.

2 Results

(i) Morphological characteristics of osteoclasts. After 20 min culturing, the osteoclasts began to spread and stick to the bone slices or the cover glasses, and acquired various shapes, like fried egg and funnel or some irregular shapes, which were characterized by the plate and filamentous pseudopods, several or even more than ten nuclei and also a number of vacuoles in cytoplasm. Similar feature was observed in the osteoclasts stained by

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