Article

Dehydroepiandrosterone Inhibited the Bone Resorption through the Upregulation of OPG/RANKL

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The plasma level of dehydroepiandrosterone (DHEA) is decreases gradually along with aging. The beneficial effects of DHEA as an anti-aging steroid, such as the stimulatory effect on immune system, anti-diabetes mellitus, anti-atherosclerosis, anti-dementia, anti-obesity and anti-osteoporosis have been demonstrated in experiment both *in vitro* and *in vivo*. It is important to investigate the effective mechanism of DHEA in therapeutics for postmenopausal osteoporosis. Having isolated and cultured osteoblasts (OBs) and osteoclasts (OCs), we analysed the effect of DHEA on osteoblastic viability, regulation of DHEA on the expression of osteoprotegerin (OPG)/receptor activator of NF-κB ligand (RANKL) mRNA in OBs, and then observed the action of DHEA on bone resorption of OCs in the presence or absence of OBs. The results showed that DHEA improved viability of OBs within the concentration range of 10^{-8}-10^{-6} M, especially at the concentration of 10^{-7} M. DHEA could apparently increase the ratio of OPG/RANKL mRNA in OBs. In the presence of OBs, DHEA could decrease the number and area of absorption lacuna of specula. We concluded, therefore, only in the presence of OBs, DHEA could inhibit the bone resorption of OCs, which may be mediated by OPG/RANKL of OBs. *Cellular & Molecular Immunology*. 2006;3(1):41-45.

Key Words: dehydroepiandrosterone, OPG/RANKL, osteoblast

Introduction

Dehydroepiandrosterone (DHEA) and DHEA-S are naturally produced steroids synthesized in the adrenal cortex, gonads, brain, and gastrointestinal tract, and are known to have chemo-preventive and anti-proliferative actions on tumors (1). The plasma concentrations of DHEA and DHEA-S are increased during adrenarche, and thereafter decrease steadily after puberty throughout life (2). Although DHEA and DHEA-S have few intrinsic androgenic actions, they have recently attracted widespread attention owing to their beneficial effects on obesity, anti-aging, lipid metabolism, immunity, diabetes mellitus, memory retention and osteoporosis (3-5). DHEA significantly improved both the overall well being and the scores for depression and anxiety. Treatment with DHEA for 1 year in 14 postmenopausal women increased significantly the bone mineral density of the femur, and also the serum osteocalcin levels, while the bone alkaline phosphatase levels were decreased (6).

However, the biochemical and molecular mechanisms of DHEA efficiency have not been clarified yet. In the present study, the bone resorption of osteoclasts co-cultured with or without osteoblasts would be analyzed after being treated with DHEA. The molecular mechanisms of the action involving the regulation of OPG/RANKL would be elucidated.

Materials and Methods

Animals and reagents

Newborn BALB/c mice were obtained from Animal Center of Chinese Academy of Science and used for preparing osteoblastic cells and osteoclasts. The bone tissue slices of cattle were kindly provided from Bone Metabolic Department (Research Center of Geriatric Medicine, Institute of Radiation Medicine, Fudan University, Shanghai, China).

DHEA, Alkaline Phosphatase staining Kit, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazo-lium bromide (MTT), toluidine blue, proteinase E, trypsin and collagen I/II were purchased from SIGMA Co. One-step RT-PCR kit was provided by the Gene Co. Fetal bovine serum (FBS) and minimum essential medium (α-MEM) were purchased from Gibco Co. Propidium iodide (PI) staining kit was purchased from BD Biosciences.

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Osteoblastic cell culture

Murine osteoblastic cells were isolated from calvariae of 8-12 neonatal BALB/c littermates by enzymatic digestion as previously described (7, 8). The osteoblastic cells were collected and seeded in 60 mm dish in α-MEM containing 10% of FBS and 100 U/ml of penicillin and 100 μg/ml of streptomycin. Half million cells of the osteoblasts at the second passage were cultured in 25 cm^2 culture flask, or seeded in 24- or 96-well plate and cultured for 12 hours at 37°C in 95% humidified air plus 5% CO_2.

The osteoblastic phenotype derived from calvaria was confirmed by staining for the presence of alkaline phosphatase with nitroblue tetrazolium salt and 5 bromo-4-chloro-3-indolylphosphate (NBT/BCIP). Besides, OPG is a specific soluble marker of osteoblasts.

Osteoclast culture and bone resorption assay

Osteoclasts were prepared from tibiae and femurs from 2~4 day-old BALB/c mice by dissecting and scraping the diaphysis into bone fragments after flushing marrow cavity with PBS. Bone fragments in α-MEM were blew, and agitated for 2 min, followed by 30 s silence, and then the 1 ml superior cell suspension was transferred into 24-well plate in which the bone tissue slice or cover glass laid. After 30 min culture, the α-MEM containing 10% FBS was changed with 2 ml for washing away the cells unattached to the wall, and then 2 ml medium with or without OBs suspension (10^6/ml) was added into every well. The medium was changed every day for 3 days, and DHEA was added at the beginning of culture and at the medium change. At the end of cultures, the cells were subjected to a tartrate-resistant acid phosphatase (TRAP) staining using leukocyte acid phosphatase assay in accordance with manufacturer's protocol and reference (9, 10). The TRAP positive multi-nucleated cells containing three or more nuclei were counted as osteoclast-like cells (OCLs). After counting number of the OCLs, the bone tissue slices were prepared. The area and number of bone resorption lacunae (with typical form, darity border, blue-purple staining) were observed by Nikon ACT-1 and counted by using Global Lab image system (Imager-Pro Plus 4.1, America).

Detection of cell proliferation by MTT

The proliferation test of OBs was performed according to the literature (11). In brief, the OBs of passage 1 from neonatal mice were cultured in a 96-multiwell plate at different concentration (10^{-10}-10^{-5} M) of DHEA for 24 h, 48 h or 72 h, then were rinsed with PBS, afforded with serum-free MEM 100 μl, MTT 10 μl in every plate for 4 h at 37°C, followed with 100 μl of lyses solution (20% SDS, 50% Dimethyl-formamide, 2% acetic acid glacial) in every plate for 2 h at 37 °C. The absorbance of medium at the wave length of 570 nm was measured at once by spectrophotometer (Bio-Rad) after transient agitation.

The OBs of passage 1 seeded in the 6-well plate were afforded with or without 10^{-7} M of DHEA for 3 days, and then were re-suspended in a 500 microliter volume of PBS and chill well on ice. The cold cell suspension was rapidly pipeted into the cold ethanol and mixed by forcing air bubbles through the suspension. After remained on ice for 15 minutes, the cell suspension was filtered to remove the debris and centrifuged into pellet. The pellet was incubated at 37℃ with PI stain solution for 15 minutes, and analyzed by flow cytometry.

Reverse transcription-PCR (RT-PCR)

Total RNA was isolated from OBs by TRIZOL (Invitrogen) according to the provided protocol. For the reverse transcription reaction and PCR, 5 μg of RNA together with 2.2 μM oligo (dT), 0.2 mM dNTPs, 1 pM of each primer, 20 U of reverse transcriptase, 50 U of RNasin, PCR buffer and 0.5 U of *Taq* polymerase was performed according to the one-step Robust RT-PCR kit. Two negative controls were included: one without the RNA sample, and the other without the reverse transcriptase.

The PCR was performed on a Gene Amp PCR system 2,400 (Perkin-Elmer) in a final volume of 50 μl, and were electrophoresed on a 1.2% agarose gel and visualized under the UV light after ethidium bromide staining. For semi-quantitative estimation, the gel was analyzed with Alpha Imager 2000. The corresponding primers used are listed below with their annealing temperatures and 35 cycles. OPG (635 bp): Forward 5'-TGA GTG TGA GGA AGG GCG TTAC-3', Reverse 5'-TTC CTC GTT CTC TCA ATC TC-3' (60°C); RANKL (750 bp): Forward 5'-ATC AGA AGA CAG CAC TCA CT-3', Reverse 5'-ATC TAG GAC ATC CAT GCT AAT GTT C-3' (55°C); β-actin (240 bp): Forward 5'-GGG CAC AGT GTG GGT GAC-3', Reverse 5'-CTG GCA CCA CAC CTT CTA C-3' (55°C) (9).

Statistical analysis

The data were analyzed using the SPSS for Windows (version 11.0, SPSS Inc). The cell proliferation was analyzed using the unpaired Student's *t* test and the Chi-square test. Dose-response relation of OPG/RANKL mRNA was analyzed for group differences using One-way ANOVA model. Differences were considered to be significant when *p*

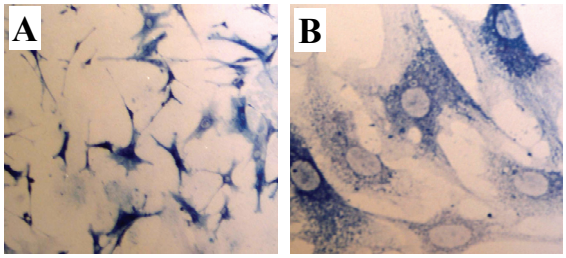

Figure 1. Evaluation of the primary cultured OBs derived from calvaria. The calvariae-derived OBs were evaluated with ALP kit, whose purity was over 90 percent (A, original magnification 50×). ALP staining is filled not in the nucleus but in the cytoplasm, and the nucleus lies in one side of OB. Every cell has 3-4 synapses that may connect with each other (B, 200×).

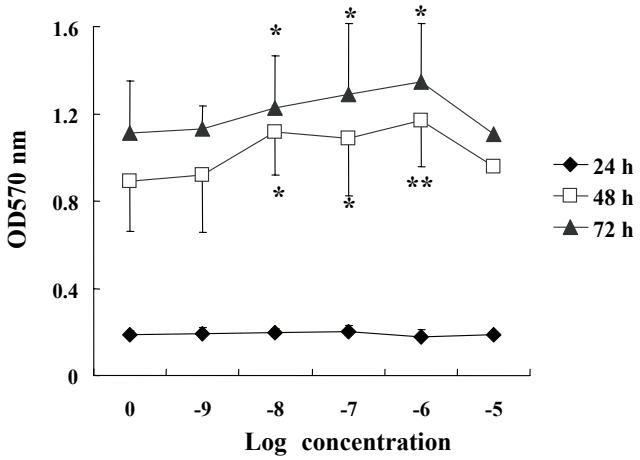

Figure 2. Stimulative effect of DHEA on OB viability *in vitro*. The OBs of passage 1 were treated with or without the different concentrations of DHEA (10^{-9}~10^{-5} M) for 24 h, 48 h and 72 h. The data represent mean ± SD of 12 plates per group. *$p < 0.05$, **$p < 0.01$ compared to the control.

value was equal to or less than 0.05.

Results

Evaluation of the primary cultured OBs derived from calvaria

The calvariae-derived OBs were evaluated with ALP kit. ALP staining is filled not in the nucleus but in the cytoplasm, and the nucleus lies in one side of OB. Every cell has 3-4 synapses that may connect with each other. The purity of OBs was over 90 percent (Figure 1), which meets the need of this experiment.

DHEA promoted the osteoblastic proliferation

DHEA improved viability of OBs within the concentrations ranging 10^{-8}~10^{-6} M ($p < 0.05$, $p < 0.01$, respectively). DHEA in the concentration of 10^{-9} or 10^{-5} M, however, had no effect on the OBs (Figure 2). Further study of cell proliferation by FCM shows that the 10^{-7} M of DHEA increased the S-phase and G_2/M phase of cell cycle, and then improved the proliferation index ($p < 0.01$, Table 1).

Table 1. Regulation of DHEA on the DNA content of OBs *in vitro*

Group	Cell cycle			PI value
	G_0/G_1	S	G_2/M	
DHEA	63.5 ± 5.1	27.5 ± 4.4	9.0 ± 1.2	36.5*
Control	83.09 ± 2.8	15.2 ± 3.5	2.3 ± 1.2	16.9

The OBs of passage 1 were treated with or without 10^{-7} M of DHEA for 3 days, then were analyzed by FCM. *$p < 0.01$ compared to the control.

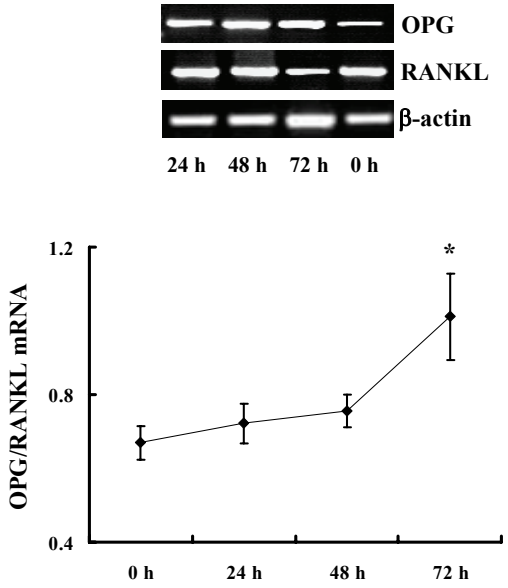

Figure 3. Regulation of DHEA on the expression of OPG/RANKL mRNA in OBs. The OBs of passage 1 were incubated with DHEA (10^{-7} mol/L) for 24 h, 48 h and 72 h, and the mRNA level of OPG/RANKL was analyzed. OPG and RANKL use the similar actin mRNA levels. *$p < 0.01$ compared to the control by ANOVA.

DHEA upregulated the expression of OPG/RNAKL in osteoblasts

The OBs of passage 1 were incubated with DHEA (10^{-7} M) for 24 h, 48 h and 72 h, and the mRNA levels of OPG and RANKL were analyzed. Compared with the mRNA levels of OPG/RANKL in 24 h and 48 h, the ratio of OPG/RANKL in 72 h was significantly increased ($p < 0.01$) (Figure 3).

DHEA indirectly inhibited the resorption of osteoclasts

The OCLs characterized by several nuclei were evaluated by TRAP (Figure 4), and the bone resorption lacunae which shows the color of purple and blue was stained by toluidine

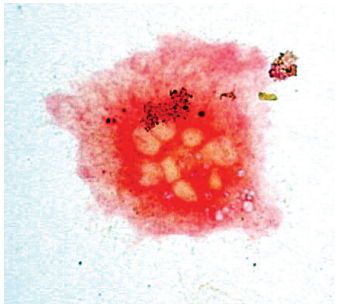

Figure 4. The evaluation of murine OCLs by TRAP (LM × 800). The cells were subjected to a tartrate-resistant acid phosphatase (TRAP) staining using leukocyte acid phosphatase assay. The TRAP positive multinucleated cells containing three or more nuclei were counted as osteoclast-like cells (OCLs).

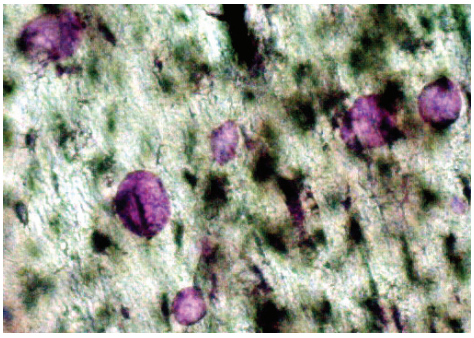

Figure 5. The staining for bone resorption lacuna by toluidine blue (LM × 200). The bone tissue slices were prepared by toluidine blue, whose area and number of bone resorption lacunae were characterized by typical form, darity border and blue-purple staining.

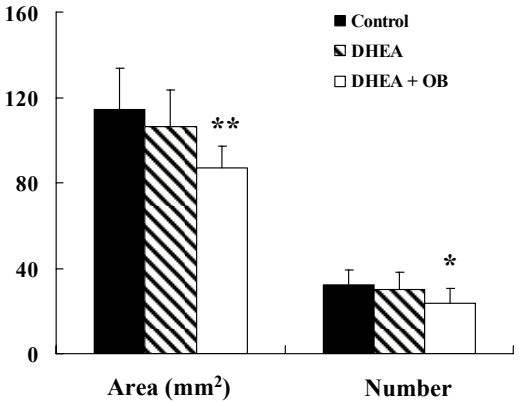

Figure 6. Analysis of the number and area of bone resorption lacuna. In the absence or presence of OBs, the OCLs were treated with DHEA (10^{-7} M) for 3 days, then the number and area of bone resorption lacuna were evaluated. *p < 0.05 and **p < 0.01 compared to the control and DHEA only.

blue (Figure 5). DHEA could apparently decrease the number and area of bone resorption lacuna in the co-culture of OBs and OCs than that only in the presence of OCs ($p < 0.05$ and $p < 0.01$, respectively), which shows that DHEA, without OBs, had no impact on the bone resorption of OCs (Figure 6).

Discussion

The functions and interactions of OBs and OCs are intimately linked. During skeletal development and throughout life, the cells from OB lineage synthesize and secrete molecules that in turn initiate and control OC differentiation (12, 13). This is a direct and crucial interaction that has been well established *in vivo*. For example, two cell-surface proteins, RANK (receptor activator of NF-κB ligand) and its partner RANKL (for 'RANK ligand'), are also key regulators of OC formation and function. RANK is present on OC precursor cells, and when activated, promotes OC maturation by increasing the expression of specific genes, including the gene encoding c-Fos (14). RANKL is produced by and resides on the surface of OBs. So, when an OC precursor encounters an OB, the resulting interaction between RANK and RANKL stimulates the OC precursor to mature into a fully differentiated, bone-resorbing OC. OBs also produce osteoprotegerin (OPG), a soluble 'decoy' receptor that binds to RANKL and prevents it from binding to RANK. This effectively inhibits RANKL-mediated OC maturation. Many signals that regulate OC maturation do so indirectly by controlling the production of RANKL or osteoprotegerin by OBs. It has been indicated that calcitonin and RANKL act directly on human osteoclasts to inhibit or stimulate osteoclast bone-resorbing activity, respectively, and that PTH, 1,25(OH)2D3, and OPG are more likely to influence osteoclast activity indirectly. The balance between the osteoclast-promoting RANKL and osteoclast-inhibiting OPG can therefore regulate the number and activity of OCs (15).

In the present study we found that DHEA could directly act on OBs to enhance the level of OPG/RANKL mRNA, DHEA, however, could not directly inhibit the bone resorption of OCs, but indirectly act on OCs through promoting OPG/RANKL production of OBs.

The importance of the distinction between cell number and the function of individual cell has often been obscured by using the vague term "activity". Manolagas et al. proved that the balance between bone resorption and bone formation depended more on the number of cells carrying out these processes than on their individual capacities. A dramatic increase in the number of osteoblasts, bone formation rate, and BMD could be accounted for by a dramatic decrease in the prevalence of osteoblast apoptosis (16). The increased bone formation caused by daily PTH injections is associated with increased osteoblast numbers, the only other agent known to increase bone mass by rebuilding bone, prostaglandin E, also increases the lifespan of mature osteoblasts by reducing the prevalence of their apoptosis (17). DHEA improved not only the OBs proliferation but also the cell viability of OBs. The increased number of OBs population can augment bone mass.

In humans, circulating levels of adrenal androgens, including dehydroepiandro-sterone, are strongly associated with bone density in aging women (18). There are three possible mechanisms of DHEA effect. First is its direct action *via* DHEA receptor on the target gene. Second is its indirect action, and DHEA is converted enzymatically to testosterone and/or estradiol in peripheral tissues. The last possibility is that the hydrophobic DHEA molecule may alter the cell function after binding to some macromolecules such as enzyme proteins (19). Recent data indicated that the skeletal actions of androgens might be partially mediated *via* the estrogen receptor after conversion to estrogens by the action of aromatase (20). However, there is evidence that androgens have a direct effect on bone, as suggested by the presence of androgen receptors (AR) in human and rat

osteoblast-like cell lines as well as normal human osteoblast-like cells *in vitro* (21).

In conclusion, only in the presence of OBs, DHEA can inhibit the bone resorption of OCs, which may be mediated by OPG/RANKL of OBs. The exact mechanism of DHEA for inhibiting bone resorption involving ERβ, however, needs further research.

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