Peptide 11R-VIVIT stimulates osteoblastogenesis through regulating the expression of nuclear factor of activated T cells cytoplasmic 1

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Abstract: Osteoporosis is characterized by the imbalance of two relatively independent processes-osteoblastogenesis and osteoclastogenesis. Calcineurin (Cn)/nuclear factor of activated T cells (NFAT)(Cn/NFAT) signaling pathway is involved in these two processes in bone metabolism, but its potential as a target to treat osteoporosis needs to be defined. The aim of this study is to investigate the inhibition of polypeptide 11R-VIVIT on Cn/NFAT signaling pathway. Rat calvaria (RC) cells were prepared from experimental model of osteoporosis in rat. 11R-VIVIT was used to treat cultured RC cells from wide type (WT) rat or from osteoporosis (OP) rat, we then measured the expressions of NFATc1, osteopontin (OPN), osteocalcin (OC), cytokines, NFκB subunit p65 by real time PCR, western blot or immunofluorescence. Then ALP expression and staining, and alizarin red S (ARS) staining were employed to study the osteoblastodifferentiation. 11R-VIVIT regulates the expression of NFATc1, and some other molecules in Cn/NFAT signaling pathway, such as OPN and OC, at transcriptional level and protein level. Further, it can regulate the expression of inflammatory cytokine like IL-1β, IL-10 and TNF-alpha and NFκB activity. Further, 11R-VIVIT can accelerate osteoblastodifferentiation of RC cells demonstrated by ALP and ARS staining. 11R-VIVIT can stimulate the bone formation by decreasing NFATc1 expression and regulating the expression of inflammation related molecules.

Key words: 11R-VIVIT; Osteoporosis; Cytokines; Differentiation.

Introduction

Osteoporosis is a disease with reduced bone mass and deteriorated bone micro-architecture, clinically characterized by increased skeletal fragility and bone ach. As a multifactorial and incurable disease, osteoporosis roughly comes from the imbalance of two relatively independent processes-osteoblastogenesis and osteoclastogenesis. Around 200 million people are at risk of osteoporosis world wide, which make it a very serious health concern. Bisphosphonates (1) are widely used for the therapy of osteoporosis with robust efficacy in preventing fractures, rare but serious adverse events associated with bisphosphonates, such as atypical femur fractures, osteonecrosis of the jaw, and esophageal cancer, have been encountered. Therefore, alternative therapy to be defined.

Marrowmesenchymal stem cells (MSCs) can differentiate into osteoblasts, fat cells, chondrocytes, muscle cells and stromal cells, and play a dominant role in pathogenesis of osteoporosis (2). Abnormal MSCs are attributable, in a great extent, to compromised osteoblastogenesis, resulting in delayed fracture healing and decreased callus quality in osteoporosis (3), which implies that the inhibition of osteoporotic differentiation be the center for developing drugs against osteoporosis. Fetal rat calvaria cell line (RC) mimics the functions of MSC in many different ways, such as osteoblastodifferentiation, and RC cell line is easy to be prepared and cultured (4). Thus, RC is widely used to study the pathogenesis and treatment of bone metabolism disorders like osteoporosis.

Lately, calcineurin/nuclear factor of activating T cell (Cn/NFAT) signaling pathway was found to play a critical role in bone metabolism, such as in process of osteogenesis, for example, molecule NFAT cytoplasmic 1 (NFATc1) stimulates in Cn/NFAT signaling pathway inhibits fos related antigen (Fra-2) (5), by thus, to inhibit the formation of osteoprogenitor cell. In another study performed in MSCs, Cyclosporin A (CsA) can promote osteogenesis process mediated by blocking the Cn/NFAT signaling pathways (6, 7). It should be pointed out that CsA and FK506 (7, 8), two classic Cn/NFAT inhibitors, not only inhibit molecule NFAT, but the expression of Cn, which may cause progressive renal failure, hyperglycemia, and nervous lesion and increase the risk of malignancy (9). To avoid these adverse effects, Arambruf (10) constructed VIVIT polypeptide (I: ileucine, V: valine, T: threonine) based on the Cn/NFAT interaction region to block specifically Cn/NFAT signaling pathway. Later, Noguchi (11) upgraded and synthesized cell permeable NFAT inhibitor-11R-VIVIT polypeptide (R, Arginine). 11R-VIVIT can only specifically block the expression of relevant molecules downstream of Cn/NFAT signaling pathway, no effect on the expression of NFATc1 stimulated in Cn/NFAT signaling pathway (6, 7).
healing and osteoblastogenesis in osteoporosis needs to be defined.

In the current study, with RC cell (mimic of MSCs) as cellular model in vitro, we found that 11R-VIVIT regulates the expression of genes in Cn/NFATc1 signaling pathway, thus plays a role in osteoblastogenesis, which shape itself as a potential drug against osteoporosis.

**Materials and Methods**

**Rat model of osteoporosis**

Construct of rat model of osteoporosis was based on the method described previously (14). Wistar rat were obtained from the Institute of Laboratory Animal Sciences, Chinese Academy of Medical Sciences (Shanghai, China). All of the animal related experiments were approved by The Institutional Animal Care and Use Committee of Hangzhou Normal University.

**Fetal rat calvaria (RC) digestion and preparation of calvaria cells**

RC cell line, one mimic of MSC models, was prepared and cultured as described previously (15). Briefly, calvariae from 24-hour-old newborn healthy rats or rats with osteoporosis were collected, minced, and digested in a collagenase mixture (300 mg/ml collagenase, 4.5 U/ml elastase, 9.7 U/ml DNAase, 0.12 mM chondroitin sulfate, 100 mM sorbitol, 111.2 mM KCl, 1.3 mM MgCl₂, 13 mM glucose, 21.3 mM Tris-HCl, pH 7.4, 0.5 mM ZnCl₂) at 37°C. The cell mixture were plated and grown in modified essential medium (MEM) containing 15% fetal calf serum (FCS) and antibiotics (100 μg/ml penicillin G; Sigma, St. Louis, MO, USA), 50 μg/ml gentamycin (Sigma, St. Louis, MO, USA), 300 ng/ml fungizone (Flow Laboratories, Mississauga, Ontario, Canada). RC cells were then harvested with trypsin next day, counted and seeded as the density of 5000/cm².

**Preparation of RC cell nuclear protein**

The isolation and preparation of RC cell nuclear protein was performed according to standard protocol. Briefly, RC cells grown in large petri dish were lysed with lysis buffer 1 (20 mM Tris pH 7.5–8.0 100 mM NaCl 300 mM sucrose 3 mM MgCl₂) supplemented with protease inhibitors (Roche Diagnostics Indianapolis, IN, USA) and subjected to centrifuge. The pellet was resuspended in lysis buffer 2 (20 mM Tris pH 8.0 300 mM NaCl 2 mM EDTA pH 8.0) followed by another centrifugation. Aliquot supernatant were stored at -70°C for use.

**Western Blot Analysis**

Western blots were performed as described previously (16). Briefly, cells were lysed at 4°C in RIPA buffer (150 mM NaCl, 0.5%, sodium deoxycholate, 50 mM Tris- HCl, pH 8.0, 0.1% SDS, 0.1% NP40) supplemented with protease inhibitors (Roche Diagnostics Indianapolis, IN, USA). The homogenates were centrifuged, the supernatants of whole cell lysates and nuclear protein from previous preparation were collected for western blots probed with polyclonal NFATc1 antibody (1:2000 dilution, Santa Cruz Biotech, CA, USA), monoclonal NFkB subunit p65 antibody (1:3000 dilution, Santa Cruz Biotech, CA, USA), OC antibody (1:2000 dilution, Santa Cruz Biotech, CA, USA), OPN (1:2000 dilution, Santa Cruz Biotech, CA, USA) or Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) monoclonal antibody (1:5000 dilution, Santa Cruz Biotech, CA, USA), followed with corresponding horse radish peroxidase-conjugated secondary antibody (1:1000; Amersham, Piscataway, NJ, USA), visualized using enhanced chemiluminescence (ECL) according to the manufacturer's instructions (Amersham Bioscience, Piscataway, NJ, USA). Here, GAPDH functions as internal control for equal loading.

**Real-time PCR assay**

Total RNA from RC cells was extracted with TRIzol reagent (Invitrogen, Carlsbad, CA, USA), reverse transcribed using the Thermo-script™ RT-PCR System (Invitrogen, Carlsbad, CA, USA) and purified with RNeasy Mini Kit (Qiagen, Germantown, MD, USA). Real time PCRs were performed using iQ SYBR Green Supermix kit (Bio-Rad, Hercules, CA, USA) with the iCycler sequence detection system (Bio-Rad, Hercules, CA, USA) with specific primers for NFATc1, Alkaline phosphatase (ALP), Osteocalcin (OC), OPN, interleukin (IL)-1beta, tumor necrosis factor (TNF)-α and IL-10 and GAPDH as shown in Table 1. Here, GAPDH acts as internal control.

**Immunofluorescence assay**

To study the regulation of 11R-VIVIT on the expression of p65, one subunit of NFkB, immunostainings were performed according to the standard protocol with primary antibody-p65 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and Alexa Fluor® 488 secondary antibody (Molecular Probes, Carlsbad, CA) as described previously (17). Samples were mounted in Prolong Gold Antifade Reagent and analyzed by ZEISS AXIOSKOP 2 PLUS Microscope (Carl Zeiss MicroImaging, Inc. Thornwood, NY).

**Osteogenic differentiation induction**

RC cells were plated in 2 mlMEM medium in a 6-well plate. After overnight incubation, osteogenic differentiation was induced performed by supplementing medium with 10 mM β-glycerophosphate and 250 μM ascorbic acid-2-phosphate (18). ALP expression and calcium deposit were used as early and late markers for osteogenesis and were detected by real time PCR, ALP and Alizarin Red Staining, respectively.

**ALP staining**

ALP staining was carried out based on the protocol described previously (19). Briefly, RC cells were washed with PBS and stained with 5-Bromo-4-chloro-3-indolyl phosphate/Nitrobluetetrazolium (BCIP/NBT, Sigma-Aldrich, ST. Louis, MO, USA) for 60 min in the dark. ALP positive cells were visualized by light microscope (dark purple color). To quantify the bone formation, analytical FCM was performed using the bright channel on FCM Canto™ (BD Biosciences, San Jose, CA, USA).
RC cells were isolated and prepared from rat with osteoporosis or wild type rat, and then subjected to 11R-VIVIT treatments. (A) In RC cells from rat with OP showed lower NFATc1 expression at transcriptional level compared to RC cells from WT rat, however, 11R-VIVIT stimulates significantly the mRNA level of NFATc1 in WT rat RC cells, further, 11R-VIVIT can rescue the compromised NFATc1 expression in OP rat RC cells to normal level. (B) By western blot, we found that there is significant increase of the expression of NFATc1 at protein level in comparison with that from WT rat. On the contrary, 11R-VIVIT can regulate positively the expression of NFATc1 compared to WT rat. Further, 11R-VIVIT can rescue the decreased expression of NFATc1 in OP. Western blot was quantified by scanning densitometry. Values are representative of three independent experiments, each bar represents mean ± S.E., *: p<0.05, **: P<0.01, NS: no significance.

Alizarin Red S (ARS) staining
ARS staining and analysis were performed according to the methods described previously (20). Briefly, ARS stock solution was prepared by dissolving 1mg of alizarin red S in 100ml of 1% KOH. After two weeks of culture in osteogenic induction medium, RC cells were washed, fixed then stained with 40mM freshly ARS solution (PH=4.2). Calcium deposits can be visualized by red color under light microscope. To quantify the staining, ARS Staining Quantification Assay (ARed-Q) method (ScienCell Research Laboratories, Carlsbad, CA, USA) was used based on the manufacturer instruction. Destained ARS dye was quantified by measuring the absorbance at 405 nm.

Statistical analysis
Values are expressed as mean ± SEM with unpaired two-tailed Student’s t test by InStat v3.06 (GraphPad, San Diego, CA) software. P< 0.05 was considered statistically significant.

Results
11R-VIVIT inhibits the expression of NFATc1
To study the effect of 11R-VIVIT on osteoblastogenesis, we first treated the RC cells with 11R-VIVIT for 48 hr, we found that the expression of NFATc1 is increased in OP patients, but decreased in 11R-VIVIT treated WT RC cells at mRNA level, further, 11R-VIVIT can restore the inhibition of NFATc1 expression in RC cells from OP patients (Figure 1A). To confirm our result, we did the western blots which came out with very similar results, OP increases but 11R-VIVIT haunts the expression of NFATc1 at protein level, and 11R-VIVIT can resume the enhanced expression of NFATc1 in OP to normal level (Figure 1B). The right panel in Figure 1B showed the percentage of density compared to control.

11R-VIVIT regulates the expression of down-stream molecules in Cn/NFAT signaling pathways
To further investigate the effect of inhibition of NFATc1 by IIR-VIVIT on the pathogenesis of osteoporosis, we then examined the expression of some molecules such as OC and OPN, which are important for osteoblastogenesis, by both real time PCR and western blot (Figure 2A and B). We found very opposite expression pattern of these molecules with NFATc1. Compared to control, 11R-VIVIT increases the expression of OC and OPN, but significant lower expressions were seen in OP patients. Further, 11R-VIVIT can restore their expression to normal level, even lower level at protein level.

11R-VIVIT regulates the expression of cytokines related to osteoclastogenesis
Given the fact that cytokines play important roles in the onset and development of osteoblastogenesis and osteoclastogenesis, we examined the production of inflammatory cytokines at mRNA level by real time PCR (Figure 3). We found that 11R-VIVIT decreased the pro-inflammatory cytokines IL-1beta and TNF-alpha, can resume the enhanced expression of IL-1 and TNF-alpha in OP. Further, 11R-VIVIT can increase the production of anti-inflammatory cytokine IL-10 and restore its compromised expression in OP. Our data indicates that 11R-VIVIT may be involved in the regulation of inflammatory cytokines.

NF-kB activity
To study further how 11R-VIVIT regulates the expression of NFATc1 and affects the expression of inflammatory cytokines.
genes, cytokines and even osteoblastogenesis, we then investigated the status of NFkB by real time PCR, western blot and immunofluorescence (Figure 4A, 4B and 4C). We found that 11R-VIVIT cannot change the expression of p65 at both transcriptional level and protein level in all experimental groups. Western blot demonstrated that p65 is predominantly localized to the cytosolic pool, only basal level of p65 translocate to nucleus in RC cells. After 11R-VIVIT treatment, more p65 undergo translocation to nucleus demonstrated by western blot and immunostaining, indicating that NFkB is crucial for the regulation of 11R-VIVIT in osteoblast differentiation, even though that the expression of total NFkB is not changed.

11R-VIVIT accelerate bone formation

ALP is an early and sensitive marker for osteogenesis. By immunostaining, we found that 11R-VIVIT can increase significantly the quantity of ALP at transcriptional level (Figure 5A), indicating increased osteogenesis at early stage, further it can restore compromised ALP quantity in OP patients (Figure 5B). Figure 5C shows the percentage of area of bones. Further, calcium deposit is a late marker for osteogenesis. ARS staining, indicator of calcium deposit can reflect the extent of osteogenesis. In Figure 6, we can see that 11R-VIVIT can stimulate the osteogenesis dramatically in RC cells in comparison with control-treated counterpart. In opposite, there is compromised bone formation in OP patients, but 11R-VIVIT can restore the osteogenic process in OP patients (Figure 6A). The percentage of the area of the bone was analyzed by software (Figure 6B).

Discussion

In the current study, we demonstrated that 11R-VIVIT decreases the expression of NFATc1, by which...
Human MSCs were treated under six different conditions (sample 1 – 6) to induce osteogenesis for 18 days. ScienCell’s Alizarin Red S staining quantification assay was applied to the treated samples and controls cultured in the hMSC growth medium without induction. (A) Typical ARS staining for controls (top) and treated samples (bottom); (B) ARS quantification using the assay. Data are pooled from three independent experiments. *: P<0.05, **: P<0.01, NS: no significance.

Figure 6. ARS staining and quantification of human mesenchymal stem cell (hMSC) osteogenesis Human MSCs were treated under 6 different conditions (sample 1 – 6) to induce osteogenesis for 18 days. ScienCell’s Alizarin Red S staining quantification assay was applied to the treated samples and controls cultured in the hMSC growth medium without induction. (A) Typical ARS staining for controls (top) and treated samples (bottom); (B) ARS quantification using the assay. Data are pooled from three independent experiments. *: P<0.05, **: P<0.01, NS: no significance.

 regulates the expression and function of osteoblast-differentiation related genes, inflammatory cytokines and NFkB, resulting in osteoblastic differentiation. This study shed some light on the mechanistic insight into the perspective of treatment of osteoporosis.

Bone formation and reabsorption are dynamic and elaborated processes resulted from coupled activation of bone-forming osteoblasts (21) and bone-resorbing osteoclasts (22). Osteoporosis is characterized by imbalanced processes, in which bone resorption dominates over bone formation (23). In process of bone metabolism, Cn/NFAT signaling pathway plays critical roles in bone metabolism in OP, evidenced by the facts that 11R-VIVIT can also activate nuclear factor-kB (NFκB) pathway to inhibit apoptosis of osteoclast (43). TNFa is involved in tumor-induced bone resorption and non-tumor-induced osteopenia (44-46). It stimulates formation of osteoclasts by including either proliferation of osteoclast-precursor cells or activation of differentiated osteoclasts (47). Linkage was established between TNFa and osteoporosis by sib-pair analysis (48) and single nucleotide polymorphism (SNP) assay (49). Receptor activator of NFκB (RANK) pathway, a new TNF family pathway, plays important roles in bone metabolism. RANK binds to its ligand RANKL, leading to osteoclasts differentiating and maturing, increased bone loss. Interleukin 10 (IL-10) is another susceptibility gene associated with the pathogenesis of osteoporosis with polymorphisms (39). Interleukin-10 inhibits osteoclastogenesis by reducing NFATc1 expression and preventing its translocation to the nucleus (50). Interleukin-10 inhibits bone resorption: a potential therapeutic strategy in periodontitis and other bone loss diseases (40). Besides, some other cytokines were identified involved in the onset and development of osteoporosis. IL-6 promotes the differentiation of osteoclasts from its precursor (51); transforming growth factor β1 (TGF-β1) is linked to pathogenesis for its polymorphism (39). Study found increased IL-31 in osteoporosis (52).

In conclusion, 11R-VIVIT increase osteoblast differentiation and restore the compromised bone metabolism in OP, evidenced by the facts that 11R-VIVIT regulates osteoblast related genes, inflammatory related cytokines and NFκB pathways.

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