Studies on the Physiological Action of Active Vitamin D_3 on Osteoporosis Treatment

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Abstract

Osteoporosis is a common disease in elderly which is defined as a low bone mineral density (BMD) and easy to undergo bone fractures. Bone fractures reduce QOL and life expectancy of osteoporosis patient so that preventing bone fractures is the aim of osteoporosis treatment. However, not a few people broke their bones even under adequate osteoporosis treatments. So, establishment of more effective osteoporosis treatment to prevent bone fractures are required. Active vitamin D_3 (VD_3) analogs and bisphosphonates (BPs) are used for osteoporosis treatment. Active VD_3 and BPs reduced bone resorption and corrected excessive bone turnover. BPs bind to bone surface and are incorporated into osteoclast during bone resorption so that induce apoptosis of osteoclasts. However, the mechanisms of anti-bone resorptive effects of active VD_3 had not been elucidated. In this study, I demonstrated that active VD_3 directly inhibited osteoclastogenesis via IFN-β cascade. Active VD_3 increased IFN-β expression at the early stage of osteoclastogenesis and inhibited human bone marrow derived osteoclast progenitor cells. Antibody against IFN-β diminished the inhibitory effect of active VD_3 on inhibition of osteoclastogenesis. And inhibitory effect of VD_3 on osteoclastlgenesis was also reduced in IFN-β receptor deficient mice osteoclast progenitor cells. In rat model for postmenopausal osteoporosis, combination treatment of early phase osteoclastogenesis inhibitor active VD_3 and late phase inhibitor BP showed a synergistic effect on bone strength through reduction of bone resorption. Interestingly, different from an additive effect on reduction of bone resorption, combination treatment with active VD_3 and BP exhibited no difference in bone formation between monotherapy and combination therapy. My findings of molecular mechanisms of inhibitory effect of active VD_3 on osteoclast differentiation and *in vivo* effects of combination therapy of active VD_3 and BP with additive effect on reducing bone resorption without severe suppression of bone formation may help us to investigate the effective treatment of osteoporosis using active VD_3.

Abbreviations

ADL：activity of daily living

ALF：alfacalcidol

ALN：alendronate

AP-1：activator protein-1

BMD：bone mineral density

BP：bisphosphonate

CFU-GM：granulocyte macrophage colony forming units

DXA：dual-energy X-ray absorptiometry

ELD：eldecalcitol

ES/BS：erosion surface per bone surface

c-Fos：c-FBJ osteosarcoma

GM-CSF：granulocyte macrophage colony-stimulating factor

IFN：interferon

IL-3：interleukin-3

M-CSF：macrophage colony-stimulating factor

MS/BS：mineralizing surface per bone surface

NFATc1：nuclear factor of activated T cells c1

NF-κB　：nuclear factor κB

OPG：osteoprotegerin

OVX：ovariectomized

QOL：quality of life

RANK：receptor activator of NF-κB

RANKL：RANK ligand

SFC：stem cell factor

TNF：tumor necrosis factor

VD_3：vitamin D_3

VDR：vitamin D receptor

VDRE：vitamin D response element

General Introduction

Osteoporosis is a common disease in elderly which is defined as a low bone mineral density (BMD) and easy to undergo bone fractures. It is estimated, over 80 years old, about a half in women and ten percent of men have non-traumatic bone fractures or low BMD less than 70% that of young adult mean (Yoshimura et al. 2010) and diagnosed as osteoporosis. It is well known that bone fractures affect life prognosis or activity of daily living (ADL) in elder people, therefore the prevention of bone fracture with osteoporotic treatment has benefits not only in bone itself but also in a quality of life (QOL) in elder people.

Bone is a dynamic tissue so that old bone matrix are replaced by new bone component. The matrix are resorbed by osteoclasts, which are differentiated from monocytes, and re-constructed by mesenchymal osteoblasts. Bone resorption by osteoclast releases bone forming molecules from bone matrix which as IGF-1, and stimulates osteoblast differentiation and bone formation. Not only secreted molecules from bone matrix, cell-to-cell interaction with osteoblast, for example, interaction of ephrinB2-EphB4 also stimulate bone matrix production (Zhao et al. 2006). On the other hand, activated osteoblast stimulates osteoclastogenesis or bone resorption by osteoclasts. Osteoblasts express receptor activator of NF-κB (RANK) ligand (RANKL) on its membrane or secrete soluble form of RANKL. RANKL binds to RANK on osteoclast surface and stimulates osteoclast differentiation or bone resorption. RANKL-RANK signal stimulates transcription factors such as nuclear factor κB (NF-κB) and c-FBJ osteosarcoma (Fos) which a component of activator protein 1 (AP-1). NF-κB and AP-1 induce upregulation of transcription factor nuclear factor of activated T cells (NFAT) c1. NFATc1 is a key transcription factor to activate osteoclast differentiation and bone resorption. So, bone resorption by osteoclast induces osteoblast differentiation and stimulation, and stimulated osteoblasts induce osteoclastogenesis. This bone resorption and formation cycle is called “remodeling”.

Postmenopausal osteoporosis is induced by accelerated bone resorption because of estrogen deficiency. The lack of estrogen signaling in osteoclast results in prolongation of its life span and increases number of osteoclasts and activates bone resorption (Nakamura et al. 2007). Activated bone resorption accelerates bone formation. This excessive remodeling cycle result in bone loss, because bone formation required much more time than bone resorption so that bone formation can not catch up with bone resorption in high bone turnover situations.

For osteoporosis treatment, various drugs are used. Among them, anti-bone resorptive drugs are mainly used. BPs are most popular drugs in this field. BPs bind to bone surface and incorporated into osteoclast during bone resorption. BPs induce apoptosis of osteoclast to inhibit bone resorption. Because BPs effect is so strong, sometimes bone remodeling suppressed severely. A low bone remodeling turnover accumulate micro damages in bone and it is considered as a cause of atypical fracture. Atypical fracture occurs suddenly in femoral midshaft in patients who received a long term BPs medication.

Active VD_3 analogs are other anti-bone resorptive drugs. In addition to the effect on reduction of bone resorption, active VD_3 analogs increase calcium absorption of small intestine so that support bone formation. Though active VD_3 and its analogs reduce bone resorption and increases BMD in animals or osteoporosis patients, the role of active VD_3 in osteoclast differentiation is not fully understood.

Bone fractures in elderly accompanied by other dysfuctions. Lumbar fractures cause bending posture and intestinal mal-function. Fractures of femurs are the major cause of bedridden and reduction of life expectancy. Therefore, preventing bone fracture is important not only for activity of daily living (ADL) but quality of life (QOL). In present osteoporosis treatment, not a few bone fractures occurred in the patients who undergo osteoporosis treatment so that more effective treatment to prevent bone fractures would be desired.

In this study, I investigated the effect of active VD_3 on osteoclast differentiation using human bone marrow-derived osteoclast precursor cells, and elucidated the molecular mechanism of the direct effect of active VD_3 on human osteoclastogenesis. Next, I revealed that the effectiveness of osteoporosis treatment with VD_3 derivative, eldecalcitol (ELD), and BP, alendronate (ALN), which has different mechanism on osteoclastogenesis inhibition, using a rat osteoporosis model.

Chapter 1

Active VD_3 inhibits human osteoclastogenesis directly via IFN-beta.

One-Alpha, 25-dihydroxy vitamin D_3 [1α,25$(OH)_2D_3$], an active form of VD_3, plays a critical role in calcium and bone metabolism. Although active VD_3 decrease osteoclast number and bone resorption of osteoporosis patients, the action of active VD_3 on osteoclastogenesis is still unclear. Besides usage for anti-bone resorptive drug on osteoporosis, VD_3 are used to obtain osteoclast in osteoblast-monocyte co-culture system. Osteoclast precursors express RANK, a receptor of RANKL, and RANKL-RANK signaling stimulates osteoclast differentiation. The active VD_3 upregulates a expression of RANKL in osteoblasts and enhances osteoclastgenesis of monocyte in co-culture. Therefore, there is a discrepancy of active VD_3 actions between anti-bone resorptive effect *in vivo* and enhancement of osteoclastogenesis *in vitro*. So it is important to elucidate the precise effect of active VD_3 on osteoclast differentiation to investigate the effective approach to osteoporosis therapy.

In osteoporosis patients, the expression of RANKL is abundant in osteoblasts and bone resorption-formation remodeling cycle is highly accelerating. To estimate the effect of active VD_3 on osteoclastogenesis in the circumstance of osteoporosis patients *in vitro*, the active VD_3 effect should be assessed in osteoclastogenesis accelerating condition, in other words, monocyte monoculture stimulate with abundant RANKL protein. For this purpose, I investigate the effect of active VD_3 on osteoclastogenesis in human bone marrow derived monocyte stimulated with RANKL. Human monocytes differentiated into osteoclast in medium with abundant RANKL which mimic the accelerating circumstance of high bone turnover in osteoporosis patients and active VD_3 was added in the medium to asses its effect on osteoclastogenesis.

Here I show that active VD_3 significantly inhibited human osteoclast formation at the early stage of differentiation in a concentration-dependent manner. Active VD_3 upregulated the expression of interferon-ß (IFN-ß), a strong inhibitor of osteoclastogenesis in osteoclast progenitors. Inhibitory effects of active VD_3 on osteoclastogenesis was diminished by treatment with a neutralizing antibody against IFN-ß. And bone marrow of IFN-ß receptor deficient mice are partially resistant to active VD_3 treatment. Thus, my study may provide a molecular basis for the treatment of human bone diseases by active vitamin D_3 through regulation of the IFN-ß.

Introduction

Bone is rigid tissue consist of bone matrix and minerals. And bone is constantly renewed by bone resorbing osteoclasts and bone forming osteoblasts. This bone resorption-formation cycle called bone remodeling. Bone remodeling helps to maintain the bone integrity to repair damages. It is considered that bone formation was caused by bone formation stimulating molecule which is released from resorbed bone matrix or secreted from osteoclasts. Osteoporosis is defined as low BMD and fragility of bone. In osteoporosis patients, both bone resorption and bone formation are accelerated. This excessive bone resorption and formation cycle called "high bone turnover". Though bone formation is activated, high bone turnover result in bone loss because bone resorptoin is much faster than bone formation. So inhibition of bone resorption is a main strategy for osteoporosis treatment.

Osteoclasts are bone-resorbing multinuclear cells derived from hematopoietic stem cells (Miyamoto et al. 2003). Recent studies have shown that the interaction between receptor activator of nuclear factor kappa-B (RANK) and RANK-ligand (RANKL) is essential for osteoclast differentiation and activation (Yasuda et al. 1998, Kong et al. 1999, Dougall et al. 1999). RANK signaling stimulates tumor necrosis factor (TNF) receptor-associated factor 6 (TRAF6) and c-Fos, a component of the AP-1 transcription factor complex, which in turn activates various downstream molecules such as tartrate-resistant acid phosphatase (TRAP) (Naito et al. 1999, Takayanagi et al. 2002a) (Figure 1). c-Fos is an essential transcription factor for osteoclast differentiation, and mice deficient in c-Fos exhibit a complete lack of osteoclast formation and severe osteopetrosis (Johnson et al. 1992). Nuclear factor of activated T cells (NFAT) 2, also called NFATc1, is a downstream transcription factor of c-Fos in the RANKL/RANK axis and is also essential for osteoclastogenesis (Matsuo et al. 2004, Asagiri et al. 2005). Although c-Fos positively regulates osteoclast differentiation by inducing NFATc1 and Fos-related antigen (Fra)-1, both of which are essential transcription factors for osteoclast differentiation (Takayanagi et al. 2002a, Matsuo et al. 2004, Matsuo et al. 2000), it also induces interferon-beta (IFN-ß), a strong inhibitor of osteoclast differentiation, and it negatively regulates osteoclastogenesis through one of the IFN-α/ßreceptor components, IFNAR1, in a negative feedback manner (Takayanagi et al. 2002b).

Patients with osteoporosis exhibit excessive bone resorption as a consequence of accelerated osteoclast differentiation and activation. A depletioln of estrogen signaling, lifespan of osteoclasts are elongated result in acceleration of one resorption (Nakamura et

al. 2007). Extensive bone resorption brings immature bone formation, which results in decreased BMD and attenuated bone strength. Administration of active VD_3 analogues to osteoporosis patients reduces bone resorption and bone fracture frequency (Sairanen et al. 2000, Aloia et al. 1988). Ovariectomized rodents have been utilized as models of postmenopausal osteoporosis, and treatment with active VD_3 analogues has been demonstrated to reduce osteoclast formation and bone resorption, thereby increasing BMD and bone strength *in vivo* (Shiraishi et al. 2000, Uchiyama et al. 2002). Interestingly, active VD_3 has been used to induce osteoclast formation in a coculture system of bone marrow and osteoblastic cells. It has been shown that active VD_3 acts on osteoblasts to upregulate the expression of RANKL, an essential trans-membrane ligand for osteoclastogenesis, while downregulating the expression of osteoprotegerin (OPG), a decoy receptor of RANKL that prevents osteoclastogenesis (Yasuda et al. 1998). Thus, active VD_3 has been considered to be an osteoclast-inducing factor, although it inhibits osteoclast formation and increases BMD in osteoporosis patients.

In this study, I show that human osteoclastogenesis induced by macrophage colony-stimulating factor (M-CSF) and RANKL from bone marrow-derived osteoclast progenitor cells was strongly inhibited by active VD_3 at an early stage of differentiation in a concentration-dependent manner. Active VD_3 treatment upregulated IFN-β expression in osteoclast progenitor cells. The inhibitory effect of active VD_3 on human osteoclastogenesis was restored by addition of an antibody against IFN-β. And bone marrow cells of IFN-β receptor deficient mice are partially resistant to active VD_3 treatment. Thus, my data suggest a molecular basis for the treatment of activated osteoclast-induced bone diseases such as osteoporosis by active VD_3 through the upregulation of IFN-β in the early stage of osteoclast differentiation. The elucidation of the effect of active VD_3 on osteoclastogenesis may help us to investigate a effective treatment of osteoporosis.

Materials and methods

Isolation of human bone marrow CFU-GM cells

The study was approved by an Institutional Ethical Review Board (Keio Hospital #16-17-1), and informed consent was obtained from all study subjects. Human bone marrow-derived colony-forming unit granulocyte macrophage (CFU-GM) cells were generated as follows. Human bone marrow was obtained from patients undergoing routine hip replacement surgery. Cells were diluted 1:1 with Dulbecco's phosphate-buffered saline (Invitrogen, Carlsbad, CA, USA) and filtered through a 70-μm nylon mesh cell strainer (BD Bioscience, Franklin Lakes, NJ, USA). The cell suspension was carefully layered on histopaque-1077 (Sigma-Aldrich, St. Louis, MO, USA) and centrifuged at 440g for 30 min at room temperature. The cell layer at the interface was transferred into a fresh tube as bone marrow mononuclear cells. Bone marrow mononuclear cells were cultured in methylcellulose semisolid medium (MethoCult H-4534; Stem cell Technologies, Vancouver, BC, Canada) containing 1% methylcellulose, 30% fetal bovine serum (FBS), 1% bovine serum albumin, 10^{-4} M 2-mercaptoethanol, 2 mM L-glutamine, 50 ng/mL recombinant human (rh) stem cell factor, 10 ng/mL rh GM-CSF, and 10 ng/mL rh IL-3 in 35-mm Petri dishes. Cultures were maintained in a humidified atmosphere at 5% CO2 at 37°C for 2 weeks to obtain CFU-GM cells.

Osteoclast differentiation of human CFU-GM cells

Human CFU-GM cells were seeded in 96-well plates (2.0×10^4 cells/well) and cultured for 6 days in alpha-minimum essential medium (α-MEM) supplemented with 10% FBS, 30 ng/mL M-CSF (Wako, Osaka, Japan) and 30 ng/mL RANKL (Wako) in the absence or presence of 10^{-10} M, 10^{-9} M, 10^{-8} M $1\alpha,25(OH)_2D_3$ (Wako). To examine the role for IFN-β in osteoclastogenesis of human CFU-GM cells, 500 unit/mL sheep polyclonal antibody against human IFN-β (PBL Biomedical Laboratories, New Brunswick, NJ, USA) was added in the culture medium. The culture medium was changed to fresh medium every other day. Osteoclastogenesis was evaluated by TRAP staining as described below.

Tartrate-resistant acid phosphatase (TRAP) staining

Cultured cells were fixed with 10% formalin in PBS for 10 min at room temperature. After treatment with ethanol/ acetone (50:50 vol/vol) for 1 min, the cells were air dried and

incubated for 30 min at room temperature with TRAP-staining solution: 0.1 M sodium acetate (pH 5.0) containing 0.01% naphthol AS-MX phosphate (Sigma-Aldrich) as a substrate, and 0.03% red violet LB salt (Sigma-Aldrich) as a stain for the reaction product in the presence of 50 mM sodium tartrate. TRAP-positive multinuclear cells containing more than three nuclei were counted as osteoclasts.

RNA extraction, RT-PCR, and quantitative real-time PCR analysis

Total RNA was isolated from osteoclast progenitors or osteoclasts using an RNeasy Mini kit (Qiagen, Valencia, CA, USA), and total RNA was reverse transcribed using Reverscript IV (Wako). Reverse transcription-polymerase chain reaction (RT-PCR) analysis was performed using the following primer sets: IFN-ß, 5'-TCA TCT AGC ACT GGC TGG AA-3'–5'-TTT CAA AAT CTT CTA GTG TCC TTT CA-3' ; b-actin, 5'-TCC TGT GGC ATC CAC GAA ACT A-3'-5' -CTC GGC CAC ATT GTG AAC TTT G-3'. PCR reactions were performed using TITANIUM Taq PCR Kit (Clontech, Mountain View, CA, USA).

Osteoclast differentiation of mouse bone marrow cells

All mice were maintained under pathogen-free conditions and cared for in accordance with the guidelines of Keio University School of Medicine. Bone marrow cells were isolated from 8- to 12-week-old IFN-α/ßreceptor 1 (IF-NAR1)-deficient or wild-type mice and cultured in α-MEM supplemented with 10% FBS for 5h. Non-adherent cells were harvested as osteoclast progenitor cells, seeded in 96-well plates (2.0×10^4 cells/well), and cultured for 6 days in α-MEM supplemented with 10% FBS, 30 ng/mL M-CSF, and 30 ng/mL RANKL in the presence or absence of $1\alpha,25(OH)_2D_3$. The culture medium was changed to fresh medium on day 3. Osteoclastogenesis was evaluated by TRAP staining.

Statistics

P values were calculated by unpaired Student's t test. P values less than 0.05 were considered significant and are indicated by asterisks.

Results

The effect of active VD_3 on human osteoclastogenesis

I first asked whether active VD_3 directly affects human osteoclastogenesis from osteoclast progenitor cells. Human osteoclast progenitor cells were prepared by cultivation of human bone marrow mononuclear cells in methylcellulose semisolid culture medium. Human osteoclastogenesis induced by M-CSF and RANKL in CFU-GM cells was significantly inhibited by active VD_3 in a concentration-dependent manner (Figure 2A,2B), indicating that active VD_3 inhibits osteoclast differentiation in human bone marrow-derived cells.

To understand the inhibitory mechanism of active VD_3 on osteoclastogenesis, I subdivided the culture period into three parts (days 0–2, days 2–4, and days 4–6) to assess which stage was critical for inhibition of osteoclastogenesis by active VD_3. Osteoclastogenesis was strongly inhibited when active VD_3 was added to the culture medium at the earlier stage of differentiation (Figure 2C), which suggests that active VD_3 affects molecules that act at an early rather than a later period of osteoclast differentiation in the presence of M-CSF and RANKL.

IFN-β expression in osteoclast progenitor cells stimulated with active VD_3

Next, I tried to elucidate the mechanism underlying inhibition of osteoclastogenesis by active VD_3. I found that the expression of IFN-β, a strong inhibitor of osteoclastogenesis (Takayanagi et al. 2002b), was significantly upregulated by treatment with active VD_3 in osteoclast progenitor cells (Figure 3A,3B). To analyze the role of IFN-β in the inhibition of osteoclastogenesis by active VD_3, an antibody against IFN-β (500 unit/mL) was added to cultures with active VD_3. Osteoclastogenesis inhibited by active VD_3 was significantly restored by treatment with the antibody against IFN-β (Figure 3C), indicating that active VD_3 negatively regulates osteoclastogenesis through the upregulation of IFN-β in osteoclast progenitor cells.

The osteoclast differentiation of bone marrow cells from IFNARI-deficient mice stimulated with active VD_3

I tried to confirm the inhibitory effect of active VD_3 on osteoclastogenesis through an IFN-β cascade. To this end, I utilized an animal model deficient in IFNARI, one of the IFN-α/β receptor components, to inhibit the IFN-β-induced signals. The inhibition of

osteoclast differentiation by active VD_3 was severe in wild-type cells compared with IFNARI-deficient cells (Figure 4). Taken together, my results demonstrate a novel mechanism of the direct inhibition of osteoclast differentiation by active VD_3 through autocrine secretion of IFN-β.

Active VD_3 act to osteoclast progenitor cells and upregulated IFN-β expression and inhibited osteoclastogenesis

Finally, I investigated whether active VD_3 can induce IFN-β and inhibit osteoclast differentiation in osteoclast progenitor cells which are not committed in osteoclast. Isolated CFU-GM were cultured in the medium with SCF, GM-CSF and IL-3, and active VD_3 was added in the culture medium for 2 days. Cytokines and active VD_3 were washed out and osteoclastogenesis was induced with M-CSF and RANKL. The osteoclastogenesis of CFU-GM was inhibited by active VD_3 treatment (Figure 5A,5B). The IFN-β mRNA expression in CFU-GM cells was increased after 2-day's active VD_3 stimulation (Figure 5C).

Discussion

Because not a few people are insufficiet for VD_3 nutrition among osteoporosis patients, the clinical effects of active VD_3 analogues have been considered to occur via correction of active VD_3 deficiency. However, a recent study reported that administration of active VD_3 analogues to osteoporosis patients under native VD_3 supplementation also increased BMD (Matsumoto et al. 2005), suggesting that active VD_3 and its analogues have antiosteoporotic effects in addition to correction of VD_3 deficiency. Here I provide a possible mechanism underlying the antiosteoporotic effect of active VD_3 in which active VD_3 inhibits osteoclastogenesis by upregulating IFN-β expression.

Active VD_3 downregulates PTH transcription in parathyroid cells (Okazaki et al. 1988, Demay et al. 1992) and upregulates intestinal calcium absorption, thereby prevents BMD reduction (Gallagher et al. 1979). In spite of its usage for osteoporosis therapy in humans, it was reported that active VD_3 is an osteoclast-inducing factor in human cells (Takahashi et al. 1989). Active VD_3 has also been used as an osteoclastogenesis-stimulating agent in co-cultures of murine osteoclast precursor cells with calvarial osteoblasts. Active VD_3 upregulates RANKL and downregulates the expression of OPG in osteoblasts (Yasuda et al. 1998), and this reciprocal regulation of RANKL and OPG is critical for osteoclastogenesis. Thus, active VD_3 has been considered to be an osteoclast-inducing factor through osteoblast-mediated activity. The regulation of the balance between direct inhibition of osteoclast progenitor cells by active VD_3 and indirect stimulation of osteoclastogenesis through osteoblasts is still unclear, but my results suggest that administration of active VD_3 or its analogues to osteoporosis patients could directly inhibit osteoclastogenesis in circumstances with high bone turnover rates. *In vitro* experiment of human CFU-GM osteoclast precursor cells cultured with abundant RANKL mimics the circumstance of accelerating osteoclastogenesis in osteoporosis patients. My result showed the direct inhibitory effect of active VD_3 on osteoclastgenesis through autocrine secretion of IFN-β in this circumstance. Active VD_3 is a steroid hormone so that binds to its nuclear receptor, vitamin D receptor (VDR). Active VD_3 and VDR complex binds to vitamin D response element (VDRE) in the target genes and controls is mRNA expression. Because I could not find any putative VDRE in 50-flanking region of the IFN-β gene, the regulation of IFN-β

expression by active VD_3 is likely indirect. Further study is needed to elucidate the molecular mechanisms of the regulation of IFN-ß expression by active VD_3.

Here, I showed that active VD_3 inhibited human osteoclastogenesis through IFN-ß axis. This result may enhance the investigation of osteoporosis treatment using active VD_3 and its analogs.

Chapter 2

The effect of combination treatment of active VD_3 and BP on bone remodeling in ovariectomized rats.

Eldecalcitol (ELD), an active VD_3 analog, and alendronate (ALN), one of the nitrogen containing BPs are used for osteoporosis treatment because of its anti-bone resorptive effects. Osteoporosis is defined as a low BMD and bone fragility. The purpose of osteoporosis treatment is preventing bone fracture. However, not a few people under went bone fracture who receive appropriate osteoporosis treatment. The bone fractures induce low QOL and poor life expectancy so that establishment of more effective treatment for osteoporosis is required. I showed in chapter 1 that active VD_3 inhibits the osteoclast differentiation at an early stage. On the other hand, BPs binds to bone surface and incorporated into osteoclast during bone resorption result in induction apotosis of mature osteoclast. I supposed that combination treatment of these two drugs which have different mechanisms in inhibition of bone resorption may exhibit superior effect on osteoporosis treatmet than mono-therapy of these drugs. To investigate this hypothesis, I administered either vehicle, ALN (0.2 mg/kg), ELD (15 ng/kg), or a combination of ALN and ELD to the ovariectomized (OVX) rats and measured bone strength and biological activities. The treatment started 2 weeks after surgery and continued for 12 weeks. A combination treatment of ELD and ALN showed significant increase in BMD of both lumber spine (L2-L4) and femur midshaft compared to mono-therapy groups. Erosion surface of lumber spine was reduced in ALN or ELD mono-therapy groups and the combination treatment group showed synergistic effect on erosion surface reduction. In endosteal surface of femur, only combination treatment group showed significant decrease in erosion surface. When it comes bone strength, combination treatment increased compression strength of lumber than both mono-therapy groups and 3-point bending strength of femur than ALN mono-therapy group.

On the other hand, it is considered that “atypical fracture”, a bone fracture of femur midshaft without surgical trauma, is a consequence of “adynamic bone” which showed excessive reduction of bone turnover induced by strong inhibition of bone resorption by anti-bone resorptive agents. In combination treatment of ALN and ELD, different from synergistic effect on reduction of erosion surface, mineralize surface, a parameter for bone formation, in lumber were not changed in all three treatment groups suggested that this combination treatment regimen has mild effect on reduction of bone formation, while it

showed an additive effect on inhibition of bone resorption.

In conclusion, the combination treatment of ELD, an active VD_3, and ALN, one of bisphosphonates, has synergistic effect on increasing in BMD and bone strength through the reduction of bone resorption without severe suppression of bone formation. This combination regimen for osteoporosis treatment may helpful in reduction of bone fracture frequency in osteoporosis patients without severe suppression of bone formation.

Introduction

Osteoporosis is characterized by a reduction in bone mass with age, contributing to a loss in bone strength (No authors 1991). 1α,25(OH)$_2$D$_3$ (active VD$_3$) and its derivartives are used in osteoporosis treatment. Preclinical studies have shown clearly that active VD$_3$ metabolites can fully prevent estrogen deficiency-induced loss of cancellous and cortical bone by suppression of bone resorption (Erben et al. 1992, Erben et al. 1998, Shiraishi et al. 2000, Weber et al. 2001). In OVX rat model of osteoporosis, the novel active VD$_3$ derivative [ELD; 1α,25-dihydroxy-2ß-(3-hydroxypro-pyloxy) vitamin D$_3$] suppress bone resorption and increase bone mineral density (BMD) (Uchiyama et al. 2002). In osteoporosis patients, ELD suppresses bone resorption markers and increases lumbar and hip BMD following 1 year treatment (Matsumoto et al. 2005). A recent randomized, active comparator, double-blind study has shown that ELD has superior effects over alfacalcidol (ALF), a prodrug for active vitamin D$_3$, in preventing vertebral and wrist fractures in osteoporotic patients with sufficient vitamin D supply, with a safety profile similar to that of ALF (Matsumoto et al. 2011). In spite of the inhibitory effect on osteoclast differentiation in humans or animals, active VD$_3$ increases the expression of RANKL from osteoblasts *in vitro* which activate a differentiation and bone resorptive activity of osteoclast. Because of this *in vitro* capacity of acceleration in osteoclast differentiation, I wondered how active VD$_3$ reduce osteoclast number and bone resorption *in vivo*. In chapter 1, I demonstrated that active VD$_3$ increased the expression of IFN-ß in osteoclast precursor cells and inhibited osteoclastogenesis at the eary stage in RANKL abundant circumstance. My result made it possible to propose the regimens of osteoporosis treatment with combined medication with other bone resorptive drugs which have different mechanism to reduse bone resorption.

Bisphosphonates (BPs) such as alendronate (ALN) and risedronate are the most popular first-line drugs for the therapy of osteoporosis (Black et al. 1996, Harris et al. 1999). The target of BPs are mature osteoclasts because BPs binds to the bone surface and incorporate into mature osteoclasts during bone resorption. BPs are potent inhibitors of the bone resorptive capacity of mature osteoclasts and also may induce osteoclast apoptosis (Fleisch 1998), and they prevent estrogen deficiency-induced bone loss in animals (Azuma et al. 1998, Ito et al. 2002).

In clinical, not a few bone fractures occured in the patients who receive appropriate osteoporosis treatments. The bone fractures worsen the QOL, ADL or life expectancy in

elder people so that establishment of more effective regimens of osteoporosis treatment are required to prevent bone fractures. As I demonstrated in chapter 1, active VD_3 inhibits osteoclastogenesis at the early stage, on the other hand, BPs are absorbed into mature osteoclast and induce apoptosis of mature osteoclasts. The target cells of active VD_3 and BPs are distinct from each other, so that the combination therapy of these two drugs may have an additive effect on reduction of bone fractures.

In this study, the effects of combination therapy of eldecalcitol (ELD), an active VD_3 derivative, and alendronate (ALN), a one of the nitrogen containing BPs, on BMD, bone strength and also physiological effects using OVX rat which is the model for post menopausal osteoporosis.

Materials and methods

Reagents

1α,25-Dihydroxy-2β-(3-hydroxypropyloxy)vitamin D_3 (eldecalcitol:ELD) and alendronate (ALN) were synthesized in our laboratories. ELD was dissolved in medium-chain triglyceride (MCT) and diluted to the given concentrations. ALN was dissolved in sterile physiological saline and diluted to the given concentrations.

Animal experiments

The experimental protocol was approved by the Institutional Animal Care and Use Committee of Chugai Pharmaceutical Co., Ltd.

Eight-month-old female Wistar–Imamichi rats were purchased from the Imamichi Institute for Animal Reproduction (Ibaraki, Japan). The rats were kept in individual stainless steel wire cages, and were allowed free access to standard commercial rodent chow (CE-2; CLEA Japan, Inc. Tokyo, Japan) and tap water. Animals were ovariectomized (OVX) or sham-operated (SHAM). Animals received daily doses of both vehicles (saline and MCT), or 0.2 mg/kg of ALN and MCT, or 0.015 μg/kg of ELD and saline, or a combination of ALN and ELD. Both compounds and their vehicles were administered orally via gavage daily for 12 weeks at a dose volume of 1 mL/ kg from 2 weeks after surgery. The dosage of each drug was selected based on that used clinically; 0.2 mg/kg and 0.015 μg/kg were comparable to the clinical dose for ALN and ELD, respectively. In order to prevent interference with ALN absorption, ELD or MCT vehicle was administered in the morning (between 08:00 and 10:00) and ALN or saline was given in the afternoon (between 14:00 and 16:00). The body weight of each rat was measured weekly, After the administration of drug or vehicle at 12 weeks, all rats were autopsied. and the lumbar vertebrae (L2–L5) and bilateral femurs were excised. The 5th lumbar vertebra (L5) and the left femur, which were used for measurement of mechanical strength, were stored frozen at −80 °C, and the other vertebrae (L2–L4) and right femur, which were used for measurement of BMD and bone histomorphometry parameters, were fixed in 70% ethanol.

Measurement of mechanical properties

Using a mechanical strength analyzer (TK-252CC; Muromachi Kikai Co., Ltd., Tokyo, Japan), the mechanical strength of the lumbar vertebra (L5) and left femur was measured

using a compression test (Mosekilde et al. 1993) and a 3-point bending test (Katsumata et al. 1995), respectively. For the compression test, planoparallel surfaces were obtained by removing the cranial and caudal ends of the vertebral specimen. From the vertebral body, a central cylinder with planoparallel ends and a height of approximately 5 mm was obtained. A compression force was applied to the specimen in a craniocaudal direction using a steel disk at a deformation rate of 2.5 mm/min. The ultimate compressive load (N) was calculated as the mechanical properties directly from the load–displacement curve. For the 3-point bending test, the left femur was placed on a special holding device with supports located 12 mm apart. The bending force was applied with the cross head at a rate of 20 mm/min, until a fracture occurred. From the load–displacement curve, the ultimate compressive load (N) was obtained.

Measurement of bone mineral density

The average BMD (mg/cm^2) of the lumbar vertebrae (L2–L4) was measured by dual-energy X-ray absorptiometry (DCS-600EX, Aloka Co., Ltd., Tokyo, Japan). The BMD of the right femur was measured with the DCS-600EX, with scans starting in the most proximal area and ending in the most distal area. During data analysis, the femur was divided into 10 equal segments (D1–D10) along its major axis. The mean values of the BMD for the 3 most proximal scanned areas (D1–D3), those for the next 4 scanned areas (D4–D7), and those for the 3 most distal areas (D8–D10) were calculated as the densities of the proximal, middle, and distal parts of the femur, respectively.

Bone histomorphometry

The 3rd lumbar vertebral body (L3) and the diaphysis of the right femur were fixed in 70% ethanol, and stained according to the method of Villanueva (Villanueva 1974). After dehydration with ethanol and acetone, the samples were embedded in methyl methacrylate (Wako Pure Chemical Industries, Osaka, Japan).

Midsagittal sections of L3, 5 μm thick, were obtained with a microtome (Supercut 2050; Reichert-Jung, Heidelberg, Germany) and prepared for measurement. A measuring field (~2.5 mm^2) encompassing most of the cancellous bone in the L3 vertebra was used. The cancellous bone within 1.7 mm from the growth plates was excluded from the measurements in the vertebra. The image of the specimen, observed under a fluorescence microscope and recorded with a video camera (DK-3000; Hitachi), was processed using a plotter (Cosmozone

1SA; Nikon, Tokyo, Japan) to measure the parameters. For the femoral diaphysis, 20- to 30-μm-thick cross-cut ground sections were obtained with a microgrinding machine system (KG4000; EXAKT, Norderstedt, Germany) and prepared for measurement. The image of the specimen, observed under a fluorescence microscope and recorded with a video camera, was processed using a plotter (Cosmozone 1SA; Nikon) to measure the total cross-sectional area.
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Structural analysis using micro-computed tomography (micro-CT)

In order to investigate the effects of ALN and ELD combination therapy on the trabeculae in the cancellous tissue of the lumbar vertebral body, 3-dimensional trabecular analysis was performed by micro-CT (μCT 40; Scanco Medical, Zurich, Switzer- land). The μCT 40 is equipped with a microfocus X-ray tube with a focal spot of 10 μm, producing a fan beam that is detected by a charge-coupled device array with a turntable that can be shifted automatically in the axial direction. The filtered 40-kVp X-ray spectrum peaks at 25 keV, allowing excellent bone-versus-marrow contrast. The whole spinal body was scanned in 250 slices (thickness, 13 μm) in the dorsoventral direction. On the original 3D images, morphometric indices were directly determined from the binarized volume of interest (VOI) (Augat et al. 1998).

Statistical analysis

All data are presented as means±standard deviation (SD). Statistical analysis was performed by analysis of variance (ANOVA) on the SAS statistical analysis software package (SAS institute Inc., Cary, NC, USA). The statistical differences between individual groups were analyzed by ANOVA followed by Tukey's multiple comparison tests. The values of $p<0.05$ were considered significant for all statistical analyses.

Results

The effect of combination therapy on bone mineral density and strength in lamber spine and femur midshaft

I invesitigated the effect of combination therapy of ALN and ELD on osteoporosis treatment using OVX rat model. After 12 weeks treatment of ALN and ELD, OVX rats were treated with ALN, ELD alone or their comination for 12 weeks and BMD of lumbar (L2-L4) which contains a lot of cancellous bone and femur (midshaft) which consist of cortical bone were measured using DXA. OVX rats treated with vehicle showed low BMD in both lumbar and femur midshaft compared to sham operated control rats. Rats with 12-weeks treatment of ALN or ELD alone showed significantly higher BMD than OVX treated with vehicle rats in both of lumbar spine and femur midshaft (Figure 6). The BMD of combination treatment of ALN and ELD group was much more higher than both mono-therapy groups in lumbar spine, and than ALN mono-therapy group in femur midshaft (Figure 6). Micro CT images of lumbar spine showed the reduction of cortical and cancellous bone reduction in OVX vehicle treated group (Figure 7). The cross- and longitudinal-section of lumbar spine (L4) revealed that mono-therapy of ALN or ELD increased number of trabecular bone and cortical thickness. Moreover, the μCT images of lumber spine from rats got the combination treatment of ALN and ELD showed more solid structure of cancellous and cortical bone. (Figure 7).

A compresson test of lumbar spine was adopted to assess a bone strength of lumbar and 3-point bending test to femur midshaft. The compression strength of lumbar spine in OVX vehicle treated group was significnatly decreased compared with sham operated group (Figure 8A). The max load of lumbar spine of ALN treated group showed tendency of restoration and significant increase in ELD treated group. A combination treatment of ALN and ELD showed significant increase in max load of lumbar than both mono-therapy groups. The OVX rats treated with vehicle appear to decline in femur bone strength compared to sham operated group and rats treated with ELD alone or combination treatment of ALN and ELD showed significantly increase of femur bone strength than OVX vehicle treatment rats. There are statistically significance between the femur bone strength of combination therapy group and each mono-therapy groups.

The effect of combination treatment of ALN and ELD in bone turnover

To elucidate the mechanisms of the effectiveness of combination treatment in BMD and bone strength of both lunbar spine and femur midshaft, I investigated the bone turnover of cancellous and cortical bone. The proportion of eroded surface (ES/BS) on lunbar cancellous bone was increased in OVX vehicle treated group than sham operated control group. Both mono-therapy groups exhibit significant decrease of ES/BS and combination treatment showed an additive effect on reduction of ES/BS in lumbar cancellous bone (Figure 9A). On femur cortical bone, ES/BS was not changed between sham, OVX, and mono-therapy groups but significantly decreased on combination therapy group compared to OVX group (Figure 9B). Though the additive effect on reduction of bone resorption, combination treatment of ALN and ELD did not show an additive effect on the proportion of bone mineralizing surface of cancellous bone (Figure 9C).

Discussion

The different and complementary modes of action of anti-osteoporotic drugs allow a considerable range of combination therapies for treating osteoporosis. In chapter 2, I demonstrated that in an OVX rat model of osteoporosis, the combination treatment of ALN and ELD clearly improved the mechanical properties of the lumbar spine and midshaft femur by additively suppressing bone resorption while maintaining bone formation compared to each mono-therapy. Previous reports have demonstrated that the anti-resorptive effects of ALN and ELD are mediated through different pathways: BPs inhibit the bone resorptive capacity of mature osteoclasts and also may induce osteoclast apoptosis (Azuma et al. 1998), while active VD_3 analogs reduce osteoclast number by suppressing osteoclast differentiation as I reported in chapter 1., ELD mono-therapy increased the mechanical properties as well as the BMD of the lumbar vertebrae and midshaft femur. Moreover, in agreement with previous studies (Black et al. 1996, Ito et al. 2002, Sato et al. 1996, Ma et al. 2003), this study showed that prophylactic administration of ALN to OVX rats over a 12-week period could prevent estrogen depletion-induced bone loss in the femur and lumbar vertebrae by depressing the elevated bone turnover. Ovariectomized animals were widely used for the model of postmenopausal osteoporosis, so my data suggest that combination treatment of ALN and ELD may also have superior effect than mono-therapy in clinical use. The combination treatment of these drugs decreased bone resorption, which is the main effect of these drugs, whereas there was no significant difference in MS/BS, a bone formation parameter, compared to mono-therapy. Therefore, the present study suggested that the major mechanism underlying the anti-osteoporotic effect of ELD on vertebral cancellous bone and on the midshaft femur of OVX rats is induced by inhibiting bone resorption while maintaining bone formation..

The detailed micro-CT analyses indicated that the increase in mechanical strength of the lumbar vertebra after ELD treatment was primarily due to the restoration of trabecular bone and the increase in thickness of cortical bone. The bone strength was affected not only BMD but also bone quality. It is considered that these morphological changes in cancellous and cortical bone may contribute to increase the bone strength by ALN and ELD combination treatment. As I mentioned, the combination treatment increased bone strength of femur. Bone fractures of femur in osteoporosis patients are risk for bedridden so that application of this combination treatment may improve QOL and life expectancy of

osteoporosis patients. These findings suggest that the combination treatment of ALN and ELD has a therapeutic advantage over either ALN or ELD mono-therapy for treating osteoporosis.

Based on my results, I concluded that with respect to the improvement of mechanical strength of the lumbar spine as well as the midshaft femur, the combination treatment of ALN and ELD was more beneficial than mono-therapy with either drug alone and the suppressive effect of ALN and ELD on bone resorption was considered to be additive, and the bone formation was not affected by ALN and ELD co-administration.

General discussion

Here, I demonstrated that the inhibitory effect of active VD_3 on osteoclastogenesis. Active VD_3 act to early osteoclast progenitor cells and upregulated the expression of IFN-β, which is a strong inhibitor for osteoclast differentiation. Some anti-bone resorptive drugs are used in osteoporosis treatment but main target of these drugs is mature osteoclast. So my data revealed that active VD_3 has distinct mechanisms to regulate the osteoclast differentiation or its activity from other drugs using in osteoporosis treatment. This finding of new mechanism of anti-osteoclastogenesis effect of active VD_3 may make advantage in investigation of effective osteoporosis treatment.

I also demonstrated the additive effect of ELD, an active VD_3 analogue, and ALN, one of BPs, in OVX rats. The combination treatment these drugs showed additive effect on reduction of bone resorption, increasing BMD and bone strength. Moreover, though ELD and ALN combination treatment had additive effect on reduction of bone resorption, no effect was observed in bone formation compared to mono-therapy. Usually, too much suppression of bone resorption cause “freezing bone” characterized by no bone formation. This phenomenon thought to the cause of accumulation of bone micro-damage. It is considered that micro-damages of bone induce sudden bone fractures in femur midshaft which called “atypical fracture”. Atypical fractures in osteoporosis patients are hard to repair because of severe suppression of bone turnover. In this respect, ALN and ELD combination treatment has characteristic effect on controlling bone turnover from other osteoporosis treatment by suppression of bone resorption because strong inhibition of bone resorption usually accompanied with suppression of bone formation.

In conclusion, I revealed the novel mechanism of active VD_3 in inhibition of osteoclastogenesis through IFN-β at the early stage. And also demonstrated that the combination treatment of ELD, an active VD_3 analog, and ALN, one of BPs, which have different mechanisms in suppressing bone resorption, effectively inhibited bone resorption of OVX rats and increased BMD and bone strength both lumbar spine and femur midshaft. Though the combination therapy strongly reduced bone resorption in OVX rats, bone formation was not changed compared to sham operated rats or OVX rats treated with ALN or ELD alone. Based on my results, a clinical study of ALN and ELD combination treatment for osteoporosis patients is ongoing. It is expected that this regimen of strong inhibition of bone resorption with mild suppression of bone formation will help to reduce bone fractures and maintain QOL and life expectancy in osteoporosis patients.

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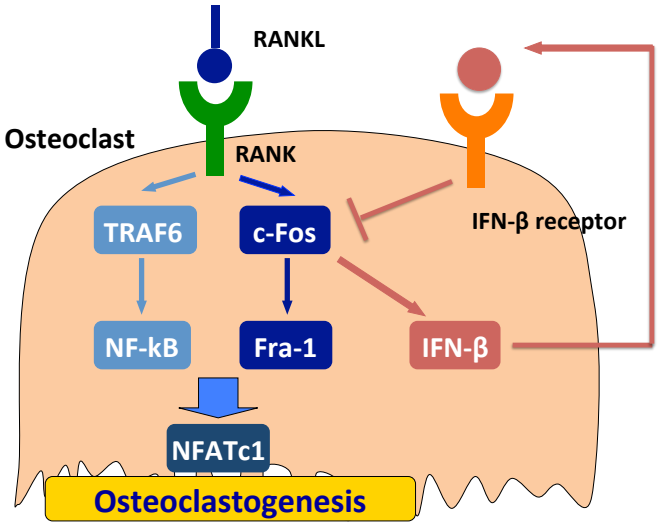

Figure 1 Signal transduction of RANKL induced osteoclastogenesis. RANKL binds to its receptor RANK and stimulates TRAF6 and c-Fos pathways. c-Fos not only accelerates but also inhibits osteoclastogenesis through expression of IFN-ß secretion. Secreted IFN-ß binds to its receptor in osteoclast and inhibits protein translation of c-Fos.

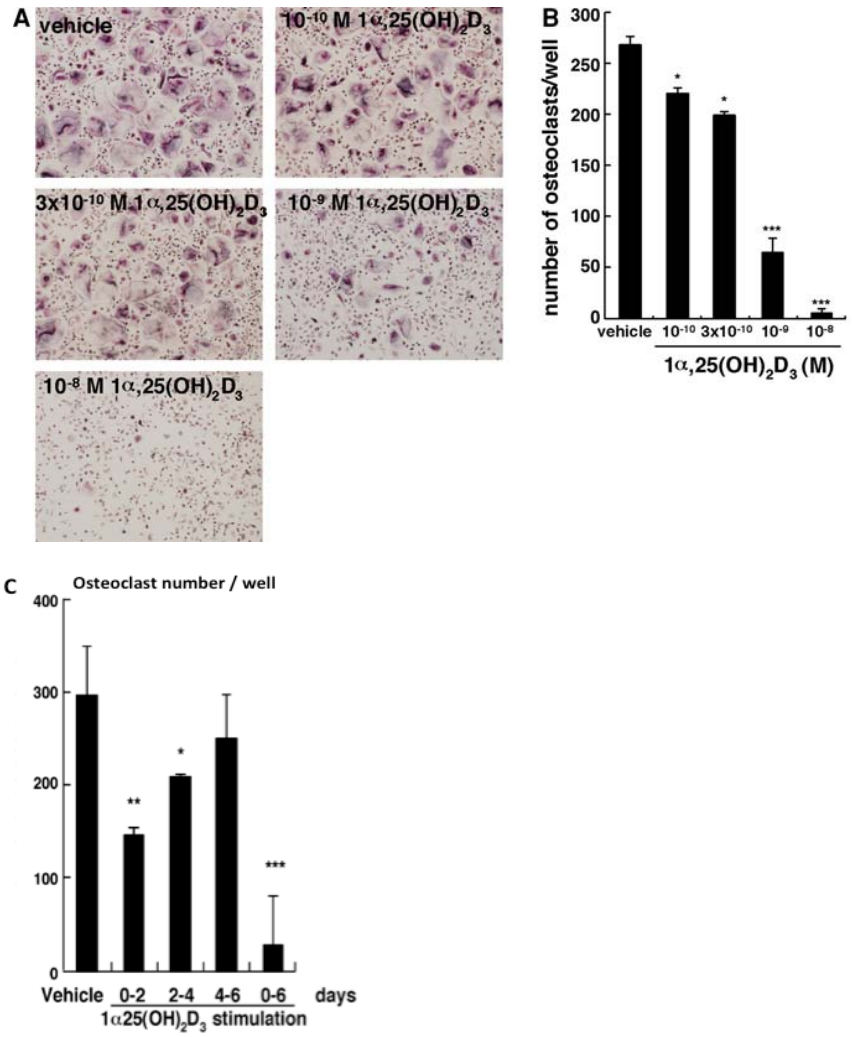

Figure 2 One-Alpha, 25-dihydroxy vitamin D_3 (acrive VD_3) inhibited osteoclast formation in colony-forming unit granulocyte macrophage (CFU-GM) cells. Human CFU-GM cells were cultured for 6 days with M-CSF (30 ng/ml) and receptor activator of nuclear factor kappa-B ligand (RANKL) (30 ng/ml) in the presence or absence of the indicated concentrations of active VD_3. Mature osteoclasts were stained with TRAP (A) and the number of multinuclear osteoclasts were shown (B). (C) Acrive D_3 was added in culture medium in indicated period. Cells were subjected to TRAP staining. Multinuclear TRAP-positive cells containing more than three nuclei were scored as osteoclasts. Vehicle, 0.1% EtOH. Data are mean number ± SD of osteoclasts (*$P < 0.05$, ***$P < 0.001$)

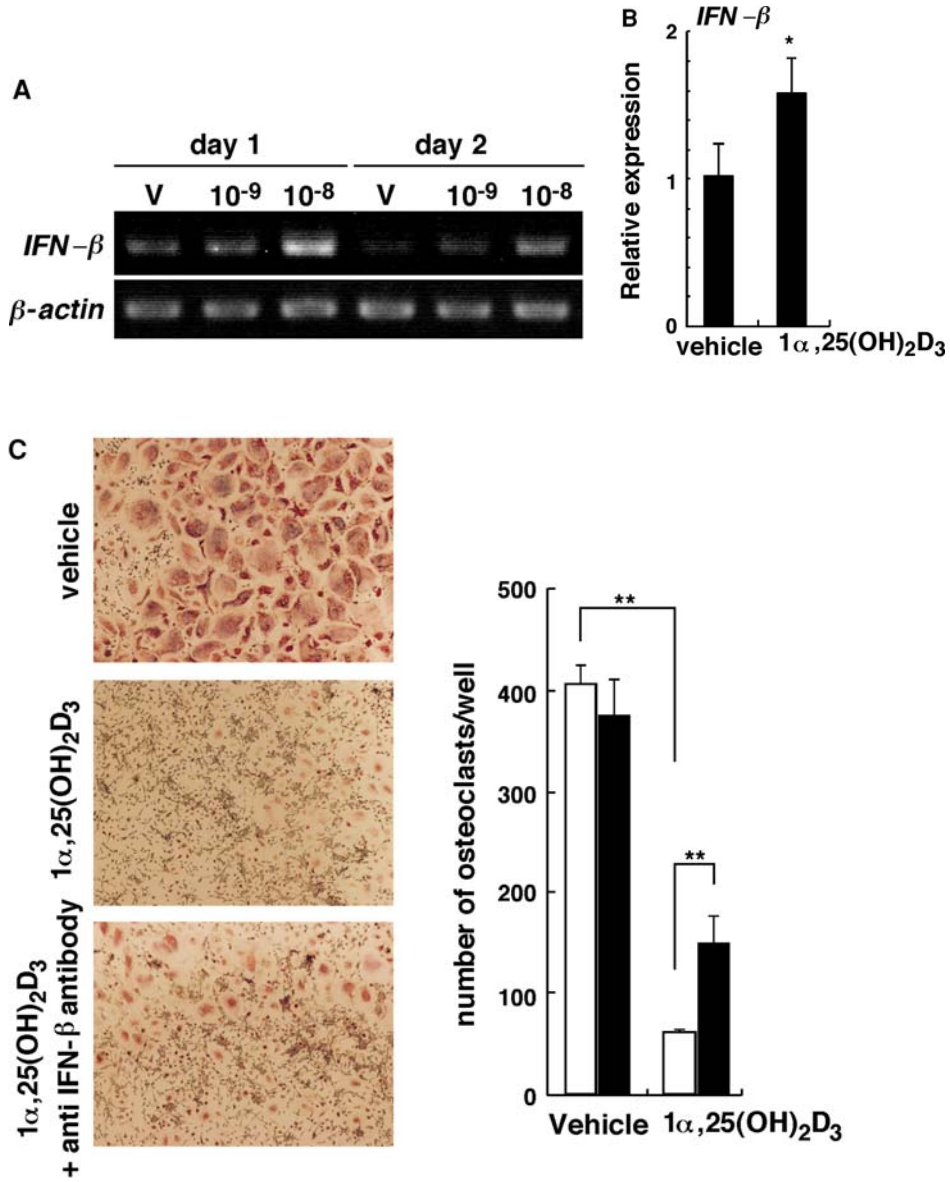

Figure 3 Interferon-beta (IFN-ß) induced by 1α,25(OH)$_2$D$_3$ inhibits human osteoclastogenesis.

Human CFU-GM cells were cultured with M-CSF (30 ng/ml) and RANKL (30 ng/ml) in the presence or absence of the indicated concentrations of 1α,25(OH)$_2$D$_3$ for 1–2 days. Then, total RNA was isolated and RT-PCR analysis was undertaken to detect the expression of IFN-ß (A). ß-actin expression is shown as an internal control. IFN-ß expression was upregulated by active VD$_3$ treatment in human osteoclast progenitor cells. V, vehicle (0.1% EtOH). Human CFU-GM cells were cultured with M-CSF (30 ng/ml) and RANKL (30 ng/ml)

in the presence or absence of 10^{-8} M active VD_3 for 2 days. Total RNA was then isolated and real-time PCR analysis performed to detect the expression of IFN-ß relative to GAPDH (B) (*P < 0.05). Human CFU-GM cells were cultured with M-CSF (30 ng/ml) and RANKL (30 ng/ml) in the presence or absence of 10^{-8} M $1\alpha,25(OH)_2D_3$ with (black column) or without (white column) antibody against IFN-ß for 6 days and stained with TRAP. Multinuclear TRAP-positive cells containing more than three nuclei were scored as osteoclasts (C). Left, representative data; right, data are mean number ± SD osteoclasts (**P < 0.01). Osteoclastogenesis inhibited by 10^{-8} M acitive VD_3 was significantly rescued by the antibody against IFN-ß. Vehicle, 0.1% EtOH.

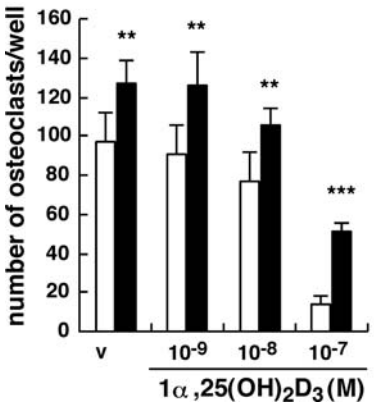

Figure 4 IFN-α/ß receptor 1-dependent inhibition of osteoclastogenesis by active VD_3. Osteoclast progenitor cells isolated from wild-type (white column) or IFNAR-deficient (black column) mice were cultured with M-CSF (30 ng/ml) and RANKL (30 ng/ml) in the presence or absence of the indicated concentrations of active VD_3 for 5 days and stained with TRAP. Multinuclear TRAP-positive cells containing more than three nuclei were scored as osteoclasts. Data are mean number ± SD of osteoclasts (**P ¥<0.01, ***P ¥<0.001). V, vehicle, 0.1% EtOH.

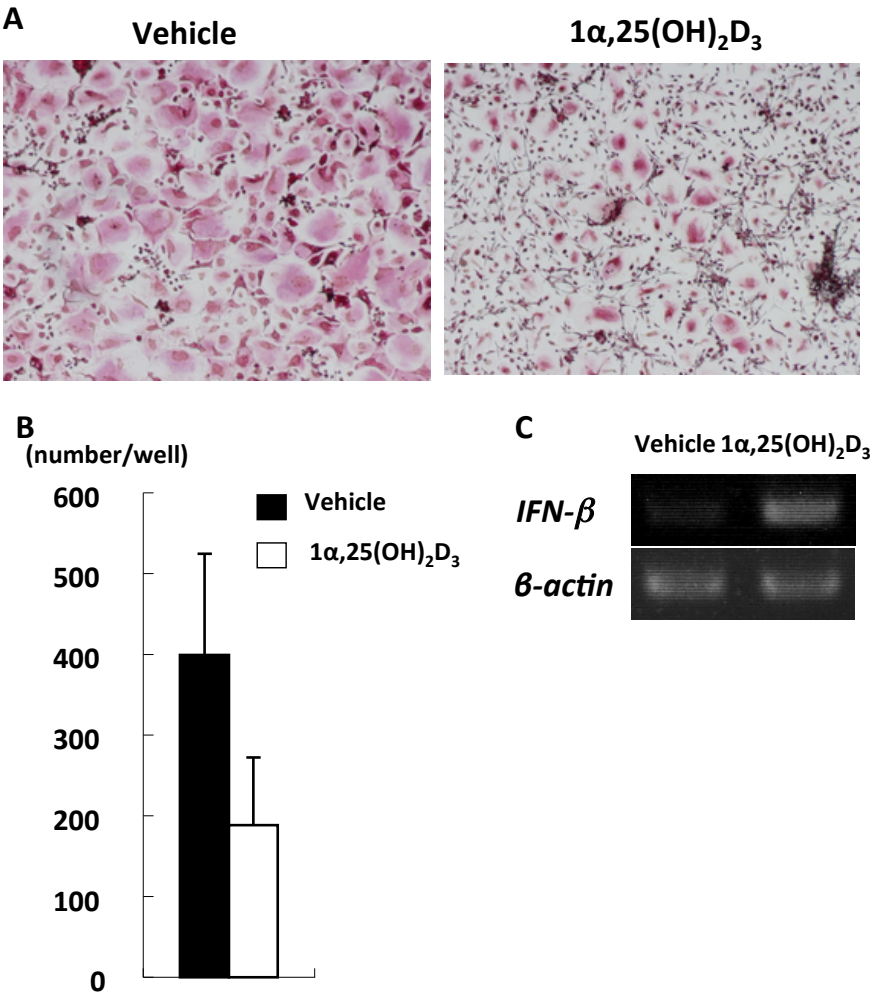

Figure 5　Active VD_3 act to osteoclast progenitor cells and upregulated IFN-β expression and inhibited osteoclastogenesis.

Osteoclast precursor cells were stimulated with active VD_3 for 2 days with SCF, GM-CSF and IL-3. Then active VD_3 and cytokines were washed out and osteoclastogenesis was induced by M-CSF and RANKL. Osteoclasts were visualized by TRAP staining (A). Active VD_3 stimulation to osteoclast precursor cells inhibited osteoclastogenesis. Number of TRAP positive multinuclear cells were shown (B). mRNA of IFN-β was increased in osteoclast precursor cells after 2-day's activeVD_3 stimulation (C).

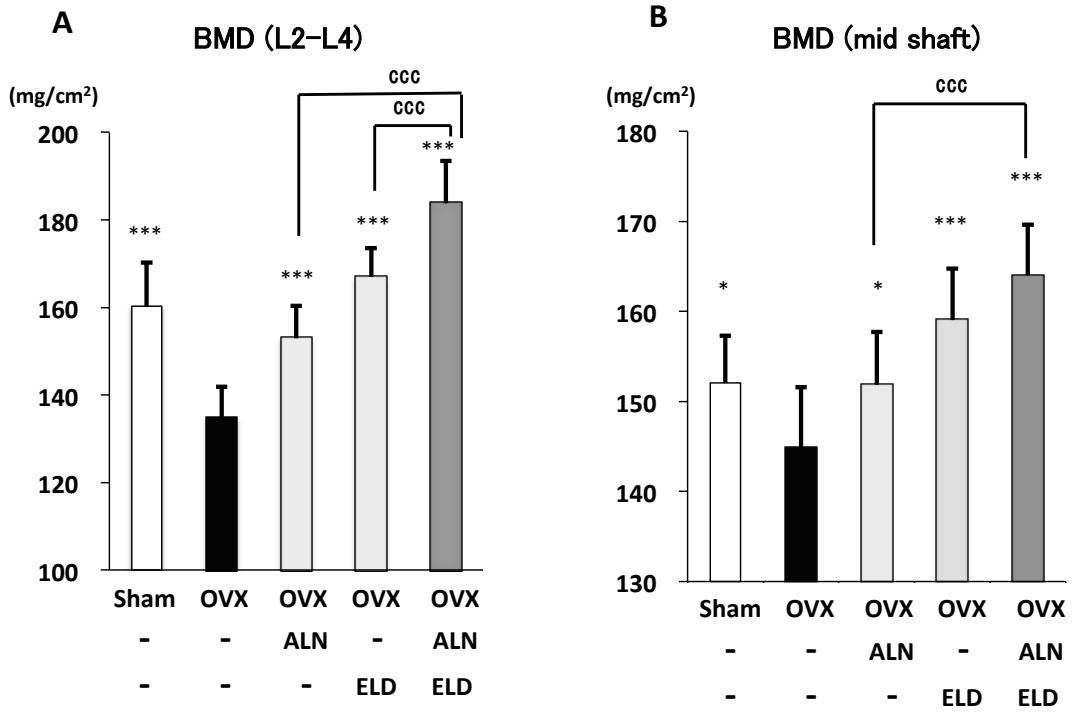

Figure 6　The bone mineral density (BMD) measured by dual-energy X-ray absorptiometry in the Lumber spine (L2-L4) (A) and the middle femur (B) after treatment with ELD and ALN, alone or in combination for 12 weeks in OVX rats. Each column and vertical bar represents the mean ± SD.***: $p < 0.001$ vs. OVX control group; ccc: $p < 0.001$ vs. mono-therapy. The statistical differences between individual groups were analyzed by ANOVA followed by Tukey's multiple comparison tests.

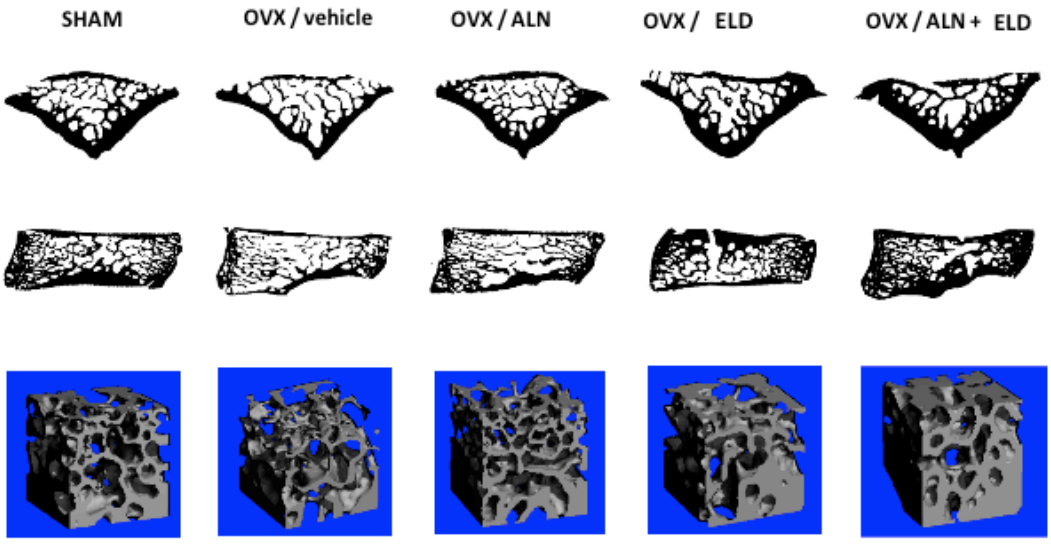

Figure 7　Micro-CT images of lumbar vertebra (L4) in ovariectomized (OVX) rats treated with eldecalcitol (ELD) and alendronate (ALN), alone or in combination. ALN or ELD alone prevented the bone loss and deterioration of cancellous bone structure induced by OVX. Note that co-administration of ALN and ELD improved the density of the trabecular bone network and thickened the cortical region of vertebral bodies.

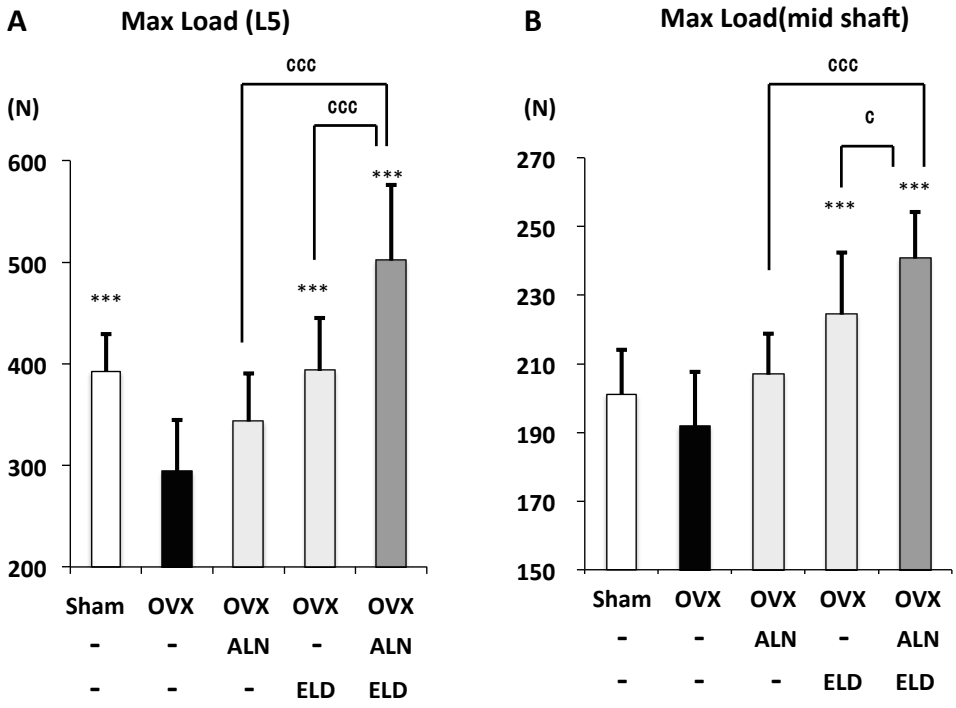

Figure 8　A bone strength of lumber spine (L5)(A)and the middle femur (B) after treatment with eldecalcitol (ELD) and alendronate (ALN), alone or in combination, for 12 weeks in ovariectomized (OVX) rats. Each column and vertical bar represents the mean ± SD.***: $p < 0.001$ vs. OVX control group; c:$p < 0.05$ vs. mono-therapy ;ccc: $p < 0.001$ vs. mono-therapy. The statistical differences between individual groups were analyzed by ANOVA followed by Tukey's multiple comparison tests.

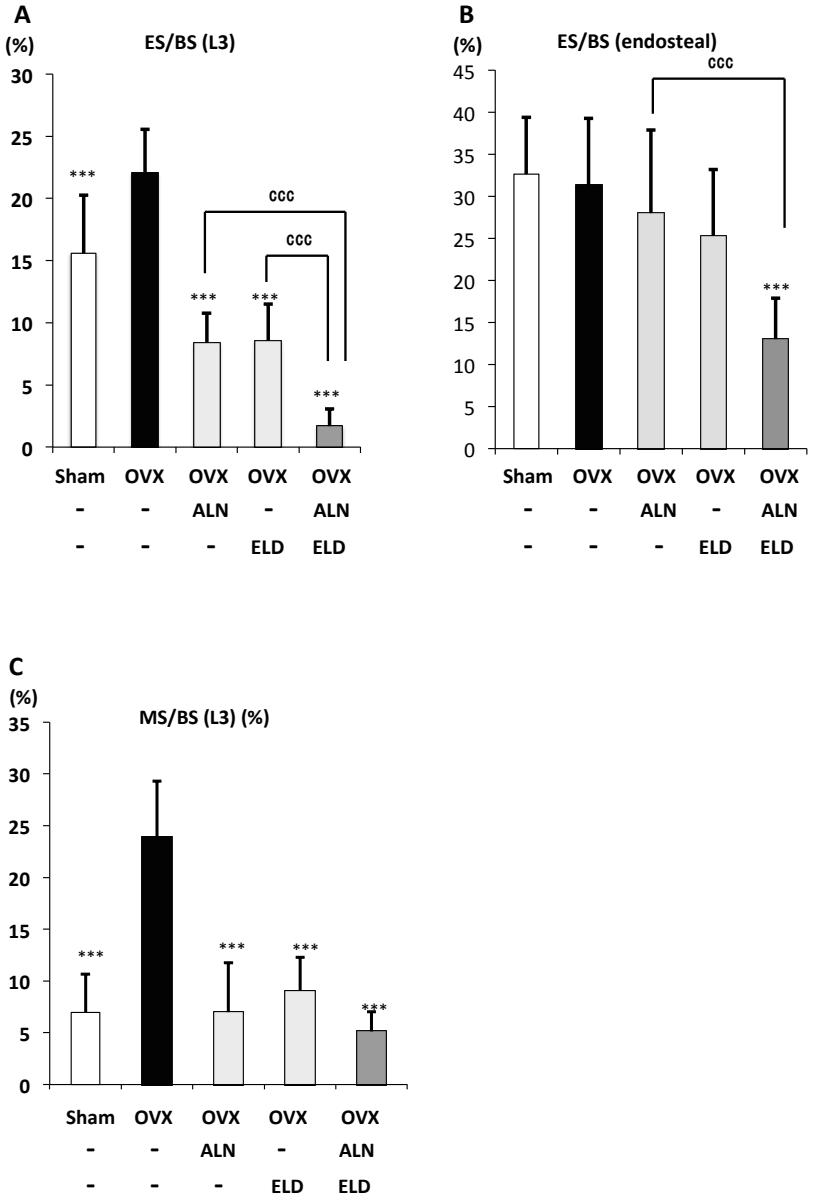

Figure 9 Bone formation and resorption parameters. ES/BS in L3 and endosteal of femur midshaft and MS/BS in lumbar are shown. Each column and vertical bar represents the mean ± SD.***: $p < 0.001$ vs. OVX control group;ccc: $p < 0.001$ vs. mono-therapy. The statistical differences between individual groups were analyzed by ANOVA followed by Tukey's multiple comparison tests.