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# In Vitro에서 골 형성과 흡수에 대한 Endothelin-1의 영향

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# Effects of Endothelin-1 on Bone Formation and Resorption in Vitro

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## Abstract

**Background**: Balanced regulation of the receptor activator of nuclear factor-kB (RANK) ligand (RANKL) and osteoprotegerin (OPG) by osteoblasts is important for osteoclastogenesis. Sex hormones, glucocorticoids, and prostaglandin E2 (PGE2) are known to modulate osteoblast proliferation and osteoclast formation. Endothelin (ET)-1 is a mitogen as well as a strong vasoconstrictor.It stimulates proliferation of osteoblasts, but its effects on differentiation are controversial. In addition, little is known about ET-1 regulation of osteoclast formation. Thus, the present study was undertaken to investigate whether ET-1 canregulate the expression of RANKL and OPG genes in osteoblasts and affect RANKL-induced osteoclastogenesis.

**Methods**: Osteoblasts were derived from neonatal calvariae and monocytic preosteoclasts from the bone marrow of adult mice, respectively. Cells were cultured in a-minimum essential medium containing 10 nM ET-1. The gene expressions of RANKL and OPG in osteoblasts and RANK in preosteoclasts were measured by real-time RT-PCR. Mineralization by osteoblasts was determined by Alizarin-red staining. Osteoclastogenesis was examined using tartrate-resistant acid phosphatase (TRAP) staining and resorption pit assay.

**Results**: Osteoblasts expressed both ETA and ETB receptors (ETAR and ETBR). ET-1 (10 nM) increased osteoblast proliferation 1.6-fold compared with the control after 3 days in culture and stimulated differentiation, which was indicated by increased formation of mineralized matrix. Proliferation and differentiation of osteoblasts was blocked by 1 uM BQ123, an ETAR antagonist. ET-1 suppressed RANKL gene expression by 50% but did not affect OPG gene expression, and thus reduced the ratio of RANKL to OPG mRNA. PGE2 production by osteoblasts was increased by ET-1. In preosteoclast cultures, ET-1 had little effect on RANK mRNA expression and suppressed RANKL-induced osteoclastogenesis by decreasing the number of TRAP-positive osteoclasts and the resorbed areas.

**Conclusions**: These findings demonstrate that ET-1 can increase bone formation by stimulating proliferation and differentiation of osteoblasts and decrease bone resorption by suppressing RANKL-induced osteoclastogenesis as well as suppressing RANKL mRNA expression.

**Key words** : endothelin-1, osteoblast, osteoclast, osteoprotegerin, receptor activator of nuclear factor-kB ligand

## Introduction

Bone remodeling is an organized process in which old or damaged boneis removed by osteoclasts and replaced with newly formed bone by osteoblasts with little change in the shape of the bone. Because the remodeling cycle consecutively progresses, direct interactions between osteoclasts and osteoblasts are important, and are controlled by both systemic and local factors. The major regulators mediating interactions between osteoblasts and osteoclasts are osteoprotegerin (OPG), receptor activator

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of nuclear factor-κB ligand (RANKL), and its cognate receptor RANK[1, 2]. OPG is secreted by osteoblasts. As a RANKL decoy receptor, OPG binds to RANKL and neutralizes the effect of RANKL and thus blocks osteoclastogenesis[3]. RANKL is expressed as a membrane-bound or soluble protein secreted by osteoblasts. RANKL induces osteoclastogenesis when it interacts with its receptor RANK on the membranes of osteoclasts. A variety of systemic or local osteotropic factors such as hormones, growth factors, cytokines, and prostaglandins modulate bone remodeling by regulating OPG and/or RANKL expression of osteoblasts. For example, parathyroid hormone, prostaglandin E2 (PGE2), and glucocorticoids enhance bone resorption by increasing RANKL, with or without decreasing OPG[4-7], while sex hormones and mechanical stress reduce it by decreasing RANKL[8-10].

Endothelin (ET)-1 is a potent vasoconstrictor and a mitogen in a variety of cell types. It has been detected in bone cells, including osteoblasts and osteoclasts[11, 12]. Recent studies indicate that in osteoblasts, ET-1 stimulates mitogenesis, protein synthesis of collagen and non-collagen peptides, and PGE2 production[13, 14),15]. Depending on the assay system used, it also either decreased or increased alkaline phosphatase activity, osteocalcin production, and mineralization[16-19]. ET-1 initiates its actions by binding to its receptors, $ET_A$ receptor ($ET_AR$) or $ET_B$ receptor ($ET_BR$). Knockout mice of ET-1 and $ET_AR$ display similar phenotypes, causing hypoplasia of the facial bones, suggesting that the ET-1/ $ET_AR$ axis is involved in bone formation. Other evidence that ET-1 plays a role in new bone formation is found in studies of cancer bone metastasis[20, 21]. For example, prostate cancers are the most common causes of osteoblastic bone metastasis, followed by breast cancers. Nelson et al.[22] showed plasma concentration of ET-1 was significantly higher in advanced prostate cancer with bone metastases than in organ-confined prostate cancers. Yin et al.[23] demonstrated that in mice inoculated with ZR-75-1 breast cancer cells, which secrete ET-1 but not PTHrP, tumors produced osteoblastic bone metastasis. However, the actions of ET-1 on osteoclasts or osteoclastogenesis are little known. One report demonstrated that ET-1 reduced the motility of mature osteoclasts[24].

The aims of the present study were to reassess whether ET-1 stimulates osteoblast differentiation, to assess whether ET-1 indirectly influences osteoclast formation by regulating the expression of RANKL, OPG, and/or RANK genes, and to examine whether ET-1 directly modulates RANKL-induced osteoclastogenesis.

## Materials and Methods

### *Culture of osteoblasts and preosteoclasts*

Calvariae were harvested from neonatal C57BL mice within 3 days of delivery according to the method of Perkins et al. with slight modifications[25]. The calvariae were cut into small pieces and treated with serial digestion of 0.1% collagenase type 1A (Sigma, St. Louis, MO, USA) in PBS for 20 min on a rocking platform. At the end of each 10-min digestion, the supernatant was removed and collagenase activity was neutralized with fetal bovine serum (FBS, GIBCO, Gaithersburg, MD, USA). Fractions 2-5 were pooled to yield primary osteoblasts. The cells were cultured in a-minimum essential media (a-MEM, GIBCO) supplemented with 10% FBS, 100 IU/ ml penicillin and 100 ug/ml streptomycin at 37C in a humidified atmosphere with 5% CO2. Bone marrow cells were obtained from femora and tibiae of adult C57BL mice according to the method of Suda et al[2]. The bone ends were cut and the marrow cavity was flushed out into a Petri dish by injecting á-MEM using a 21-gauge needle. The bone marrow cells were washed twice and incubated in á-MEM containing 10% FBS and M-CSF (10 ng/ml) for 24 hr in a T-75 flask. After 24 hr, non-adherent

mononuclear preosteoclasts were prepared, resuspended and further incubated in á-MEM with M-CSF (50 ng/ml) for 3 days. To differentiate into mature osteoclasts, RANKL (50 ng/ml) was added and cells were cultured for more than 7 days.

### *Mineralization assay*

Osteoblastic cells seeded in a 24-well plate were incubated in a medium containing vehicle, ET-1 (10 nM), ET-1+BQ123 (1 uM), and PGE2 (0.1 uM) for 21 days. To stimulate mineralization, vitamin C and β-glycerophosphate were added to the medium for the last 7 days. Mineralized matrix was visualized through Alizarin red-S staining. For this, the cells were fixed in 70% ethanol for 1 hr at room temperature and stained with 40 mM Alizarin red-s solution (pH 4.2) for 10 min. After washing, red images were obtained using microscopy.

### *PGE2 ELISA*

Primary cultured or MC3T3 osteoblastic cells were cultured with vehicle or ET-1 (10 nM) in serum-free a-MEMfor 24 hr. The amount of PGE2 in the culture medium was determined using an ELISA kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions.

### *Tartarate-resistant acid phosphatase (TRAP) staining and resorption pit assay*

Preosteoclasts were plated in 48-well plates and treated with vehicle, ET-1 (10 nM), RANKL (50 ng/ml), ET-1 +RANKL for up to 7 days. M-CSF was maintained throughout the culture period. These cells were then fixed in citrate-buffered acetone for 10 min and stained with an acid phosphatase leukocyte kit (Sigma) for 1 hr at 37℃. Multinucleated mature osteoclasts with which TRAP were stained was visualized using inverted microcopy. For resorption assays, an OAAS plate (Ostech, Seoul, Korea) was used. The bottom of the OAAS plate was coated with calcium-phosphate particles for easy detection of bone resorption. Preosteoclasts were cultured in an OAAS plate and treated according to the same method as TRAP staining. They were lysed using 0.6% perchlorate solution and images of the resorbed areas were obtained under light microscopy.

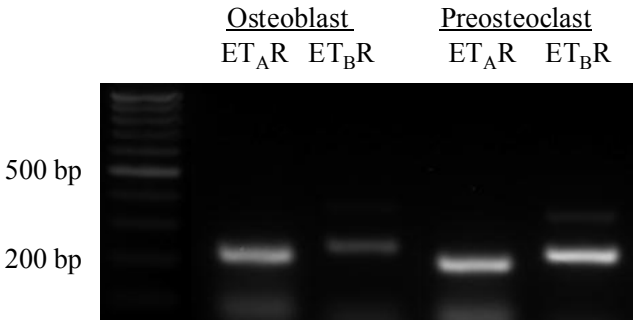



Fig. 1. Expression of endothelin (ET) receptor mRNA in primary osteoblasts and preosteoclasts. RT-PCR products were separated by 2% gel electrophoresis. Expected sizes for $ET_AR$ and $ET_BR$ are 204 and 231 bp, respectively.

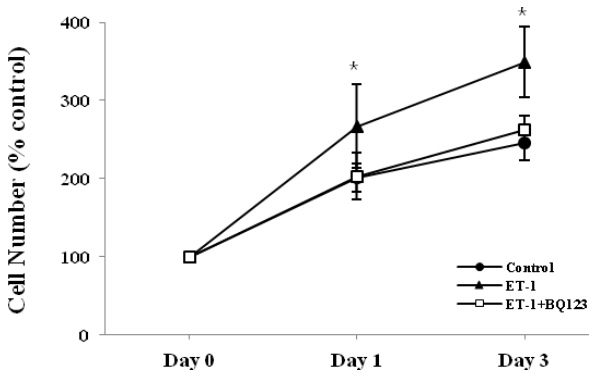



Fig. 2. Changes in osteoblast proliferation by ET-1and an $ET_AR$ antagonist BQ123. ET-1 (10 nM) was added in 0.5% serum-containing medium and cell numbers were counted using hemocytomer at days 1 and 3 of culture. Osteoblasts were seeded at 5.5 x $10^3$ in a 12-well plate. Data are mean ± SD of 3 wells. * $p<0.05$ vs. the control.

### *Quantitative real-time RT-PCR*

Osteoblastic cells were cultured in a 6-well plate and total RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. After spectrophotometric determination of

Table 1. Primer sequences used for real-time and conventional RT-PCR

| Gene | Sequence | Size | GenBank# |
|---|---|---|---|
| ETBR | F 5'-AGCTGGTGCCCTTCATACAG-3'<br>R 5'-GGGGCTTTCCTTTGTAGTCC-3' | 231 | NM 007904 |
| ETAR | F 5'-TCGAGAAGTGGCAAAGACTG-3'<br>R 5'-AGAGCTATTGGGTTTATGCAAGA-3' | 204 | XM 134499 |
| RANKL | F 5'-ATTTGCACACCTCACCATCA-3'<br>R 5'-GTGCTCCCTCCTTTCATCAG-3' | 291 | NM 057149 |
| OPG | F 5'-TGTGTATTGCAGCCCAGTGT-3'<br>R 5'-CAGGGTGCTTTCGATGAAGT-3' | 240 | NM 008764 |
| RANK | F 5'-CTACACAGGCAGTGGGAACA-3'<br>R 5'-TGGCTGACATACACCACGAT-3' | 306 | MN 009399 |
| β-actin | F 5'-GACGGCCAGGTCATCACTAT-3'<br>R 5'-CTTCTGCATCCTGTCAGCAA-3' | 216 | NM 007393 |

total RNA concentrations, reverse transcription was performed in a total volume of 40 ul containing 1 to 2 ug RNA and random hexamer using the SuperScript II first-strand synthesis system (Invitrogen). PCR was run in a LightCycler in a 20 ul reaction which contained 5 ul of 5-fold diluted cDNA, 2 nM primer, and 2x SYBR II master buffer (TakaraKorea, Seoul, Korea). Reaction conditions are: 95℃ for 10 sec followed by 45 cycles at 95℃ for 5 sec, 60℃ for 10 sec, 72℃ for 10 sec and 80℃ (fluorescence detection) for 5sec. The relative mRNA expression was calculated using a comparative method as described in ABI user bulletin #2. Before applying the method, it was confirmed that the relative PCR efficiencies of targets (OPG, RANKL, RANK) and reference (β-actin) were approximately equal. Primers were generated using Primer 3 software (S. Rosen and HJ Skaletsky, Whitehead Institute, MIT). All primers used were designed to include either one or two introns, and their sequences are presented in Table 1.

### *Statistical analysis*

Data are expressed as mean ± SD. Unpaired Student's t-tests were performed and p-values less than 0.05 were considered statistically significant.

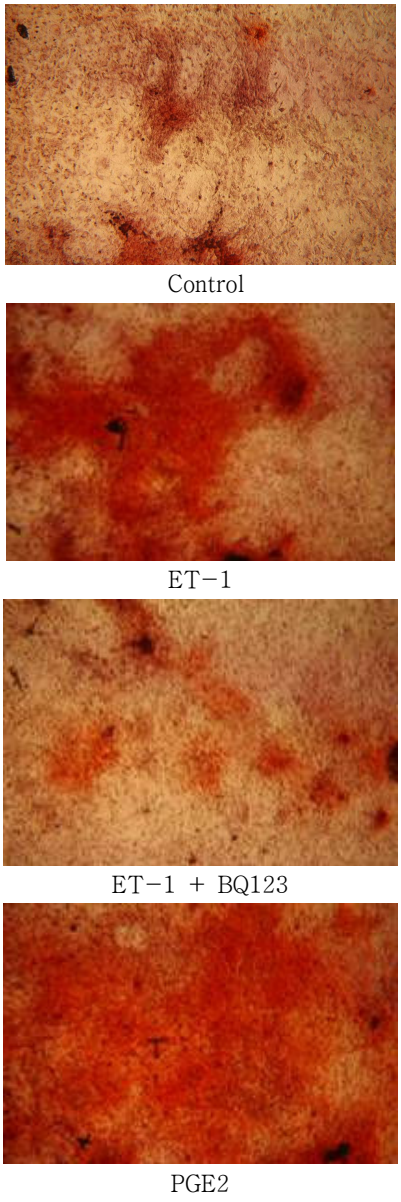



Fig. 3. Mineralized matrix formation by osteoblasts. Concentration of ET-1 (10 nM) was maintained throughout the culture period (21 days); cells were stained with Alizarin red on day 21 of culture. Note that ET-1 greatly increased mineralization compared to the control and was completely reversed by $ET_A$ receptorblocker, BQ123 (1 uM),. PGE2 (0.1 uM) was used as a positive control. (Magnification, 40X.)

## Results

### *Effects of ET-1 on osteoblast proliferation and differentiation*

Fig. 1. shows the expression of ET receptors in primary cultured osteoblasts and bone marrow monoocytic preosteoclasts. Osteoblasts and preosteoclasts both expressed $ET_AR$ and $ET_BR$ genes. In osteoblasts, the intensity of $ET_BR$ gene expression was weaker than that of $ET_AR$. In preosteoclasts, however, expression levels of $ET_AR$ and $ET_BR$ genes were similar. Fig. 2. illustrates changes in osteoblast proliferation by treatment with ET−1 and an $ET_AR$ blocker, BQ123. Exposure to 10 nM ET−1 increased osteoblast proliferation by 1.4−fold at 1 day after culture and 3.4−fold at 3 days after culture, respectively, compared to vehicle treatment. BQ123 (1 uM) completely abolished the proliferative effect of ET−1. Differentiated osteoblasts produce alkaline phosphatase activity at early stages and make mineralized matrix at later stages. To examine the effect of ET−1 on later stage differentiation, mineralized matrix formation was evaluated 21 days of culture. As seen in Fig. 3, treatment with ET−1 accelerated mineralization compared to vehicle treatment, but addition of BQ123 reversed it. These findings suggest that the proliferation and differentiation of osteoblasts are mediated through $ET_AR$.

PGE2 is a well−known differentiation factor of osteoblasts. PGE2 (0.1 uM) greatly increased mineralized matrix formation. ET−1 also increased PGE2 production by 1.8−fold in primary osteoblastic cells and by 6.5−fold in MC3T3 cell lines, compared with vehicle treatment (Fig. 4), implying that ET−1 can stimulate osteoblast differentiation by increasing PGE2 production via an $ET_AR$ pathway.

### *Expressions of OPG, RANKL, and RANK mRNAs*

To determine whether ET−1 can indirectly modulate osteoclastotogenesis by regulating the expression of OPG, RANKL, or RANK genes in osteoblasts, the expressions of these genes were quantitatively analyzed by real−time RT−PCR. As shown in Fig. 5A, the level of RANKL mRNA was reduced to approximately half of the control level by 24 hr treatment of 10 nM ET−1, but OPG mRNA

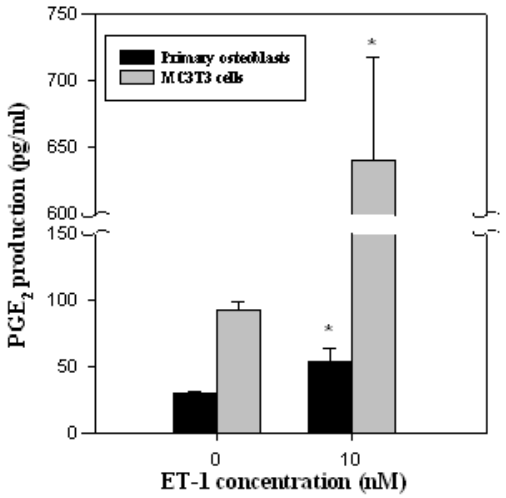



Fig. 4. Effect of ET−1 on PGE2 production in osteoblastic cells. Cells were incubated in serum−and phenol red −free medium for 24 hr. Values are mean ± SD of three wells. * p <0.05 vs. the matched control (0 nM).

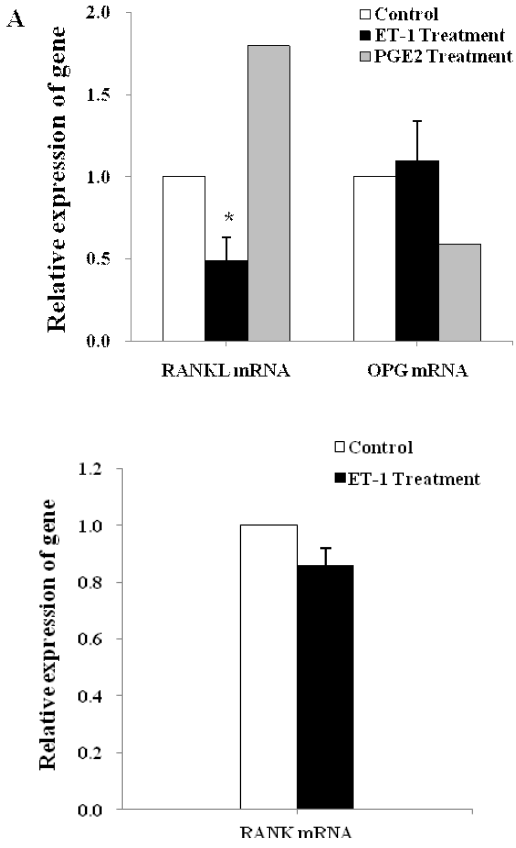
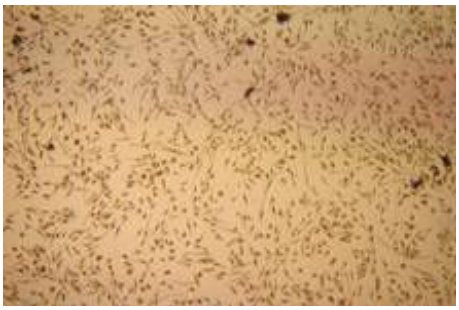
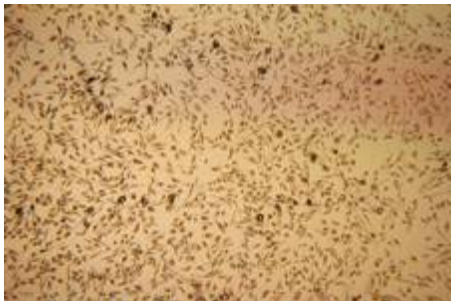



Fig. 5. Real−time RT−PCR analysis of the mRNA expression for RANKL, OPG, and RANK in osteoblasts (upper panel) and preosteoclasts (lower panel). Osteoblasts were treated with ET−1 (10 nM) or PGE2 (0.1 uM)for 24 hr and preosteoclasts with ET−1 (10 nM) only. The relative expression was normalized with b−actin. Values are mean ±SD of three separate experiments or mean of two experiments. *p <0.05 vs. the control.

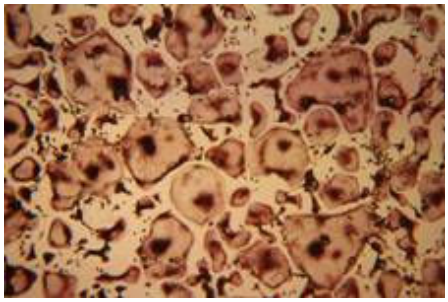
was not changed. Therefore, the ratio of RANKL to OPG was decreased, suggesting the inhibition of osteoclastogenesis. PGE2, which is known to stimulate RANKL expression and suppress OPG expression in osteoblasts, was used as a positive control. Fig. 5B illustrates RANK mRNA expression in preosteoclasts; ET-1 (10 nM) nearly affected the RANK gene expression.



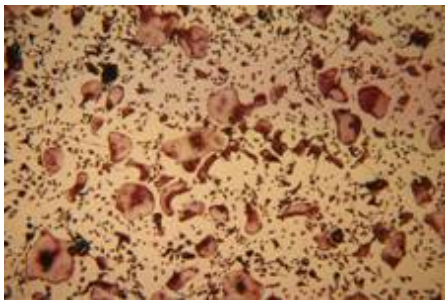
Control



ET-1



RANKL



RANKL + ET-1

Fig. 6. Tartrate-resistant acid phosphatase (TRAP) staining showing osteoclastogenesis. Bone marrow-derived mononuclear preosteoclasts were incubated with ET-1(10 nM), RANKL (50 ng/ml) or RANKL+ET-1 for 7 days in the presence of M-CSF and then stained with TRAP stain kit. Note that the number, size, and multinuclearity of osteoclasts were increased by RANKL treatment, but was greatly reduced by co-treatment of RANKL plus ET-1. (Magnification, 100X.)

### *TRAP staining and resorption pit formation*

RANKL is indispensible for osteoclast differentiation. As shown in Fig. 6, when RANKL (50 ng/ml) was added, most preosteoclasts differentiated into multinucleated osteoclasts. ET-1 (10 nM) had no effect on osteoclast differentiation. However, co-treatment with RANKL moderately reduced the size and number of multinucleated osteoclasts, suggesting that ET-1 acts as an inhibitory factor in RANKL-induced osteoclastogenesis. A resorption pitassay, which is a definite determinant of osteoclastogenesis, again confirmed TRAP findings because resorbed areas from RANKL treatment were also reduced by co-treatment of ET-1 (Fig. 7).

## Discussion

An accumulating body of evidence indicates that ET-1 stimulates cell proliferation including osteoblasts, vascular smooth muscle cells, and cancer cells[16, 26-29]. This was also demonstrated in our study (Fig. 2). However, the effects of ET-1 on osteoblast differentiation are controversial. Von Schroeder et al.[26] increased mineralization, as well as alkaline phosphatase (ALP) activity, in rat calvarial culture. In human trabecular cell culture, Kasperk et al[30] reported that ET-1 stimulated ALP activity. However, other researchers[16, 18, 19] showed that ET-1 decreased either ALP activity or mineralization in mouse MC3T3 cell line and human osteoblastic cell line. We observed that ET-1 stimulated osteoblast differentiation by bone matrix assay (Fig. 3), in which formation of mineralized bone matrices represents alate step of differentiation. It is therefore likely that depending on cell types and culture conditions, ET-1 plays either stimulatory or inhibitory role in osteoblast differentiation. The differentiation process is

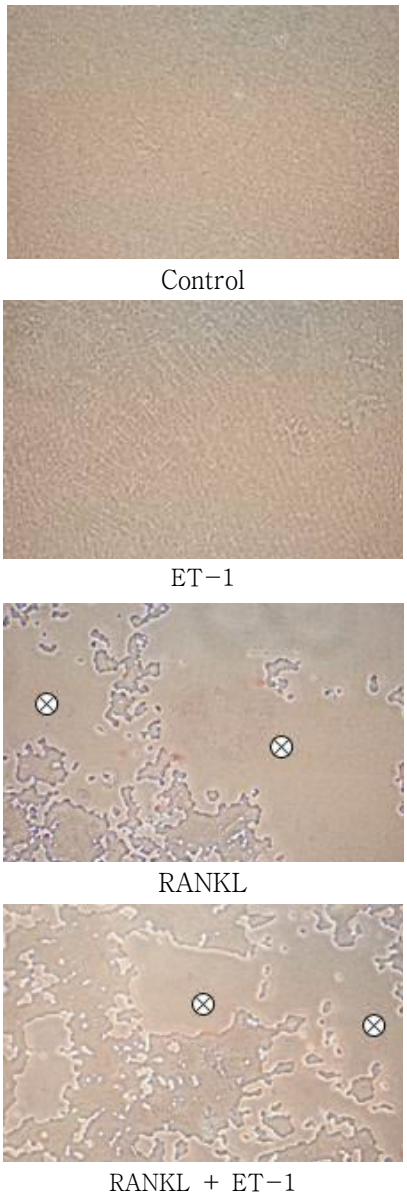



Fig. 7. Resorption pit formation by mature osteoclasts. Mononuclear preosteoclasts were cultured in a calcium phosphate crystal−coated OAAS plate. After treatment with ET−1(10 nM), RANKL (50 ng/ml) or RANKL+ET−1 for 7 days in the presence of M−CSF, cells attached to the plate were washed away and resorbed areas were visualized using light microscopy. Crossed circles indicate resorbed areas. Note that RANKL−induced resorption areas were reduced by treatment with ET−1. (Magnification, 100X.)

expected to occur through the $ET_AR$ because an $ET_AR$ antagonist BQ123moderately reversed an increase in the formation of mineralized matrices. Many factors are involved in the differentiation process, and PGE2 is known as one of the strong mediators to stimulate ALP activity and mineralization [ ], but the mechanisms whereby it stimulates the differentiation process are not fully elucidated. In the present study PGE2 production was greatly increased by ET−1, suggesting that it may mediatethe differentiation process.

Osteoclastogenesis is tightly coupled with the OPG/RANKL/RANK system. Although RANKL is essential for osteoclastogenesis, OPG as a decoy receptor of RANKL neutralizes the action of RANKL. It is believed that the balanced production of OPG and RANKL by osteoblasts is more critical for osteoclastogenesis than changes in each of the two[31]. PTH, PGE2, and glucocorticoids can indirectly stimulate osteoclastogenesis through up−regulation of RANKL and down−regulation of OPG[4−7],whereas estrogen, testosterone, and mechanical stress can indirectly inhibit osteoclastogenesis by decreasing RANKL expression with little effect on OPG expression[8−10]. We demonstrated that ET−1 decreased RANKL mRNA expression by 50%, meanwhile it did not affect OPG mRNA expression (Fig. 5). The ratio of RANKL to OPG gene expression was decreased and was therefore tipped toward the inhibition of osteoclastogenesis.Although ET−1 stimulated PGE2 production in osteoblasts, it appears that this amount of PGE2 had little effect on the change in RANKL/OPG ratio because 0.1 uM PGE2 greatly increased rather than decreased the ratio (Fig. 5).

Most osteoclastogenesis occurs via RANKL−dependent pathways[2, 32]. Our study showed that ET−1 did not directly induce osteoclast formation from preosteoclasts. However, when preosteoclasts were differentiated into osteoclasts in a RANKL−containing medium, ET−1 decreased the size as well as multinuclearity of osteoclasts and reduced TRAP activity and resorption pit formation (Figs. 6 and 7). Therefore, it is clear that ET−1 acts as an

inhibitory factor during RANKL-induced osteoclastogenesis. Although the mechanisms by which ET-1 inhibits RANKL-induced osteoclastogenesis have not beenelucidated, there is a possibility that ET-1 directly inhibits the actions of osteoclasts. Alam et al.[24] demonstrated that ET-1 inhibited bone resorption by a direct effect on cell motility in isolated rat osteoclasts. Another possibility is that PGE2, produced by ET-1, indirectly plays some roles in suppression of osteoclastogenesis. In fact, Mano et al. and Okamoto et al.[33, 34] have reported that PGE2 directly inhibits bone-resorbing activity or cell motility of isolated osteoclasts[33, 34]. The RANKL-RANK interaction is also important in RANKL-induced osteoclastogenesis[2, 32]. In this situation, downstream RANK signaling is more critical for osteoclast differentiation than the RANKL-RANK interaction in as far as the interaction is not blocked[2, 32]. As shown in Fig. 5, ET-1 had little effect on the expression of RANK mRNA in preosteoclasts and thus an apparently reduced RANKL-RANK interaction due to decreased RANKL expression would not have had an influence on osteoclastogenesis.

In conclusion, ET-1 stimulated the proliferation and differentiation of osteoblasts via an $ET_AR$ pathway and decreased the ratio of RANKL/OPG mRNA, thereby indirectly inhibitingosteoclastogenesis. RANKL-induced osteoclast formation and its activity were greatly suppressed by ET-1. These findings demonstrate that ET-1 is involved in both bone formation and bone resorption.

## 국문초록

**In vitro에서 골 형성과 흡수에 대한 endothelin-1의 영향**

**배경**: 조골세포에서 생성되는 receptor activator of nuclear factor-kB ligand (RANKL)과 osteoprotegerin (OPG) 사이의 균형은 파골세포 형성에 매우 중요하다. Enodothelin-1(ET-1)은 강력한 혈관수축제일뿐만 아니라 다양한 세포에서 세포 증식을 촉진시키는 물질이다. 그러나 ET-1의 조골세포의 분화에 대한 영향은 상반될 뿐만 아니라 ET-1이 조골세포의 RANKL과 OPG의 생성을 변화시켜 간접적으로 파골세포 형성에 영향을 주는지 혹은 파골세포의 분화에 직접적으로 영향을 주는지에 대해서는 연구된 바 없다. 따라서 본 연구에서는 ET-1에 의한 조골세포의 분화와 RANKL 및 OPG 유전자 발현이 파골세포 형성에 미치는 영향을 조사하였다.

**재료와 방법**: 조골세포는 생쥐의 두개골로부터 그리고 파골전구세포는 골수로부터 각각 분리하였다. 이들 세포들을 10 nM ET-1이 포함된 α-MEM 배지에서 배양하였다. 유전자의 발현은 real-time RT-PCR을 통해 정량적으로 측정하였다. 조골세포의 분화는 alizarin-red 염색으로, 파골세포의 형성과 활성은 tartrate-resistant acid phosphatase 염색과 골 흡수와(resorption pit)의 크기로 평가하였다.

**결과**: ET-1은 조골세포의 증식과 분화를 촉진시켰으며, 이는 $ET_A$ 수용체를 통해 이루어졌다. 조골세포에서 ET-1은 PGE2 생성을 크게 증가시켰을 뿐만 아니라 RANKL 유전자 발현을 대조군에 비해 50% 감소시켰다. 그러나 OPG 유전자의 발현에 영향을 주지 않았다. 파골전구세포에서 ET-1은 RANK 유전자의 발현에 영향을 주지 않았지만 RANKL에 의해 유도된 파골세포 형성과 활성은 억제시켰다.

**결론**: ET-1은 조골세포의 증식과 분화를 촉진시키고 RANKL mRNA 발현을 감소시켜 파골세포 형성을 간접적으로 억제한다. 또한 RANKL에 의해 유도되는 파골세포 형성과 활성을 직접적으로 억제한다. 이러한 결과로 미루어 ET-1은 골형성은 촉진하고 골흡수는 억제할 것으로 사료된다.

**중심단어**: endothelin-1, osteoprotegerin, receptor activator of nuclear factor-kB ligand, 조골세포, 파골세포, 석회화, 골흡수와,

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