International Journal of Oral Biology, Vol. 30, No. 2, June 30 2005, p. 47~57

International Journal of Oral Biology

Effects of Leptin on Osteoclast Generation and Activity

Seon-Yle Ko*, Sang-Rae Cho, Se-Won Kim[1], and Jung-Keun Kim

Dept. of Oral Biochemistry and [1]Dept. of Dental Pharmacology, Dankook University, Cheonan, Choongnam, Korea.

(Received April 12, 2005 ; Accepted June 8, 2005)

Leptin, the product of the obese gene, is a circulating hormone secreted primarily from adipocytes. Several results suggest that leptin is important mediators of bone metabolism. The present study was undertaken to determine the effects of leptin on anti-osteoclastogenesis using murine precursors cultured on Ca-P coated plates and on the production of osteoprotegerin (OPG) in osteoblastic cells. Additionally, this study examined the possible involvement of prostaglandin E_2 (PGE_2)/protein kinase C (PKC)-mediated signals on the effect of leptin on anti-osteoclastogenesis to various culture systems of osteoclast precursors. Osteoclast generation was determined by counting tartrate-resistant acid phosphatase positive [TRAP (+)] multinucleated cells (MNCs). Osteoclastic activity was determined by measuring area of resorption pits formed by osteoclasts on Ca-P coated plate. The number of 1,25-dihydroxycholecalciferol ($1,25[OH]_2D_3$)- or PGE_2- induced TRAP (+) MNCs in the mouse bone marrow cell culture decreased significantly after treatment with leptin. The number of receptor activator of NF-kB ligand (RANKL)-induced TRAP (+) MNCs in M-CSF dependent bone marrow macrophage (MDBM) cell or RAW264.7 cell culture decreased significantly with leptin treatment. Indomethacin inhibited osteoclast generation induced by $1,25[OH]_2D_3$ and dexamethasone, however, no significant differences were found in the leptin treated group when compared to the corresponding indomethacin group. Phorbol 12-myristate 13-acetate (PMA), a PKC activator, inhibited osteoclast generation induced by $1,25[OH]_2D_3$. The number of TRAP (+) MNCs decreased significantly with treatment by PMA at concentrations of 0.01 and 0.1 μM in culture. Leptin inhibited PMA-mediated osteoclast generation. Isoquinoline-5-sulfonic 2-methyl-1-piperazide dihydrochloride (H7) had no effect on osteoclast generation induced by $1,25[OH]_2D_3$. Cell culture treatment with leptin resulted in no significant differences in osteoclast generation compared to the corresponding H7 group. Indomethacin showed no significant effect on TRAP (+) MNCs formation from the RAW264.7 cell line. PMA inhibited TRAP (+) MNCs formation induced by RANKL in the RAW264.7 cell culture. H7 had no effect on osteoclast generation from the RAW264.7 cell line. There was no difference compared with the corresponding control group after treatment with leptin. $1,25[OH]_2D_3$- or PGE_2-induced osteoclastic activity decreased significantly with leptin treatment at a concentration of 100 ng/ml in mouse bone marrow cell culture. Indomethacin, PMA, and H7 significantly inhibited osteoclastic activity induced by $1,25[OH]_2D_3$ in mouse bone marrow cell culture. No significant differences were found between the leptin treated group and the corresponding control group. The secretion of OPG, a substance known to inhibit osteoclast formation, was detected from the osteoblasts. Treatment by leptin resulted in significant increases in OPG secretion by osteoblastic cells. Taken these results, leptin may be an important regulatory cytokines within the bone marrow microenvironment.

Keywords: Leptin, Osteoclast

Introduction

Bone is highly specialized, dynamic organ that undergoes continuous regeneration. Once the skeleton has reached maturity, regeneration continues in the form of periodic replacement of old bone with new at the same location (Frost, 1973). This process is called remodeling. Bone remodeling is a coupled process in which bone resorption is normally followed by new bone formation. Early in life, the

*Corresponding author: Seon-Yle Ko, Ph.D. Dept. of Oral Biochemistry, School of Dentistry, Dankook University, San 29-1, Anseo-dong, Cheonan, Choongnam, 330-716, Korea. Tel.: +82-41-550-1934, Fax.: +82-41-552-7648, E-mail: syko@dku.edu

rate of bone formation exceeds bone resorption with a net increase in bone mass, while late in life, bone resorption exceeds bone formation with net loss of bone. During some pathological processes, such as in osteoporotic patients, bone remodeling is uncoupled, and bone resorption is not followed by new bone formation. Osteoporosis is one of the most common diseases, occurring predominantly in post-menopausal women and aging men. Low body weight has long been recognised as one of the common clinical association of osteoporosis (Felson *et al.*, 1993). Research performed on mice lacking leptin and the leptin receptor, demonstrated that leptin signaling deficiency also results in increased bone formation and bone density (Ducy *et al.*, 2000). The effect of leptin on bone formation is probably mediated by the hypothalamus because intracerebroventricular infusion of leptin to ob/ob mice causes a decrease in bone density at a dose that does not have any effect on body weight.

Leptin, a 16 kDa non-glycosylated protein that is encoded by the obese (ob) gene and produced in adipocytes, was originally described to be involved in appetite suppression, energy expenditure, and body weight regulation (Huang and Cai, 2000). In addition to its role as a hormonal regulator of body weight, leptin is now implicated as a regulatory molecule in lipid metabolism (Shimabukuro *et al.*, 1997), haematopoeisis (Gainsford *et al.*, 1996), insulin action (Cohen *et al.*, 1996), ovarian function (Barash *et al.*, 1996), reproduction (Considine and Caro, 1999), immune function (Lord *et al.*, 1998), sympathetic activation (Collins *et al.*, 1996), gastrointestinal function (Bado *et al.*, 1998), brain development (Steppan and Swick, 1999) and angiogenesis (Sierra-Honigmann *et al.*, 1998).

Several lines of evidence suggest that leptin also plays a role in bone metabolism. Clinical studies have shown that obesity protects against osteoporosis (Ribot *et al.*, 1994; Albala *et al.*, 1996) that both bone mass (Reid *et al.*, 1992; Felson *et al.*, 1993), and serum leptin (Considine *et al.*, 1996; Solin *et al.*, 1997) are related positively to body fat mass (Pasco *et al.*, 2001). Higher bone density in ob/ob or db/db mice could be result of an increase in osteoblast function, a decrease in osteoclast function, or a combination of the two situation. Ducy *et al.* (2000) conducted experiments to examine these possible etiologies. *In vitro* studies have demonstrated a direct effect by leptin on human marrow stromal cells, stimulating osteoblast differentiation and mineralization of bone matrix (Thomas *et al.*, 1999). In human fetuses that are at 18-35 weeks gestation, a negative correlation between serum leptin and a serum bone resorption marker was shown, suggesting that leptin might inhibit bone resorption and increase bone mass (Ogueh *et al.*, 2000). Additionally, leptin was found to enhance cortical bone formation in obese ob/ob mice (Liu *et al.*, 1997), and increase the number of human bone nodules *in vitro* (Iwaniec *et al.*, 1998). Holloway *et al.*, (2002) reported that leptin inhibits osteoclast generation in cultures of human peripheral blood mononuclear cells and murine spleen cells.

The present study was undertaken to determine the effects of leptin on anti-osteoclastogenesis in a model using murine osteoclast precursors or the RAW264.7 cell line. Hsu *et al.* (1999) recently showed that RAW264.7 cell, mouse myeloid cell line, can be differentiated into osteoclasts with high yield by stimulation with RANKL. Osteoclasts, which are present only in bone, are multinucleated giant cells (Nijweide *et al.*, 1986; Mundy and Roodman, 1987). Mature osteoclasts express intense tartrate-resistant acid phosphatase (TRAP) activity and abundant calcitonin receptors. In bone marrow, mature osteoclasts are found in small numbers in a nonproliferative state because osteoclasts are multinucleated giant cells formed by the fusion of mononuclear cells. The formation of mature osteoclasts is dependent on two chemical mediators : monocyte/macrophage colony-stimulating factor (M-CSF) and a ligand for the receptor activator of NF-kB (RANKL). M-CSF is an integral part of the proliferation and/or differentiation of osteoclast precursors (Kodama *et al.*, 1991). The osteopetrotic *op/op* mice have a severe deficiency of osteoclasts and macrophages. The deficiency is caused by the absence of functional M-CSF, which can be cured by injections of M-CSF (Begg *et al.*, 1993). Several findings have established that RANKL (Anderson *et al.*, 1997), also referred to as osteoprotegerin ligand (OPGL) (Burgess *et al.*, 1999) and TNF-related activation-induced cytokine (TRANCE) (Wong *et al.*, 1997), is a new member of the tumor necrosis factor (TNF) ligand family (Yasuda *et al.*, 1998), and is expressed by osteoblasts/stromal cells as a membrane associated protein (Takahashi *et al.*, 1999; Suda *et al.*, 1999). RANKL-deficient mice showed severe osteopetrosis and completely lacked osteoclasts (Kong *et al.*, 1999). Therefore, both M-CSF and RANKL are essential factors for osteoclast differentiation. Osteoprotegerin (OPG), is a soluble decoy receptor for RANKL, which is produced by many cell types, including osteoblastic cells, that results in the inhibition of osteoclastogenesis *in vivo* and *in vitro* (Udagawa *et al.*, 2000). Osteoclasts differentiate from hemopoietic precursors of the monocyte/macrophage lineage (Chambers, 1992; Suda *et al.*, 1992; Roodman, 1999). Leptin activates cells of this lineage (Santos-Alvarez *et al.*, 1999).

Taken together, these results suggest that leptin is an important mediator of bone metabolism. The present study was undertaken to determine the effects of leptin on anti-osteoclastogenesis using murine osteoclast precursors cultured on calcium-phosphate coated plates and on the production of OPG in osteoblastic cells *in vitro*. Additionally, this study examined the possible involvement of PGE_2/PKC-mediated signals on the effect of leptin on anti-osteoclastogenesis to various culture systems of osteoclast precursors.

Materials and Methods

Animals and Reagents

Six- to 8-week old male ICR mice were used. Media and fetal bovine serum (FBS) and other cultural reagents were purchased from Gibco laboratories (Grand Island, NY, USA). Recombinant human macrophage-colony stimulating factor (M-CSF), recombinant human receptor activator of nulear factor kB (RANKL), recombinant human osteoprotegerin (OPG), and recombinant human transforming growth factor-β, (TGF-β) were purchased from PeproTech EC Ltd (London, UK). Recombinant human leptin and 1,25-dihydroxycholecalciferol ($1,25[OH]_2D_3$) were purchased from Biomol (PA, USA). Dexamethasone, Prostaglandin E_2 (PGE_2), indomethacin, phorbol 12-myristate 13-acetate (PMA), and isoquinoline-5-sulfonic 2-methyl-1-piperazide dihydrochloride (H7) were purchased from Sigma-Aldrich Co. (MO, USA).

Bone marrow cell culture

Mice were sacrificed by cervical dislocation. Femur and tibia were removed aseptically, freed of adherent soft tissue, and cut across their epiphysis. The marrow cavity was flushed with 1 mL α-minimun essential medium (α-MEM) by slow injection using 26G needle. Collected marrow tissue were mixed by pipetting and marrow cells were filtered with 70 μm nylon mesh (Cell strainer, Falcon, USA) for single cell dispersion.

The filtered cells were collected by centrifugation, and seeded at 4×10^5 cells/well in 96-well plate. Media were changed at the second and fifth day and cultures were maintained up to 7 days in the presence of 10^{-8} M $1,25[OH]_2D_3$, 10^{-7} M dexamethasone and various concentrations of leptin and other mediators with α-MEM/10% FBS.

RAW264.7 cell culture

Mouse monocytic cell line, RAW264.7 cells, were maintained with Dulbecco's modified eagle media (DMEM, Gibco) containing 10% FBS. RAW264.7 cells were cultured in 100 mm culture dish and maintained at CO_2 incubator. When reached confluency, cells were either subcultured at a ratio of 1:10. RAW264.7 cells were plated at 5×10^3 cells/well of 96-well plate with α-MEM /10% FBS. Media were changed at third day and cultures were maintained up to 5 days in the presence of 50 ng/ml RANKL and various concentrations of leptin with α-MEM/10% FBS.

M-CSF dependent bone marrow macrophage (MDBM) cell culture

Mice were sacrificed by cervical dislocation. Femur and tibia were removed aseptically, freed of adherent soft tissue, and cut across their epiphysis. The marrow cavity was flushed with 1 mL enzyme solution containing 0.01% collagenase (type II, Gibco, USA), 0.05% trypsin (Sigma, USA) and 0.5 mM EDTA (Sigma, USA) by sterile needle. Collected marrow tissue was digested with enzyme solution for 30 min. After digestion, released cells were collected and washed with α-MEM and treated with distilled water for 5 second to remove red blood cells. After washing, marrow cells were filtered with 40 μm nylon mesh (Cell strainer, Falcon, USA) for single cell dispersion. Filtered cells were cultured in α-MEM containing 10% FBS, 5 ng/ml M-CSF at 6×10^6 cells in 100 mm culture dish for 24 hrs. After 24-hour culture, nonadherent cells were collected and seeded at 5×10^4 cells/well in α-MEM containing 10% FBS, 10 ng/ml M-CSF and 1 ng/ml TGF-β, in 96-well plate. Media were changed at the third and sixth day and cultures were maintained up to 8-9 days in the presence 10 ng/ml M-CSF, 1 ng/ml TGF-β, 50 ng/ml RANKL, various concentrations of leptin and other mediators with α-MEM/10% FBS.

Enzyme histochemistry for tartrate-resistant acid phosphatase (TRAP)

After culture, adherent cells were rinsed with a phosphate buffered saline (PBS), fixed with citrate-acetone-formaldehyde for 5 minutes, and stained for TRAP by incubating the cells for 1 hour at 37°C in acetate buffer (pH 5, Sigma), containing naphthol AS-BI phosphate and fast Garnet GBC solution, in the presence of 7 mM tartrate buffer (pH 5, Sigma). TRAP-positive cells appeared dark violet. TRAP (+) multinucleated cells (MNCs) containing three or more nuclei were counted as osteoclast-like cells by light microscope.

Measurement of resorption pit

Bone marrow cells or RAW264.7 cells were cultured on the calcium-phosphate coated OAAS™ plate (OCT Inc. Korea) in the presence of leptin. After culture, cells were removed and total resorption area was measured by image analysis program. To measure resorption area, culture media were removed from the culture plate on completion of culture. Cultured cells detached with 5% sodium hypochlorite for 5 mins, and washed again with distilled water. Total resorption pit area was captured by microscope and digital camera. Resorption pit at total well surface were measured by image analyzing program, Image pro plus™ (version 3.0, Media Cybernetics Inc., USA).

Measurement of OPG secretion

Well-characterized osteoblastic cell lines, MG63 cells, were used in this study. MG63 cells were cultured in DMEM containing 10% FBS. Cells were cultured in 75 cm^2 culture flask and maintained at 37°C in a humidified atmosphere of 95% air and 5% CO_2. When they reached confluency, cells were subcultured at a ratio of 1:10 by

suspending cells with trypsin and EDTA solution. The MG63 cells were seeded at 10^4 cells/well in DMEM containing 10% FBS in a 96-well culture plate. At confluency, the medium was changed to 0.1 mL of serum-free DMEM containing leptin. The supernatants were harvested after 24 hrs of culture. Subsequently, the amount of OPG was measured by spectrophotometry using Human OPG/OCIF ELISA kit (OCT Inc. Korea).

Statistical analysis

All data represent the mean values obtained from a minimum of two independent experiments with at least four replicates in each experiment. Data were analyzed using Student's *t* test. A *P* value less than 0.05 was taken as a significant difference between the pairs. The values are presented as mean± S.E.

Results

The goal of the present study was to determine the effects of leptin on anti-osteoclastogenesis using murine precursors cultured on calcium-phosphate coated plates and on the regulation of OPG in osteoblastic cells. Additionally, this study examined the possible involvement between PGE_2/PKC-mediated signals and leptin on anti-osteoclastogenesis in various osteoclast culture systems.

Effects of leptin on the TRAP (+) MNCs formation

Numerous TRAP (+) MNCs were genereated after mouse bone marrow cells were cultured in the presence of 10^{-8} M $1,25[OH]_2D_3$ and 10^{-7} M dexamethasone for 7 days. The number of $1,25[OH]_2D_3$-induced TRAP (+) MNCs in the mouse bone marrow cell culture were decreased significantly after treatment with leptin at concentrations of 1, 10, 100, and 1000 ng/ml (Fig. 1). Numerous TRAP (+) MNCs were generated from mouse bone marrow cells after being cultured for 7 days in the presence of 10^{-6} M PGE_2 and 10^{-7} M dexamethasone. The number of PGE_2-induced TRAP (+) MNCs was decreased significantly with treatment by leptin at 1, 10, 100, and 1000 ng/ml concentrations (Fig. 2).

MDBM cells cultured for 8 days with 10 ng/ml M-CSF, 1 ng/ml TGF-β and 50 ng/ml RANKL generated substantial numbers of TRAP (+) MNCs. The number of RANKL-induced TRAP (+) MNCs was decreased significantly with leptin treatment at 1, 10, 100, and 1000 ng/ml (Fig. 3).

Mouse monocytic cells, RAW264.7 cells, were cultured with RANKL in the presence or absence of leptin for 5 days. After culture, many TRAP (+) MNCs were observed. Fig. 4 shows osteoclast formation in response to leptin in RAW264.7 cells cultured in the presence of RANKL. The number of RANKL-induced TRAP (+) MNCs was decreased significantly when treated with leptin at 100 and 1000 ng/ml.

Indomethacin, a cyclooxygenase inhibitor, added to the bone marrow cell culture, inhibited osteoclast generation induced by $1,25[OH]_2D_3$ and dexamethasone, however, no significant differences were found in the leptin treated group when compared to the corresponding indomethacin group (Fig. 5).

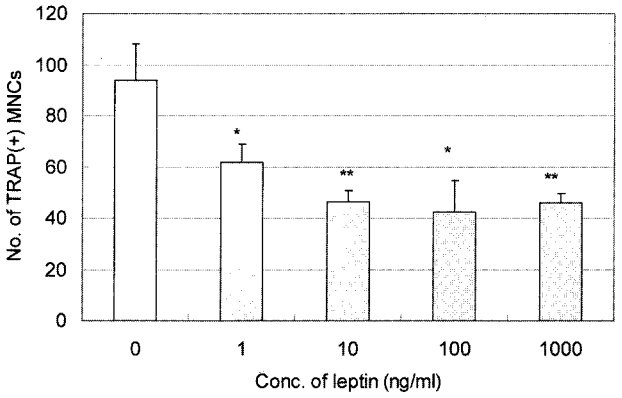

Fig. 1. Effect of leptin on the $1,25[OH]_2D_3$-induced osteoclast generation in the mouse bone marrow cell culture.
The osteoclast precursor cells were plated at a density 4×10^5 cells/well in a 96-well plate, cultured for 7 days in the presence of 10^{-8} M $1,25[OH]_2D_3$, 10^{-7} M dexamethasone and various concentrations of leptin. After culturing, the TRAP (+) multinucleated cells containing three or more nuclei were counted as osteoclasts. The data represent a mean ± S.E. of 5 experiments and are expressed as a ratio to the control.
*: Significantly different from the control, $p<0.05$
**: Significantly different from the control, $p<0.01$

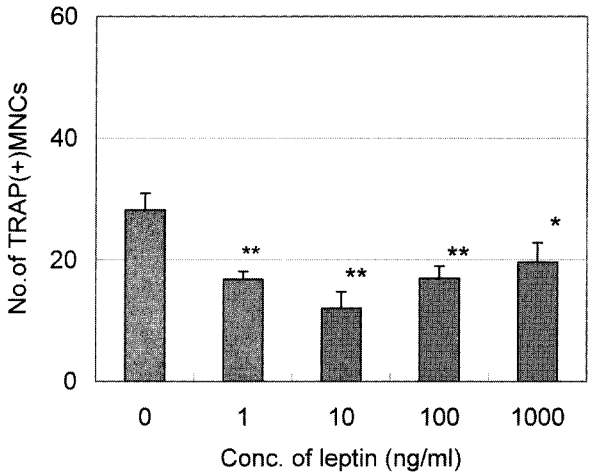

Fig. 2. Effect of leptin on the PGE_2-induced osteoclast generation in the mouse bone marrow cell culture.
The osteoclast precursor cells were plated at a density 4×10^5 cells/well in a 96-well plate, cultured for 7 days in the presence of 10^{-6} M PGE_2, 10^{-7} M dexamethasone and various concentrations of leptin. After culturing, the TRAP (+) multinucleated cells containing three or more nuclei were counted as osteoclasts. The data represent a mean ± S.E. of 5 experiments and are expressed as a ratio to the control.
*: Significantly different from the control, $p<0.05$
**: Significantly different from the control, $p<0.01$

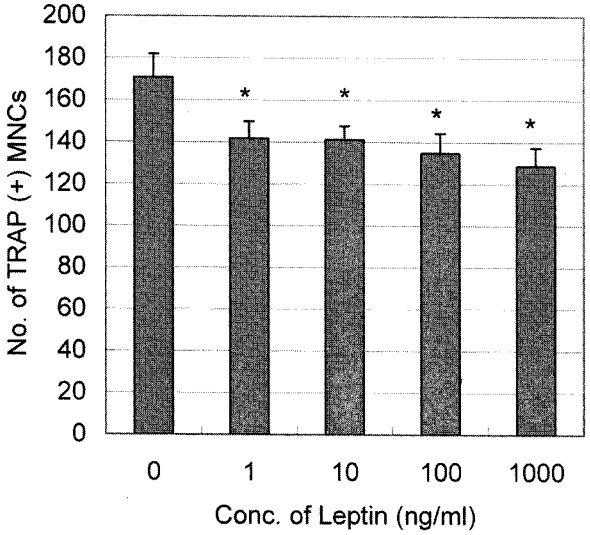

Fig. 3. Effect of leptin on the RANKL-induced osteoclast generation in the MDBM cell culture.
MDBM cells were plated at a density 5×10^4 cells/well in a 96-well plate, cultured for 8 days in the presence of 10 ng/ml M-CSF, 30 ng/ml RANKL and various concentrations of leptin. After culturing, the TRAP (+) multinucleated cells containing three or more nuclei were counted as osteoclasts. The data represent a mean ± S.E. of 5 experiments and are expressed as a ratio to the control.
* : Significantly different from the control, $p<0.05$

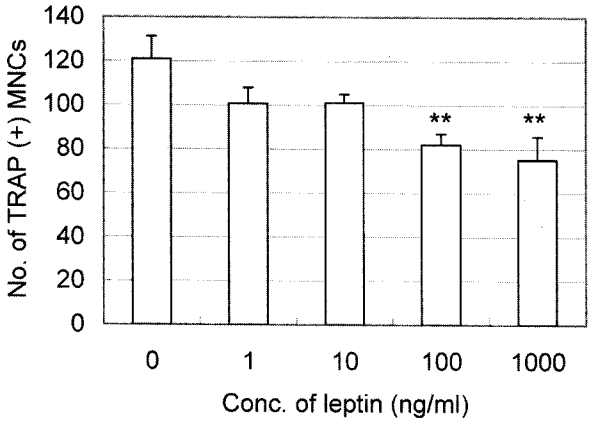

Fig. 4. Effect of leptin on the RANKL-induced osteoclast generation in the RAW264.7 cell culture.
RAW264.7 cells were plated at a density 5×10^3 cells/well in a 96-well plate, cultured for 5 days in the presence of 30 ng/ml RANKL and various concentrations of leptin. After culturing, the TRAP (+) multinucleated cells containing three or more nuclei were counted as osteoclasts. The data represent a mean ± S.E. of 5 experiments and are expressed as a ratio to the control.
** : Significantly different from the control, $p<0.01$

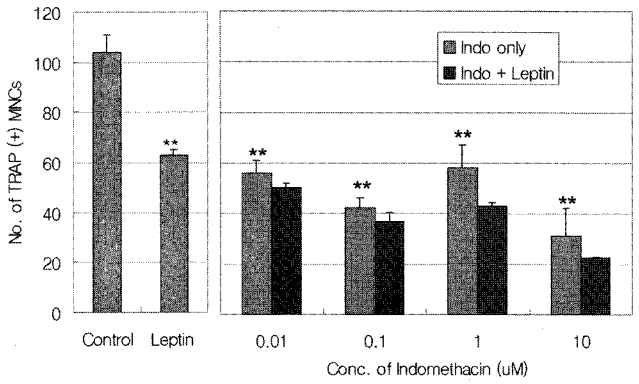

Fig. 5. Effect of leptin on the PGE_2-mediated osteoclast generation in the mouse bone marrow cell culture.
The osteoclast precursor cells were plated at a density 4×10^5 cells/well in a 96-well plate, cultured for 7 days in the presence of 10^{-8} M $1,25[OH]_2D_3$, 10^{-7} M dexamethasone and various concentrations of indomethacin with or without 100 ng/ml leptin. After culturing, the TRAP (+) multinucleated cells containing three or more nuclei were counted as osteoclasts. The data represent a mean ± S.E. of 5 experiments and are expressed as a ratio to the control. Indo : indomethacin, Leptin : 100 ng/ml leptin
** : Significantly different from the control, $p<0.01$

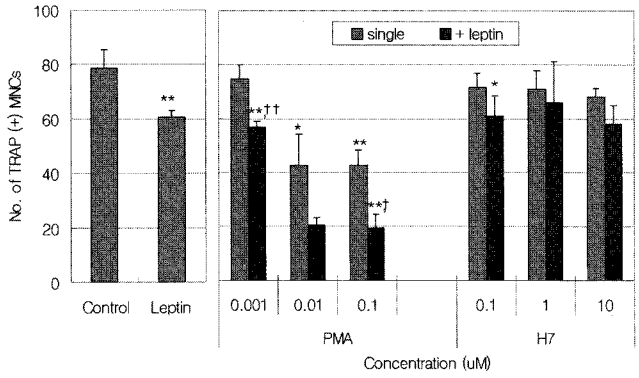

Fig. 6. Effect of leptin on the PKC-mediated osteoclast generation in the mouse bone marrow cell culture.
The osteoclast precursor cells were plated at a density 4×10^5 cells/well in a 96-well plate, cultured for 7 days in the presence of 10^{-8} M $1,25[OH]_2D_3$, 10^{-7} M dexamethasone and various concentrations of PMA or H7 with or without 100 ng/ml leptin. After culturing, the TRAP (+) multinucleated cells containing three or more nuclei were counted as osteoclasts. The data represent a mean ± S.E. of 5 experiments and are expressed as a ratio to the control. Leptin : 100 ng/ml leptin
* : Significantly different from the control, $p<0.05$
** : Significantly different from the control, $p<0.01$
†: Significantly different from the corresponding single group, $p<0.05$
††: Significantly different from the corresponding single group, $p<0.01$

PMA, a PKC activator, added to the bone marrow cell culture inhibited osteoclast generation induced by 1,25 $[OH]_2D_3$ and dexamethasone. The number of TRAP (+) MNCs was decreased significantly with treatment by PMA at concentrations of 0.01 and 0.1 μM in culture. Leptin inhibited PMA-mediated osteoclast generation (Fig. 6).

H7, a PKC inhibitor, was added to the bone marrow cell culture, and it had no effect on osteoclast generation induced by $1,25[OH]_2D_3$ and dexamethasone. Cell culture treatment with leptin resulted in no significant differences in osteoclast

gereration compared to the corresponding H7 group (Fig. 6).

Indomethacin showed no significant effect on TRAP (+) MNCs formation from the RAW264.7 cell line. PMA inhibited TRAP (+) MNCs formation induced by RANKL in the RAW264.7 cell culture. H7 had no effect on osteoclast generation from the RAW264.7 cell line. There was no difference compared with the corresponding control group after treatment with leptin (Fig. 7).

Effects of leptin on the osteoclastic activity

Extensive resorption of calcium-phosphate crystals was observed after culture. $1,25[OH]_2D_3$-induced osteoclastic activity decreased significantly with leptin treatment at a concentration of 100 ng/ml in mouse bone marrow cell culture (Fig. 8). PGE_2-induced osteoclastic activity decreased significantly with leptin treatment at concentrations of 100 and 1000 ng/ml in mouse bone marrow cell culture (Fig. 9).

Indomethacin, PMA, and H7 significantly inhibited osteoclastic activity induced by $1,25[OH]_2D_3$ in mouse bone marrow cell culture. No significant differences were found between the leptin treated group and the corresponding control group (Fig. 10).

RANKL-induced osteoclastic activity decreased with leptin treatment in the RAW264.7 cell line culture (Fig. 11). Indomethacin showed no effect on the osteoclastic activity in the RAW264.7 cell. PMA showed an inhibitory effect, while H7 had no effect on osteoclastic activity induced by RANKL in the RAW264.7 cell culture. No significant differences were found between the leptin treated and corresponding control groups (Fig. 12).

Effect of leptin on the OPG production

The secretion of OPG, a substance known to inhibit osteoclast formation, was detected from the osteoblasts using ELISA analysis. Treatment by leptin resulted in significant increases in OPG secretion by osteoblastic cells (Fig. 13). A 126 % increase in the secretion level of OPG protein was observed at a leptin concentration of 100 ng/ml (Fig. 13).

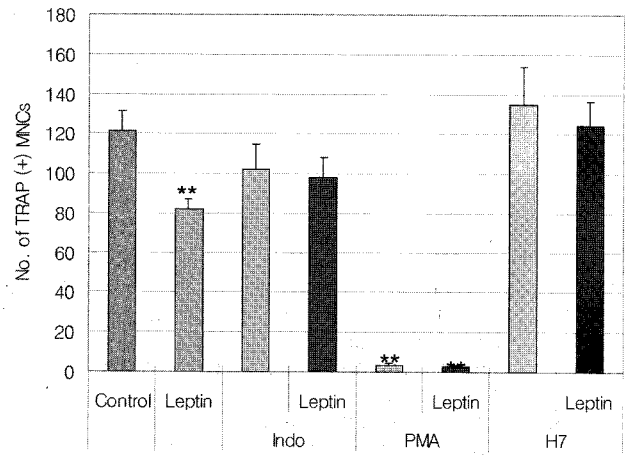

Fig. 7. Effect of leptin on the PGE_2/PKC-mediated osteoclast generation in the RAW264.7 cell culture.
RAW264.7 cells were plated at a density 5×10^3 cells/well in a 96-well plate, cultured for 5 days in the presence of 30 ng/ml RANKL and various treatment substances. After culturing, the TRAP (+) multinucleated cells containing three or more nuclei were counted as osteoclasts. The data represent a mean ± S.E. of 5 experiments and are expressed as a ratio to the control. Leptin : 100 ng/ml leptin, Indo : 10^{-5} M indomethacin, PMA : 10^{-7} M PMA, H7 : 10^{-5} M H7
**: Significantly different from the control, $p<0.01$

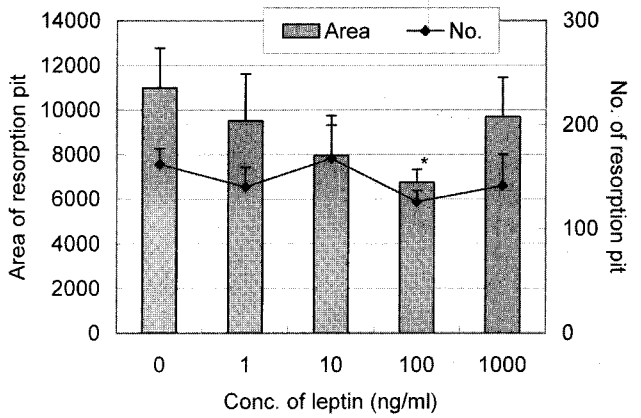

Fig. 8. Effect of leptin on the $1,25[OH]_2D_3$-induced osteoclast activity in the mouse bone marrow cell culture.
The osteoclast precursor cells were plated at a density 4×10^5 cells/well in a 96-well plate, cultured for 7 days in the presence of 10^{-8} M $1,25[OH]_2D_3$, 10^{-7} M dexamethasone and various concentrations of leptin. After culturing, the total area of the resorption pit was measured. The data represent a mean ± S.E. of 5 experiments and are expressed as a ratio to the control.
*: Significantly different from the control, $p<0.05$

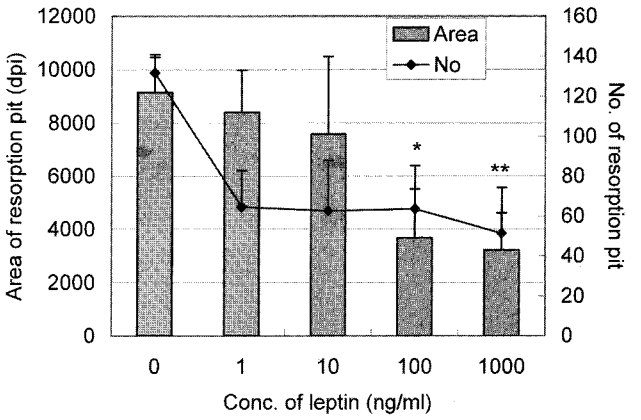

Fig. 9. Effect of leptin on the PGE_2-induced osteoclast activity in the mouse bone marrow cell culture.
The osteoclast precursor cells were plated at a density 4×10^5 cells/well in a 96-well plate, cultured for 7 days in the presence of 10^{-6} M PGE_2, 10^{-7} M dexamethasone and various concentrations of leptin. After culturing, the total area of the resorption pit was measured. The data represent a mean ± S.E. of 5 experiments and are expressed as a ratio to the control.
*: Significantly different from the control, $p<0.05$
**: Significantly different from the control, $p<0.01$

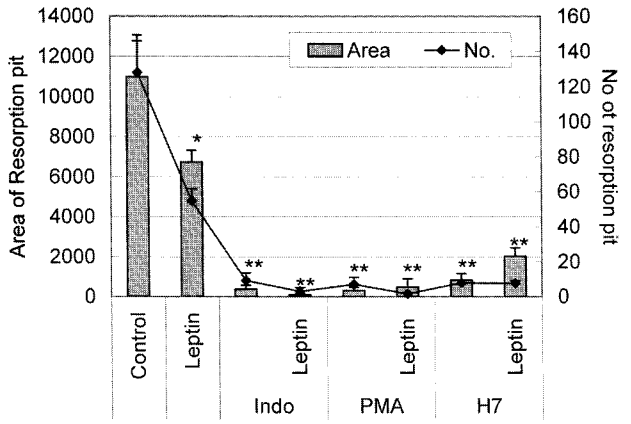

Fig. 10. Effect of leptin on the PGE_2/PKC-mediated osteoclast activity in the mouse bone marrow cell culture.
The osteoclast precursor cells were plated at a density 4×10^5 cells/well in a 96-well plate, cultured for 7 days in the presence of 10^{-8} M $1,25[OH]_2D_3$, 10^{-7} M dexamethasone and various treatment substances. After culturing, the total area of the resorption pit was measured. The data represent a mean °æ S.E. of 5 experiments and are expressed as a ratio to the control. Leptin : 100 ng/ml leptin, Indo : 10^{-5} M indomethacin, PMA : 10^{-7} M PMA, H7 : 10^{-5} M H7
*: Significantly different from the control, $p<0.05$
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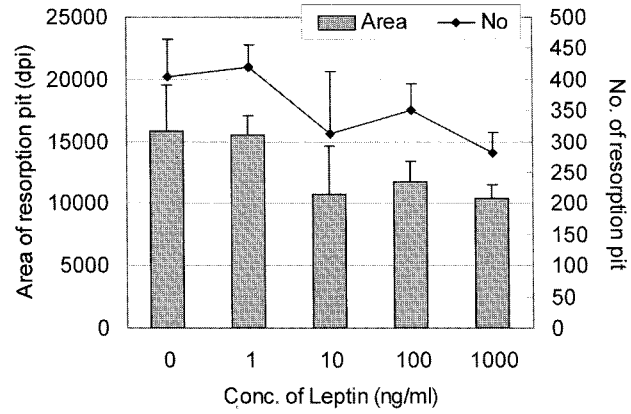

Fig. 11. Effect of leptin on the RANKL-induced osteoclast activity in the RAW264.7 cell culture.
RAW264.7 cells were plated at a density 5×10^3 cells/well in a 96-well plate, cultured for 5 days in the presence of 30 ng/ml RANKL and various concentrations of leptin. After culturing, the total area of the resorption pit was measured. The data represent a mean ± S.E. of 5 experiments and are expressed as a ratio to the control.
**: Significantly different from the control, $p<0.01$

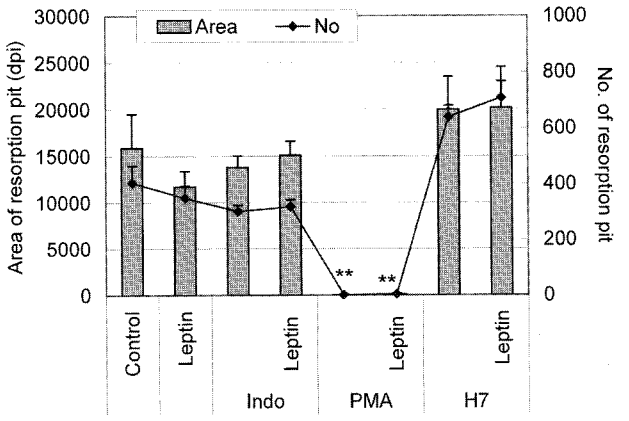

Fig. 12. Effect of leptin on the PGE_2/PKC-mediated osteoclast activity in the RAW264.7 cell culture.
RAW264.7 cells were plated at a density 5×10^3 cells/well in a 96-well plate, cultured for 5 days in the presence of 30 ng/ml RANKL and various treatment substances. After culturing, the total area of the resorption pit was measured. The data represent a mean °æ S.E. of 5 experiments and are expressed as a ratio to the control. Leptin : 100 ng/ml leptin, Indo : 10^{-5} M indomethacin, PMA : 10^{-7} M PMA, H7 : 10^{-5} M H7
**: Significantly different from the control, $p<0.01$

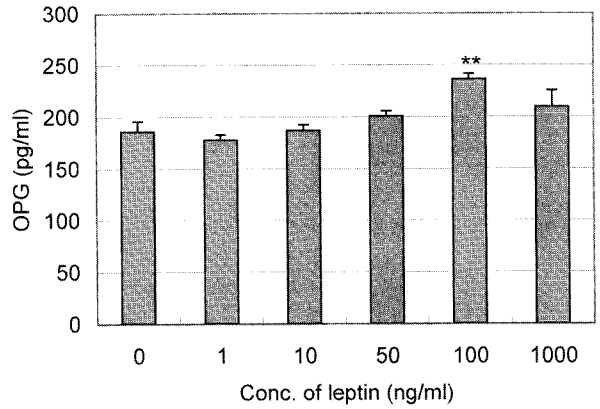

Fig. 13. Effect of leptin on the OPG production on osteoblasts.
The MG63 cells were treated with various concentrations of leptin in serum-free medium. The supernatants were harvested after 24 hrs of culture. OPG amount were measured by spectrophotometry using Human OPG/OCIF ELISA kit.
**: Significantly different from the control, $p<0.01$

Discussion

Osteoporosis is a bone disease that manifests in postmenopausal women and the elderly and is characterized by a shift in bone balance toward resorption (Eastel, 1999). The result of this imbalance is low bone density and a higher risk of fracture. In several studies obesity is associated with a lower risk of osteoporosis (Ravn *et al.*, 1999). A protective effect of overweight/obesity on BMD has been ascribed to high body mass promoting increased mechanical load on weight-bearing bones. In postmenopausal women, the main source of estrogen is produced via the conversion of androstenedione to estrone in adipose tissue. Consequently, lean women have less capacity to produce estrogen (Hoover *et al.*, 1996).

A distinct explanation for the association between body mass and osteoporosis may be related to the plasma protein leptin originating from the obese gene (Klein *et al.*, 1996). It has been shown in the natural leptin knockout model (ob/ob

mice) that leptin administration increases BMD, as well as limb length (Steppan *et al.*, 2000). Recently, it was shown that leptin induced differentiation of human bone marrow stromal cells into osteoblasts, while impeding the maturation of adipocytes (Thomas *et al.*, 1999). Gordeladze *et al.* (2002) reported that leptin modulates osteoblastic cell function, by acting in different stages of osteoblast recruitment and maturation, possibly by binding to differently expressed receptor isoforms. Other reports assert that leptin inhibits bone formation through a central nervous regulatory loop (Anselme *et al.*, 2000; Fleet, 2000), claiming that there is no leptin signaling in osteoblastic cells. Expression of leptin messenger RNA has been associated primarily with adipoytes (Stein *et al.*, 1996), but it also has been found in human placental syncytiotrophoblasts (Tartaglia, 1997), gastric epithelium (Laharrague *et al.*, 1998), activated rat hepatic stellate cells (Lengauer *et al.*, 1998), and activated murine muscle cells (Matkovic *et al.*, 1997). Moreover, it also has been found that leptin and the leptin receptor are expressed in murine fetal cartilage and bone (Klein *et al.*, 1998). These results indicate that leptin may act via autocrine and endocrine mechanism in the regulation of bone metabolism.

The present study was undertaken to determine the effects of leptin on anti-osteoclastogenesis and on the production of OPG in osteoblastic cells *in vitro*. This study examined the possible involvement between PGE_2/PKC-mediated signals and leptin on anti-osteoclastogenesis. Osteoclast generation was measured by examining the number of TRAP (+) multinucleated cells (MNCs) after culturing osteoclast precursor cells on 96-well plates. It is well known that TRAP is a phenotypic marker of osteoclasts (Minkin, 1992).

Numerous TRAP (+) MNCs were generated after mouse bone marrow cells were cultured in the presence of 10^{-8} M $1,25[OH]_2D_3$ or 10^{-6} M PGE_2 for 7 days. Maximum leptin concentrations used in this study are rather high (1 μg/ml) in comparison with physiological levels. Nonetheless, this range of concentrations is quite common in other *in vitro* studies (Ranganathan *et al.*, 1998; Thomas *et al.*, 1999), probably because of the fact that part of the leptin activity is blocked by the serum component of the medium used in *in vitro* studies. The number of $1,25[OH]_2D_3$- or PGE_2-induced TRAP (+) MNCs in the mouse bone marrow cell culture decreased significantly after treatment with leptin at concentrations of 1, 10, 100, and 1000 ng/ml (Fig. 1, 2). These results suggested that leptin is anti-osteoclastogenesis factor. Consistent with these observations are the results of other investigators. Cornish *et al.* (2002) have shown that leptin is weak anti-osteoclastogenesis factors. Maor *et al.* (2002) reported that leptin apparently acts as a skeletal growth factor with a direct peripheral effect on the skeletal growth centers. These results were in accordance with the report of Holloway *et al.* (2002) which showed that leptin inhibited osteoclastogenesis in culture of peripheral blood mononuclear cells.

The number of RANKL-induced TRAP (+) MNCs decreased significantly with leptin treatment at 1, 10, 100, and 1000 ng/ml in culture of MDBM cells (Fig. 3). The number of RANKL-induced TRAP (+) MNCs decreased significantly with leptin treatment at 100 and 1000 ng/ml in culture of RAW264.7 cells (Fig. 4). Results from the observation suggested that leptin acted directly on osteoclast precursors.

To examine the possible involvement of PGE_2/PKC-mediated signals on the effect of leptin on anti-osteoclastogenesis, indomethacin, cyclooxygenase inhibitor, was added to the bone marrow cell culture. Indomethacin inhibited osteoclast generation induced by $1,25[OH]_2D_3$ and dexamethasone, however, no significant differences were found in the leptin treated group when compared to the corresponding indomethacin group (Fig. 5). These results showed that PGE_2 was not used as mediator in this process. Phorbol 12-myristate 13-acetate (PMA), a PKC activator, added to the bone marrow cell culture inhibited osteoclast generation induced by $1,25[OH]_2D_3$ and dexamethasone. The number of TRAP (+) MNCs decreased significantly with treatment by PMA at concentrations of 0.01 and 0.1 μM in culture. Leptin inhibited PMA-mediated osteoclast generation (Fig. 6). These results are consistent with the report that PMA significantly inhibited PTH-induced TRAP (+) MNCs formation in MS1/spleen cell cocultures (Kondo *et al.*, 2002). Mooga and Dempster (1998) reported that PKC, an enzyme inhibitor (PKC-I), had no effect by itself on osteoclastic bone resorption. PMA, a PKC activator, caused a dose-responsive inhibition of bone resorption. However, these results were different with the report of Liu *et al.* (1998). They showed that PKA activator 8-br and the PKC activator TPA each stimulated TRAP(+) MNCs formation in a dose-dependent manner in MS1/spleen cell coculture. Takekoshi *et al* (2001) reported that leptin stimulates catecholamine synthesis in a PKC-dependent manner in cultured porcine adrenal medullary chromaffin cells. These results showed that PKC was used as mediator in leptin process.

Isoquinoline-5-sulfonic 2-methyl-1-piperazide dihydrochloride (H7), a PKC inhibitor had no effect on osteoclast generation induced by $1,25[OH]_2D_3$ and dexamethasone. Cell culture treatment with leptin resulted in no significant differences in osteoclast gereration compared to the corresponding H7 group (Fig. 6).

Extensive resorption of calcium-phosphate crystals was observed after culture. $1,25[OH]_2D_3$-induced osteoclastic activity decreased significantly with leptin treatment at a concentration of 100 ng/ml in mouse bone marrow cell culture (Fig. 8). PGE_2-induced osteoclastic activity decreased significantly with leptin treatment at concent-rations of 100 and 1000 ng/ml in mouse bone marrow cell culture (Fig. 9). In contrast to the results here, Cornish *et al.* (2002) reported that leptin had no effect in two models of mature osteoclast activity. Pit formation on bovine bone by isolated mature rat

osteoclasts was unaffected by leptin. These results seem to be from the fact that osteoclast precursors cells were used in the culture system examined in this experiment.

To confirm that leptin could act indirectly on RANKL, we analyzed the effect of OPG. OPG is an osteoblast-secreted decoy receptor that inhibit osteoclast differentiation and activation. OPG was investigated as a candidate mediator of the leptin effect because of its known potent inhibitory effect on osteoclastogenesis (Takahashi *et al.*, 1999). We found that OPG expression was stimulated by leptin in osteoblastic cells. Results from the observation suggested that leptin acted indirectly on osteoclast precursors.

Taken these results, leptin may be an important regulatory cytokines within the bone marrow microenvironment. Those data suggested that leptin acts both directly and indirectly on osteoclast precursors. In addition, leptin's dual local role of reducing resorption while increasing osteoblast differentiation (Gordeladze *et al.*, 2002) suggest that it may have an important bone-conserving function. The results of this experiment suggest that a possible signaling pathway in which leptin plays a significant role, is in the regulation of the expression of OPG, but the precise molecular mechanisms associated with the anti-osteoclastogenesis effects of leptin have yet to be defined. The possibility needs to be explored further whether pharmacological manipulation of the leptin pathway may be a novel approach to treat osteoporosis.

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