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Regucalcin Stimulates Osteoclast-Like Cell Formation in Mouse Marrow Cultures

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Abstract The effect of regucalcin, a regulatory protein in intracellular signaling, on osteoclastic cell formation in mouse bone marrow culture is investigated. The bone marrow cells were cultured for 7 days in an α-minimal essential medium containing either vehicle or regucalcin (10^{-10}–10^{-8}M). Osteoclast-like cell formation was estimated by staining for tartrate-resistant acid phosphatase (TRACP), a marker enzyme of osteoclasts. The presence of regucalcin (10^{-10}–10^{-8}M) caused a remarkable increase in osteoclast-like multinucleated cells (MNCs). The effect of regucalcin in stimulating osteoclast-like cell formation was significantly inhibited in the presence of calcitonin (CT; 10^{-9}M), 17β-estradiol (10^{-9}M), β-cryptoxanthin (CX; 10^{-6}M), or zinc sulfate (10^{-4}M), which is an anti-bone resorbing factor. The effect of regucalcin on osteoclast-like cell formation was not significantly blocked in the presence of cycloheximide, an inhibitor of protein synthesis, or 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB), an inhibitor of transcriptional activity. The effect of parathyroid hormone (10^{-7}M), 1,25-dihydroxyvitamin D_3 (10^{-7}M), prostaglandin E_2 (10^{-5}M), or tumor necrosis factor-α (10 ng/ml) in increasing osteoclast-like cell formation was significantly enhanced in the presence of regucalcin (10^{-8}M). Moreover, when rat femoral-diaphyseal or -metaphyseal tissues were cultured for 48 h in the presence of regucalcin (10^{-10}–10^{-8}M), the diaphyseal or metaphyseal calcium content was significantly decreased in the presence of regucalcin (10^{-10}–10^{-8}M) in vitro. The consumption of glucose and the production of lactic acid in culture medium by the diaphyseal or metaphyseal tissues was significantly raised in the presence of regucalcin (10^{-10}–10^{-8}M). This study demonstrates that regucalcin directly stimulates osteoclast-like cell formation in mouse marrow culture in vitro, and that the protein stimulates bone resorption in rat femoral tissues in vitro. J. Cell. Biochem. 94: 794–803, 2005.

Key words: regucalcin; osteoclastogenesis; bone resorption; RANKL; parathyroid hormone

Regucalcin was discovered in 1978 as a calcium-binding protein that does not contain EF-hand motif of Ca^{2+}-binding domain [Yamaguchi and Yamamoto, 1978; Yamaguchi, 1988; Shimokawa and Yamaguchi, 1993b]. The name regucalcin was proposed for this Ca^{2+}-binding protein, which may regulate Ca^{2+} effect on liver cell function [Yamaguchi and Mori, 1988; Yamaguchi, 1992]. In recent years, regucalcin has been demonstrated to play a multifunctional role as a regulatory protein in intracellular signaling process in many cells [reviewed in Yamaguchi, 2000a,b].

The gene of regucalcin is highly conserved in vertebrate species [Misawa and Yamaguchi, 2000]. The regucalcin gene is localized on chromosome X [Shimokawa et al., 1995; Bhattacharya et al., 2002]. AP1 and NFI-A1 have been found to be transcriptional factors for the enhancement of regucalcin gene promoter activity [Murata and Yamaguchi, 1998; Misawa and Yamaguchi, 2002]. Regucalcin messenger ribonucleic acid (mRNA) and its protein are greatly present in liver and kidney cortex [Shimokawa and Yamaguchi, 1992; Yamaguchi and Isogai, 1993]. The expression of regucalcin mRNA is mediated through Ca^{2+}-signaling mechanism [Shimokawa and Yamaguchi, 1993a; Murata and Yamaguchi, 1999].

Regucalcin has been shown to play a role in the maintenance of intracellular Ca^{2+}

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homeostasis and the inhibitory regulation of various Ca^{2+}-dependent protein kinases, protein phosphates, and nitric oxide synthase [Yamaguchi, 2000a,b; Izumi et al., 2003]. Interestingly, regucalcin is translocated from cytoplasma to nucleus in cells [Tsurusaki et al., 2000], and the protein has been demonstrated to regulate the enhancement of nuclear DNA and RNA syntheses in proliferative cells [Tsurusaki and Yamaguchi, 2002a,b]. Recent studies have shown that overexpression of regucalcin modulates tumor-related gene expression [Tsurusaki and Yamaguchi, 2003], and that it has a suppressive effect on proliferation of the cloned rat hepatoma H4-II-E cells [Misawa et al., 2002]. Moreover, endogenous regucalcin has a suppressive effect on cell death and apoptosis induced by stimulation of various signaling factors in the cloned rat hepatoma H4-II-E cells [Izumi and Yamaguchi, 2004]. Regucalcin may play a physiologic role in the maintenance of homeostasis of cellular response for cell stimulation.

Regucalcin transgenic rats is generated to determine a regulatory role of endogenous regucalcin in vivo using a DNA fragment containing the regucalcin/pCXN2 [Yamaguchi et al., 2002a]. Bone loss has been found to induce in regucalcin transgenic rat, suggesting that the protein plays a role in the regulation of bone metabolism [Yamaguchi et al., 2002b]. Moreover, regucalcin is expressed in rat bone marrow cells [Yamaguchi et al., 2004], and the osteoclastogenesis in bone marrow cells isolated from the transgenic rats is stimulated [Uchiyama and Yamaguchi, 2004a]. However, whether exogenous regucalcin has a direct stimulatory effect on osteoclast-like cell formation in bone marrow cells isolated from normal rats is unknown.

This study was undertaken to determine the effect of regucalcin on osteoclast-like cell formation in mouse bone marrow culture in vitro. Moreover, whether the effect of various bone-resorbing factors on osteoclastic cell formation is modulated by regucalcin was investigated to know a role of regucalcin in the regulation of osteoclast formation. We found that regucalcin has a direct stimulatory effect on osteoclast-like cell formation in mouse marrow culture and it can enhance the effect of bone-resorbing factors on osteoclastic cell formation, and that the protein stimulates bone resorption in rat femoral tissues in vitro.

MATERIALS AND METHODS

Chemicals

Dulbecco's modified Eagle's medium (DMEM; high glucose, 4.5 g/dl) and penicillin–streptomycin solution (5,000 U/mg penicillin; 5,000 μg/ml streptomycin solution) were obtained from Gibco Laboratories (Grand Island, NY). α-Modification of Eagle's minimum essential medium (α-MEM) was obtained from Flow Laboratories, Inc. (McLean, VA). Bovine serum albumin (fraction V), fetal bovine serum (FBS), macrophage colony-stimulating factor (M-CSF; mouse), receptor activator of NF-κB ligand (RANKL; mouse), prostaglandin E_2 (PGE_2), 1,25-dihydrocyvitamin D_3 (VD_3), tumor necrosis factor-α (TNFα), parathyroid hormone (1–34) (PTH human), 17β-estradiol (E_2), cycloheximide, and 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB) were obtained from Sigma Chemicals (St. Louis, MO). Synthetic [$Asu^{1,7}$] eel calcitonin (CT) was supplied by Asahi Chemical Industry Co., Ltd. (Shizuoka, Japan). β-Cryptoxanthin (CX) was obtained from Extrasynthese (Lyon-Nord, France). Zinc sulfate and other chemicals were reagent grade from Wako Pure Chemical Industries (Osaka, Japan). All water was glass distilled.

Animals

Male mice (ddY strain; 6 weeks old) and rats (Wistar strain; 4 weeks old) were obtained from Japan SLC (Hamamatsu, Japan). The animals were fed commercial laboratory chow (solid) containing 1.1% phosphorus and 0.012% zinc, and given distilled water. Animals were killed by exsanguinations.

Isolation of Regucalcin

Regucalcin is markedly expressed in rat liver cytosol [Shimokawa and Yamaguchi, 1992; Yamaguchi and Isogai, 1993] from which it was isolated. Regucalcin in the cytosol fraction (the supernatant of 105,000*g*) of rat liver homogenate was purified to electrophoretic homogeneity by gel filtration on Sephadex G-75 and G-50 followed by ion-exchange chromatography on diethylaminoethyl (DEAE)-cellulose, as reported previously [Yamaguchi and Yamamoto, 1978].

Marrow Culture

Bone marrow cells were isolated from mice, as reported elsewhere [Mundy and Roodman,

1987; Takahashi et al., 1998]. Briefly, bone ends of the femur were cut off, and the marrow cavity were flushed with 1 ml of α-MEM. The marrow cells were washed with α-MEM and cultured in the same medium containing 10% heat-inactivated FBS at 1.0×10^7 cells/ml in 24-well plates (0.5 ml/well) in a water-saturated atmosphere containing 5% CO_2 and 95% air at 37°C. The cells were cultured for 3 days; then 0.2 ml of the old medium was replaced with fresh medium and cultures were maintained for an additional 4 days. Various concentrations of regucalcin were added to the culture medium containing either vehicle, PTH (10^{-7}M), PGE_2 (10^{-5}M), VD_3 (10^{-7}M), TNFα (10 ng/ml), or RANKL (10 ng/mg) plus M-CSF (10 ng/ml) with an effective concentration at the beginning of the cultures and at the time of medium change. In separate experiments, the respective media contained either CT, E_2, CX, or zinc sulfate.

Enzyme Histochemistry

After being cultured for 7 days, cells adherent to the 24-well plates were stained for tartrate-resistant acid phosphatase (TRACP), a marker enzyme of osteoclasts [Burstone, 1958; Minkin, 1982]. Briefly, cells were washed with Hanks' balanced salt solution and fixed with 10% neutralized formalin-phosphate (pH 7.2) for 10 min. After the culture dishes were dried, TRACP staining was applied according to the method of Burstone [1958]. The fixed cells were incubated for 12 min at room temperature (25°C) in acetate buffer (pH 5.0) containing naphthol AS-MX phosphate (Sigma) as a substrate and red violet LB salt (Sigma) as a stain for the reaction product, in the presence of 10 mM sodium tartrate. TRACP-positive multinucleated cells (MNCs) containing three or more nuclei were counted as osteoclast-like cells.

Bone Tissue Culture

Femoral-diaphyseal and -metaphyseal tissues from 4-week-old male rats were removed aseptically. The diaphyseal or metaphyseal tissues were cultured in a 35-min dish in 2.0 ml of medium consisting of DMEM (high glucose, 4.5%) supplemented with 0.25% bovine serum albumin (fraction V) plus antibiotics with either regucalcin (10^{-10}–10^{-8}M), bone-resorbing factor (PTH; 10^{-7}M), or vehicle (sterile 0.1% ethanol) [Yamaguchi et al., 1987]. Cultures were maintained at 37°C in a water-saturated atmosphere containing 5% CO_2 and 95% air for 48 h.

Bone Calcium

The bone tissues were dried for 16 h at 120°C, weighed, and then digested with nitric acid at 120°C [Yamaguchi et al., 1987]. Calcium was determined by atomic absorption spectrophotometry. The bone calcium content was expressed as milligram of calcium per gram of dry bone.

Medium Glucose and Lactic Acid

The concentration of glucose in the medium, where bone tissues were cultured for 48 h, was determined by the colorimetric method using *o*-toluidine [Hyvarimen and Nikkila, 1962]. The dry weight of the cultured bone tissue was measured after extraction with 5% trichloroacetic acid, acetone, and diether ether. The medium glucose consumed by bone culture for 48 h was expressed as milligram glucose per gram of dry bone tissue. Likewise, the medium lactic acid was measured by the enzymatic method [Noll, 1965]. Data were expressed as milligram of lactic acid per gram of dry bone tissue.

Statistical Methods

Data are expressed as mean ± SEM. Statistical differences were analyzed using Student's paired *t*-test. Also, we used an ANOVA test to compare between-treatment groups. P values <0.05 were considered to indicate statistically significant differences.

RESULTS

Effect of Regucalcin on Osteoclast-Like Cell Formation

The effect of regucalcin on osteoclast-like MNC formation in the mouse marrow culture is shown in Figure 1. Mouse marrow cells were cultured for 7 days in a medium containing either vehicle or regucalcin (10^{-10}–10^{-8}M). The picture of osteoclast-like cells formed in the presence of either vehicle (none), regucalcin (10^{-8}M), PTH (10^{-8}M), or M-CSF (10 ng/ml) plus RANKL (10 ng/ml) showed the same morphologic patterns (Fig. 1).

The number of TRACP-positive MNCs was significantly increased in the presence of regucalcin (10^{-10}–10^{-8}M) (Fig. 2). TRACP-positive MNCs were not formed appreciably in the

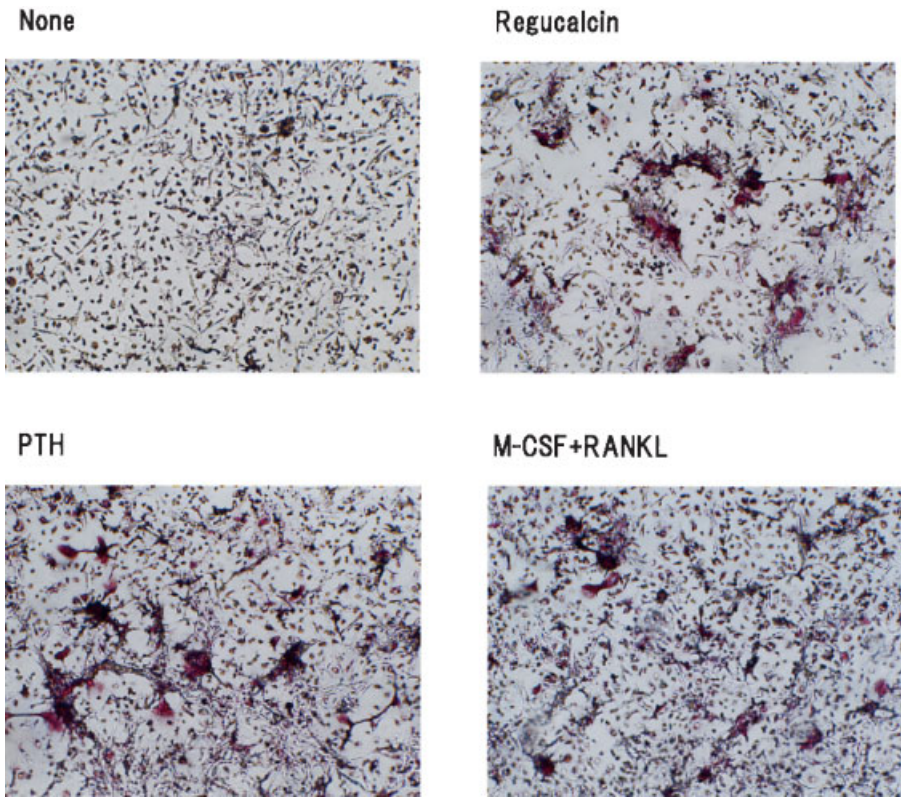

Fig. 1. The formation of osteoclast-like cells in mouse marrow cells cultured in the presence of regucalcin. The marrow cells were cultured for 7 days in a medium containing either vehicle (none), regucalcin (10^{-8}M), PTH (10^{-8}M), or M-CSF (10 ng/ml) plus RANKL (10 ng/ml). Cells were fixed and stained for TRACP.

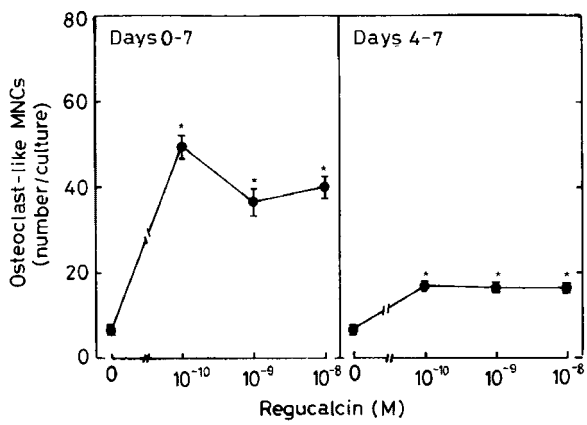

Fig. 2. Effect of regucalcin on osteoclast-like cell formation in mouse marrow culture. Mouse marrow cells were cultured for 7 days in a medium containing either vehicle or regucalcin (10^{-10}–10^{-8}M). In separate experiments, mouse marrow cells were cultured for 3 days in a medium without regucalin addition, and then the medium was changed, and the cells were cultured for an additional 4 days in a medium containing either vehicle or regucalcin (10^{-10}–10^{-8}M). Cells were then fixed and stained for TRACP, and the number of TRACP-positive MNCs was scored. Each value is the mean ± SEM of six cultures. *, $P < 0.01$ compared with the control (none) value.

control culture without regucalcin at any incubation time. Meanwhile, the cells were cultured for 3 days in a medium without regucalcin addition, and then the medium was changed, and the cells were cultured for an additional 4 days in a medium containing either vehicle or regucalcin (10^{-10}–10^{-8}M). The presence of regucalcin (10^{-10}–10^{-8}M) caused a significant increase in TRACP-positive MNCs, although its effect in stimulating osteoclast-like cell formation was weakened.

The effect of regucalcin on osteoclast-like cell formation in the presence of M-CSF in mouse marrow culture is shown in Figure 3. The cells were cultured for 7 days in a medium containing either vehicle or regucalcin (10^{-10}–10^{-8}M) in the presence of M-CSF (10 ng/ml). The presence of regucalcin (10^{-10}–10^{-8}M) caused a significant increase in TRACP-positive MNCs in the presence of M-CSF. The effect of regucalcin (10^{-9} or 10^{-8}M) was weakened when the protein was added at 4 days after culture of marrow cells in the presence of M-CSF (10 ng/ml).

The effect of regucalcin on osteoclast-like cell formation in the presence of both M-CSF and

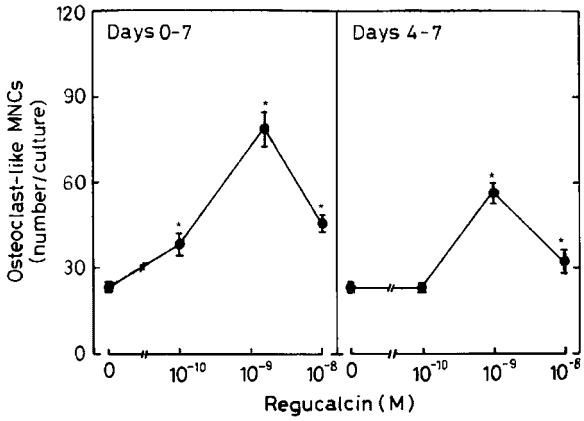

Fig. 3. Effect of regucalcin on osteoclast-like cell formation in the presence of M-CSF in mouse marrow culture. Mouse marrow cells were cultured for 7 days in a medium containing either vehicle or regucalcin (10^{-10}–10^{-8}M) in the presence of M-CSF (10 ng/ml). In separate experiments, mouse marrow cells were cultured for 3 days in a medium without regucalcin addition, and then the medium was changed, and the cells were cultured for an additional 4 days in a medium containing either vehicle or regucalcin (10^{-10}–10^{-8}M) in the presence of M-CSF (10 ng/ml). Cells were then fixed and stained for TRACP, and the number of TRACP-positive MNCs was scored. Each value is the mean ± SEM of six cultures. *, $P < 0.01$ compared with the control (none) value.

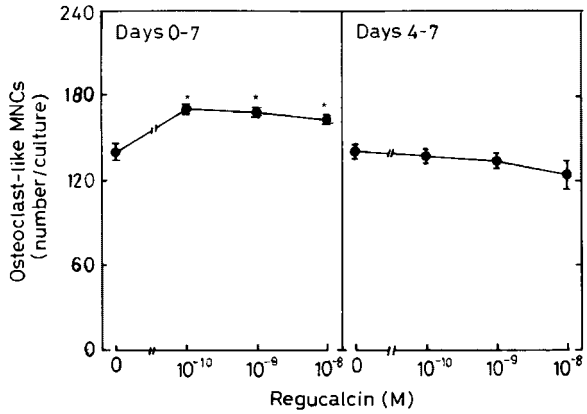

Fig. 4. Effect of regucalcin on osteoclast-like cell formation in the presence of both M-CSF and RANKL in mouse marrow culture. Mouse marrow cells were cultured for 7 days in a medium containing either vehicle or regucalcin (10^{-10}–10^{-8}M) in the presence of M-CSF (10 ng/ml) plus RANKL (10 ng/ml). In separate experiments, mouse marrow cells were cultured for 3 days in a medium containing M-CSF (10 ng/ml) plus RANKL (10 ng/ml) without regucalcin addition, and then the medium was changed, and the cells were cultured for an additional 4 days in a medium containing either vehicle or regucalcin (10^{-10}–10^{-8}M) in the presence of M-CSF (10 ng/ml) plus RANKL (10 ng/ml). Cells were then fixed and stained for TRACP, and the number of TRACP-positive MNCs was scored. Each value is the mean ± SEM of six cultures. *, $P < 0.01$ compared with the control (none) value.

RANKL in mouse marrow culture is shown in Figure 4. Mouse marrow cells were cultured for 7 days in a medium containing either vehicle or regucalcin (10^{-10}–10^{-8}M) in the presence of M-CSF (10 mg/ml) plus RANKL (10 mg/ml). TRACP-positive MNCs were markedly increased in the presence of M-CSF plus RANKL. This increase was significantly enhanced by the addition of regucalcin (10^{-10}–10^{-8}M). Also, TRACP-positive MNCs were significantly increased by the addition of regucalcin at 4 days after culture of marrow cells in the presence of M-CSF (10 mg/ml) plus RANKL (10 mg/ml).

The effect of various factors on regucalcin-increased osteoclastic-like cell formation in mouse marrow culture is shown in Figure 5. Mouse marrow cells were cultured for 7 days in a medium containing either vehicle or regucalcin (10^{-8}M) in the presence or absence of CT (10^{-4}M), E_2 (10^{-9}M), CX (10^{-6}M), or zinc sulfate (10^{-4}M), which is known to inhibit osteoclast-like cell formation. The effect of regucalcin in increasing TRACP-positive MNCs was significantly inhibited in the presence of CT, E_2, CX, or zinc.

The effect of cycloheximide, an inhibitor of protein synthesis, or DRB, an inhibitor of transcriptional activity, on the regucalcin-increased osteoclast-like cell formation in mouse marrow culture is shown in Figure 6. Mouse marrow cells were cultured for 7 days in a medium

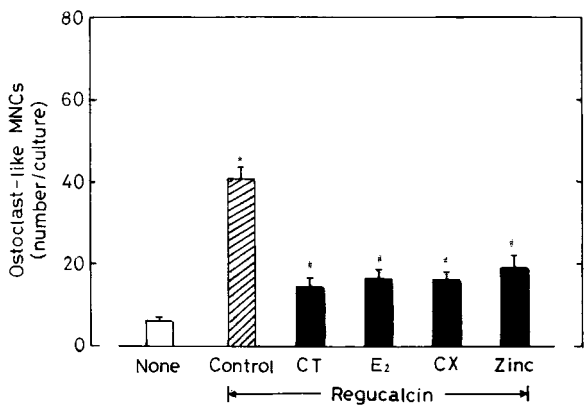

Fig. 5. Effect of various factors on regucalcin-stimulated osteoclast-like cell formation in mouse marrow culture. Mouse marrow cells were cultured for 7 days in a medium containing either vehicle, calcitonin (CT; 10^{-9}M), 17β-estradiol (E_2; 10^{-9}M), β-cryptoxanthin (CX; 10^{-6}M), or zinc sulfate (10^{-4}M) in the absence or presence of regucalcin (10^{-8}M). Cells were then fixed and stained for TRACP, and the number of TRACP-positive MNCs was scored. Each value is the mean ± SEM of six cultures. *, $P < 0.01$ compared with the control (none) value. #, $P < 0.01$ compared with the value obtained from regucalcin alone.

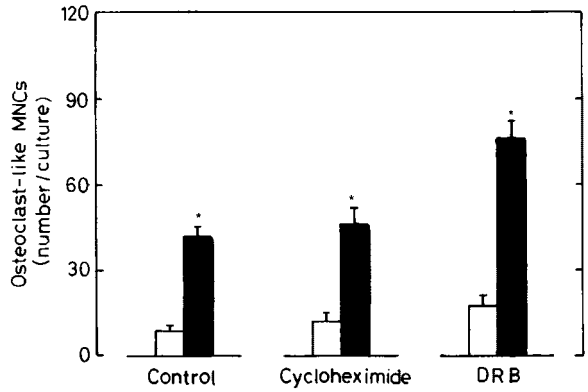

Fig. 6. Effect of cycloheximide or DRB on regucalcin-stimulated osteoclast-like cell formation in mouse marrow culture. Mouse marrow cells were cultured for 7 days in a medium containing either vehicle or regucalcin (10^{-8}M) in the presence or absence of cycloheximide (10^{-7}M) or DRB (10^{-6}M). Cells were then fixed and stained for TRACP, and the number of TRACP-positive MNCs was scored. Each value is the mean ± SEM of six cultures. *, $P < 0.01$ compared with the control (none) value. White bars, none; black bars, regucalcin.

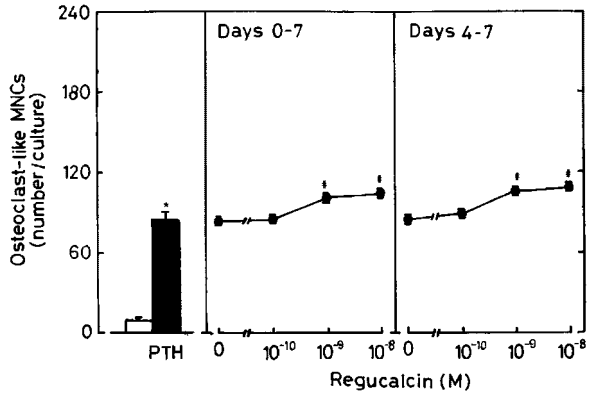

Fig. 7. Effect of regucalcin on PTH-stimulated osteoclast-like cell formation in mouse culture. Mouse marrow cells were cultured for 7 days in a medium containing either vehicle or PTH (10^{-7}M) in the presence or absence of regucalcin (10^{-10}–10^{-8}M). In separate experiments, mouse marrow cells were cultured for 3 days in a medium containing PTH (10^{-7}M) without regucalcin addition, and then the medium was changed, and the cells were cultured for an additional 4 days in a medium containing either vehicle or regucalcin (10^{-10}–10^{-8}M) in the presence of PTH (10^{-7}M). Cells were then fixed and stained for TRACP, and the number of TRACP-positive MNCs was scored. Each value is the mean ± SEM of six cultures. *, $P < 0.01$ compared with the control (none) value. #, $P < 0.01$ compared with the value obtained from PTH alone. White bars, none; black bars, PTH.

containing either vehicle or regucalcin (10^{-8}M) in the presence or absence of cycloheximide (10^{-7}M) or DRB (10^{-6}M). The effect of regucalcin in increasing TRACP-positive MNCs was not significantly inhibited in the presence of cycloheximide or DRB.

Effect of Regucalcin on Various Bone-Resorbing Factors-Increased Osteoclast-Like Cell Formation

Mouse marrow cells were cultured for 7 days in a medium containing either vehicle or bone-resorbing factors in the presence or absence of regucalcin (10^{-10}–10^{-8}M). The effect of PTH (10^{-7}M) (Fig. 7), PGE_2 (10^{-5}M) (Fig. 8), D_3 (10^{-7}M) (Fig. 9), or TNFα (10 ng/ml) (Fig. 10) in increasing TRACP-positive MNCs was significantly enhanced in the presence of regucalcin (10^{-8}M). The effect of PTH or TNFα was significantly enhanced in the presence of regucalcin (10^{-9}M). The enhancing effect of regucalcin (10^{-8}M) on the bone-resorbing factors-increased TRACP-positive MNCs was also seen when the protein was added at 4 days after culture with a medium containing bone-resorbing factors (Figs. 7–10).

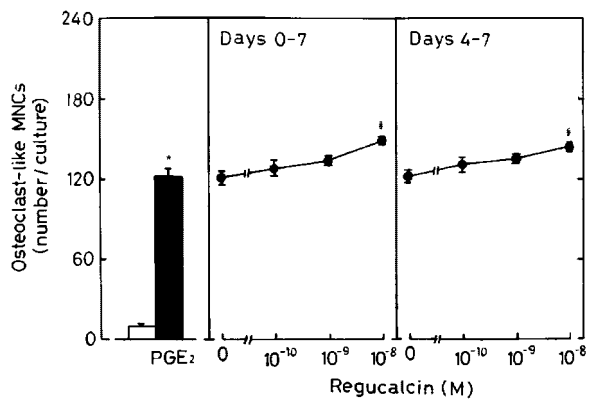

Fig. 8. Effect of regucalcin on PGE_2-stimulated osteoclast-like cell formation in mouse culture. Mouse marrow cells were cultured for 7 days in a medium containing either vehicle or PGE_2 (10^{-5}M) in the presence or absence of regucalcin (10^{-10}–10^{-8}M). In separate experiments, mouse marrow cells were cultured for 3 days in a medium containing PGE_2 (10^{-5}M) without regucalcin addition, and then the medium was changed, and the cells were cultured for an additional 4 days in a medium containing either vehicle or regucalcin (10^{-10}–10^{-8}M) in the presence of PGE_2 (10^{-5}M). Cells were then fixed and stained for TRACP, and the number of TRACP-positive MNCs was scored. Each value is the mean ± SEM of six cultures. *, $P < 0.01$ compared with the control (none) value. #, $P < 0.01$ compared with the value obtained from PGE_2 alone. White bars, none; black bars, PGE_2.

Effect of Regucalcin in Bone Tissue Culture

Rat femoral-diaphyseal and -metaphyseal tissues were cultured for 48 h in a medium containing either vehicle or regucalcin (10^{-10}–10^{-8}M) in the presence or absence of PTH (10^{-7}M) (Fig. 11). The presence of regucalcin

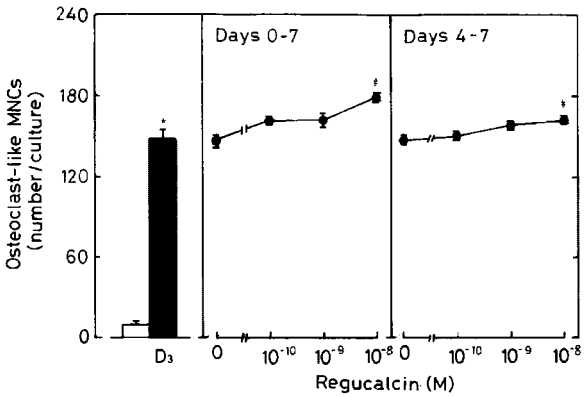

Fig. 9. Effect of regucalcin on 1,25-dihydroxyvitamihn D_3 (D_3)-stimulated osteoclast-like cell formation in mouse culture. Mouse marrow cells were cultured for 7 days in a medium containing either vehicle or D_3 (10^{-7}M) in the presence or absence of regucalcin (10^{-10}–10^{-8}M). In separate experiments, mouse marrow cells were cultured for 3 days in a medium containing D_3 (10^{-7}M) without regucalcin addition, and then the medium was changed, and the cells were cultured for an additional 4 days in a medium containing either vehicle or regucalcin (10^{-10}–10^{-8}M) in the presence of PTH (10^{-7}M). Cells were then fixed and stained for TRACP, and the number of TRACP-positive MNCs was scored. Each value is the mean ± SEM of six cultures. *, $P < 0.01$ compared with the control (none) value. #, $P < 0.01$ compared with the value obtained from D_3 alone. White bars, none; black bars, D_3.

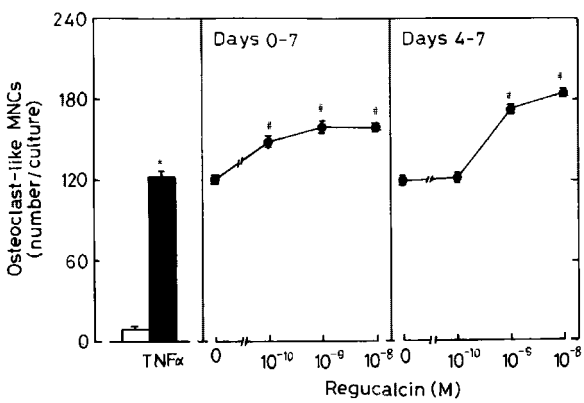

Fig. 10. Effect of regucalcin on TNFα-stimulated osteoclast-like cell formation in mouse culture. Mouse marrow cells were cultured for 7 days in a medium containing either vehicle or TNFα (10 ng/ml) in the presence or absence of regucalcin (10^{-10}–10^{-8}M). In separate experiments, mouse marrow cells were cultured for 3 days in a medium containing TNFα (10 ng/ml) without regucalcin addition, and then the medium was changed, and the cells were cultured for an additional 4 days in a medium containing either vehicle or regucalcin (10^{-10}–10^{-8}M) in the presence of TNFα (10 ng/ml). Cells were then fixed and stained for TRACP, and the number of TRACP-positive MNCs was scored. Each value is the mean ± SEM of six cultures. *, $P < 0.01$ compared with the control (none) value. #, $P < 0.01$ compared with the value obtained from TNFα alone. White bars, none; black bars, TNFα.

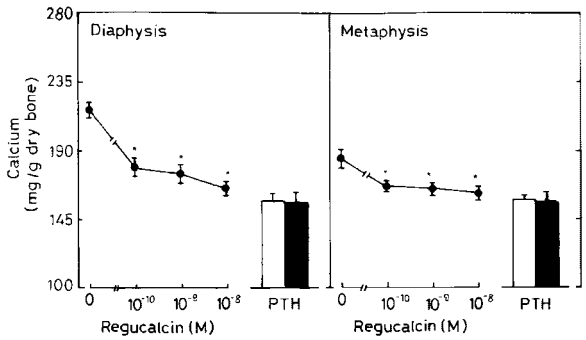

Fig. 11. Effect of regucalcin on calcium content in rat femoral-diaphyseal or -metaphyseal tissues in vitro. Bone tissues were cultured for 48 h in a medium containing either vehicle or regucalcin (10^{-10}–10^{-8}M). In separate experiments, bone tissues were cultured for 48 h in a medium containing either PTH (10^{-7}M) or PTH (10^{-7}M) plus regucalcin (10^{-8}M). Each value is the mean ± SEM of six rats. *, $P < 0.01$ compared with the control (none) value. White bars, PTH alone; black bars, PTH plus regucalcin.

(10^{-10}–10^{-8}M) caused a significant decrease in calcium content in the diaphyseal or metaphyseal tissues. Bone calcium content was significantly decreased by the addition of PTH (10^{-7}M). The effect of PTH in decreasing bone calcium content was not significantly altered in the presence of regucalcin.

The effect of regucalcin on medium glucose consumption in rat femoral-diaphyseal and -metaphyseal tissues is shown in Figure 12. The consumption of medium glucose in femoral-diaphyseal or -metaphyseal tissues was significantly increased in the presence of regucalcin

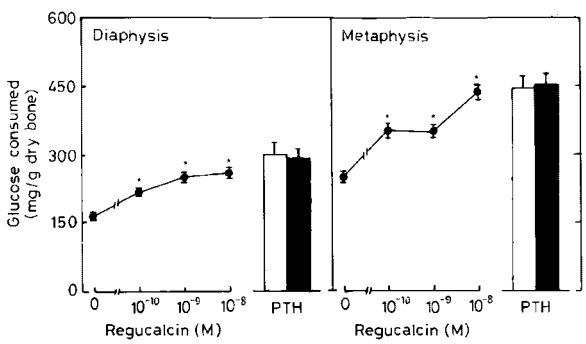

Fig. 12. Effect of regucalcin on medium glucose consumption by rat femoral-diaphyseal and -metaphyseal tissues in vitro. Bone tissues were cultured for 48 h in a medium containing either vehicle or regucalcin (10^{-10}–10^{-8}M). In separate experiments, bone tissues were cultured for 48 h in a medium containing either PTH (10^{-7}M) or PTH (10^{-7}M) plus regucalcin (10^{-8}M). Each value is the mean ± SEM of six rats. *, $P < 0.01$ compared with the control (none) value. White bars, PTH alone; black bars, PTH plus regucalcin.

(10^{-10}–10^{-8}M). Culture with PTH (10^{-7}M) caused a significant increase in glucose consumption by bone tissues. The effect of PTH in stimulating bone glucose consumption was not significantly enhanced in the presence of regucalin (10^{-8}M).

The effect of regucalcin on the production of lactic acid in rat femoral-diaphyseal or -metaphyseal tissues is shown in Figure 13. The production of lactic acid by femoral-diaphyseal or -metaphyseal tissues was significantly increased in the presence of regucalcin (10^{-10}–10^{-8}M). Bone lactic acid production was significantly increased by culture with PTH (10^{-7}M). The effect of PTH was not significantly enhanced in the presence of regucalcin (10^{-8}M).

DISCUSSION

Bone loss is induced in regucalcin transgenic rats in vivo [Yamaguchi et al., 2002b]. Regucalcin has been expressed in rat bone marrow cells [Yamaguchi et al., 2004]. Bone marrow cells, which were isolated from regucalcin transgenic rats with increasing age, were cultured without regucalcin addition. In this case, osteoclast-like cell formation was significantly enhanced as compared with that of wild-type animals [Uchiyama and Yamaguchi, 2004a]. Whether exogenous regucalcin stimulates osteoclastic cell formation, however, is unknown. This study clearly demonstrated that the addition of regucalcin stimulates osteoclast-like cell formation in mouse marrow culture in vitro. This finding may support the view that overexpression of regucalcin stimulates osteoclastic bone resorption in regucalcin transgenic rats in vivo.

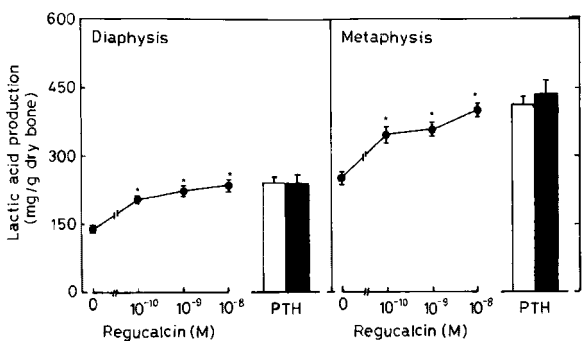

Fig. 13. Effect of regucalcin on lactic acid production by rat femoral-diaphyseal or -metaphyseal tissues in vitro. Bone tissues were cultured for 48 h in a medium containing either vehicle or regucalcin (10^{-10}–10^{-8}M). In separate experiments, bone tissues were cultured for 48 h in a medium containing either PTH (10^{-7}M) or PTH (10^{-7}M) plus regucalcin (10^{-8}M). Each value is the mean ± SEM of six rats. *, $P < 0.01$ compared with the control (none) value. White bars, PTH alone; black bars, PTH plus regucalcin.

It is well established that RANKL plays a key role in development of osteoclasts from preosteoclasts where their differentiation is enhanced in the presence of M-CSF [Tanaka et al., 2003; Zaidi et al., 2003]. RANKL is secreted from osteoblasts. PTH, D_3, or PGE_2, which are bone-resorbing factors, stimulates the expression of RANKL in osteoblasts. RANKL binds to RANK (receptor for RANKL) in preosteoclasts and stimulates differentiation to osteoclasts. Regucalcin significantly increased osteoclast-like cell formation, when mouse marrow cells were cultured for 7 days in the presence of both RANKL and M-CSF. However, such an effect was not observed by the addition of regucalcin at the later stage of culture in the presence of both RANKL and M-CSF. The effect of regucalcin in stimulating osteoclast-like cell formation was also seen by its addition at the later stage of culture in the absence of both RANKL and M-CSF. It is speculated that regucalcin enables the process of differentiation to osteoclasts of preosteoclasts. In addition, regucalcin had an osteoclastogenic effect in splenocytes (data not shown), suggesting that the protein acts on preosteoclasts in marrow culture system.

The effect of regucalcin in stimulating osteoclast-like cell formation was significantly inhibited in the presence of cycloheximide, an inhibitor of protein synthesis, or DRB, an inhibitor of transcriptional activity, suggesting that the effect of regucalcin is not resulted from a newly synthesized protein component. Moreover, the culture with regucalcin (10^{-8}M) was not resulted change in the expression of RANKL or OPG in bone marrow culture using RT-PCR analysis (data not shown). The effect of regucalcin in stimulating osteoclastic cell formation may not be involved in the enhancement of RANKL expression or the suppression of OPG expression in bone marrow culture system. It is speculated that regucalcin may stimulate osteoclastogenesis independently on the mechanism through RANKL/OPG. Regucalcin may bind to preosteoclasts and stimulate differentiation to osteoclasts. This remains to be elucidated.

The effect of various bone-resorbing factors (PTH, PGE_2, D_3, or TNFα) in stimulating osteoclast-like cell formation in mouse marrow

culture was significantly enhanced in the presence of regucalcin. Interestingly, the effect of TNFα was markedly enhanced in the presence of regucalcin. TNFα is an autocrine factor in osteoclasts, promoting their differentiation, and mediates RANKL's induction of osteoclastogenesis [Zou et al., 2001]. It is speculated that regucalcin may modulate the effect of TNFα in osteoclastogenesis.

The stimulatory effect of regucalcin on osteoclast-like cell formation in mouse marrow culture was significantly inhibited in the presence of various anti-bone resorbing factors (CT, E_2, CX, or zinc). It is well known that CT or 17β-estradiol (E_2) inhibits osteoclastic bone resorption. Zinc sulfate [Kishi and Yamaguchi, 1994] or CX [Uchiyama and Yamaguchi, 2004b] has an inhibitory effect on osteoclastic bone resorption in vitro. Thus, regucalcin-induced osteoclastogenesis was inhibited by various anti-bone resorbing factors.

Whether regucalcin can stimulate bone resorption using rat femoral-diaphyseal or -metaphyseal tissues in vitro was examined. Regucalcin was found to have a stimulatory effect on bone resorption in bone tissue culture in vitro; the protein caused a significant decrease in femoral-diaphyseal or -metaphyseal calcium content and a significant increase of medium glucose consumption or lactic acid production by the bone tissues. Such an effect was also seen in the presence of PTH, which is a physiologic important bone-resorbing hormone. We also cultured whole bone marrow cells on a dentine slice in the presence of regucalcin (10^{-8}M), and a significant increase in the number of pits formed on dentine slice was observed (data not shown). These observations support the view that regucalcin may stimulate osteoclastic bone resorption in vitro. Regucalcin may have a role as bone-resorbing factor.

In conclusion, it has been demonstrated that regucalcin has a stimulatory effect on osteoclastogenesis in mouse marrow culture in vitro.

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