

α -Lipoic acid suppresses osteoclastogenesis despite increasing the receptor activator of nuclear factor κ B ligand/osteoprotegerin ratio in human bone marrow stromal cells

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Abstract

Growing evidence has shown a biochemical link between increased oxidative stress and reduced bone density. Although α -lipoic acid (α -LA) has been shown to act as a thiol antioxidant, its effect on bone cells has not been determined. Using proteomic analysis, we identified six differentially expressed proteins in the conditioned media of α -LA-treated human bone marrow stromal cell line (HS-5). One of these proteins, receptor activator of nuclear factor κ B ligand (RANKL), was significantly up-regulated, as confirmed by immunoblotting with anti-RANKL antibody. ELISA showed that α -LA stimulated RANKL production in cellular extracts (membranous RANKL) about 5-fold and in conditioned medium (soluble RANKL) about 23-fold, but had no effect on osteoprotegerin (OPG) secretion. Despite increasing the RANKL/OPG ratio, α -LA showed a dose-dependent

suppression of osteoclastogenesis, both in a coculture system of mouse bone marrow cells and osteoblasts and in a mouse bone marrow cell culture system, and reduced bone resorption in a dose-dependent manner. In addition, α -LA-induced soluble RANKL was not inhibited by matrix metalloprotease inhibitors, indicating that soluble RANKL is produced by α -LA without any posttranslational processing. In contrast, α -LA had no significant effect on the proliferation and differentiation of HS-5 cells. These results suggest that α -LA suppresses osteoclastogenesis by directly inhibiting RANKL-RANK mediated signals, not by mediating cellular RANKL production. In addition, our findings indicate that α -LA-induced soluble RANKL is not produced by shedding of membranous RANKL.

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Introduction

There is increasing evidence of a biochemical link between increased oxidative stress and reduced bone density. The levels of 8-iso-prostaglandin (PG) $F_2\text{-}\alpha$, a biomarker of oxidative stress, were found to be negatively associated with both bone mineral density and quantitative ultrasound (Basu *et al.* 2001). In addition, dietary and endogenous antioxidants were markedly decreased in osteoporotic women (Maggio *et al.* 2003). Furthermore, the administration of various natural or synthetic antioxidants, such as vitamins C and E (Leveille *et al.* 1997, Wang *et al.* 1997, Hall & Greendale 1998, Melhus *et al.* 1999), N-acetyl cysteine (Lean *et al.* 2003), melatonin (Koyama *et al.* 2002) and dietary flavonoids (Chen *et al.* 2003), has been shown to be of benefit in the prevention and/or attenuation of osteoporosis.

Recently, α -lipoic acid (α -LA) has received considerable attention due to its activity as a thiol antioxidant. α -LA and its reduced form, dihydrolipoic acid, reduce oxidative stress by scavenging free radicals in membrane and aqueous domains, by chelating transition metals in biological systems, by preventing membrane lipid peroxidation and protein damage through the redox regeneration of other antioxidants such as vitamins C and E, and by increasing intracellular glutathione (Packer *et al.* 1995, Packer 1998). α -LA may also be effective in the prevention and treatment of oxidative stress, including those associated with ischemia-reperfusion injury (Roy *et al.* 1997), diabetes (Jacob *et al.* 1996, Henriksen *et al.* 1997, Ziegler *et al.* 1999), and neurodegenerative diseases (Packer *et al.* 1997). The ability of α -LA to protect against osteoporosis has not been addressed. We therefore tested

the effects of α -LA on osteoblast-lineage cells and osteoclasts, and observed a dissociation of receptor activator of nuclear factor κ B (NF- κ B) ligand (RANKL) expression and osteoclastogenesis. That is, α -LA markedly suppressed osteoclast formation despite increasing RANKL production by bone marrow stromal cells (BMSCs). We also found that the α -LA-induced up-regulation of soluble RANKL was not due simply to the shedding of membranous RANKL.

Materials and Methods

Reagents and materials

α -LA was purchased from Duchefa (Haarlem, Netherlands) and Merck. Pro-Prep protein extraction solution was purchased from Calbiochem (Darmstadt, Germany) and Intron (Seoul, Korea). Rabbit anti-RANKL polyclonal antibody and horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The cell death detection ELISA was purchased from Roche Applied Science, and an ELISA kit for osteoprotegerin (OPG) was purchased from Duoset, R&D Systems (Minneapolis, MN, USA). Macrophage-colony stimulating factor (M-CSF) and soluble RANKL were obtained from Peprotech EC (London, UK), and the leukocyte acid phosphatase assay kit was purchased from Sigma-Aldrich.

Culture of human BMSCs

Conditionally immortalized human BMSCs, HS-5 cells, obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA), were seeded onto 100 mm plastic culture dishes at a density of 1×10^6 cells/dish and cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS) (Gibco), 100 units/ml penicillin, and 100 μ g/ml streptomycin at 37 °C in a humidified atmosphere with 5% CO₂. The medium was changed twice weekly from the second week onward, and cells grown to 70~80% confluence were subcultured using 0.25% trypsin and 1 mM EDTA-4Na (Gibco).

Counting of viable cells and the cell death detection ELISA

HS-5 cells were seeded onto six-well plates at a density of 1×10^5 /well and cultured for 2~3 days in DMEM containing 10% FBS. Cells at 70~80% confluence were incubated in the presence or absence of 0.1 or 0.5 mM α -LA for 2 days in DMEM containing 0.1% FBS, and, following trypsinization, the live cells were counted in 0.01% trypan blue using a hemacytometer.

The cell death detection ELISA was used to evaluate apoptosis and necrosis activity in HS-5 cells according to

the manufacturer's protocol. Briefly, cells were seeded onto 96-well plates at a density of 5×10^3 /well, and cultured for 2 days in DMEM containing 0.1% FBS. The cells were cultured in the presence or absence of 0.1 or 0.5 mM α -LA for 24 h, the plates were centrifuged at 450 g for 10 min at room temperature, and 200 μ l lysis buffer were added to each well for 30 min at room temperature. Immunoreagent complex (80 μ l) was added to 20 μ l cell lysate (for apoptosis) or supernatant (for necrosis) on streptavidin-coated wells. After shaking for 2 h, each microtiter well was washed three times with 200 μ l incubation buffer. Specific antibody binding was detected with HRP-conjugated secondary antibody and measured with 2,2'-azino-di-(3-ethylbenzthiazoline-6-sulfonic acid (Sigma-Aldrich) at 405 nm.

Alkaline phosphatase (ALP) activity assay

HS-5 cells were seeded onto six-well plates at a density of 1×10^5 /well and cultured for 2~3 days in DMEM containing 10% FBS. Cells at 70~80% confluence were incubated for 24 or 48 h in the presence or absence of 0.1 or 0.5 mM α -LA in DMEM containing 0.1% BSA (Sigma-Aldrich) and 50 nM 1 α ,25-dihydroxy vitamin D₃ (Calbiochem). The cells were washed with PBS and ALP activity was measured using the *p*-nitrophenyl phosphate hydrolysis method (Steppan *et al.* 2000). The ALP activity in each sample was normalized relative to total cellular protein content, which was determined by the Bradford method.

Proteomics protocol

Sample preparation HS-5 cells were seeded onto 100 mm dishes at a density of 1×10^6 /dish and cultured for 2 days in DMEM containing 10% FBS. Following two washes with warm Hanks' balanced salt solution (HBSS), the cells were incubated in the presence or absence of 0.1 mM α -LA for 24 h in serum-free DMEM. The conditioned medium (CM) from three dishes was pooled, centrifuged at 340 g at 4 °C for 10 min to remove dead or released cells, and incubated with 10% (vol/vol) trichloroacetic acid (Sigma-Aldrich) for 1 h on ice. After centrifugation, each pellet was washed in ice-cold acetone containing 20 mM dithiothreitol (DTT) (Bio-Rad), and solubilized in 2-DE lysis buffer, consisting of 7 M urea, 2 M thiourea, 4% CHAPS, 100 mM DTT, 25 mM Tris-HCl (pH 7.1), 50 mM KCl, 0.2% bio-Lyte 3-10 (7-10) (Bio-Rad), 210 U DNase (Sigma-Aldrich) and protease inhibitor cocktail (1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride, 0.8 μ M aprotinin, 21 μ M leupeptin, 36 μ M bestatin, 15 μ M pepstatin A, 14 μ M E-64) (Sigma-Aldrich). Protein concentration was estimated using the Bio-Rad protein assay kit, and the samples were stored at -80 °C until use.

Two-dimensional PAGE (2D-PAGE) and image analysis

First-dimensional isoelectric focusing (IEF) was performed using linear 17 cm pH 4–7 and 7 cm pH 7–10 immobilizing the pH gradient (IPG) strips (Bio-Rad, Richmond, CA, USA) following the manufacturer's protocol with minor modifications. For pH 4–7 IPG strips, each sample containing up to 250 μ g protein was diluted to 350 μ l with rehydration solution (8 M urea, 4% CHAPS, 10 mM DTT, 0.2% bio-Lyte 3–10, 0.002% bromophenol blue) and passively applied to the strips by overnight rehydration. IEF was performed sequentially at 250 V for 25 min, 1000 V for 1 h, 8000 V for 5 h, and 8000 V for 75 000 Vh on a Protean IEF Cell (Bio-Rad). For pH 7–10 IPG strips, 100 μ g protein were rehydrated overnight in modified buffer, consisting of 8 M urea, 4% CHAPS, 10 mM DTT, 0.6% bio-lyte 7–10, 1.2% Destreak Reagent (Amersham Biosciences) and 0.02% bromophenol blue, and the same buffer, also containing 3.5% DTT, was added to the cathodic side of a paper wick. IEF was performed sequentially at 250 V for 15 min, 1000 V for 1 h, 4000 V for 2 h, and 4000 V for 22 000 Vh.

Prior to SDS-PAGE, the IPG strips were equilibrated for 15 min in equilibration solution (6 M urea, 2% SDS, 0.05 M Tris-HCl, pH 8.8, 20% glycerol, 2% DTT), and then for 15 min in the same buffer containing 2.5% iodoacetamide (Bio-Rad). The strips were transferred to 13% uniform polyacrylamide gels and electrophoresed in 17 cm Protean Plus Dodeca cells or 7 cm Mini-Protean 3 Dodeca cell at 5 mA/gel for 1 h and at 20 mA/gel thereafter. Proteins were visualized by silver staining (Shevchenko *et al.* 1996), and the stained 2D gels were imaged using VersaDoc imaging system (Bio-Rad) and analyzed using PDquest software (Bio-Rad).

In-gel tryptic digestion of proteins

Each silver-stained spot was washed twice with water for 5 min, and each gel piece was incubated for 5 min with acetonitrile (Sigma-Aldrich). The liquid was removed, and each gel piece was destained for 5 min in a 1:1 (vol/vol) mixture of 30 mM potassium ferricyanide/50 mM sodium thiosulfate (Sigma-Aldrich). Each piece was washed four times with 50% methanol/10% acetonitrile for 10 min, treated sequentially with 50 mM NH_4HCO_3 and acetonitrile, and dried under vacuum. Each gel piece was re-swollen in 0.1 M NH_4HCO_3 and 10 mM DTT for 45 min at 56 °C, and incubated for 30 min in 55 mM iodoacetamide and 0.1 M NH_4HCO_3 at room temperature in the dark. Each particle was washed for 5 min each with 0.1 M NH_4HCO_3 /acetonitrile (1:1 vol/vol) and acetonitrile, dried in a vacuum centrifuge, and rehydrated overnight at 37 °C in digestion buffer containing 50 mM NH_4HCO_3 , 0.1% *n*-octyl β -D-glucopyranoside (Sigma-Aldrich), and 12.5 ng/ μ l trypsin. Peptides were extracted from the gel pieces with 5% acetonitrile/2% trifluoroacetic acid (Sigma-Aldrich), and each solution was treated for 10 min in a bath sonicator.

Matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) analysis

Target preparation was carried out by the solution phase nitrocellulose method (Landry *et al.* 2000). Briefly, 40 mg/ml α -cyano-4-hydroxycinnamic acid (Sigma-Aldrich) and 20 mg/ml nitrocellulose (Amersham Biosciences) were prepared separately in acetone and mixed with 2-propanol at a ratio of 2:1:1. Two microliters of this mixture were added to 2 μ l of each peptide sample solution, and 1 μ l was spotted onto a MALDI plate and allowed to remain for 5 min. The plate was washed with 5% formic acid and then with water. The dried spots were analyzed in a Voyager-DE STR MALDI-TOF mass spectrometer (Applied Biosystems, Foster city, CA, USA). Internal standards for calibration were a des-Arg1-bradykinin peak (*m/z* 904.4681), an angiotensin peak (*m/z* 1296.6853) and a neurotensin peak (*m/z* 1672.9715). Proteins were identified by peptide mass fingerprinting using the search program MS-Fit (<http://prospector.ucsf.edu/ucshtml4-0/msfit.htm>), ProFound (http://129-85-19-192/profound_bin/WebProFound.exe), and Mascot (http://www.matrixscience.com/cgi/search_form.pl?FORMVER=2&SEARCH=PMF).

Immunoblotting for RANKL in CM from human BMSCs

Following 2D-PAGE, the proteins were electrophoretically transferred to nitrocellulose membranes in transfer buffer (18.3 mM Tris, 150 mM glycine, 20% methanol) using a Trans-Blot Cell (Bio-Rad) at 100 mA for 1.5 h with cooling. After blocking with TTBS (100 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.05% Tween 20) containing 5% skim milk for 1 h at room temperature, the membranes were incubated at room temperature for 45 min with rabbit polyclonal antibody against RANKL and then with HRP-conjugated goat anti-rabbit IgG. Specific binding was visualized using an enhanced chemiluminescence kit (Amersham Biosciences).

ELISA for RANKL and OPG

HS-5 cells were seeded onto 60 mm dish at a density of 3×10^5 /plate, and cultured for 2 days in DMEM containing 10% FBS. The cells were incubated in the presence or absence of 0.1 or 0.5 mM α -LA for 24 h in the same medium, and the CM and cellular extracts were prepared with Pro-Prep protein extraction solution according to the manufacturer's protocol. Each well of a microtiter plate (Nunc, Roskilde, Denmark) was coated with 100 μ l cellular extract overnight at 4 °C, followed by incubation with 200 μ l blocking buffer (0.3% BSA in TTBS) for 1 h at room temperature. Each well was incubated with 100 μ l goat anti-RANKL polyclonal antibody, in blocking buffer (1:1000) for 2 h at room temperature. Specific primary antibody binding was detected with HRP-conjugated rabbit anti-goat antibodies (Santa Cruz) and was measured

using 3,3',5,5'-tetramethylbenzidine solution (Calbiochem) at 450 nm absorbance in an ELISA reader (Spectra, Minneapolis, MN, USA). When we used normal goat IgG (AD-108-C; R&D Systems) instead of the anti-RANKL antibody as a primary antibody in CM containing known concentrations of human recombinant RANKL (R&D Systems), their immunoreactivities were negligible. The standard curve was generated using recombinant human RANKL at concentrations from 0 to 50 pM, and sensitivity was 0.2 pM. Primary mouse osteoblasts were also used in the experiments. A similar method was used to measure OPG concentration, employing a specific ELISA kit (Duoset, R&D Systems) according to the manufacturer's protocol. Each measurement was normalized relative to total cellular protein content, determined using Coomassie reagent (Pierce, Rockford, IL, USA).

Coculture and mouse bone marrow cell (BMC) culture

Mouse BMCs and osteoblasts were cocultured as described (Lee *et al.* 2002). Briefly, after BMCs were obtained by flushing tibiae from 6- to 8-week-old ICR mice, the cells were incubated overnight, and nonadherent BMCs were collected. Primary osteoblasts were obtained by growing calvarial cells from newborn ICR mice for 1 day in α -MEM (Sigma-Aldrich) supplemented with 10% FBS, 100 units/ml penicillin and 100 μ g/ml streptomycin. Into each well of a 48-well plate were seeded 2×10^5 BMCs and 2×10^4 osteoblasts, and the cells were cultured for 5 days in α -MEM containing 10^{-8} M $1\alpha,25$ -dihydroxy vitamin D₃ and 10^{-6} M PGE₂ (Calbiochem), in the presence or absence of various concentrations of α -LA. Alternatively, 1.5×10^6 BMCs in α -MEM containing 10% FBS were placed in each well of a 48-well plate. The cells were differentiated by culturing them for 5 days in the presence of 30 ng/ml M-CSF and 50 ng/ml soluble RANKL, in the presence or absence of various concentrations of α -LA. The complete culture medium including M-CSF and soluble RANKL was changed every 3 days. All experiments were approved by the Institutional Animal Care and Use Committee at the Asan Institute for Life Sciences, Seoul, Korea.

Tartrate-resistant acid phosphatase (TRAP) assay

Cytochemical staining of TRAP-positive cells was performed using the leukocyte acid phosphatase assay kit following the manufacturer's procedure. The multinucleated TRAP-positive cells (nuclei number > 3) were scored under a light microscope.

Resorption assay

Mouse BMCs (3×10^4 cells/well) were plated on whale dentine slices (Supplied by Dr N Takahashi, Institute for Oral Science, Matsumoto Dental University, Japan), and

treated with M-CSF (30 ng/ml) and RANKL (100 ng/ml). After the culture period, osteoclasts were removed from the dentine slices by ablation using cotton tips, and the dentine slices were stained with 1% toluidine blue (Sigma-Aldrich). Total areas of resorbed pits were analyzed by the Image Pro-Plus program version 4.0 (Media Cybernetics, Silver Spring, MD, USA).

Statistics

All experiments were repeated at least five times, with each point consisting of triplicate measurements. All data are expressed as means \pm S.E.M. The significance of differences between two groups was assessed by the Mann-Whitney *U*-test, whereas differences among three or more groups were tested by ANOVA with post-hoc analysis using Duncan's multiple range test. Dose-dependent relationships were examined by Spearman's rank correlation analysis. Statistical significance was defined as a *P* value less than 0.05. The SPSS 10.0 package (SPSS Inc., Chicago, IL, USA) was used for statistical procedures.

Results

Lack of effect of α -LA on proliferation and differentiation of HS-5 cells

To examine the direct effect of α -LA on HS-5 cells, we measured cell viability. Incubation of HS-5 cells with 0.1 or 0.5 mM α -LA for 48 h did not affect cell viability (104.0 ± 12.6 and $94.0 \pm 11.4\%$ respectively, compared with viability in the absence of α -LA, Fig. 1A). The cell death detection ELISA confirmed that α -LA did not increase apoptosis or necrosis of HS-5 cells (Fig. 1B). We also investigated the effects of α -LA on ALP activity in HS-5 cells. Incubation of cells with 0.1 or 0.5 mM α -LA for 24 or 48 h did not affect ALP activity. At 24 h, activities were 25.0 ± 1.4 and 23.4 ± 0.6 μ mol/mg protein for 0.1 and 0.5 mM α -LA respectively, vs 23.2 ± 1.0 μ mol/mg protein in the untreated control; at 48 h, these activities were 44.4 ± 1.2 , 45.2 ± 1.6 and 48.7 ± 1.4 μ mol/mg protein respectively.

α -LA altered protein secretion from HS-5 cells

We performed proteomic analysis to determine the ability of α -LA to alter the secretion by HS-5 cells of proteins of molecular mass 10–250 kDa into CM. More than five independent gels were performed to ensure that the protein profiles were reproducible. Image analysis showed that a total of 11 protein spots were expressed differentially more than twice (Fig. 2). All were excised and analyzed by MALDI-TOF mass spectrometry (MS) after in-gel tryptic digestion. Three of these spots could not be readily identified by MALDI-TOF MS, primarily because the

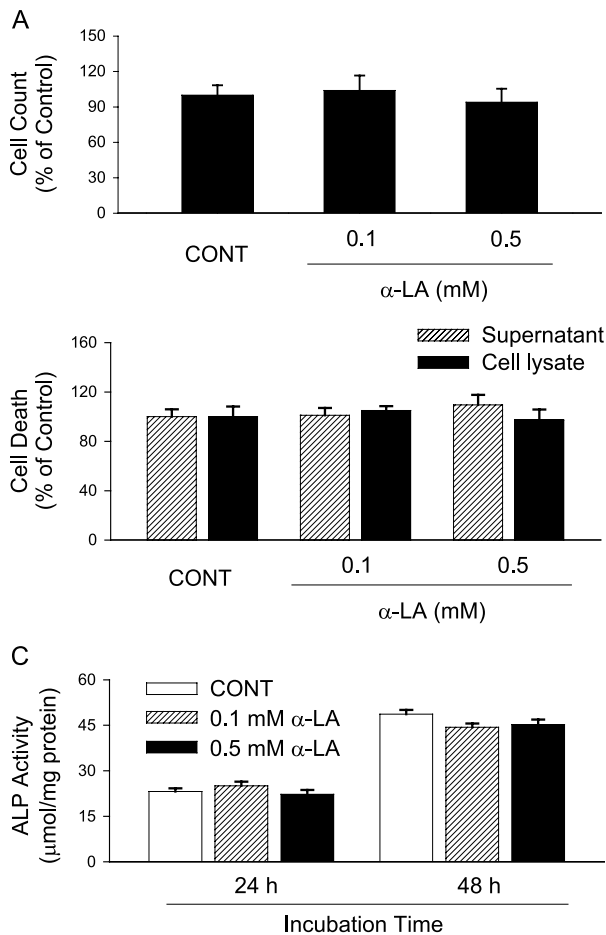


Figure 1 α -LA does not affect proliferation and differentiation of human BMSCs. (A) HS-5 cells (1×10^5) were cultured for 2–3 days in DMEM containing 10% FBS, and incubated with 0, 0.1 or 0.5 mM α -LA for 2 days in DMEM containing 0.1% FBS, and the living cells were counted in a hemacytometer. (B) HS-5 cells (5×10^3) were cultured for 2 days in DMEM containing 0.1% FBS, and incubated with 0, 0.1 or 0.5 mM α -LA for 24 h. A cell death detection ELISA was used to evaluate the percentage of cells that had undergone apoptosis (in the cell lysate) and necrosis (in the supernatant) according to the manufacturer's protocol. (C) HS-5 cells were incubated with the indicated concentration of α -LA for 24 or 48 h, and ALP activity was measured and normalized relative to total cellular protein content.

amounts of proteins in the individual spots were too small to allow analysis, whereas data on two of the spots were not found in our database. We were successful, however, in obtaining database results for the MALDI-TOF mass spectrum on the remaining six spots (Table 1). Interestingly, we found that secretion of RANKL, a critical mediator in the formation and differentiation of osteoclasts (Spot M5), was increased after treatment of cells with α -LA. When we performed immunoblotting with anti-RANKL antibody on the CM from HS-5 cells, we found that anti-RANKL antibodies were bound to the spot

thought to be RANKL, thus confirming their identity (data not shown).

α -LA stimulated expression of secreted and cellular RANKL without affecting OPG secretion

We measured the concentrations of RANKL and OPG in CM and/or cellular extracts of HS-5 cells cultured for 24 h in the presence or absence of α -LA. ELISAs showed that incubation with 0.1 or 0.5 mM α -LA increased cellular RANKL levels 2.7-fold ($P < 0.01$) and 4.8-fold ($P < 0.001$) respectively (Fig. 3A). We also found that incubation of HS-5 cells with 0.1 or 0.5 mM α -LA markedly increased RANKL secretion into the CM at 11.3- and 22.9-fold respectively ($P < 0.001$ for each, Fig. 3B). Other RANKL-inducing agents, such as $1\alpha,25$ -dihydroxy vitamin D₃, interleukin (IL)-6, PGE₂ and extracellular calcium, increased cellular RANKL but not soluble RANKL (Fig. 3A). α -LA, however, had no effect on OPG secretion by HS-5 cells (Fig. 3C). Also in mouse primary osteoblasts, α -LA significantly increased cellular and secreted RANKL levels (Fig. 3D and E).

α -LA-induced secretion of soluble RANKL was not inhibited by matrix metalloproteinase (MMP) inhibitors

We determined the role of MMPs on α -LA-induced stimulation of soluble RANKL secretion. HS-5 cells were incubated for 1 h with various MMP inhibitors, following which they were incubated in the presence or absence of α -LA for 24 h, and the secretion of soluble RANKL into the CM was measured by ELISA (Fig. 4). We found that none of the MMP inhibitors tested inhibited the α -LA-induced secretion of soluble RANKL.

α -LA reduced osteoclastogenesis from mouse BMCs and bone resorption

Since α -LA stimulated the production of membranous and soluble RANKL in HS-5 cells and primary mouse osteoblasts, and RANKL is a critical mediator of osteoclast formation and differentiation from monocytic cells (Lacey *et al.* 1998, Yasuda *et al.* 1998, Hsu *et al.* 1999), we tested the effects of α -LA on osteoclastogenesis from mouse BMCs using the TRAP assay. Using a coculture system of mouse BMCs and osteoblasts, we found that incubation with α -LA for 5 days reduced formation of TRAP-positive osteoclasts in a dose-dependent manner, from 288 ± 15 to 120 ± 9 (0.05 mM), to 102 ± 7 (0.1 mM), to 32 ± 2 (0.25 mM), to 18 ± 3 (0.5 mM) ($P < 0.01$ for trend, Fig. 5A). When we tested the effects of α -LA on BMCs cultured with soluble RANKL, we found that culture for 5 days with these same α -LA concentrations dose-dependently reduced formation of TRAP-positive cells, from 176 ± 8 to 48 ± 3 (0.05 mM), to 21 ± 6 (0.1 mM) to 2 ± 0.1 (0.25 mM) ($P < 0.01$ for trend), and there were no

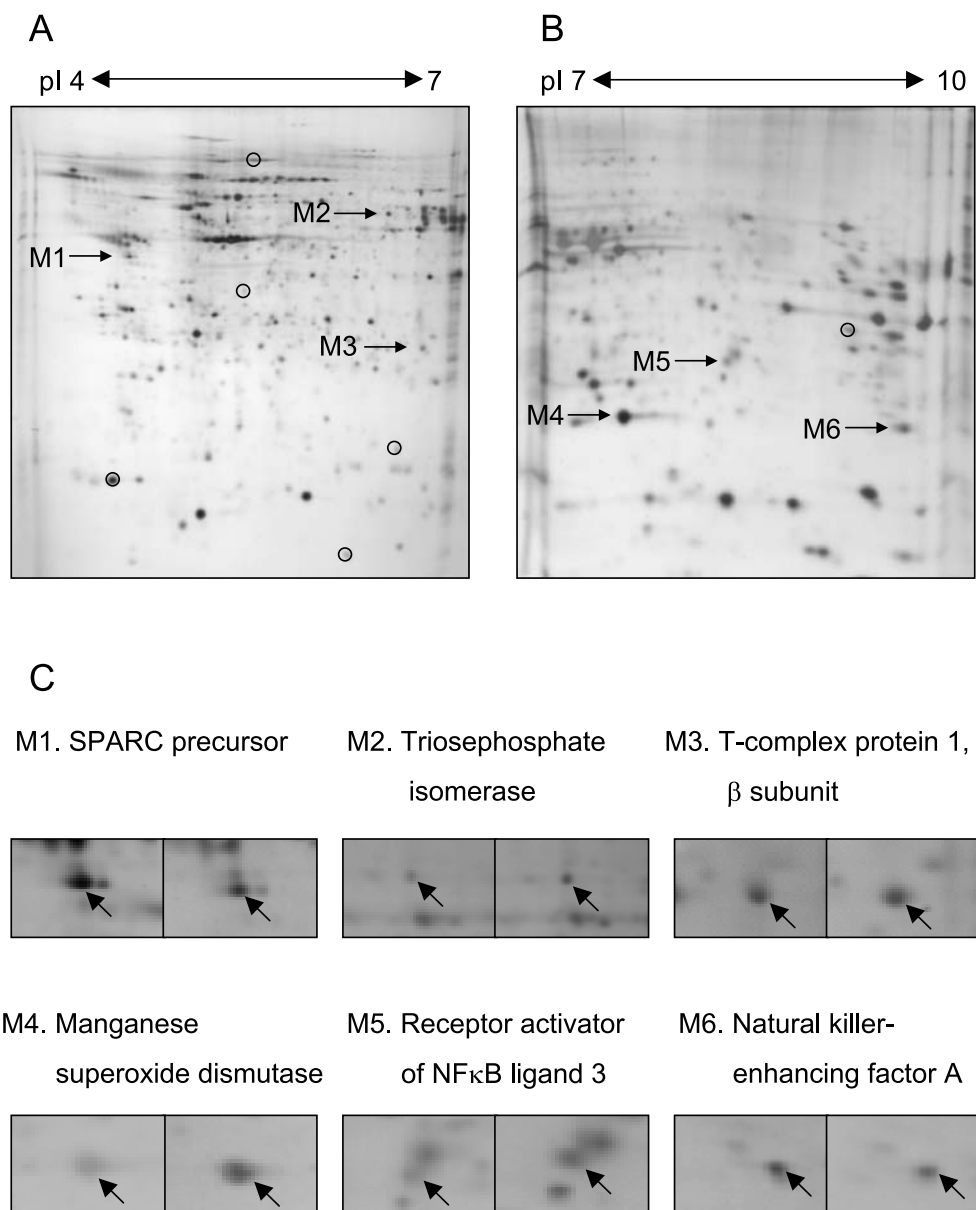


Figure 2 Two-dimensional gel electrophoresis protein profiles of CM from HS-5 cells following α -LA treatment. HS-5 cells (1×10^6) were cultured for 2 days in DMEM containing 10% FBS, washed twice with warm HBSS, and incubated with 0 or 0.1 mM α -LA for 24 h in serum-free DMEM. Proteins were separated in the first dimension on pH 4–7 (A) and pH 7–10 (B) immobilized pH gradient strips, followed by SDS-PAGE on 13% gels and silver staining. Proteins differentially expressed by levels of 2-fold or more are indicated with arrows, and their identities are shown in Table 1. Five of the differentially expressed spots were not readily identified by MALDI-TOF MS or they were not found in our database (circled spots). (C) Insets show expanded images of each proteins indicated by the same markers.

TRAP-positive cells after treatment with 0.5 mM α -LA (Fig. 5B). In addition, α -LA reduced bone resorption in a dose-dependent manner (Fig. 5C). Incubation with 0.1, 0.5 and 1.0 mM α -LA for 7 days reduced resorbed bone area to 46.6 ± 4.3 , 30.9 ± 8.7 and $12.8 \pm 2.3\%$ respectively of the control levels.

α -LA was reported to inhibit adhesion molecule expression in human aortic endothelial cells and monocyte adhesion (Zhang & Frei 2001); thus we investigated whether α -LA can affect adhesiveness of BMCs. BMCs were allowed to attach to fibronectin-coated culture plate in the presence or absence of various concentrations of

Table 1 Identification of differentially expressed proteins into culture media in the presence or absence of 0.1 mM α -LA for 24 h. Proteins differentially expressed 2-fold or greater between control and α -LA-treated HS-5 cells were identified by MALDI-TOF MS

Spots	Protein name	Accession #	MW gel (kDa)	pI gel	Sequence coverage (%)	Peptide match	Expression level (fold vs control)	Comment on function
M1	SPARC (secreted protein acidic and rich in cysteine) precursor: osteonectin	P09486	34 632	4.7	28	8	0.3 \pm 0.1	Regulates cell growth through its modulation of cell-matrix interactions; may be associated with cell de-adhesion and antiproliferation
M2	Triosephosphate isomerase	P00938	26 670	6.4	51	12	2.8 \pm 1.7	Important in several metabolic pathways; glycolytic enzyme that catalyzes the interconversion of D-glyceraldehyde 3-phosphate to dihydroxyacetone phosphate
M3	T-complex protein 1, β subunit	P78371	57 489	6.0	33	15	3.8 \pm 1.6	Chaperone protein involved in the folding of cytoskeletal proteins
M4	Manganese superoxide dismutase	14488599	22 089	6.9	56	10	2.3 \pm 0.3	Maintaining the integrity of mitochondrial enzymes susceptible to direct inactivation by superoxide; converts superoxide radicals (O_2^-) to hydrogen peroxide; present only within mitochondria
M5	Receptor activator of NF κ B ligand 3	BAA97258	22 150	8.6	37	5	3.8 \pm 1.0	Induces osteoclastogenesis by binding to its receptor, RANK
M6	Peroxioredoxin 1; natural killer-enhancing factor A; thioredoxin-dependent peroxide reductase 2	4505591	22 111	8.3	15	3	0.5 \pm 0.1	Member of the peroxiredoxin family of antioxidant enzymes; plays an antioxidant protective role in cells; may have a proliferative effect

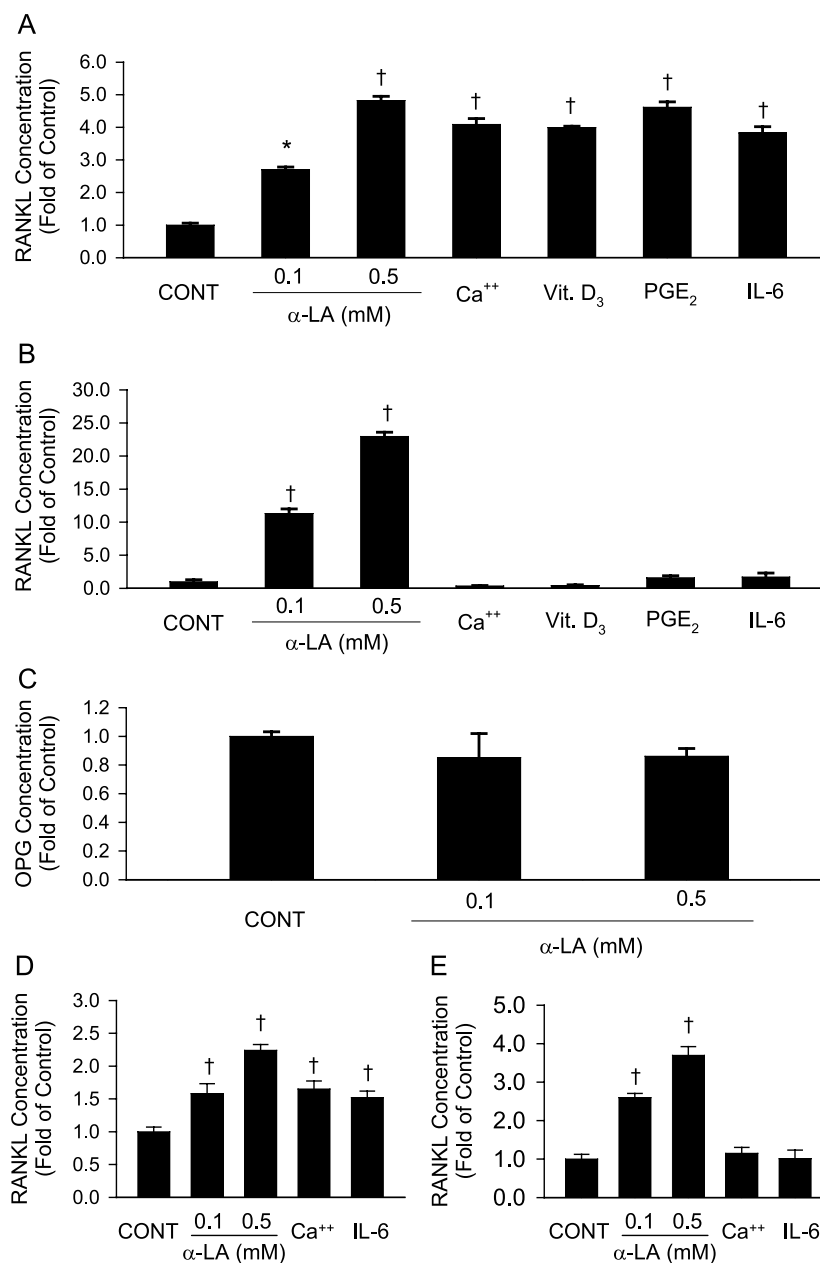


Figure 3 α -LA stimulates production of cellular and secreted RANKL in human BMSCs without affecting OPG production. HS-5 cells (3×10^5) were cultured for 2 days in DMEM containing 10% FBS and treated with 0, 0.1 or 0.5 mM α -LA for 24 h, and RANKL concentrations in the cellular extracts (A) and CM (B), and OPG concentration in the CM (C), were measured by ELISA. As positive controls for RANKL, cells were treated with $1\alpha,25$ -dihydroxy vitamin D_3 (10^{-8} M), IL-6 (100 ng/ml), PGE_2 (10^{-7} M) or extracellular calcium (10 mM). Cellular (D) and secreted RANKL (E) were also determined in mouse calvarial osteoblasts. The concentrations of secreted proteins in the control cultures (i.e. in the absence of α -LA) were defined as 100%. * $P < 0.01$; † $P < 0.001$ compared with α -LA-untreated control.

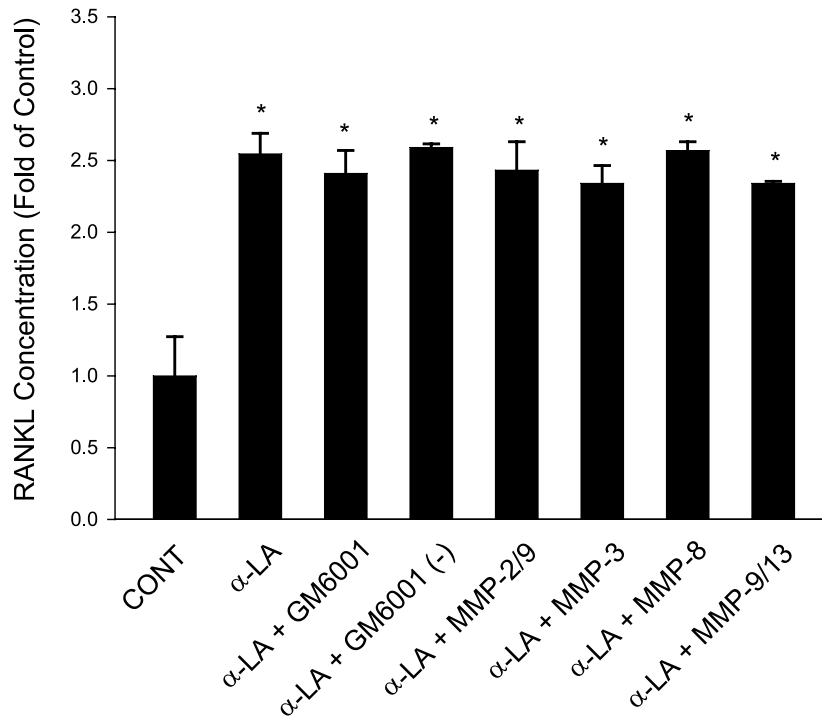


Figure 4 MMP-independent release of soluble RANKL from HS-5 cells by α -LA. A broad spectrum MMP inhibitor (GM6001), and its negative control (GM6001 (-)), or specific MMP inhibitors (Calbiochem) were added to the medium for 1 h at concentrations of 10 μ M. After treatment with 0.1 mM α -LA for 24 h, RANKL concentrations in the CM were measured by ELISA. The concentration of RANKL in cells cultured in the absence of MMP inhibitors and α -LA was defined as 100%. * P <0.001 when compared with α -LA-untreated cells.

α -LA. However, we noted that α -LA did not affect the adhesiveness of BMCs (data not shown).

CM from α -LA-treated HS-5 cells stimulated osteoclastogenesis from mouse BMCs with reversal by α -LA

The CM was collected from HS-5 cells treated with α -LA (LA-CM) or the control vehicle (control CM) in α -MEM for 1 day, with the addition of only M-CSF. LA-CM significantly stimulated osteoclastogenesis from mouse BMCs (Fig. 6A), confirming that increased RANKL secretion from HS-5 cells by α -LA is a potent stimulator for osteoclastogenesis. The LA-CM-induced osteoclastogenesis was reversed by the treatment of α -LA in a dose-dependent manner (Fig. 6B).

Discussion

RANKL is a new member of the tumor necrosis factor (TNF)-ligand family, which is expressed as a membrane-associated protein in osteoblasts/stromal cells in response to

many bone-resorbing factors (Lacey *et al.* 1998, Yasuda *et al.* 1998). Osteoclast precursors that express RANK, a member of the TNF receptor family, recognize RANKL through cell-cell interactions with osteoblasts/stromal cells, inducing their differentiation into osteoclasts in the presence of M-CSF (Hsu *et al.* 1999). OPG, which is mainly produced by osteoblasts/stromal cells, is a soluble decoy receptor for RANKL and has been shown to function as an inhibitory factor in osteoclastogenesis (Yasuda *et al.* 1998). Therefore, the balance between RANKL and OPG (the RANKL/OPG ratio) is thought to be critical for modulation of osteoclastogenesis and bone remodeling.

Osteotropic factors such as $1\alpha,25$ -dihydroxy vitamin D_3 , parathyroid hormone (PTH), PGE_2 and IL-11, which stimulate osteoclast formation, have been shown to up-regulate the expression of RANKL mRNA, while down-regulating OPG mRNA expression (Boyle *et al.* 2003). Factors that elevate intracellular calcium, including compounds such as ionomycin and phorbol 12-myristate 13-acetate (an activator of protein kinase C (PKC)) as well as high calcium concentration in the culture medium, also induce osteoclast formation, while stimulating the

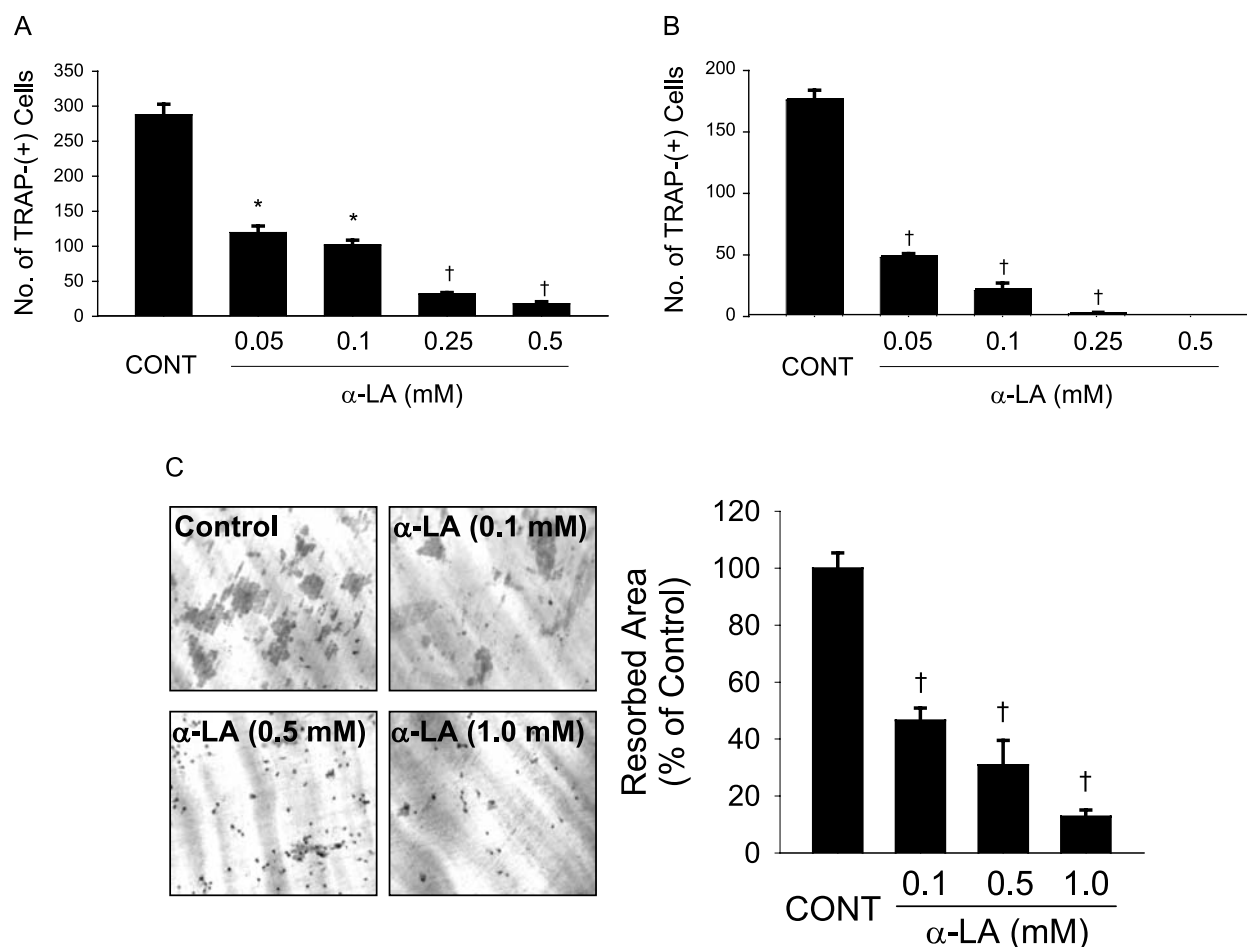


Figure 5 α -LA reduces osteoclastogenesis of mouse BMCs and bone resorption activity. Mouse BMCs (2×10^5) were cocultured with 2×10^4 calvarial osteoblasts with or without the indicated concentrations of α -LA in α -MEM containing 10% FBS, 10^{-8} M $1\alpha,25$ -dihydroxy vitamin D_3 , and 10^{-6} M PGE_2 for 5 days (A). Alternatively, mouse BMCs (1.5×10^6) were cultured with or without the indicated concentrations of α -LA in α -MEM containing 10% FBS, 30 ng/ml M-CSF and 50 ng/ml soluble RANKL (B). The cells were fixed and stained for TRAP, and the number of multinucleated TRAP-positive cells (>3 nuclei) was measured. * $P < 0.01$; † $P < 0.001$ when compared with α -LA-untreated cells. (C) The effect of α -LA on the resorption activity of osteoclasts. Mouse BMCs were incubated for 7 days in medium containing M-CSF (30 ng/ml) and RANKL (100 ng/ml) with or without α -LA on dentine slices. After removing cells, dentines were stained by 1% toluidine blue. Shown are representative photographs ($\times 40$). Resorbed area was quantified using a computer imaging software system. † $P < 0.001$ vs control.

expression of both RANKL and OPG mRNAs (Takami *et al.* 2000). At least four signals are independently involved in RANKL expression in osteoblasts/stromal cells: vitamin D receptor-mediated signals induced by $1\alpha,25$ -dihydroxy vitamin D_3 ; cAMP/PKA-mediated signals induced by PTH or PGE_2 ; gp130-mediated signals induced by IL-11; and calcium/PKC signals induced by ionomycin or extracellular calcium (Suda *et al.* 1992, Takami *et al.* 2000). We have shown here that α -LA enhanced RANKL expression without having a significant effect on OPG expression, resulting in an increased RANKL/OPG ratio, while suppressing osteoclast formation. These results indicate that α -LA directly suppresses osteoclast formation by inhibiting either the RANKL-RANK interaction or RANK-mediated signals.

Activation of NF- κ B, c-jun N-terminal protein kinase, p38, extracellular signal-regulated kinase and the Src pathway through RANK-mediated signals appears to be involved in the differentiation and activation of osteoclasts (Boyle *et al.* 2003). α -LA has been reported to suppress NF- κ B activation in cells of the monocyte lineage cells, such as the RAW 246-7 (Cohen *et al.* 2003) and MonoMac6 (Lee & Hughes 2002) cell lines and peripheral blood mononuclear cells from diabetic patients (Hofmann *et al.* 1998, 1999). We therefore hypothesized that α -LA inhibits osteoclast formation by suppressing NF- κ B activation. Consistent with this hypothesis, N-acetyl cysteine, another antioxidant, has been shown to inhibit osteoclastogenesis by suppressing NF- κ B activation in murine BMCs (Lean *et al.* 2003).

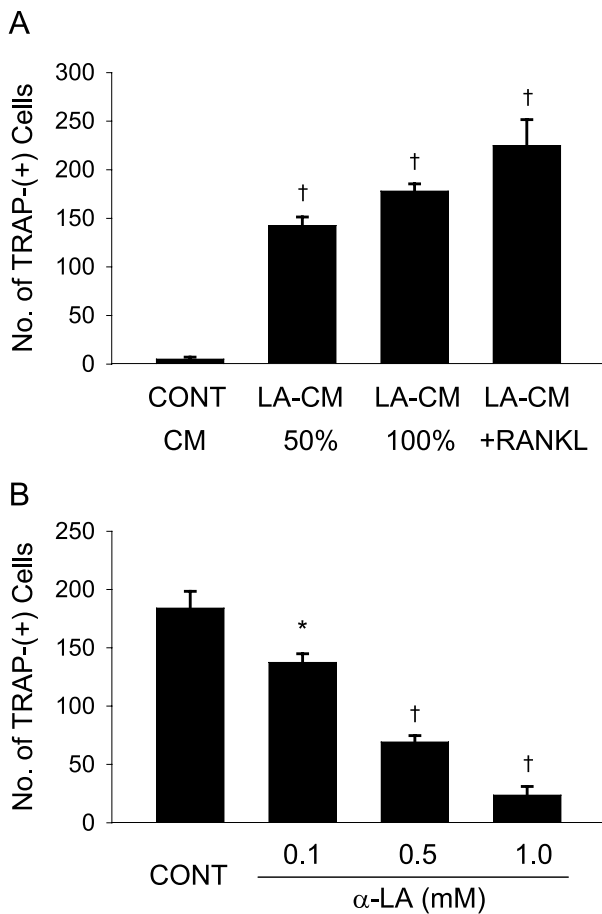


Figure 6 CM from α -LA-treated HS-5 cells stimulated mouse osteoclast formation. The CM were collected from HS-5 cells treated with 1 mM α -LA (LA-CM) or the control vehicle (control CM) in α -MEM for 1 day. (A) In the presence of M-CSF (30 ng/ml), BMCs were cultured in control CM, 50% LA-CM, 100% LA-CM plus fresh α -MEM, or 50% LA-CM plus RANKL (50 ng/ml). After 9 days, TRAP-positive multinuclear cells were determined by TRAP staining. [†] $P < 0.001$ vs control. (B) BMCs were incubated in 100% LA-CM plus M-CSF (30 ng/ml) in the presence of α -LA at the indicated concentration. TRAP-positive multinucleated cells were determined by TRAP staining. * $P < 0.01$, [†] $P < 0.001$ vs control.

RANKL is expressed as a membranous form and as a soluble form, corresponding to the C-terminal portion of membranous RANKL (Lacey *et al.* 1998, Lum *et al.* 1999, Wong *et al.* 1999). In contrast to the membranous form, the regulation of the soluble form has not been clarified. We have shown here that α -LA, but not 1 α ,25-dihydroxy vitamin D₃, PGE₂, IL-6 or extracellular calcium, stimulated soluble RANKL production in HS-5 cells. Although the mechanism of the *in vivo* production of soluble RANKL is not yet known, metalloproteases have been implicated in the shedding or release of several cell surface proteins from the plasma membrane, including cytokines, cytokine receptors, adhesion proteins, and others such as

the β -amyloid precursor protein (Hooper *et al.* 1997). TNF- α is thought to be released from the plasma membrane by the metalloprotease-disintegrin TNF- α convertase (TACE) (Black *et al.* 1997). Similarly, RANKL shedding has been shown to be catalyzed by TACE, by the membranous metalloprotease MT1-MMP or by A-disintegrin and metalloprotease 19 (Lum *et al.* 1999, Chesneau *et al.* 2003). Since shedding of many cell surface proteins can be inhibited with metalloprotease inhibitors, we tested the ability of these inhibitors to affect soluble RANKL production. We found, however, that none of the metalloprotease inhibitors tested had any effect on α -LA-induced production of soluble RANKL, indicating that the latter is not simply by shedding of membranous RANKL.

Three isoforms of RANKL in mice and human, produced by alternative splicing from the same gene, have been identified: RANKL1, the original RANKL, which possesses intracellular, transmembrane and extracellular domains; RANKL2, which has a shorter intracellular domain in mice and none in humans; and RANKL3, which lacks the intracellular and transmembrane domains and may act as a soluble form protein (Ikeda *et al.* 2001, Suzuki *et al.* 2004). The results shown here suggest that the soluble RANKL induced by α -LA in HS-5 cells is RANKL3. This is supported by observations that both the bone marrow stromal ST2 cell line and the preosteoblastic MC3T3E-1 cell line express the RANKL3 isoform (Ikeda *et al.* 2001).

In summary, we have shown here that α -LA suppresses osteoclastogenesis despite increasing the RANKL/OPG ratio in HS-5 cells, suggesting that α -LA directly inhibits osteoclastogenesis. Although the *in vivo* effect of α -LA on bone is not yet known, this compound may halt or attenuate bone loss clinically. In addition, we have shown that α -LA stimulates secretion of the soluble form of RANKL by HS-5 cells without any posttranslational processing. Further studies are required to verify the mechanism by which soluble RANKL production is regulated.

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