JOURNAL OF BONE AND MINERAL RESEARCH
Volume 17, Number 7, 2002

Osteoprotegerin Abrogates Chronic Alcohol Ingestion–Induced Bone Loss in Mice

JIAN ZHANG,[1,2] JINLU DAI,[1] DIN-LII LIN,[1] PAULA HABIB,[1] PETER SMITH,[1] JILL MURTHA,[1,2] ZHENG FU,[3] ZHI YAO,[4] YINGHUA QI,[5] and EVAN T. KELLER[1–3,6,7]

ABSTRACT

To investigate the role of osteoprotegerin (OPG) on alcohol (ethanol)-mediated osteoporosis, we measured a variety of bone remodeling parameters in mice that were either on a control diet, an ethanol (5%) diet, or an ethanol (5%) diet plus OPG administration. OPG diminished the ethanol-induced (1) decrease in bone mineral density (BMD) as determined by dual-energy densitometry, (2) decrease in cancellous bone volume and trabecular width and the increase of osteoclast surface as determined by histomorphometry of the femur, (3) increase in urinary deoxypyridinolines (Dpd's) as determined by ELISA, and (4) increase in colony-forming unit-granulocyte macrophage (CFU-GM) formation and osteoclastogenesis as determined by ex vivo bone marrow cell cultures. Additionally, OPG diminished the ethanol-induced decrease of several osteoblastic parameters including osteoblast formation and osteoblast culture calcium retention. These findings were supported by histomorphometric indices in the distal femur. Taken together, these data show that OPG diminishes ethanol-induced bone loss. Furthermore, they suggest that OPG achieves this through its ability to abrogate ethanol-induced promotion of osteoclastogenesis and promote osteoblast proliferation. (J Bone Miner Res 2002;17:1256–1263)

Key words: ethanol, osteoporosis, animal model, osteoprotegerin, RANKL

INTRODUCTION

ALCOHOLISM IS a widespread problem accompanied by many pathophysiological manifestations. For example, a large proportion of chronic alcoholics have osteopenia.[1–7] There is evidence that alcohol (ethanol) induces bone loss through both inhibition of osteoblast activity[8–10] and induction of osteoclast activity.[9,11–13]

Osteoclasts, the multinucleated giant cells that remove bone, are derived from colony-forming unit granulocyte-macrophage (CFU-GM), which branch from the monocyte-macrophage lineage during the early differentiation process. Macrophage colony–stimulating factor (M-CSF) is a primary factor that induces hematopoietic stem cells to differentiate into CFU-M. Under the stimulation of M-CSF and RANKL, the cells differentiate into prefusion osteoclasts. With the stimulation of M-CSF, RANKL, and interleukin-1 (IL-1) at this step, the prefusion osteoclasts differentiate into mature osteoclasts with osteolytic activity. The differentiation and activation of osteoclasts is regulated by a variety of factors such as hormones, growth factors, and cytokines (reviewed by Teitelbaum[14]). Most osteoclastogenic factors

The authors have no conflict of interest.

[1]Unit for Laboratory Animal Medicine, University of Michigan, Ann Arbor, Michigan, USA.
[2]Department of Pathology, University of Michigan, Ann Arbor, Michigan, USA.
[3]Program in Immunology, University of Michigan, Ann Arbor, Michigan, USA.
[4]Department of Immunology, Tianjin Medical University, Tianjin, China.
[5]Lab of Cellular and Molecular Biology, Department of Internal Medicine, Tianjin Third Municipal Hospital, Tianjin, China.
[6]Institute of Gerontology, University of Michigan, Ann Arbor, Michigan, USA.
[7]Program in Comparative Integrative Genomics, University of Michigan, Ann Arbor, Michigan, USA.

act through osteoblasts and indirectly stimulate osteoclast maturation and activation. These factors are classified into three categories based on different signal transduction pathways: the vitamin D receptor pathway activated by 1,25-dihydroxyvitamin D_3 [$1,25(OH)_2D_3$]; the protein kinase A pathway activated by parathyroid hormone (PTH), prostaglandin E_2 (PGE_2), or IL-1; and the glycoprotein 130 (gp130) pathway activated by the IL-6–type cytokines.[15] Recently, a new cytokine system was established to play a critical role in osteoclastogenesis, the RANKL, and osteoprotegerin (OPG) system.

Osteoclastogenesis occurs through the binding of RANKL, which is present on osteoblasts, to RANK, which is found on the osteoclast precursors. OPG is a protein produced by osteoblasts and bone marrow stromal cells. OPG binds RANKL, effectively blocking it from binding to RANK and thus inhibiting osteoclastogenesis (reviewed by Teitelbaum[14]).

Consistent with the result of increased bone resorption by ethanol in vitro[13] and bone histomorphometry findings of an increased bone resorptive area in alcoholics,[9,16] we have previously documented that alcohol induces osteoclast activity and bone resorption in mice.[17] Furthermore, the ethanol-induced osteoclast activity was associated with increased RANKL mRNA expression in bone marrow cells.[17] Therefore, we hypothesized that inhibition of RANKL should diminish ethanol-induced bone loss. Accordingly, we determined, in this study, if the RANKL inhibitory protein OPG inhibits alcohol-mediated bone loss in a murine model.

MATERIALS AND METHODS

Animals

Eight-week-old male C57/Bl6 mice (Charles River Laboratories, Wilmington, MA, USA) were housed individually in plastic cages under standard laboratory animal conditions. Mice were fed liquid ethanol or control diets as we have previously described.[17] Briefly, a National Research Council (NRC) liquid diet[18] that contains 100% of the vitamin and mineral requirements set by the NRC was used. The ethanol-treated group diet contained 26% of energy as ethanol, 31.5% as carbohydrates, 12.5% as protein, and 30% as fat (diet no. 710279; Dyets, Bethlehem, PA, USA) as previously described,[19] resulting in a dietary ethanol concentration of 5% (vol/vol) and blood ethanol concentrations of 9.9 mmol/liter and 7.3 mmol/liter at 5 h and 20 h postfeeding, respectively.[18] An isocaloric level of maltose was substituted for ethanol in the nonethanol control diet. Diets were prepared fresh daily. To minimize differences due to food intake, mice from both the control and the ethanol + OPG groups were matched with a mouse from the ethanol group. To accomplish this, food intake for each ethanol-fed mouse was measured and then its respective match-fed control diet–fed mouse and ethanol-fed + OPG mouse received the same volume of food the following day. Using this technique, there was no difference in body weight between groups at the end of the study (data not shown).

Treatment

As the time animals were randomized to be fed with control or ethanol diet, ethanol-fed mice were randomized to receive either injections (through intraperitoneal injection) of vehicle (1% bovine serum albumin [BSA] in 1× PBS; $n = 10$) or recombinant mouse OPG/F_c chimera (R & D Systems, Inc., Minneapolis, MN, USA) at 1 mg/kg of body weight ($n = 10$) twice a week and continued for 4 months. At the end of 4 months, animals were killed by CO_2 asphyxiation. The University of Michigan Animal Care and Use Committee approved the animal protocols.

Bone densitometry

Bone mineral density (BMD) was measured using DXA on an Eclipse Peripheral Dexa Scanner (Norland Medical Systems, Ft. Atkinson, WI, USA) using research software. To measure whole body BMD, mice were anesthetized with ketamine pre- and postethanol feeding and placed in sternal recumbency on the scanner. The mice were scanned at 10 mm/s with a resolution of 0.5 mm × 0.5 mm. BMD was determined in a window that excluded the calvarium and tail. To measure femoral BMD, the right femur was excised from soft tissue and placed on the scanner in lateral position. The femur was scanned at 5 mm/s with a resolution of 0.1 mm × 0.1 mm. BMD was determined in a window that encompassed the entire femur. Short-term BMD precision (%CV) was ~3% for both of these techniques.

Quantification of deoxypyridinolines

At the time of killing, urine was collected and frozen at −80°C until assayed. Urine creatinine was measured using a creatinine kit (Quidel, Mountain View, CA, USA) as recommended by the manufacturer. Deoxypyridinolines (Dpd's) were measured using the Pyrilinks-D kit (Quidel) as recommended by the manufacturer. A standard curve was created using a four-parameter fit generated by MetraFIT software (Quidel). Dpd was corrected for variations in urine concentration by dividing the Dpd value (nmol/liter) by the creatinine value (mmol/liter) for each sample.

Quantification of CFU-fibroblast and CFU-osteoblast and culture calcium retention

CFU-fibroblast (CFU-F) and CFU-osteoblast (CFU-OB) were measured as previously reported,[20] with minor modification. Briefly, mouse femora were flushed with 3 ml of phenol red–free α-minimum essential medium (α-MEM) to obtain marrow cells. The cells were rinsed twice and the total cell numbers per femur was determined and resuspended to obtain a single cell suspension of 1×10^7 cells/ml. For determination of CFU-F, 1.5×10^6 (150 μl) cells were seeded into 4 ml of complete media (α-MEM, 15% FBS, 50 μg/ml of ascorbic acid, and 10 mM of β-glycerophosphate) + 10 nM of dexamethasone/well of 6-well plates. Assays were performed in duplicate for each animal. One-half of the media was removed and replaced on day 5 with α-MEM, containing 30% FBS, 100 μg/ml of ascorbic acid, and 20 mM of β-glycerophosphate to result in

a final concentration of the original complete media. Dexamethasone was not added after the initial culture period. On day 10 of culture, cells were fixed, stained for ALP, and counterstained with hematoxylin. ALP^+ colonies containing $\geq$20 cells were enumerated. To determine CFU-OB, cultures were established in duplicate and maintained as for CFU-F except 2.5×10^6 cells (250 μl of 1×10^7 cells/ml) were plated per well. Media was replaced every 5 days as described previously. Cells were cultured for 25 day at which time they were fixed in 18% formaldehyde, 50% ethanol, and 32% deionized water for 1 minute and then stained using Von Kossa's method. Stained colonies were enumerated. To measure calcium content in cultures, cells were prepared and grown as for CFU-OB. Calcium content was measured using standard biochemistry (Calcium Kit; Fisher Scientific Co., Pittsburgh, PA, USA).

Quantification of osteoclast formation in bone marrow cultures

Osteoclast formation was determined as previously reported,[20] with minor modifications. Briefly, marrow cells were collected as described previously. To determine osteoclast number, the cells were plated at 1.5×10^6 cells/2 cm^2 well on a 13-mm^2 round tissue culture coverslips (4–6 replicate cultures/mouse). The cultures were maintained for 9 days in the presence of 10^{-8} M of 1,25 $(OH)_2D_3$ with replacement of half the medium every 3 days. To confirm the identification of osteoclasts, several replicates were evaluated for TRAP (Acid Phosphatase Kit; Sigma Chemical Co., St. Louis, MO, USA) and calcitonin receptors (CTR) by a previously described method.[21] Briefly, at the end of the culture period, cells were incubated with ^{125}I-salmon calcitonin (0.02 μCi) in the absence or presence of excess unlabeled salmon calcitonin (10^{-7} M) at room temperature for 2 h. Cells then were washed twice with PBS and fixed with 2.5% glutaraldehyde in PBS. The coverslips were dipped in LM-1 photographic emulsion (Eastman Kodak, Rochester, NY, USA) and maintained at 4°C for 14 days. Cells were counterstained with methyl green and then scored as mononuclear, 2–5 nuclei, or more than nuclei. Results are reported as number of multinucleated $TRAP^+$ cells and CTR^+ cells per/coverslip.

Assessment of individual CFU-F colonies for ability to support osteoclast formation

CFU-F osteoclastogenic activity was determined as previously reported[20] with minor modifications. Briefly, 100 μl of 2×10^5 bone marrow cells/ml in α-MEM was placed into wells of 96-well plates containing 100 μl of α-MEM, 30% FBS, 100 μg/ml OF ascorbic acid, 20 mM OF β-glycerophosphate in each well of 96-well plates. Cells were incubated for 5 days, at which time spleen cells were isolated from C57/BL6 mice and resuspended to 1×10^6/ml in α-MEM with 20% FBS and 20 nM of 1,25$(OH)_2D_3$. Then, 100 μl of media was removed from each well and 100 μl of the spleen cell suspension was added. On day 9, one-half of the media was removed and replaced with α-MEM with 20% FBS and 20 nM of 1,25$(OH)_2D_3$. On day 13, the cells were stained for TRAP and each well was examined microscopically for the presence of CFU-F (>20 cells) and osteoclastic cells ($TRAP^+$).

Quantification of CFU-GM

To determine CFU-GM formation, 1.25×10^5 of the bone marrow cells (i.e., 250 μl of 5×10^5 cells/ml) were seeded into 2.5 ml of a methylcellulose-based media containing CFU-GM promoting growth factors (MethoCult HCC-3534; Stem Cell Technologies, Vancouver, BC, Canada) in a 15-ml sterile test tube. The cells were vortexed and bubbles were allowed to settle. Then, 1.1 ml of cell/media mixture was placed into a 35-mm cell culture dish using a 16G 1.5-in needle. Assays were performed in duplicate. The duplicate 35-mm dishes with cells were placed into a 150-mm plate along with a covered 35-mm dish filled with 3 ml of sterile water. The cells were incubated at 37°C 5% CO_2 for 7 days, at which time CFU-GMs (defined as >50 cells) were counted.

Bone histomorphometry

The right femur was dissected from soft tissue, fixed in 10% phosphate-buffered formalin for 24 h, decalcified in 14% EDTA, and embedded in a lateral position in paraffin. 5 μM thick longitudinal sections from the middle of the femur were stained with hematoxylin and eosin or trichrome stain. Histomorphometry was performed using Bioquant Nova Imaging Software and digitizer tablet (Bioquant, Nashville, TN, USA). Histomorphometric parameters of the secondary spongiosa in the distal femoral metaphysis were measured in standard rectangular area (1.5 mm^2 at least 0.2 mm proximal to the distal femoral growth plate, to exclude primary spongiosa.[22] Parameters were measured at a magnification of 100×. The terminology used was that recommended by the Histomorphometry Nomenclature Committee of the American Society for Bone and Mineral Research.[23]

Statistics

Repeated measures analysis of covariance (ANCOVA) was used to test for significant differences in mean BMD among groups, using weight as a covariate. Factorial ANOVA was used to test for significant differences in parameters other than BMD. Fisher's least significant difference was used for post hoc analysis. Statistical significance was determined as $p \leq 0.05$.

RESULTS

Previously, we have documented that ethanol liquid diet intake induces blood ethanol levels of ~10 mmol/liter in ethanol-fed mice,[17] which represents the blood levels obtained in chronic alcoholics.[24,25] Furthermore, we have reported that exposure to ethanol at these levels results in loss of BMD and increased osteoclastogenesis.[17] However, a direct connection between osteoclastogenesis and ethanol-induced bone loss has not been established previously. To determine if OPG, an inhibitor of osteoclastogenesis, could

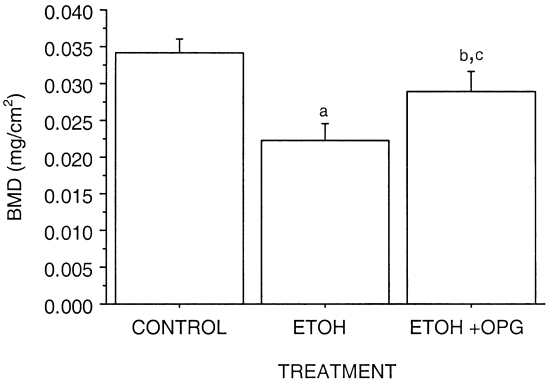

FIG. 1. OPG diminishes ethanol-induced loss of BMD in the distal femur. Mice were fed either a control diet, a 5% ethanol diet, or a 5% ethanol diet plus administration of OPG for 4 months. Femoral BMD was measured from excised left femurs 4 months postinitiation of diet. Data are reported as mean (±SD) femoral BMD. Data were analyzed using ANCOVA using bodyweight as a covariate and Fisher's least significant difference for post hoc analysis. [a]$p < 0.0001$ versus control group; [b]$p = 0.01$ versus control group; [c]$p = 0.001$ versus ETOH group. Measurements were performed in 10 individual mice per group.

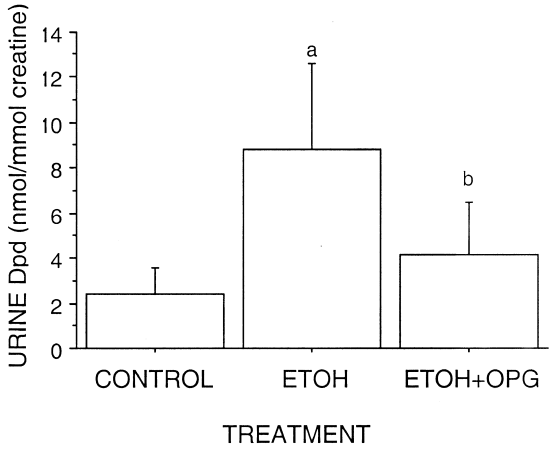

FIG. 2. OPG diminishes the ethanol-induced increase of urinary Dpd. Mice were fed either a control diet, a 5% ethanol diet, or a 5% ethanol diet plus administration of OPG for 4 months. Then, the mice were killed and urine was collected and evaluated for Dpd and creatinine levels as described in the Materials and Methods section. Data are presented as mean (±SD) urinary Dpd corrected for creatinine levels. Data were analyzed using ANOVA and Fisher's least significant difference for post hoc analysis. [a]$p = 0.005$ versus control group; [b]$p = 0.03$ versus ETOH group. Measurements were performed in 6–8 individual mice per group.

prevent the ethanol-induced decline in BMD, mice were either fed either a control diet, a 5% ethanol diet, or a 5% ethanol diet plus administration of OPG for 4 months, followed by measurement of excised right distal femoral BMD using DXA. Distal femur, as opposed to whole femur, was evaluated because it is composed primarily of trabecular bone, and changes of BMD are more readily detectable at this site than at the whole femur (J. Zhang, unpublished observation, 2000). Ethanol induced an ~17.9% loss of distal femoral bone mineral content (BMC); whereas OPG administration cut this approximately in half to 8.9% (Fig. 1). To determine if the OPG-mediated prevention of ethanol-induced BMD loss was associated with diminished bone resorption, urinary Dpd level, an indicator of bone resorption, was quantified. In agreement with the BMD data, ethanol induced a mild increase of Dpd levels, which was diminished by OPG administration (Fig. 2). These data establish that OPG administration partially protects mice from ethanol-induced bone loss. Furthermore, the Dpd data suggest that OPG mediates this effect, in part, through decreasing bone resorption.

Ethanol-induced decline of BMD is associated with both stimulation of osteoclastogenesis and increased RANKL expression in the marrow.[17] Thus, it follows that ethanol may induce osteoclastogenesis through its ability to induce RANKL. To evaluate this hypothesis, we determined the effect of OPG administration on ethanol-induced formation of both bone marrow-derived CFU-GM, which are the osteoclast precursors, and mature osteoclasts. OPG had no effect on CFU-GM formation, which was increased ~28.9% and 18.7% in ethanol-fed mice receiving either vehicle or OPG, respectively, compared with control-fed animals (Fig. 3A). In contrast, ethanol induced an ~32.7% increase of osteoclast-like cells that was completely abrogated by OPG (Fig. 3B). In fact, OPG reduced the number of osteoclast-like cells by ~56.8% below those of the control animals. To ensure that changes of CFU-GM and osteoclast-like cells were not caused by general changes in bone marrow cell number, we determined the number of cells obtained per femur. There was an average of $5.3 \pm 1.51 \times 10^7$ cells/femur and no differences were detected among the groups (data not shown). These data, taken together with our previous demonstration that ethanol induces RANKL expression in the bone marrow,[17] suggest that ethanol induces CFU-GM formation independent of RANKL, but the formation of ethanol-induced osteoclasts is dependent on RANKL expression.

RANKL is expressed on osteoblasts and bone marrow stromal fibroblasts.[26,27] Therefore, it follows that ethanol may up-regulate CFU-F's ability to induce osteoclastogenesis through increased RANKL expression. To test this hypothesis, we determined ethanol-mediated up-regulation of CFU-F–induced osteoclastogenesis and OPG's ability to inhibit this activity. Ethanol induced an ~450% increase in osteoclasts associated with CFU-F (Fig. 4). OPG diminished this by 350%. These data show that OPG can inhibit CFU-F–mediated osteoclastogenesis.

In addition to its ability to inhibit RANKL-induced osteoclastogenesis, OPG can bind TNF-related apoptosis-inducing ligand (TRAIL), a TNF-like molecule that can induce apoptosis. Thus, it is plausible that OPG diminishes loss of BMD through inhibition of osteoblast apoptosis as has been established for PTH.[28] To test this possibility, we measured the effect of ethanol ingestion and OPG administration on CFU-F formation, osteoblast colony formation, and osteoblast function. Both ethanol and OPG alone or combined had no effect on CFU-F formation (Fig. 5A). However, ethanol reduced osteoblast colony formation by 31.8%; OPG completely blocked the ethanol-induced decline of osteoblast function (Fig. 5B). In parallel to the decline in osteoblast colony formation, ethanol ingestion

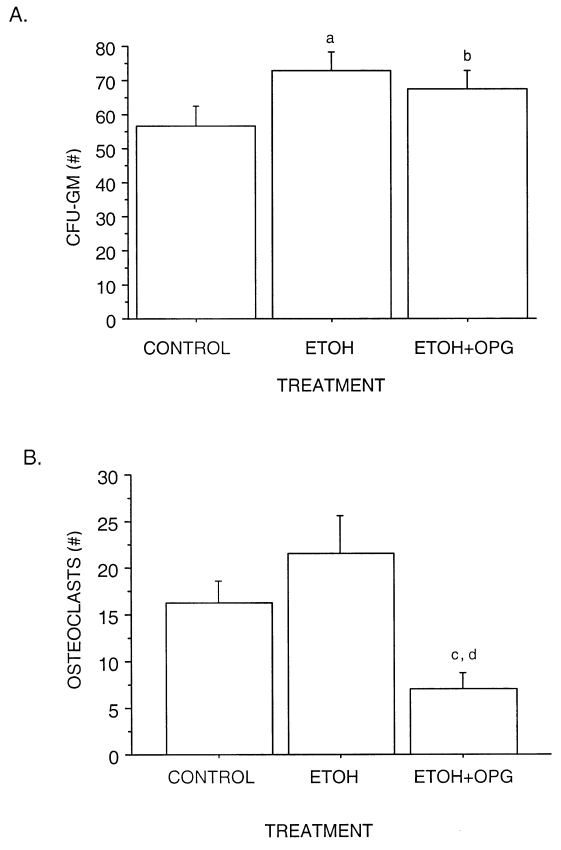

FIG. 3. OPG diminishes osteoclast production but not CFU-GM formation. Mice were fed either a control diet, a 5% ethanol diet, or a 5% ethanol diet plus administration of OPG for 4 months. The mice were then killed and bone marrow was collected from the femur. (A) CFU-GM formation was determined as described in the Materials and Methods section. Data are presented as mean (±SD) CFU-GM formed/5.5×10^5 nucleated bone marrow cells. (B) Osteoclast formation was determined in marrow cultures maintained for 9 days in the presence of 10 nM of $1,25(OH)_2D_3$. Osteoclast numbers were determined by counting cells that bound I-125-calcitonin and were $TRAPase^+$ as described in the Materials and Methods section. Data are presented as mean (±SD) osteoclasts/1.5×10^6 nucleated bone marrow cells. Data were analyzed using ANOVA and Fisher's least significant difference for post hoc analysis. (A) $^a p < 0.0001$ compared with control group; $^b p = 0.005$ compared with ETOH group. (B) $^c p = 0.038$ compared with control group; $^d p = 0.002$ compared with ETOH group. Measurements were performed on 10 individual mice per group.

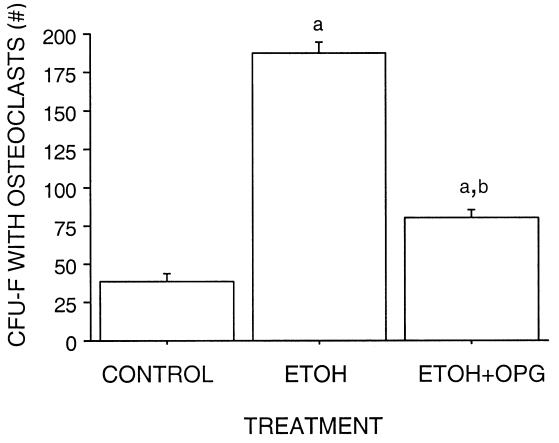

FIG. 4. OPG diminishes CFU-F's ability to support osteoclastogenesis. Mice were fed either a control diet, a 5% ethanol diet, or a 5% ethanol diet plus administration of OPG for 4 months. CFU-F cultures, from individual animals, were established in 96-well plates for 5 days and then single cell suspension from spleens of C57/BL6 mice were added to the wells. The cultures were maintained for an additional 8 days and then stained for TRAP. Each well was then examined microscopically for the presence of CFU-F (>20 cells) and osteoclast-like cells ($TRAP^+$). Data are presented as mean (±SD) CFU-F with osteoclasts. Data were analyzed using ANOVA and Fisher's least significant difference for post hoc analysis. $^a p < 0.0001$ versus control group; $^b p < 0.0001$ versus ETOH group. The mice then were killed and bone marrow was collected from the femur.

was associated with decreased calcium retention in culture and OPG blocked this decline (Fig. 5C). These data show that OPG administration has a protective effect against an ethanol-induced decline of osteoblast production and function.

To determine if these in vitro observations were reflected in vivo, bone histomorphometry was performed on the femur (contralateral to the femur used for obtaining bone marrow cells). Ethanol induced loss of cancellous bone area, which OPG prevented (Table 1). Additionally, ethanol increased the number of osteoclasts per millimeter of bone and the overall osteoclast perimeter and decreased the number of osteoblasts per millimeter of bone and the overall osteoblast perimeter (Table 1). OPG administration prevented the ethanol-induced changes (Table 1). Finally, ethanol ingestion induced a decrease of trabecular width, which OPG abrogated (Table 1). These data are consistent with the in vitro data and show that OPG diminishes ethanol-induced loss of bone at the cellular level in vivo.

DISCUSSION

This study provides strong evidence that OPG prevents an ethanol-mediated decline of BMD through inhibition of osteoclast formation. These data are consistent with previous reports that ethanol induces osteoclastogenesis and inhibits osteoblast colony formation[17] and OPG inhibits osteoclastogenesis.[29] In addition to the novel finding that OPG prevented ethanol-induced osteoclastogenesis, results from this study suggest that OPG diminishes ethanol-mediated inhibition of osteoblast formation.

OPG, a secreted soluble decoy receptor for RANKL, mediates its bone-sparing effects by binding to RANKL, which inhibits RANKL-induced osteoclastogenesis.[29] Administration of recombinant OPG has been shown to increase bone density in mice an rats,[29,30] diminish ovariectomy-mediated loss of bone volume in the proximal tibial metaphysis of mice,[29] prevent tail suspension–induced osteopenia in mice,[31] and rescue OPG-deficient mice from osteoporosis.[32] Adenoviral delivery of OPG was shown to ameliorate bone resorption in a mouse ovariectomy model of osteoporosis.[33] A single subcutaneous dose of OPG was shown to reduce bone turnover as determined by several biochemical markers such as urinary

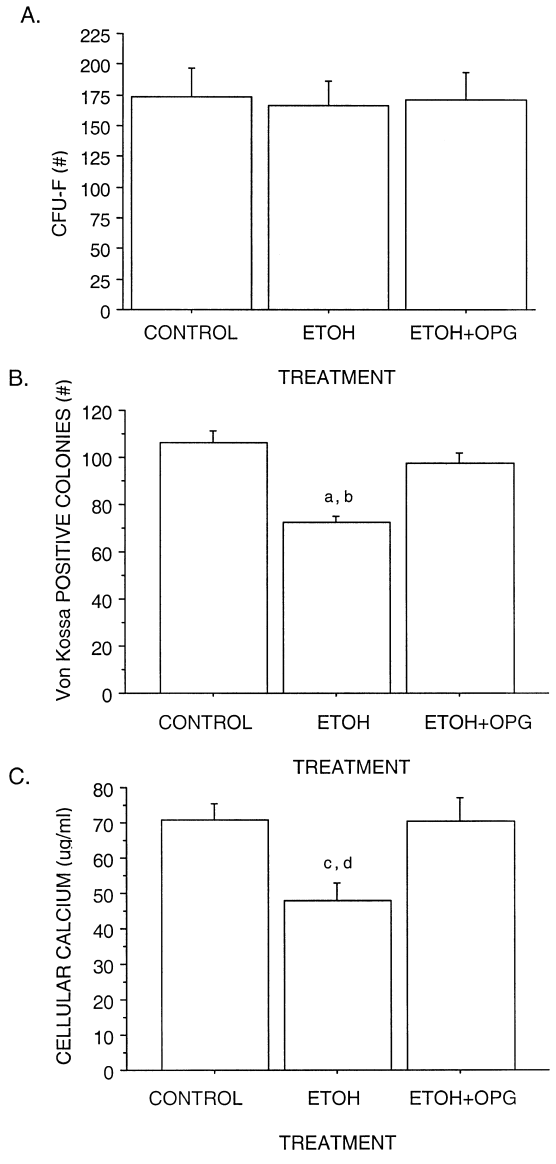

FIG. 5. OPG protects against an ethanol-mediated decline of osteoblast formation and calcium retention but not CFU-F formation in vitro. Mice were fed either a control diet, a 5% ethanol diet, or a 5% ethanol diet plus administration of OPG for 4 months. Then, the mice were killed and bone marrow was collected from the femur. (A) To assess the marrow's ability to support osteoblastogenesis, marrow cultures were maintained for 10 days in the presence of 50 μg/ml of ascorbic acid, 10 mM of β-glycerophosphate. CFU-Fs were determined by counting ALP^+ colonies as described in the Materials and Methods section. (B and C) To assess for the marrows ability to support osteoblast function, marrow cultures were maintained for 25 days in the presence of 50 μg/ml of ascorbic acid and 10 mM of β-glycerophosphate. (B) CFU-OBs were determined by counting von Kossa-positive colonies and (C) total calcium in the cultures was determined as described in the Materials and Methods section. Data are reported as mean ($\pm$SD). Data were analyzed using ANOVA and Fisher's least significant difference for post hoc analysis. (B) von Kossa $^{a}p < 0.0001$ versus control group; $^{b}p = 0.0002$ versus ETOH + OPG group (C) calcium $^{c}p = 0.006$ versus control group; $^{d}p = 0.007$ versus ETOH + OPG group. Measurements were performed on 10 individual mice per group.

N-telopeptide and Dpd in a randomized, double-blind, placebo-controlled clinical study that was conducted in postmenopausal women.[34] Furthermore, OPG administration was shown to decrease osteoclast number and osteoclast perimeter in the proximal tibial metaphysis of hypercalcemic C-26 adenocarcinoma-bearing mice.[35] OPG administration also was shown to inhibit osteoclast activity in cancer cell-induced osteoclastogenesis.[36–40] In agreement with these findings, this report shows that OPG prevented ethanol-mediated osteoporosis and that this was associated with decreased osteoclastogenesis. The absence of a treatment group that received OPG alone renders it difficult to interpret the magnitude of the impact that ethanol had on OPG's ability to modulate bone remodeling. However, the data clearly show that OPG diminished ethanol-mediated bone loss.

Several lines of evidence exist that show that in addition to decreased bone production, ethanol-mediated bone loss is associated with increased bone resorption in chronic alcoholics. For example, chronic alcoholics show increased urinary excretion of hydroxyproline[41] and histomorphometric evidence of increased resorptive surface and osteoclast number measurements.[9,16] Thus, the ability to inhibit ethanol-mediated bone resorption in alcoholics, through compounds such as OPG and bisphosphonates, should inhibit progression of osteopenia in alcoholics.

The observation that OPG diminished ethanol-induced osteoclastogenesis but not CFU-GM formation is consistent with the current knowledge of osteoclast formation. Specifically, pluripotent stem cells become committed to CFU-GM through cytokines such as IL-1 and IL-6. RANKL then acts on the CFU-GM to induce osteoclast formation. Thus, OPG, which inhibits RANKL, should have no impact on CFU-GM formation as opposed to inhibiting osteoclast formation. These results are consistent with the previous report that OPG administration to mice decreased osteoclastogenesis in bone marrow cultures derived from mice administered various cytokines and hormones [IL-1β, TNF-α, PTH, PTH-related peptide (PTHrP), and $1,25(OH)_2D_3$] that are known to increase bone resorption.[42]

We cannot readily account for the observation that OPG administration was associated with decreased ethanol-induced CFU-F–mediated osteoclastogenesis. Specifically, in this assay, the CFU-F are from the mice being treated, while the osteoclast precursors are derived from suspension of spleen from untreated mice. Thus, although it is probable that ethanol ingestion induced RANKL expression in the CFU-F in vivo, which in turn could induce osteoclastogenesis, the mechanism through which OPG administration diminished their ability to induce osteoclastogenesis once the CFU-F are grown in vitro is not clear. OPG should, in theory, not impact the development of the CFU-F, because OPG mediates its effect on RANKL protein. One possibility is that OPG forms a negative feedback mechanism on the RANKL, thus resulting in down-regulation of RANKL production from the CFU-F, thus the CFU-F from the OPG-treated group would express less RANKL, resulting in less production of osteoclasts from the splenic suspensions. Further experiments are required to clarify this activity.

The ability of OPG to prevent an ethanol-mediated decrease of osteoblast colony formation was an unexpected

TABLE 1. STATIC HISTOMORPHOMETRY OF DISTAL FEMORAL CANCELLOUS BONE IN MICE FED A CONTROL DIET, AN ETHANOL DIET, OR AN ETHANOL DIET WITH OPG ADMINISTRATION FOR 4 MONTHS

	Control	*Ethanol*	*Ethanol + OPG*
Cancellous bone area (%)	30.09 ± 0.57	24.42 ± 0.79*	29.56 ± 0.65†
Osteoclast perimeter (%)	2.32 ± 0.12	3.79 ± 0.11*	2.39 ± 0.08†
Osteoclasts/bone perimeter (mm)	1.55 ± 0.11	1.95 ± 0.17‡	1.2 ± 0.10†
Osteoblast perimeter (%)	3.68 ± 0.13	2.95 ± 0.19*	3.43 ± 0.15§
Osteoblasts/bone perimeter (mm)	3.68 ± 0.16	3.12 ± 0.11‡	3.87 ± 0.18†
Trabecular number (mm^{-1})	3.4 ± 0.30	3.7 ± 0.40	3.7 ± 0.30
Trabecular width (μM)	46.24 ± 1.77	41.49 ± 1.47‡	49.53 ± 1.63†
Trabecular spacing (μM)	434.1 ± 11.1	440.5 ± 8.7	450.1 ± 13.5

Data are presented as mean ± SEM ($n = 10$ mice/group).
* $p < 0.01$ versus control; † $p < 0.01$ versus ethanol; ‡ $p < 0.05$ versus control; § $p < 0.05$ versus ethanol.

finding. This finding was supported further by the histomorphometric observation that OPG administration abrogated ethanol's ability to decrease the osteoblast perimeter. Taken together, these observations suggest that OPG promotes bone production through a mechanism other than a direct effect on osteoclastogenesis. This postulation is in line with a previous report that describes that OPG increased the mineral phase of bone production in a murine tail suspension model.[31] The mechanism through which ethanol inhibits osteoblasts currently is not well defined but may involve induction of apoptosis. OPG's ability to bind TRAIL[43] may allow it to inhibit TRAIL-mediated apoptosis, although the affinity for TRAIL at 37°C is low.[44] Thus, OPG may mediate its effect through inhibition of TRAIL-mediated apoptosis or a similar mechanism to inhibit ethanol-mediated apoptosis of osteoblasts.

The observation that ethanol reduced CFU-OB (von Kossa positive) but not CFU-F (ALP^+) cells suggests that there is a differential effect of ethanol based on the maturity of the cell. Specifically, CFU-Fs are less differentiated cells than CFU-OBs and may have been less sensitive to the effects of ethanol. Differential effects based on the state of differentiation have been observed previously in osteoblastic cells. For example, condition media from prostate cancer cells inhibited bone nodule formation and ALP activity more rapidly in a well-differentiated osteosarcoma line than in primary fetal rat calvaria cells, which contain many undifferentiated osteoprogenitor cells.[45]

In conclusion, OPG inhibited ethanol-induced bone loss in mice. The OPG effect on bone was associated with decreasing ethanol-induced osteoclastogenesis and preventing an ethanol-induced decrease of osteoblast formation. These results suggest that in addition to decreased bone production, increased bone resorption contributes to ethanol-induced bone loss. Furthermore, these results suggest that OPG may effectively inhibit bone loss in chronic alcoholics.

ACKNOWLEDGMENT

This work was supported by the National Institutes of Health (NIH) grants R01 AG15904 (to E.T.K.) and T32 RR07008 (to E.T.K.) and the Program in Comparative Integrative Genetics and Biology, University of Michigan.

REFERENCES

1. Bikle DD 1988 Effects of alcohol abuse on bone. Compr Ther **14:**16–20.
2. Bikle DD, Genant HK, Cann C, Recker RR, Halloran BP, Strewler GJ 1985 Bone disease in alcohol abuse. Ann Intern Med **103:**42–48.
3. Feitelberg S, Epstein S, Ismail F, D'Amanda C 1987 Deranged bone mineral metabolism in chronic alcoholism. Metabolism **36:**322–326.
4. Lalor BC, France MW, Powell D, Adams PH, Counihan TB 1986 Bone and mineral metabolism and chronic alcohol abuse. Q J Med **59:**497–511.
5. Laitinen K, Lamberg-Allardt C, Tunninen R, Harkonen M, Valimaki M 1992 Bone mineral density and abstention-induced changes in bone and mineral metabolism in noncirrhotic male alcoholics. Am J Med **93:**642–650.
6. Riggs BL 1987 Pathogenesis of osteoporosis. Am J Obstet Gynecol **156:**1342–1346.
7. Slemenda CW, Christian JC, Reed T, Reister TK, Williams CJ, Johnston CC Jr 1992 Long-term bone loss in men: Effects of genetic and environmental factors. Ann Intern Med **117:**286–291.
8. Friday KE, Howard GA 1991 Ethanol inhibits human bone cell proliferation and function in vitro. Metabolism **40:**562–565.
9. Johnell O, Nilsson BE, Wiklund PE 1982 Bone morphometry in alcoholics. Clin Orthop **165:**253–258.
10. Diamond T, Stiel D, Lunzer M, Wilkinson M, Posen S 1989 Ethanol reduces bone formation and may cause osteoporosis. Am J Med **86:**282–288.
11. Farley JR, Fitzsimmons R, Taylor AK, Jorch UM, Lau KH 1985 Direct effects of ethanol on bone resorption and formation in vitro. Arch Biochem Biophys **238:**305–314.
12. Baran DT, Teitelbaum SL, Bergfeld MA, Parker G, Cruvant EM, Avioli LV 1980 Effect of alcohol ingestion on bone and mineral metabolism in rats. Am J Physiol **238:**E507–E510.
13. Cheung RC, Gray C, Boyde A, Jones SJ 1995 Effects of ethanol on bone cells in vitro resulting in increased resorption. Bone **16:**143–147.
14. Teitelbaum SL 2000 Bone resorption by osteoclasts. Science **289:**504–1508.
15. Suda T, Udagawa N, Takahashi K 1996 Osteoclast generation. In: Bilezikian JP, Raisz LG, Rodan GA (eds.) Principles of Bone Biology. Academic Press, Inc., San Diego, CA, USA, pp. 87–102.
16. Diez A, Puig J, Serrano S, Marinoso ML, Bosch J, Marrugat J, Mellibovsky L, Nogues X, Knobel H, Aubia J 1994 Alcohol-induced bone disease in the absence of severe chronic liver damage. J Bone Miner Res **9:**825–831.

17. Dai J, Lin D, Zhang J, Habib P, Smith P, Murtha J, Fu Z, Yao Z, Qi Y, Keller ET 2000 Chronic alcohol ingestion induces osteoclastogenesis and bone loss through IL-6 in mice. J Clin Invest **106:**887–895.
18. Watzl B, Lopez M, Shahbazian M, Chen G, Colombo LL, Huang D, Way D, Watson RR 1993 Diet and ethanol modulate immune responses in young C57BL/6 mice. Alcohol Clin Exp Res **17:**623–630.
19. Wang Y, Watson RR 1994 Chronic ethanol consumption prior to retrovirus infection alters cytokine production by thymocytes during murine AIDS. Alcohol **11:**361–365.
20. Jilka RL, Weinstein RS, Takahashi K, Parfitt AM, Manolagas SC 1996 Linkage of decreased bone mass with impaired osteoblastogenesis in a murine model of accelerated senescence. J Clin Invest **97:**1732–1740.
21. Lee SK, Goldring SR, Lorenzo JA 1995 Expression of the calcitonin receptor in bone marrow cell cultures and in bone: A specific marker of the differentiated osteoclast that is regulated by calcitonin. Endocrinology **136:**4572–4581.
22. Kimmel DB, Bozzato RP, Kronis KA, Coble T, Sindrey D, Kwong P, Recker RR 1993 The effect of recombinant human (1–84) or synthetic human (1-34) parathyroid hormone on the skeleton of adult osteopenic ovariectomized rats. Endocrinology **132:**1577–1584.
23. Parfitt AM, Drezner MK, Glorieux FH, Kanis JA, Malluche H, Meunier PJ, Ott SM, Recker RR 1987 Bone histomorphometry: Standardization of nomenclature, symbols, and units. Report of the ASBMR Histomorphometry Nomenclature Committee. J Bone Miner Res **2:**595–610.
24. Haeckel R, Bucklitsch I 1987 The comparability of ethanol concentrations in peripheral blood and saliva. The phenomenon of variation in saliva to blood concentration ratios. J Clin Chem Clin Biochem **25:**199–204.
25. Winek CL, Esposito FM 1987 The validity of urine alcohol analysis in drunk drivers. Leg Med 97–106.
26. Fuller K, Wong B, Fox S, Choi Y, Chambers TJ 1998 TRANCE is necessary and sufficient for osteoblast-mediated activation of bone resorption in osteoclasts. J Exp Med **188:**997–1001.
27. Nakashima T, Kobayashi Y, Yamasaki S, Kawakami A, Eguchi K, Sasaki H, Sakai H 2000 Protein expression and functional difference of membrane-bound and soluble receptor activator of NF-kappaB ligand: Modulation of the expression by osteotropic factors and cytokines. Biochem Biophys Res Commun **275:**768–775.
28. Jilka RL, Weinstein RS, Bellido T, Roberson P, Parfitt AM, Manolagas SC 1999 Increased bone formation by prevention of osteoblast apoptosis with parathyroid hormone. J Clin Invest **104:**439–446.
29. Simonet WS, Lacey DL, Dunstan CR, Kelley M, Chang MS, Luthy R, Nguyen HQ, Wooden S, Bennett L, Boone T, Shimamoto G, DeRose M, Elliott R, Colombero A, Tan HL, Trai LG, Sullivan J, Davy E, Bucay N, Renshaw-Gegg L, Hughes TM, Hill D, Pattison W, Campbell P, Boyle WJ 1997 Osteoprotegerin: A novel secreted protein involved in the regulation of bone density. Cell **89:**309–319.
30. Yasuda H, Shima N, Nakagawa N, Mochizuki SI, Yano K, Fujise N, Sato Y, Goto M, Yamaguchi K, Kuriyama M, Kanno T, Murakami A, Tsuda E, Morinaga T, Higashio K 1998 Identity of osteoclastogenesis inhibitory factor (OCIF) and osteoprotegerin (OPG): A mechanism by which OPG/OCIF inhibits osteoclastogenesis in vitro. Endocrinology **139:**1329–1337.
31. Bateman TA, Dunstan CR, Ferguson VL, Lacey DL, Ayers RA, Simske SJ 2000 Osteoprotegerin mitigates tail suspension-induced osteopenia. Bone **26:**443–449.
32. Min H, Morony S, Sarosi I, Dunstan CR, Capparelli C, Scully S, Van G, Kaufman S, Kostenuik PJ, Lacey DL, Boyle WJ, Simonet WS 2000 Osteoprotegerin reverses osteoporosis by inhibiting endosteal osteoclasts and prevents vascular calcification by blocking a process resembling osteoclastogenesis. J Exp Med **192:**463–474.
33. Bolon B, Carter C, Daris M, Morony S, Capparelli C, Hsieh A, Mao M, Kostenuik P, Dunstan CR, Lacey DL, Sheng JZ 2001 Adenoviral delivery of osteoprotegerin ameliorates bone resorption in a mouse ovariectomy model of osteoporosis. Mol Ther **3:**197–205.
34. Bekker PJ, Holloway D, Nakanishi A, Arrighi M, Leese PT, Dunstan CR 2001 The effect of a single dose of osteoprotegerin in postmenopausal women. J Bone Miner Res **16:**348–360.
35. Capparelli C, Kostenuik PJ, Morony S, Starnes C, Weimann B, Van G, Scully S, Qi M, Lacey DL, Dunstan CR 2000 Osteoprotegerin prevents and reverses hypercalcemia in a murine model of humoral hypercalcemia of malignancy. Cancer Res **60:**783–787.
36. Zhang J, Dai J, Qi Y, Lin DL, Smith P, Strayhorn C, Mizokami A, Fu Z, Westman J, Keller ET 2001 Osteoprotegerin inhibits prostate cancer-induced osteoclastogenesis and prevents prostate tumor growth in the bone. J Clin Invest **107:**1235–1244.
37. Honore P, Luger NM, Sabino MA, Schwei MJ, Rogers SD, Mach DB, O'Keefe P, F., Ramnaraine ML, Clohisy DR, Mantyh PW 2000 Osteoprotegerin blocks bone cancer-induced skeletal destruction, skeletal pain and pain-related neurochemical reorganization of the spinal cord. Nat Med **6:**521–528.
38. Luger NM, Honore P, Sabino MA, Schwei MJ, Rogers SD, Mach DB, Clohisy DR, Mantyh PW 2001 Osteoprotegerin diminishes advanced bone cancer pain. Cancer Res **61:**4038–4047.
39. Morony S, Capparelli C, Sarosi I, Lacey DL, Dunstan CR, Kostenuik PJ 2001 Osteoprotegerin inhibits osteolysis and decreases skeletal tumor burden in syngeneic and nude mouse models of experimental bone metastasis. Cancer Res **61:**4432–4436.
40. Clohisy DR, Ramnaraine ML, Scully S, Qi M, Van G, Tan HL, Lacey DL 2000 Osteoprotegerin inhibits tumor-induced osteoclastogenesis and bone tumor growth in osteopetrotic mice. J Orthop Res **18:**967–976.
41. Pepersack T, Fuss M, Otero J, Bergmann P, Valsamis J, Corvilain J 1992 Longitudinal study of bone metabolism after ethanol withdrawal in alcoholic patients. J Bone Miner Res **7:**383–387.
42. Morony S, Capparelli C, Lee R, Shimamoto G, Boone T, Lacey DL, Dunstan CR 1999 A chimeric form of osteoprotegerin inhibits hypercalcemia and bone resorption induced by IL-1beta, TNF-alpha, PTH, PTHrP, and 1, 25(OH)2D3. J Bone Miner Res **14:**1478–1485.
43. Emery JG, McDonnell P, Burke MB, Deen KC, Lyn S, Silverman C, Dul E, Appelbaum ER, Eichman C, DiPrinzio R, Dodds RA, James IE, Rosenberg M, Lee JC, Young PR 1998 Osteoprotegerin is a receptor for the cytotoxic ligand TRAIL. J Biol Chem **273:**14363–14367.
44. Truneh A, Sharma S, Silverman C, Khandekar S, Reddy MP, Deen KC, McLaughlin MM, Srinivasula SM, Livi GP, Marshall LA, Alnemri ES, Williams WV, Doyle ML 2000 Temperature-sensitive differential affinity of TRAIL for its receptors. DR5 is the highest affinity receptor. J Biol Chem **275:**23319–23325.
45. Kido J, Yamauchi N, Ohishi K, Kataoka M, Nishikawa S, Nakamura T, Kadono H, Ikedo D, Ueno A, Nonomura N, Okuyama A, Nagata T 1997 Inhibition of osteoblastic cell differentiation by conditioned medium derived from the human prostatic cancer cell line PC-3 in vitro. J Cell Biochem **67:**248–256.

Address reprint requests to:
Evan T. Keller, D.V.M., Ph.D.
Room 5304 CCGCB, Box 0940
1500 East Medical Center Drive
Ann Arbor, MI 48109-0940, USA

Received in original form August 13, 2001; in revised form January 13, 2002; accepted February 6, 2002.