HHS Public Access

Author manuscript

J Bone Miner Res. Author manuscript; available in PMC 2023 December 15.

Published in final edited form as:

J Bone Miner Res. 2006 September ; 21(9): 1350–1358. doi:10.1359/jbmr.060610.

Role of IGF-I Signaling in Regulating Osteoclastogenesis

Yongmei Wang,

Shigeki Nishida,

Hashem Z Elalieh,

Roger K Long,

Bernard P Halloran,

Daniel D Bikle

Department of Medicine, Endocrine Unit, Veterans Affairs Medical Center, and University of California, San Francisco, California, USA.

Abstract

We showed that IGF-I deficiency impaired osteoclastogenesis directly and/or indirectly by altering the interaction between stromal/osteoblastic cells and osteoclast precursors, reducing RANKL and M-CSF production. These changes lead to impaired bone resorption, resulting in high BV/TV in IGF-I null mice.

Introduction: Although IGF-I has been clearly identified as an important growth factor in regulating osteoblast function, information regarding its role in osteoclastogenesis is limited. Our study was designed to analyze the role of IGF-I in modulating osteoclastogenesis using IGF-I knockout mice (IGF-$I^{-/-}$).

Materials and Methods: Trabecular bone volume (BV/TV), osteoclast number, and morphology of IGF-$I^{-/-}$ or wildtype mice (IGF-$I^{+/+}$) were evaluated in vivo by histological analysis. Osteoclast precursors from these mice were cultured in the presence of RANKL and macrophage-colony stimulating factor (M-CSF) or co-cultured with stromal/osteoblastic cells from either genotype. Osteoclast formation was assessed by measuring the number of multinucleated $TRACP^{+}$ cells and pit formation. The mRNA levels of osteoclast regulation markers were determined by quantitative RT-PCR.

Results: In vivo, IGF-$I^{-/-}$ mice have higher BV/TV and fewer (76% of IGF-$I^{+/+}$) and smaller osteoclasts with fewer nuclei. In vitro, in the presence of RANKL and M-CSF, osteoclast number (55% of IGF-$I^{+/+}$) and resorptive area (30% of IGF-$I^{+/+}$) in osteoclast precursor cultures from IGF-$I^{-/-}$ mice were significantly fewer and smaller than that from the IGF-$I^{+/+}$ mice. IGF-I (10 ng/ml) increased the size, number (2.6-fold), and function (resorptive area, 2.7-fold) of osteoclasts in cultures from IGF-$I^{+/+}$ mice, with weaker stimulation in cultures from IGF-$I^{-/-}$ mice. In co-cultures of IGF-$I^{-/-}$ osteoblasts with IGF-$I^{+/+}$ osteoclast precursors, or IGF-$I^{+/+}$ osteoblasts with IGF-$I^{-/-}$ osteoclast precursors, the number of osteoclasts formed was only 11% and 48%,

Address reprint requests to: *Daniel D Bikle, MD, PhD, Endocrine Unit (111N), VAMC, 4150 Clement Street, San Francisco, CA 94121, USA*, daniel.bikle@ucsf.edu.

The authors state that they have no conflicts of interest.

respectively, of that from co-cultures of IGF-I$^{+/+}$ osteoblasts and IGF-I$^{+/+}$ osteoclast precursors. In the long bones from IGF-I$^{-/-}$ mice, mRNA levels of RANKL, RANK, M-CSF, and c-*fms* were 55%, 33%, 60%, and 35% of that from IGF-I$^{+/+}$ mice, respectively.

Conclusions: Our results indicate that IGF-I regulates osteoclastogenesis by promoting their differentiation. IGF-I is required for maintaining the normal interaction between the osteoblast and osteoclast to support osteoclastogenesis through its regulation of RANKL and RANK expression.

Keywords

IGF-I; osteoclastogenesis; RANKL; cell–cell interaction; cell culture

INTRODUCTION

Bone is a dynamic tissue, being continually resorbed and reformed to maintain bone mass and skeletal homeostasis. Two specialized cells are responsible for bone remodeling. osteoblasts synthesize and deposit bone matrix, whereas osteoclasts resorb bone. Therefore, bone mass is dependent on the relative function of these types of cells. An imbalance between osteoclast and osteoblast activities can result in skeletal abnormalities characterized by a net loss (osteoporosis) or gain (osteosclerosis)[1-4] IGF-I has been shown to be an important factor in coupling bone remodeling.[5-7] Skeletal IGF-I originates from two sources: de novo synthesis by bone forming cells, and the circulation and/or marrow. It acts in diverse patterns, through a combination of endocrine, autocrine, and paracrine modes of action, to regulate the functions of both osteoblasts and osteoclasts.[8-10] Compared with the comprehensive studies on the role of the IGF-I in regulating osteoblast function, information regarding its effects on osteoclasts is limited and conflicting.[5,10] As reported, osteoclasts are multinucleated cells derived from hematopoietic stem cells (HSCs).[11,12] These cells give rise to monocyte/macrophage progenitor cells and differentiate into mononuclear osteoclasts. The mononuclear osteoclasts already have bone-resorbing activity and express various osteoclastic molecules such as integrin αv β3, TRACP, calcitonin receptor (CTR), and matrix metalloproteinase 9 (MMP9).

They then fuse with each other to form multinuclear osteoclasts. The cytoskeletal system is reorganized in multi-nuclear osteoclasts to generate osteoclast-specific structures such as the sealing zone and ruffled borders and to develop a transcytosis system to discharge the resorbed bone debris.[2,13] The interactions between osteoblasts/bone marrow stromal cells and osteoclast precursors are required for osteoclast formation.[14] Two molecules that are produced by osteoblasts/marrow stromal cells are essential and sufficient to support osteoclastogenesis: macrophage colony-stimulating factor (M-CSF) and RANKL.[1,15] Many genes and factors have been shown to positively or negatively regulate osteoclastogenesis and osteoclast activation directly and/or indirectly through the interactions between the osteoblasts/bone marrow stromal cells and osteoclast precursors.[15,16] These genes and factors exert their effects at various stages of osteoclast development and activation. Disruption of these genes and factors may block the development and/or function of the mature osteoclast. Type I IGF-I receptor is present on both osteoblasts and osteoclasts,[17] suggesting that IGF-I may directly or through its action on osteoblasts/bone marrow stromal cells stimulate osteoclast formation and function.[18] Our previous

study showed that, in IGF-I null mice, the balance of remodeling between formation and resorption is altered in favor of formation,[6] resulting in high trabecular bone volume (BV/TV). Thus, IGF-I deficiency may lead to a relative decrease in bone resorption. To determine the role of IGF-I in bone resorption, we analyzed the bone structure and osteoclast morphology by histology, evaluated the direct effects of IGF-I on osteoclastogenesis in vitro, and determined the impact of IGF-I deficiency on the ability of the osteoblast to stimulate osteoclastogenesis.

MATERIALS AND METHODS

Mice

The IGF-I–deficient mice used in this study were developed by Lyn Powell-Braxton at Genentech (San Francisco, CA, USA)[19] and were backcrossed into a CD-1 genetic background. The construct used for the homologous recombination contained a neomycin cassette inserted into exon 3 of the *IGF-I* gene. Genotyping of the offspring used PCR to identify either the intact exon 3 or neomycin as described.[19] The IGF-$I^{+/+}$ and IGF-$I^{-/-}$ mice used in this study were littermates. These studies were approved by the Animal Use Committee of the San Francisco Veterans Affairs Medical Center, where the animals were raised and studied.

Histology

For histological analyses, femurs and tibias from IGF-$I^{+/+}$ and IGF-$I^{-/-}$ mice were fixed overnight at 4°C in 4% paraformaldehyde in PBS (4% PFA/PBS), rinsed in PBS, dehydrated through an ethanol series, cleared in xylene, embedded in paraffin, and cut into 5-μm sections. The sections were stained with H&E or with TRACP using a kit (produce number 387; Sigma) according to the manufacturer's instructions. Mature osteoclasts were defined as $TRACP^+$ multinucleated cells that contained at least three nuclei.

Hematopoietic osteoclast precursor culture

As a source of osteoclast precursors, spleen hematopoietic cells from 3-week-old IGF-$I^{+/+}$ and IGF-$I^{-/-}$ mice were harvested, the erythrocytes lysed with RBC lysis buffer (0.16 M NH_4Cl, 0.17 M Tris; pH 7.65) for 2 minutes at room temperature, and the remaining cells cultured in α-MEM (Mediatech, Herndon, VA, USA), supplemented with 10% heat-inactivated FBS (Atlanta Biologicals, Norcross, GA, USA), 100 U/ml penicillin/streptomycin (Mediatech), and 0.25 μg/ml fungizone (Life Technologies, Rockville, MD, USA; primary medium) overnight. Pilot experiments showed that this medium contained no detectable biologically active IGF-I. Nonadherent osteoclast precursors were transferred to 24-well plates (5×10^5/well) and cultured with RANKL 30 ng/ml (Sigma) and 10 ng/ml M-CSF (Sigma) for an additional 7 days, with addition of fresh media every 3 days. Human recombinant IGF-I (10 ng/ml) or vehicle was added when cells were transferred to 24-well plates and with each medium change. In some experiments, we determined whether the effects of IGF-I on osteoclast differentiation would be neutralized by IGF-I antibody. Mouse monoclonal IGF-I antibody (Upstate Cell Signaling Solutions, Lake Placid, NY, USA) was added to the cultures (0–15 μg/ml) from the IGF-$I^{+/+}$ mice at the same time as vehicle or IGF-I. TRACP staining was performed using a commercial kit (387 A; Sigma).

Co-culture of hematopoietic cells and osteoblasts

As a source of osteoblasts, bone marrow stromal cells from 3-week-old IGF-I$^{+/+}$ or IGF-I$^{-/-}$ mice were flushed out with a syringe and 26-gauge needle and collected in primary culture medium. The marrow cell suspension was gently drawn through an 18-gauge needle to mechanically dissociate the mixture into a uniform single cell suspension and then cultured in T-75 flasks at a density of 100 × 10^6 cells/flask. When the cultures reached 70–80% confluence, the cells (stromal/osteoblastic cells) were harvested by 1× trypsin-EDTA (0.05% trypsin, 0.53 mM EDTA; Cellgro), replated at 20,000 cells/well in 24-well plates (n = 3–6 wells), and incubated in primary medium supplemented with ascorbic acid 50 μg/ml (secondary medium) for 4 or more days, with media changes every 2 days. Spleen cells (including osteoclast precursors, stromal cell free) were prepared from a separate group of 3-week-old IGF-I$^{+/+}$ or IGF-I$^{-/-}$ mice, previously cultured for 2 days and added (1 × 10^6 cells/well) to the stromal/osteoblastic cell cultures to create stromal/osteoblastic cell-spleen cell co-cultures. The co-cultures were carried for 7 days in secondary media, with media changes every 2 days. On day 7 of the co-culture, the cells were rinsed with PBS and prepared for TRACP staining. To study the effects of addition of exogenous IGF-I on osteoblasts, stromal/osteoblastic cells were pretreated by IGF-I (10 ng/ml) at the beginning of the cell culture and up until the time when osteoclast precursors were added.

Resorption assays

Osteoclast precursors (spleen cells) were harvested as described above. Cells (5 × 10^4) were plated on dentine discs (IDS, Tyne and Wear, UK) in 96-well plates and treated with RANKL 70 ng/ml and M-CSF 10 ng/ml for 5–10 days. Media were changed every 3 days. Dentine discs were washed once, and the cells were removed with Kim Warp before staining with 1% toluidine blue in 0.5% sodium tetraborate solution. Resorption pits were visualized by light microscopy, and digital images were recorded. The resorption area was determined by NIH Imaging J software.

Quantitative real-time PCR

RNA was extracted from the long bones (bone marrow flushed out) of IGF-I$^{+/+}$ and IGF-I$^{-/-}$ mice. mRNA levels of osteoclast differentiation markers were determined by quantitative real-time PCR as described before.[20] Primers and probes are as follows: GAPDH (forward: 5′-TGCACCACCAACTGCTTAG-3′; reverse: 5- GGATGCAGGGATGATGTTC-3′; probe: 5′-CAGAAGACTGTGGATGGCCCCTC-3′), RANKL (forward: 5′-GGCCACAGCGCTTCTCAG-3′; reverse: 5′-GAGTGACTTTATGGGAACCCGAT-3′; probe: 5′-CAGCTATGATGGAAGGCTCATGGTTGGA-3′), osteoprotegrin (OPG) (forward: 5′AG-TCCGTGAAGCAGGAGTG-3′; reverse: 5′-CCATCTGGACATTTTTTGCAAA-3′; probe: 5′-TGCAAGCTGGAACCCCAGAGCC-3′), RANK (forward: 5′-GGTCTGCAGCTCTTCCATGAC-3′; reverse: 5′-GAAGAGGAGCAGAACGATGAGACT-3′; probe: 5′-ACCACCCAAGGAGGCCCAGGCTTA-3′), calcitonin receptor (forward: 5′-TCCAAGGAGGTCCAGAGTGAA-3′; reverse: 5′-TGGGCTCACTAGGGAGCAGGA-3′; probe: 5′-CGGAATCTCCGCAAAACCGAAGATG-3′), c-*fms* (forward: 5′-CATCCACGCTGCGTGAAG-3′; reverse: 5′-GGGATTCGGTGTCGCAATAT-3′;

probe: 5′-CCACCAGGAGCCAGGCTCTCCC-3′), and M-CSF (forward: 5′-TTCAAGCTCTTTCTGAACCGTGTA-3′; reverse: 5′-GCCTTGTTTTGTGCCATTAAGAAG-3′; probe: 5′-AGGCCTCAGAGGCGGGCCA-3′).

RESULTS

In vivo skeletal findings

A striking phenotype of the IGF-I$^{-/-}$ mice was an obvious dwarfism, including small body size (body weight, 3.9 ± 0.9 g in IGF-I$^{-/-}$ versus 10.9 ± 2.4 g in IGF-I$^{+/+}$; $p < 0.05$) and short limbs. Our previous study identified higher BV/TV in the IGF-I$^{-/-}$ mice than in the IGF-I$^{+/+}$ mice by μCT.[6] In this study, we further analyzed the bone structures of these mice by histological analysis. By H&E staining, tibial sections from the 3-week-old IGF-I$^{-/-}$ mice showed significantly higher trabecular bone volume (Figs. 1A and 1B) but did not exhibit severe osteopetrosis. BV/TV of IGF-I$^{-/-}$ mice was 30% higher than that of IGF-I$^{+/+}$ mice (Fig. 1C). To determine whether the high trabecular bone volume in the IGF-I$^{-/-}$ mice was caused by abnormalities in the osteoclasts, the tibial sections from the IGF-I$^{+/+}$ and IGF-I$^{-/-}$ mice were stained for the osteoclast specific marker, TRACP. TRACP^{+} cells were readily detected in the primary and secondary spongiosa of both IGF-I$^{+/+}$ and IGF-I$^{-/-}$ mice (Figs. 2A and 2B), although the number of TRACP^{+} cells/bone surface (BS) was significantly less in the IGF-I$^{-/-}$ mice (76% of WT, $p < 0.05$). The morphology and size of TRACP^{+} cells observed in the IGF-I$^{-/-}$ mice were dramatically different from those observed in the IGF-I$^{+/+}$ mice (Fig. 2B). In the IGF-I$^{+/+}$ mice, TRACP^{+} cells were predominantly (60 ± 12%) multinucleated (Fig. 2A). In contrast, significantly fewer (28 ± 5%) multinucleated TRACP^{+} cells were identified in IGF-I$^{-/-}$ mice (Fig. 2B). These results indicated that IGF-I is important for bone resorption and osteoclast formation.

Impaired osteoclastogenesis in the absence of IGF-I

The following experiments were performed to determine whether the defects in osteoclast formation in IGF-I$^{-/-}$ mice were intrinsic to the osteoclast precursors and/or to the osteoblasts. First, osteoclastogenesis was evaluated by TRACP staining of the osteoclast precursors (spleen cells) in cultures from the IGF-I$^{+/+}$ and IGF-I$^{-/-}$ mice in response to M-CSF and RANKL. In the cultures from the IGF-I$^{+/+}$ mice, TRACP^{+} mononuclear and binuclear pre-osteoclasts and a few small multinuclear osteoclasts appeared by day 3 (data not shown). Large multinucleated TRACP^{+} osteoclasts were formed by day 7 (Fig. 3A). However, in the cultures from the IGF-I$^{-/-}$ mice, fewer TRACP^{+} cells were observed (55% of IGF-I$^{+/+}$ mice, $p < 0.05$), their size was smaller than that observed in the culture from the IGF-I$^{+/+}$ mice, and most of them were mononuclear or binuclear (Fig. 3B). Very few fully differentiated multinuclear osteoclasts were observed in the cultures from IGF-I$^{-/-}$ mice (230 ± 33 nuclei/100 TRACP^{+} cells in IGF-I$^{-/-}$ versus 560 ± 49 nuclei/100 TRACP^{+} cells in IGF-I$^{+/+}$, $n = 6$ wells in each group, $p < 0.05$; Fig. 3B). We verified if the abnormalities of osteoclasts in the culture from IGF-I$^{-/-}$ mice could be rescued by IGF-I treatment. IGF-I (10 ng/ml) significantly increased the number (2.0-fold in IGF-I$^{-/-}$ versus 2.6-fold in IGF-I$^{+/+}$) and size of TRACP^{+} cells in cultures from both IGF-I$^{+/+}$ and IGF-I$^{-/-}$ mice (Figs. 3C and 3D), but in the cultures from the IGF-I$^{-/-}$ mice, the effects were weaker than those from the IGF-I$^{+/+}$ mice. The number of nuclei increased in the cultures from the IGF-I$^{-/-}$ mice (230

± 33 nuclei/100 $TRACP^+$ cells in vehicle-treated cells versus 298 ± 35 nuclei/100 $TRACP^+$ cells in IGF-I–treated cells; Fig. 3D), but was not as substantial as the increase in nuclei in the cultures from the $IGF\text{-}I^{+/+}$ mice (560 ± 49 nuclei/100 $TRACP^+$ cells in vehicle-treated cells versus 736 ± 80 nuclei/100 $TRACP^+$ cells in IGF-I–treated cells; Fig. 3C). Most of the $TRACP^+$ cells in the cultures from the $IGF\text{-}I^{-/-}$ mice were still mononuclear or binuclear (Fig. 3D). To further confirm the local effects of IGF-I on osteoclasts, we added neutralizing antibody to IGF-I to osteoclast precursors from the $IGF\text{-}I^{+/+}$ mice to test if the antibody could block osteoclastogenesis. IGF-I antibody dose-dependently blocked the basal rate of osteoclastogenesis and the stimulatory effects of IGF-I (Fig. 4A). Multinucleation was also reduced (Figs. 4B-4E). These results mimic the effects of IGF-I deficiency in vivo. Finally, we determined the effects of IGF-I on osteoclast function by measuring pit formation (Fig. 5). The resorptive pits generated by osteoclasts derived from the $IGF\text{-}I^{-/-}$ mice were very small (33% of $IGF\text{-}I^{+/+}$) compared with osteoclasts from the $IGF\text{-}I^{+/+}$ mice. IGF-I (10 ng/ml) significantly increased the resorptive areas in the cultures from both genotypes. These data indicate that the defects of osteoclastogenesis in the $IGF\text{-}I^{-/-}$ mice are at least in part intrinsic to the osteoclast lineage. Local IGF-I directly affects osteoclast progenitor differentiation in vitro. Similar results are obtained when bone marrow stromal cells were used instead of spleen cells as a source of osteoclast precursors (data not shown). The next question is whether IGF-I deficiency also alters the ability of osteoblasts to regulate osteoclastogenesis.

Osteoblastic cells from $IGF\text{-}I^{-/-}$ mice are defective in supporting osteoclast differentiation

To determine whether osteoblast induction of osteoclastogenesis is also defective in the $IGF\text{-}I^{-/-}$ mice, we performed in vitro co-culture experiments in which the generation of functional osteoclasts from the hematopoietic precursors (spleen cells) is dependent on supportive stromal cells/ osteoblasts. As shown in Fig. 6, in cultures of the osteoblast/ stromal cells from the $IGF\text{-}I^{-/-}$ mice and the osteoclast precursors (spleen cells) from the $IGF\text{-}I^{+/+}$ mice (KOOB:WTSP), the number of osteoclasts was significantly decreased to 11% of the co-cultures of the osteoblast/stromal cells and spleen cells from the $IGF\text{-}I^{+/+}$ mice (WTOB:WTSP). Osteoclast formation was less affected when $IGF\text{-}I^{+/+}$ osteoblasts were co-cultured with spleen cells from $IGF\text{-}I^{-/-}$ mice (WTOB:KOOC; number of osteoclasts was 48% of WTOB:WTSP). The defect in the ability of $IGF\text{-}I^{-/-}$ osteoblast/ stromal cells to support osteoclastogenesis was partially rescued by adding IGF-I 10 ng/ml to the osteoblast/stromal cells (number of osteoclasts was 58% of WTOB:WTOC). These results indicate that the defects in osteoclastogenesis in the $IGF\text{-}I^{-/-}$ mice involved both the osteoblasts and osteoclast precursors but appear more profound in the $IGF\text{-}I^{-/-}$ osteoblasts. The results are comparable when cells from the bone marrow are used instead of spleen cells as the source of osteoclast precursors (data not shown).

Impaired expression of osteoclastic markers in the absence of IGF-I

We assessed the expression of osteoclast-specific genes by quantitative real-time PCR in the bones from $IGF\text{-}I^{-/-}$ (KO) and $IGF\text{-}I^{+/+}$ (WT) mice. As shown in Fig. 7, mRNA levels of RANKL and its receptor RANK were significantly less in the $IGF\text{-}I^{-/-}$ mice (55% and 33% of WT, respectively), whereas its decoy receptor OPG was not significantly affected (78% WT). We found a comparable reduction (58% of WT) in RANKL mRNA in osteoblast

cultures from IGF-I$^{-/-}$ mice compared with IGF-I$^{+/+}$ mice and in RANK mRNA levels (35% of WT) in osteoclast culture. mRNA levels of M-CSF and its receptor c-*fms* were also reduced in the bone of IGF-I$^{-/-}$ mice (60% and 35% of WT, respectively), as were the mRNA levels of nuclear factor of activated T cell c1 (NFATc1; 18% of the WT). Calcitonin receptor (CTR) mRNA levels were also reduced (63% of WT), but the reduction did not reach statistical significance ($p > 0.05$). These results are consistent with the reduction of osteoclasts in the bone of IGF-I$^{-/-}$ mice, a reduction in the signaling within the osteoclast precursors that leads to osteoclast formation, and a reduction in those factors produced by the osteoblasts that stimulate osteoclastogenesis.

DISCUSSION

In this study, in vivo histological analysis showed that IGF-I$^{-/-}$ mice exhibited high trabecular bone volume associated with reduced numbers and size of osteoclasts. In vitro experiments revealed that, in the absence of IGF-I, the defects of osteoclastogenesis reside both in the osteoclast and in the osteoblast/stromal cells that induce osteoclastogenesis.

IGF-I maintains bone mass by coupling bone resorption to bone formation in vivo

Bone formation and bone resorption are coupled and under strict control for maintaining normal bone mass. A surprising observation from our previous[6,20] and current studies is that the IGF-I$^{-/-}$ mouse has a decreased bone formation rate,[20] but increased trabecular bone volume. These mice have fewer and smaller osteoclasts with fewer nuclei, suggesting that bone resorption is also reduced in these mice. IGF-I deficiency apparently decreases bone resorption more than bone formation. Such results confirm that IGF-I plays an important role in coupling bone resorption to bone formation.[21]

IGF-I directly stimulates osteoclast differentiation and function

During osteoclastogenesis, hematopoietic stem cell precursors acquire the potential to become either osteoclasts or macrophages. After proliferation, committed precursors differentiate to become either cell type. Deletion of genes encoding critical molecules that regulate osteoclastogenesis (e.g., PU.1 or c-*fos*) during this sequence results in a failure of osteoclast formation with or without an impact on macrophage production.[22,23] IGF-I seems not to be critical for the initial commitment to osteoclast or macrophage lineages, because in the presence of RANKL and M-CSF, the hematopoietic stem cell precursors from the IGF-I$^{-/-}$ mice form osteoclasts, although these osteoclasts are fewer in number with marked morphological and functional abnormalities. Thus, the defects in osteoclasts observed in the IGF-I$^{-/-}$ mice likely occurred later in their differentiation process. Takeshita et al.[24] divided the osteoclast differentiation process into three stages: the pro-osteoclast (spindle-shaped macrophage cells), the pre-osteoclast (small round mononucleated TRACP^{+} cells), and the mature osteoclast (multinucleated TRACP^{+} cells, formed by the fusion of mononuclear pre-osteoclast). IGF-I deficiency apparently affects multinucleation of these cells, because in the presence of M-CSF and RANKL, osteoclast precursors from IGF-I$^{-/-}$ mice formed only mononuclear osteoclasts. The RANK signaling pathway is important for the multinucleation of osteoclast precursor through upregulation of NFATc1 expression.[25-27] In the IGF-I$^{-/-}$ mice, absence of IGF-I was associated with decreased expression

of RANK and NFATc1, blunting the RANKL/RANK signaling transduction resulting in decreased multinucleation. Although mononuclear $TRACP^+$ cells are able to resorb bone,[16] their bone resorptive ability is lower than multinucleated osteoclasts,[28,29] resulting in smaller pit formation in dentine and high BV/TV in mice. Pre-osteoclasts and osteoclasts express the IGF-I receptor[17,30] and produce IGF-I.[31] In the hematopoietic osteoclast precursor culture system (stromal cell free), IGF-I may act directly in an autocrine manner on osteoclast precursors to stimulate their differentiation. This was shown in hematopoietic osteoclast precursor cultures from the IGF-$I^{+/+}$ mice in which blocking endogenous IGF-I by neutralizing antibody impaired multinucleation of the osteoclast, similar to that seen in the cultures from the IGF-$I^{-/-}$ mice. However, IGF-I treatment only partly restored the defect in osteoclast formation and multinucleation in the cultures from the IGF-$I^{-/-}$ mice. This may reflect a reduction in osteoclast precursors in the IGF-$I^{-/-}$ and/or a defect in signaling downstream of RANK or c-*fms* leading to reduced NFATc1 expression that can not be reversed by the acute addition of IGF-I in vitro. Our data are consistent with both possibilities, and current efforts in the laboratory are directed at resolving this issue.

IGF-I maintains the normal interaction between the osteoblast and osteoclast to support osteoclastogenesis

Osteoblast/stromal cells produce RANKL, M-CSF, IGF-I, and other local factors providing the microenvironment for osteoclast formation. In our co-culture experiments, osteoblast/stromal cells derived from IGF-$I^{-/-}$ mice lacked the ability to support the differentiation of osteoclast precursors into mature osteoclasts. Pretreating these cells with IGF-I can partly rescue this ability, indicating that IGF-I is important in maintaining the interaction between osteoblast/stromal cells and osteoclast precursors to support osteoclast precursor differentiation. As in the osteoclast precursors cultures, the inability of IGF-I to fully restore osteoclastogenesis may reflect changes in either the osteoclast precursor or the osteoblast that can not be reversed completely by the acute administration of IGF-I. Osteoblast/stromal cells may affect osteoclast formation in an IGF-I–dependent fashion in at least two ways. First, osteoblast/stromal cells produce IGF-I, and the IGF-I may act as a paracrine factor to bind to its receptors on the surface of osteoclast precursors to promote their differentiation including expression of c-*fms* and RANK. Second, osteoblast/stromal cells express the IGF-I receptor, and IGF-I may act as an endocrine/paracrine/autocrine factor to simulate RANKL and M-CSF production by the osteoblasts required for osteoclast differentiation.[32,33] The lower production of M-CSF and RANKL from osteoblast/stromal cells, combined with the reduced expression of their receptors c-*fms* and RANK in osteoclasts, leads to decreased osteoclast formation in IGF-$I^{-/-}$ mice.

In summary, we showed that, in bone remodeling, IGF-I is important in coupling bone resorption to bone formation to maintain normal bone mass. At the cellular level, IGF-I stimulates osteoclastogenesis. IGF-I is required to maintain the normal interaction between the osteoblast and osteoclast precursor to support osteoclast formation through its regulation of RANKL/RANK and M-CSF/c-*fms* expression and signaling transduction.

ACKNOWLEDGMENTS

This work was supported by National Institutes of Health Grant RO1 DK 54793 and National Aeronautic and Space Administrations Grant NN04CC67G.

REFERENCES

1. Suda T, Takahashi N, Udagawa N, Jimi E, Gillespie MT, Martin TJ 1999 Modulation of osteoclast differentiation and function by the new members of the tumor necrosis factor receptor and ligand families. Endocr Rev 20:345–357. [PubMed: 10368775]
2. Karsenty G, Wagner EF 2002 Reaching a genetic and molecular understanding of skeletal development. Dev Cell 2:389–406. [PubMed: 11970890]
3. Harada S, Rodan GA 2003 Control of osteoblast function and regulation of bone mass. Nature 423:349–355. [PubMed: 12748654]
4. Tolar J, Teitelbaum SL, Orchard PJ 2004 Osteopetrosis. N Engl J Med 351:2839–2849. [PubMed: 15625335]
5. Yakar S, Rosen CJ 2003 From mouse to man: Redefining the role of insulin-like growth factor-I in the acquisition of bone mass. Exp Biol Med (Maywood) 228:245–252. [PubMed: 12626768]
6. Bikle D, Majumdar S, Laib A, Powell-Braxton L, Rosen C, Beamer W, Nauman E, Leary C, Halloran B 2001 The skeletal structure of insulin-like growth factor I-deficient mice. J Bone Miner Res 16:2320–2329. [PubMed: 11760848]
7. Zhang M, Xuan S, Bouxsein ML, von Stechow D, Akeno N, Faugere MC, Malluche H, Zhao G, Rosen CJ, Efstratiadis A, Clemens TL 2002 Osteoblast-specific knockout of the insulin-like growth factor (IGF) receptor gene reveals an essential role of IGF signaling in bone matrix mineralization. J Biol Chem 277:44005–44012. [PubMed: 12215457]
8. McCarthy TL, Centrella M 2001 Local IGF-I expression and bone formation. Growth Horm IGF Res 11:213–219. [PubMed: 11735236]
9. Rosen CJ 2004 Insulin-like growth factor I and bone mineral density: Experience from animal models and human observational studies. Best Pract Res Clin Endocrinol Metab 18:423–435. [PubMed: 15261847]
10. Ueland T 2005 GH/IGF-I and bone resorption in vivo and in vitro. Eur J Endocrinol 152:327–332. [PubMed: 15757846]
11. Chambers TJ 2000 Regulation of the differentiation and function of osteoclasts. J Pathol 192:4–13. [PubMed: 10951393]
12. Teitelbaum SL 2000 Bone resorption by osteoclasts. Science 289:1504–1508. [PubMed: 10968780]
13. Miyamoto T, Suda T 2003 Differentiation and function of osteoclasts. Keio J Med 52:1–7. [PubMed: 12713016]
14. Udagawa N, Takahashi N, Yasuda H, Mizuno A, Itoh K, Ueno Y, Shinki T, Gillespie MT, Martin TJ, Higashio K, Suda T 2000 Osteoprotegerin produced by osteoblasts is an important regulator in osteoclast development and function. Endocrinology 141:3478–3484. [PubMed: 10965921]
15. Troen BR 2003 Molecular mechanisms underlying osteoclast formation and activation. Exp Gerontol 38:605–614. [PubMed: 12814795]
16. Boyle WJ, Simonet WS, Lacey DL 2003 Osteoclast differentiation and activation. Nature 423:337–342. [PubMed: 12748652]
17. Fiorelli G, Formigli L, Zecchi Orlandini S, Gori F, Falchetti A, Morelli A, Tanini A, Benvenuti S, Brandi ML 1996 Characterization and function of the receptor for IGF-I in human preosteoclastic cells. Bone 18:269–276. [PubMed: 8703583]
18. Hill PA, Reynolds JJ, Meikle MC 1995 Osteoblasts mediate insulin-like growth factor-I and -II stimulation of osteoclast formation and function. Endocrinology 136:124–131. [PubMed: 7828521]
19. Powell-Braxton L, Hollingshead P, Warburton C, Dowd M, Pitts-Meek S, Dalton D, Gillett N, Stewart TA 1993 IGF-I is required for normal embryonic growth in mice. Genes Dev 7:2609–2617. [PubMed: 8276243]

20. Bikle DD, Sakata T, Leary C, Elalieh H, Ginzinger D, Rosen CJ, Beamer W, Majumdar S, Halloran BP 2002 Insulin-like growth factor I is required for the anabolic actions of parathyroid hormone on mouse bone. J Bone Miner Res 17:1570–1578. [PubMed: 12211426]
21. Hayden JM, Mohan S, Baylink DJ 1995 The insulin-like growth factor system and the coupling of formation to resorption. Bone 17(2 Suppl):93S–98S. [PubMed: 8579905]
22. Tondravi MM, McKercher SR, Anderson K, Erdmann JM, Quiroz M, Maki R, Teitelbaum SL 1997 Osteopetrosis in mice lacking haematopoietic transcription factor PU.1. Nature 386:81–84. [PubMed: 9052784]
23. Grigoriadis AE, Wang ZQ, Cecchini MG, Hofstetter W, Felix R, Fleisch HA, Wagner EF 1994 c-Fos: A key regulator of osteoclast-macrophage lineage determination and bone remodeling. Science 266:443–448. [PubMed: 7939685]
24. Takeshita S, Kaji K, Kudo A 2000 Identification and characterization of the new osteoclast progenitor with macrophage phenotypes being able to differentiate into mature osteoclasts. J Bone Miner Res 15:1477–1488. [PubMed: 10934646]
25. Takayanagi H, Kim S, Koga T, Nishina H, Isshiki M, Yoshida H, Saiura A, Isobe M, Yokochi T, Inoue J, Wagner EF, Mak TW, Kodama T, Taniguchi T 2002 Induction and activation of the transcription factor NFATc1 (NFAT2) integrate RANKL signaling in terminal differentiation of osteoclasts. Dev Cell 3:889–901. [PubMed: 12479813]
26. Iwamoto K, Miyamoto T, Sawatani Y, Hosogane N, Hamaguchi I, Takami M, Nomiyama K, Takagi K, Suda T 2004 Dimer formation of receptor activator of nuclear factor kappaB induces incomplete osteoclast formation. Biochem Biophys Res Commun 325:229–234. [PubMed: 15522223]
27. Kim K, Kim JH, Lee J, Jin HM, Lee SH, Fisher DE, Kook H, Kim KK, Choi Y, Kim N 2005 Nuclear factor of activated T cells c1 induces osteoclast-associated receptor gene expression during tumor necrosis factor-related activation-induced cytokine-mediated osteoclastogenesis. J Biol Chem 280:35209–35216. [PubMed: 16109714]
28. Boissy P, Saltel F, Bouniol C, Jurdic P, Machuca-Gayet I 2002 Transcriptional activity of nuclei in multinucleated osteoclasts and its modulation by calcitonin. Endocrinology 143:1913–1921. [PubMed: 11956174]
29. Yagi M, Miyamoto T, Sawatani Y, Iwamoto K, Hosogane N, Fujita N, Morita K, Ninomiya K, Suzuki T, Miyamoto K, Oike Y, Takeya M, Toyama Y, Suda T 2005 DC-STAMP is essential for cell-cell fusion in osteoclasts and foreign body giant cells. J Exp Med 202:345–351. [PubMed: 16061724]
30. Hou P, Sato T, Hofstetter W, Foged NT 1997 Identification and characterization of the insulin-like growth factor I receptor in mature rabbit osteoclasts. J Bone Miner Res 12:534–540. [PubMed: 9101364]
31. Lazowski DA, Fraher LJ, Hodsman A, Steer B, Modrowski D, Han VK 1994 Regional variation of insulin-like growth factor-I gene expression in mature rat bone and cartilage. Bone 15:563–576. [PubMed: 7980968]
32. Rubin J, Ackert-Bicknell CL, Zhu L, Fan X, Murphy TC, Nanes MS, Marcus R, Holloway L, Beamer WG, Rosen CJ 2002 IGF-I regulates osteoprotegerin (OPG) and receptor activator of nuclear factor-kappaB ligand in vitro and OPG in vivo. J Clin Endocrinol Metab 87:4273–4279. [PubMed: 12213884]
33. Joseph BK, Marks SC Jr, Hume DA, Waters MJ, Symons AL 1999 Insulin-like growth factor-I (IGF-I) and IGF-I receptor (IGF-IR) immunoreactivity in normal and osteopetrotic (toothless, tl/tl) rat tibia. Growth Factors 16:279–291. [PubMed: 10427502]

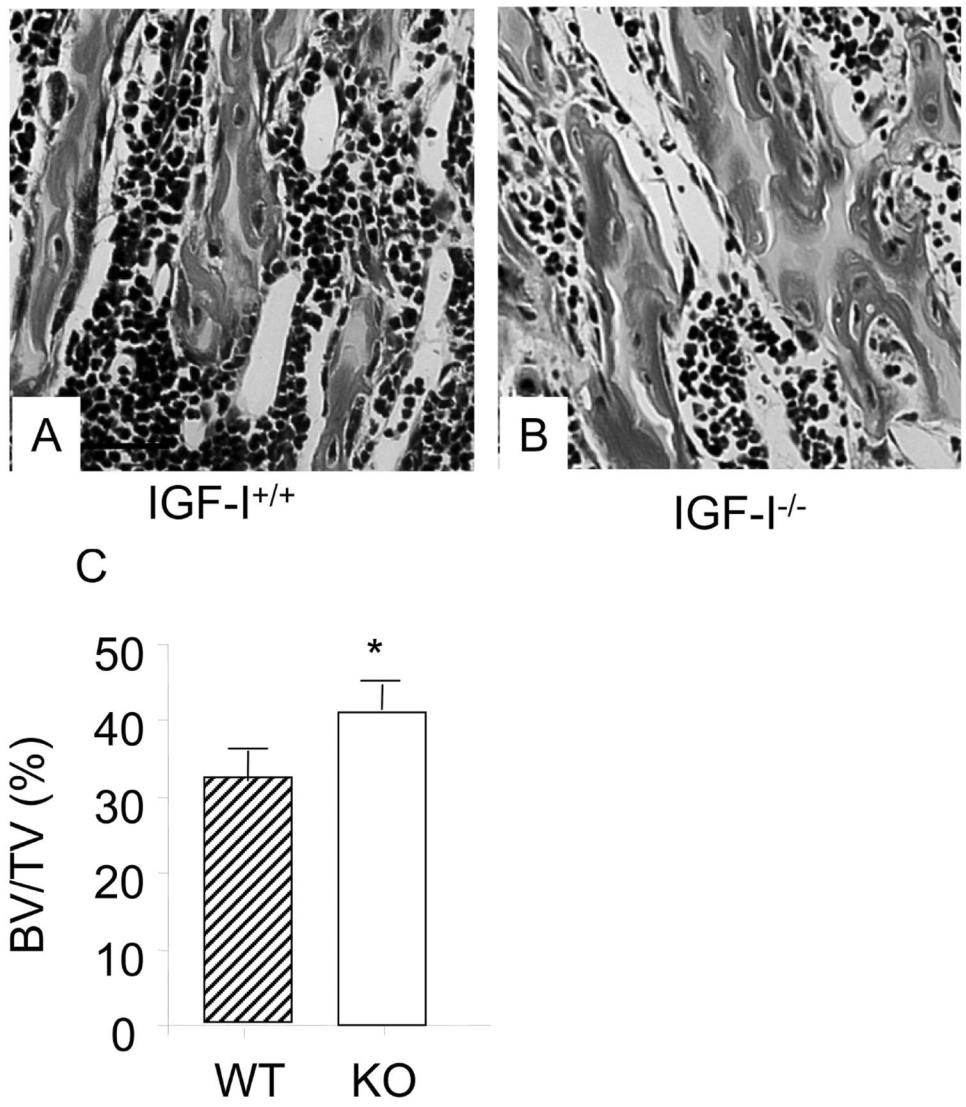

FIG. 1.

Effects of IGF-I on trabecular bone volume. Compared with (A) IGF-$I^{+/+}$, (B) IGF-$I^{-/-}$ have more trabecular bone. (c) BV/TV in IGF-$I^{-/-}$ mice (KO, open bars) is significantly higher than that in IGF-$I^{+/+}$ mice (WT, hatched bars). Results are expressed as means ± SD in C. *$p < 0.05$, kO vs. WT. Bar = 25 μm in A and B. $n = 4$ in each group.

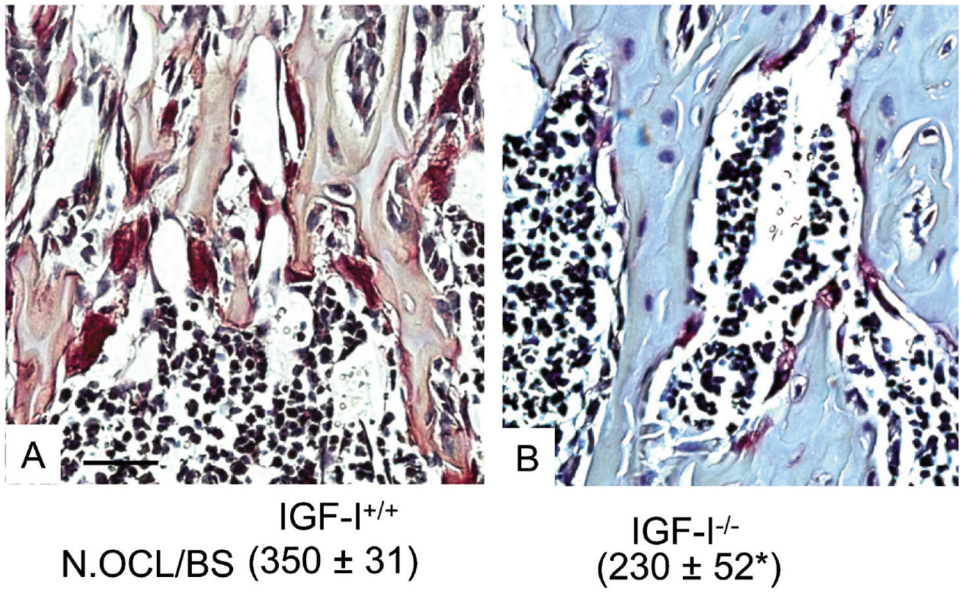

FIG. 2.

Effects of IGF-I on osteoclasts in vivo. (B) IGF-I–deficient mice (IGF-$I^{-/-}$) have fewer and smaller osteoclasts with fewer nuclei compared with (A) wildtype (IGF-$I^{+/+}$) mice. Osteoclast numbers/bone surface (N.OCL/BS) are expressed as means ± SD. $n = 3$ in each group; bar = 50 μm.

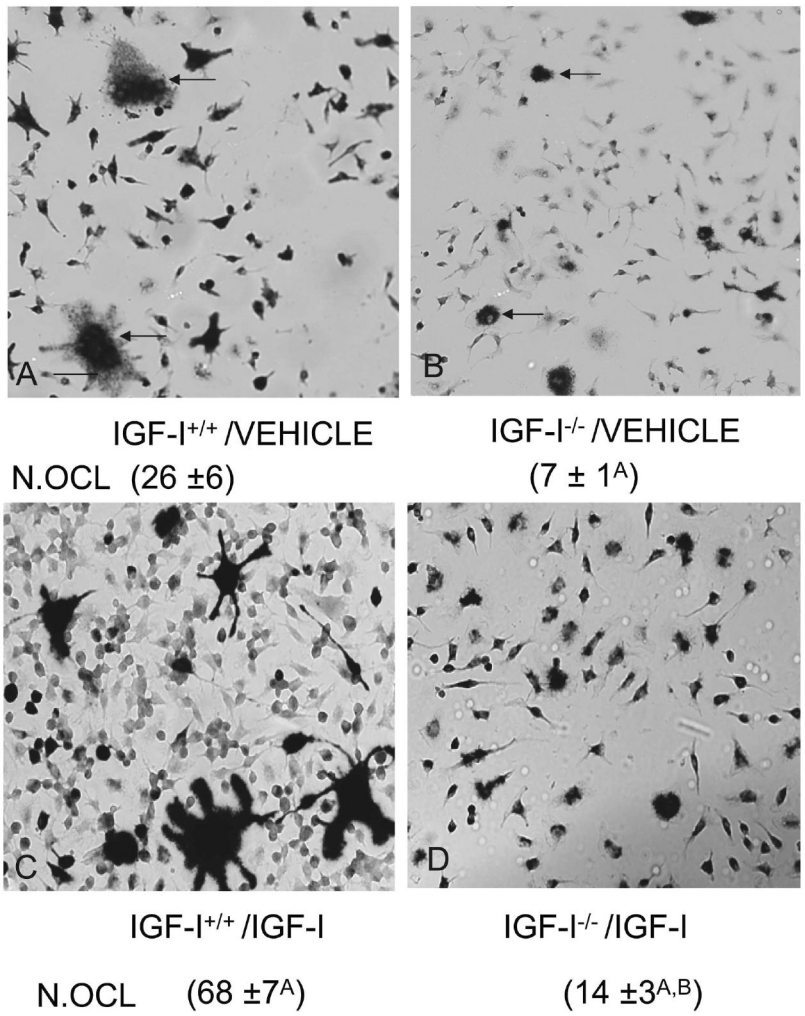

FIG. 3.

IGF-I regulates osteoclastogenesis. Osteoclast precursors (spleen cells) from (A) IGF-I$^{+/+}$ mice treated with 30 ng/ml RANKL and 10 ng/ml M-CSF readily formed multi-nucleated TRACP^{+} osteoclasts (arrows). (B) Osteoclast precursors from the IGF-I$^{-/-}$ mice formed only mononuclear or binuclear TRACP^{+} osteoclasts (arrows) and in lesser numbers. (C) IGF-I (10 ng/ml) further increased osteoclast number and size and number of nuclei in the IGF-I$^{+/+}$ cultures, (D) but was much less effective in the cultures from IGF-I$^{-/-}$ mice. Osteoclast numbers/well (N. OCL) are expressed as means ± SD. $^{A}p < 0.05$ vs. vehicle-treated IGF-I$^{+/+}$; $^{B}p < 0.05$ vs. vehicle-treated IGF-I$^{-/-}$. $n = 4$ in each group. Bar = 50 μm.

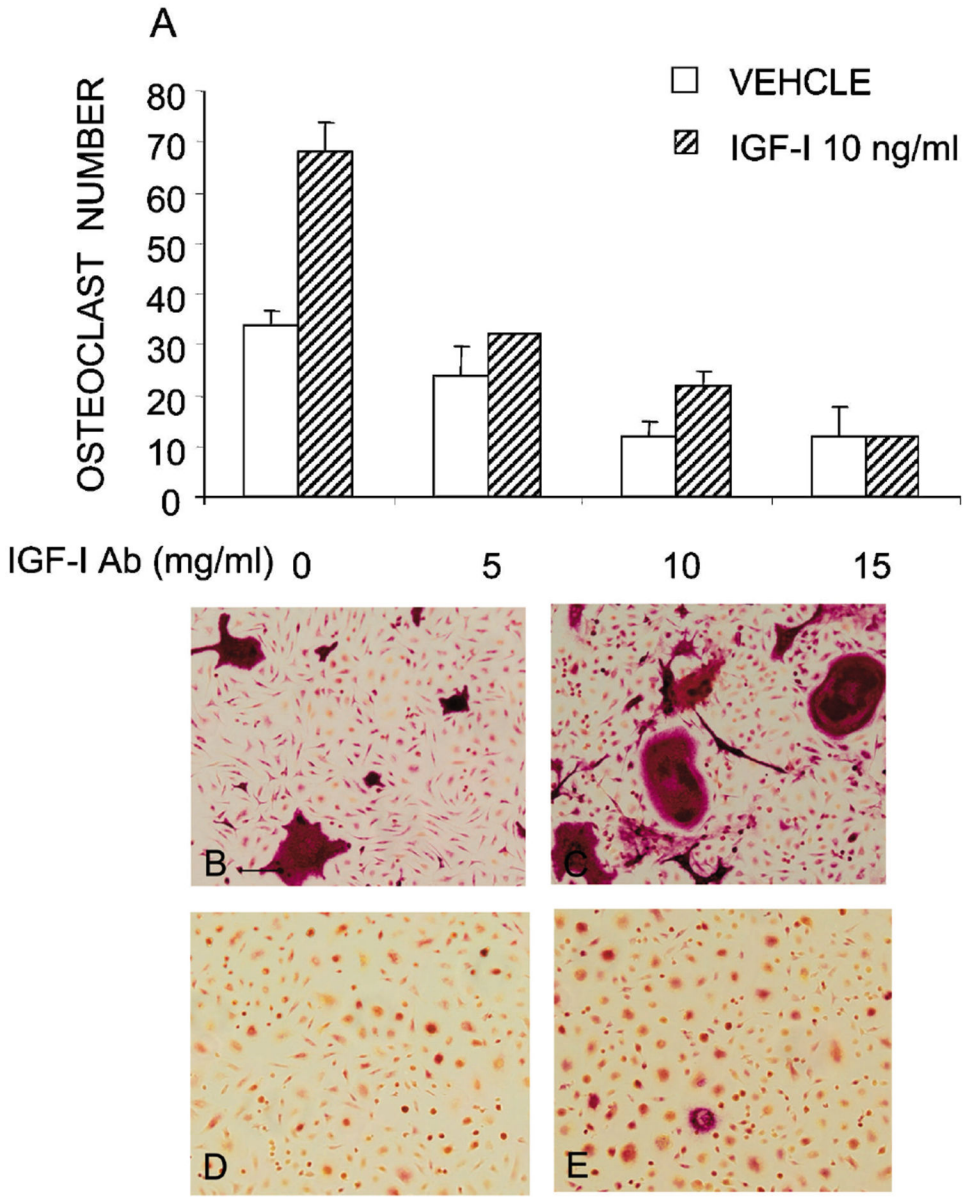

FIG. 4.

Neutralizing antibody to IGF-I blocks the effects on osteoclastogenesis. Osteoclast precursors from the wildtype mice were cultured in the presence of 50 ng/ml RANKL and 10 ng/ml M-CSF and treated with vehicle or IGF-I 10 ng/ml. (A) Neutralizing antibody to IGF-I was added to the culture at doses of 0–15 μg/ml. IGF-I antibody dose-dependently blocked the local effects of IGF-I on osteoclastogenesis in both vehicle (open bars) and IGF-I treated (hatched bars) cultures. Results are expressed as means ± SD. (B–E) Representative pictures of blocking effects of neutralizing antibody to IGF-I at the dose of 15 μg/ml in (B and D) vehicle or (C and E) IGF-I–treated cultures. (D and E) Multinucleation was also blocked by IGF-I antibody. $n = 4$ wells in each group.

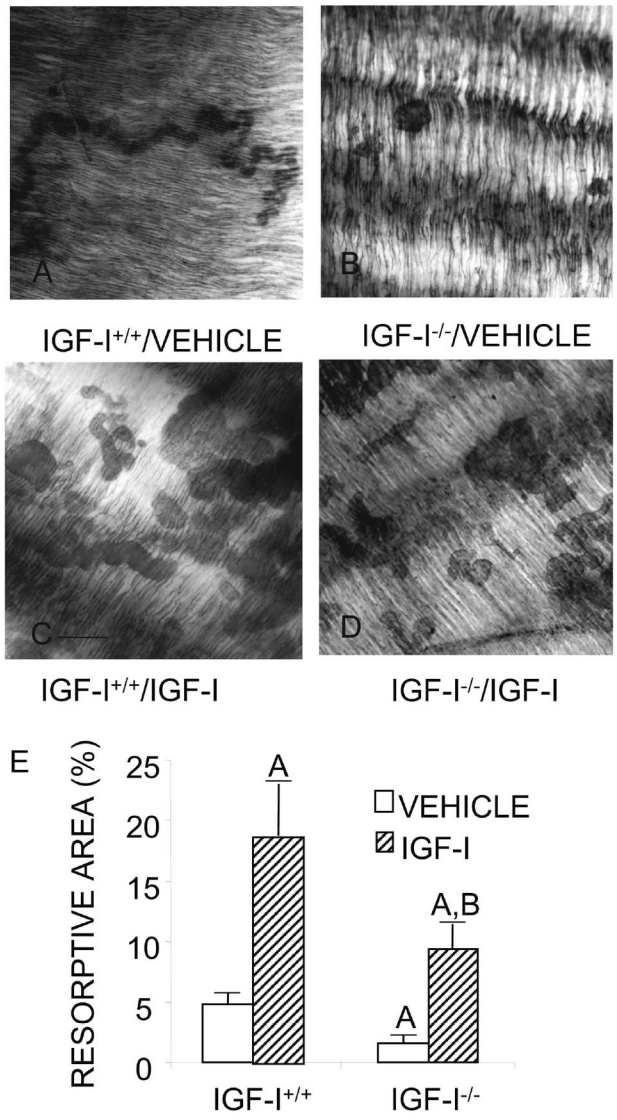

FIG. 5.

IGF-I stimulates osteoclast function. Osteoclast precursors from (A and C) IGF-$I^{+/+}$ and (B and D) IGF-$I^{-/-}$ mice were cultured on whale dentine slices in the presence of (C and D) IGF-I 10 ng/ml or (A and B) vehicle for 10 days. Bone resorptive ability was decreased in the osteoclasts from the IGF-$I^{-/-}$ mice. IGF-I (10 ng/ml) significantly increased bone resorptive ability of osteoclasts from both genotypes. (E) Resorptive areas (%) are expressed as means ± SD. $^{A}p < 0.05$ vs. IGF-$I^{+/+}$; $^{B}p < 0.05$ vs. IGF-$I^{-/-}$. $n = 3$ in each group. Bar = 100 μm.

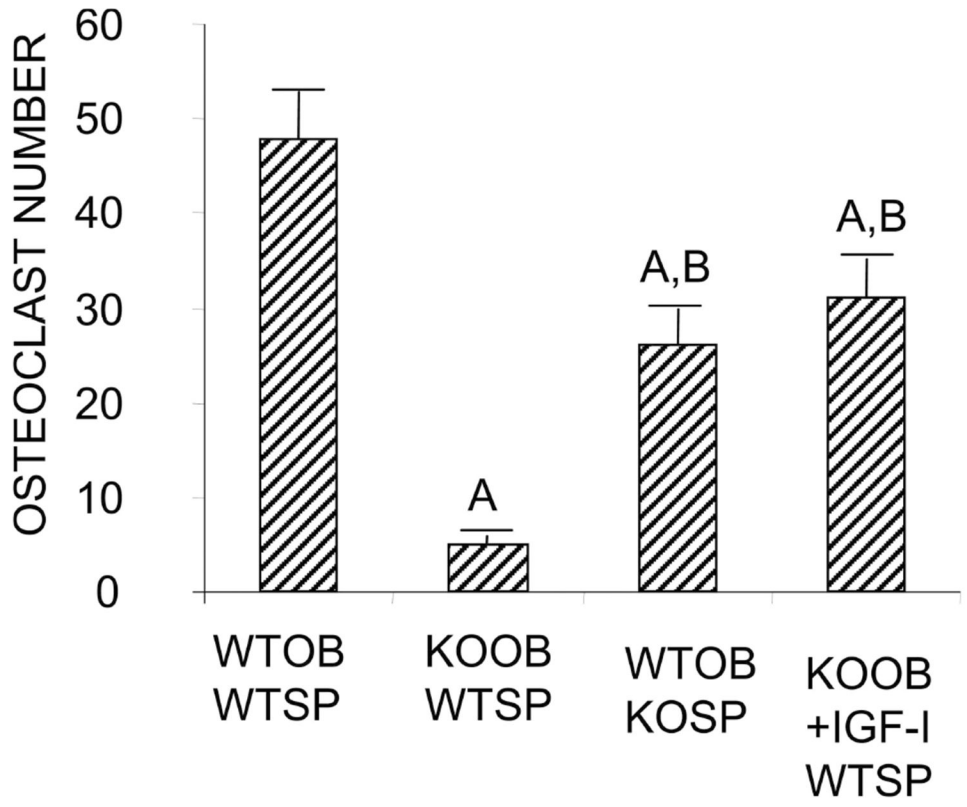

FIG. 6.

IGF-I is required for maintaining the interaction between osteoblasts (OBs) and osteoclast precursors (spleen cells [SPs]). The following sets of co-cultures were performed: IGF-$I^{+/+}$ (WT) osteoblasts and osteoclast precursors (WtOB:WTSP); IGF-$I^{-/-}$ (KO) osteoblasts and WT osteoclast precursors (KOOB-:WTSP); WT osteoblasts and KO osteoclast precursors (WTOB- :KOSP); KO osteoblasts pretreated by IGF-I 10 ng/ml for 9 days (KOOB + IGF-I) and WT osteoclast precursors (KOOB + IGF-I:WTSP). The number of osteoclasts formed in these cultures was determined. Osteoclast numbers per well are expressed as means ± SD. $^{A}p < 0.05$ vs. WTOB:WTSP; $^{B}p < 0.05$ vs. KOOB:WTSP. $n = 4$ wells in each group.

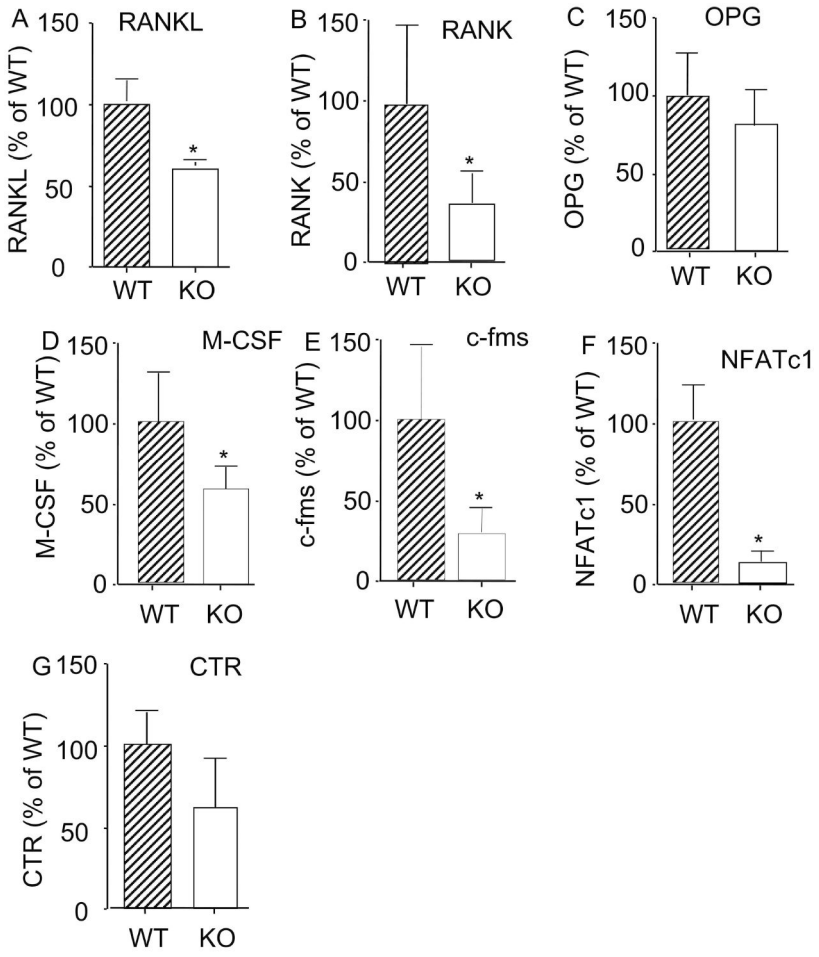

FIG. 7.

IGF-I deficiency decreases bone resorption markers. RNA was extracted from the long bones (bone marrow flushed out) of IGF-$I^{+/+}$ and IGF-$I^{-/-}$ mice. The mRNA levels of (A) RANKL, (B) RANK, (C) OPG, (D) M-CSF, (E) c-*fms*, (F) NFATc1, and (G) CTR were detected by quantitative real-time PCR. Results are expressed as percentage of wildtype, and all values have been normalized to GAPDH mRNA levels in the same sample. *$p < 0.05$ comparing IGF-$I^{-/-}$ mice (KO, open bars) vs. IGF-$I^{+/+}$ mice (WT, hatched bars). $n = 8$ in WT group; $n = 9$ in KO group.