Attenuation of Bone Mass and Increase of Osteoclast Formation in Decoy Receptor 3 Transgenic Mice*

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Decoy receptor 3 (DcR3), a soluble receptor for FasL, LIGHT, and TL1A, induces osteoclast formation from monocyte, macrophage, and bone stromal marrow cells. However, the function of DcR3 on bone formation remains largely unknown. To understand the function of DcR3 in bone formation in vivo, transgenic mice overexpressing DcR3 were generated. Bone mineral density (BMD) and bone mineral content (BMC) of total body were significantly lower in DcR3 transgenic mice as compared with wild-type controls. The difference in BMD and BMC between DcR3 transgenic and control mice was confirmed by histomorphometric analysis, which showed a 35.7% decrease in trabecular bone volume in DcR3 transgenic mice in comparison with wild-type controls. The number of osteoclasts increased in DcR3 transgenic mice. In addition, local administration of DcR3 (30 μg/ml, 10 μl, once/day) into the metaphysis of the tibia via the implantation of a needle cannula significantly decreased the BMD, BMC, and bone volume of secondary spongiosa in tibia. Local injection of DcR3 also increased osteoclast numbers around trabecular bone in tibia. Furthermore, coadministration of soluble tumor necrosis factor receptor inhibitor/Fc chimera (TNFRSF1A) but not osteoprotegerin inhibited the action of DcR3. In addition, in an assay of osteoclast activity on substrate plates, DcR3 significantly increased the resorption activity of mature osteoclasts. Treatment with higher concentrations of DcR3 slightly increased nodule formation and alkaline phosphatase activity of primary cultured osteoblasts. These results indicate that DcR3 may play an important role in osteoporosis or other bone diseases.

Bone is a complex tissue composed of several cell types that are continuously undergoing a process of renewal and repair termed “bone remodeling.” Two major cell types responsible for bone remodeling are osteoclasts, which resorb bone, and osteoblasts, which form new bone (1).

Osteoclasts are cells derived from the monocyte-macrophage lineage that have an important role in modeling bone during skeletal growth and in remodeling bone during adult life (2). Increased osteoclast activity or uncoupling of osteoclastic bone resorption from bone formation results in focal or generalized bone loss and is a characteristic feature of bone diseases such as osteoporosis, Paget disease of bone, and cancer-associated bone disease (3). The importance of osteoclastic bone resorption in the pathogenesis of these diseases is reflected by the fact that the most successful drug treatment for bone disease works by inhibiting bone resorption (4). Recent findings indicated that two key molecules, macrophage colony-stimulating factor (M-CSF)3 and the receptor for activation of NF-κB ligand (RANKL), are essential and sufficient to promote osteoclastogenesis (5). On the other hand, several systemic hormones and cytokines, including glucocorticoid (6, 11), interleukin-1 (7), interleukin-6 (8), interleukin-11 (9), transforming growth factor-β (10), and tumor necrosis factor-α (TNF-α) (12, 13), have been shown to play roles in the differentiation of osteoclast progenitors into mature multinucleated osteoclasts. The pleiotropic cytokine TNF-α has been implicated in the pathogenesis of osteoclastogenesis via the activation of TNF receptor 1 (TNFR1) (14, 15).

Decoy receptor 3 (DcR3) was discovered in a search of the human genome data base for sequences with homology to the TNF receptor superfamily (16, 17), as well as in a screen to identify novel secreted proteins (18). It competes for the binding of the following ligands to their respective receptors: FasL with Fas; LIGHT with *Herpesvirus* entry mediator and lymphotoxin-β receptor; and TNF-like molecule 1A (TL1A) with death receptor 3 (DR3) (19). There is strong evidence that DcR3 is overexpressed in various tumors, including lung and colon cancers, gastrointestinal tract tumors, virus-associated lymphomas, malignant gliomas, and pancreatic cancers (16, 18, 20). In addition, the overexpression of DcR3 has been reported in cases of silicosis or systemic lupus erythematosus (21), as well as in bacterial antigen-stimulated monocytes and myeloid dendritic cells (22). DcR3 can block the effects of its known ligands (FasL, LIGHT, and TL1A) and contributes to tumor growth by impeding the immune response as well as inducing angiogenesis (23).

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3 The abbreviations used are: M-CSF, macrophage colony-stimulating factor; DcR3, decoy receptor 3; ALP, alkaline phosphatase; RANKL, receptor for activation of NF-κB ligand; TNF-α, tumor necrosis factor-α; TNFRSF1A, soluble TNF receptor inhibitor/Fc chimera; BMD, bone mineral density; BMC, bone mineral content; OAAS, osteoclast activity assay substrate; TRAP, tetratrate-resistant acid phosphatase; OPG, osteoprotegerin; ELISA, enzyme-linked immunosorbent assay; RT, reverse transcription.
On the other hand, DcR3 can also act as a regulator for the differentiation and maturation of myeloid cells, possibly through interaction with novel ligand(s) (24, 25).

In a previous study, Yang et al. (25) demonstrate that human DcR3 induces osteoclastogenesis from the cultures of monocyte, macrophage, and bone marrow cells. However, the regulatory action of DcR3 on bone remodeling in animals remains largely unknown. Here we report that DcR3 transgenic mice show a decrease of bone mineral density (BMD), bone mineral content (BMC), and bone volume, accompanied by an increase of osteoclast formation. The in vivo effect of DcR3 may play an important role in osteolytic bone diseases.

**EXPERIMENTAL PROCEDURES**

**Materials**—α-MEM, fetal bovine serum, penicillin, and streptomycin were obtained from Invitrogen. The osteoclast activity assay substrate (OAAS) was obtained from OCT USA, Inc. (Torrance, CA). Recombinant human M-CSF, RANKL, and osteoprotegerin (OPG) and ELISA kits for mouse TNF-α and RANKL and were obtained from R&D Systems (Minneapolis, MN). Human soluble TNF receptor inhibitor/Fc chimera (TNFRSF1A) was obtained from GenScript Corp. (Piscataway, NJ). Vitamin C, β-glycerophosphate, and alizarin red-S were obtained from Sigma. The ELISA kit for C-terminal telopeptides of type-I collagen was obtained from Nordic Bioscience (Herlev, Denmark). The human DcR3 ELISA kit was obtained from Anawrahta Biotech (Taipei, Taiwan).

**Generation of DcR3 Transgenic Mice and Histological Examination**—The DcR3 heterozygous transgenic mice were generated from a BALB/c background for at least six generations as described previously (26). The coding sequence of human DcR3 was subcloned into pPGK-Neo-bpA to regulate the expression of DcR3 ubiquitously under the control of PGK promoter. DcR3 transgenic mice were screened by PCR using the primers pGKP (sense, 5’-GCCAATAGCAGCTTGCTC-3’) and DcR3–207 (anti-sense, 5’-TAGGTGGGTGTTTGCCAC-3’), which amplify a 350-bp fragment from the pPGK- DcR3 transgene. The DcR3 transgene-negative littermates were used as wild-type mice.

BMD and BMC of full-body (body weight, 22–28 g) were determined by dual-energy x-ray absorptiometer (XR-26; Norland, Fort Atkinson, WI) using a mode for small subjects. A coefficient of variation of 0.7% was calculated from daily measurements of BMD on a lumbar phantom for more than 1 year. At the end of the program, the tibia was fixed and decalcified and then embedded in paraffin. Serial sections (5 μm) were cut longitudinally, and the sections were stained with Mayer’s hematoxylin-eosin solution. Images of the growth plate and proximal tibia were photographed using Olympus microscope IX70. For measurement of the osteoclast number, the sections were stained with tartrate-resistant acid phosphatase (TRAP). Bone volume was calculated using image analysis software (Image-pro plus 3.0) and expressed as the percentage of bone area. All measurements were done in a single blind fashion.

**Local Administration of DcR3 into Tibia in Young Rats**—Local injection of DcR3 into tibia of young rats was performed as

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**FIGURE 1. Attenuation of BMD and BMC in DcR3 transgenic mice.** BMD (A) and BMC (B) of whole body were measured using a dual-energy x-ray absorptiometry scanner. Note that BMD and BMC decreased in DcR3 transgenic mice (n = 9). C, the expression of human DcR3 in various tissues of DcR3 transgenic mice was determined by immuno-blotting using an anti-DcR3 monoclonal antibody (n = 3). The blood sample from wild-type (WT) control mice was also used for comparison. D, osteoclast activity was assessed from serum samples by measuring the level of terminal telopeptide of type-I collagen (COL1) using an ELISA kit (n = 5). E, RNA was extracted from adult calvaria, reverse-transcribed, and amplified with specific primers using real-time PCR (n = 3). Expression of mRNA for ALP was calculated by the relative standard curve method and normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Data are presented as mean ± S.E. *, p < 0.05 as compared with wild-type mice.
Attenuation of Bone Mass by DcR3

described previously (27, 28). Male Sprague-Dawley rats weighing 75–90 g were used. Implantation of a cannula (22-gauge needle) was done from the posterolateral side into the proximal tibial metaphysis in both limbs of rats anesthetized with trichloroacetaldehyde. The cannula had its outer end in the subcutaneous tissue. DcR3 (30 μg/ml, 10 μl) was percutaneously injected into the proximal tibia through the cannula (once/day) for 7 consecutive days. The same volume of vehicle was injected into the contralateral side for comparison. The rats were sacrificed, and tibiae were removed on day 14. BMD and BMC of the tibia were measured with the same dual-energy x-ray absorptiometer as described above. At the end of the program, the tibia sections were stained with hematoxylin and eosin or TRAP as mentioned above.

Detection of DcR3 Protein by Western Blot—To detect the tissue distribution of DcR3 protein in transgenic mice, tissues were harvested and lysed in cell lysis buffer (27). Total lysate at 30 μg were fractionated by SDS-PAGE and subjected to Western blot analysis, using the anti-DcR3 monoclonal antibody (clone 3H5) (26).

Real-time RT-PCR with SYBR Green Detection—RNA from 2-month-old calvaria was extracted with TRizol (MBio Inc, Taipei, Taiwan) following the manufacturer’s instructions. 5 μg of RNA were reverse-transcribed into cDNA using SuperScript III first-strand synthesis system for RT-PCR (Invitrogen). Real-time PCR amplification was performed on a 7900 instrument (Applied Biosystems). Real-time RT-PCR fluorescence detection was performed in 96-well plates using SYBR Green PCR Master Mix (Applied Biosystems). Real-time PCR primers are as follows: alkaline phosphatase (ALP), 5′-GGTGCCAAGCTGGGAAAGACAC-3′ and 5′-CCCACCCGCTATTCCAAAC-3′; galderaldehyde-3-phosphate dehydrogenase, 5′-ATCCCATCA-CCCTTCCTCAGGAG-3′ and 5′-CCCTTCACCCACCTTGTGATG-3′.

Osteoclast Generation—Bone marrow cells were prepared by removing marrow from the femurs and tibiae of adult mice and flushing the bone marrow cavity with α-MEM, which was supplemented with 20 mM HEPES and 10% heat-inactivated fetal bovine serum, 2 mM glutamine, penicillin (100 units/ml), and streptomycin (100 μg/ml). The non-adherent cells (hemato-poietic cells) were collected after 24 h and used as osteoclast precursors. Cells were seeded at 1 × 10⁶ cells/0.5 ml/well in 24-well plates in the presence of human recombinant soluble RANKL (50 ng/ml) and M-CSF (20 ng/ml). The culture medium was replaced every 3 days. Osteoclast formation was measured by TRAP staining on days 2, 4, 6, and 8, respectively. In brief, adherent cells were fixed with 10% formaldehyde in phosphate-buffered saline for 3 min and then stained with Naphthol AS-MX phosphate and tartrate solution for 1 h at 37°C. Osteoclast-like cells in each well were scored by counting the number of TRAP-positive and multinucleated cells containing more than three nuclei.

Assay of Resorbing Activity of Osteoclast—Osteoclast precursors were isolated from mice long bones as mentioned above. The cells were resuspended in complete α-MEM medium and plated into a calcium phosphate apatite-coated OAAS plate at 1 × 10⁶ cells/0.5 ml/well. The cells were cultured in the presence of M-CSF (20 ng/ml) plus RANKL (50 ng/ml). After 8 days of culture, the remaining cells in the plate were lysed using 1 N NaOH. Five images per well were obtained using inverted microscope (× 200), and the resorbed area was measured using an image analyzer.

Isolation of Mature Osteoclasts—Mature osteoclasts were isolated as described previously (29). Long bones were isolated from 6-day-old rabbits (body weight, 70–90 g). After removal of muscle and cartilage, the bones were minced in α-MEM (once/day) for 7 consecutive days. The same volume of vehicle was injected into the contralateral side for comparison. The rats were sacrificed, and tibiae were removed on day 14. BMD and BMC of the tibia were measured with the same dual-energy x-ray absorptiometer as described above. At the end of the program, the tibia sections were stained with hematoxylin and eosin or TRAP as mentioned above. Detection of DcR3 Protein by Western Blot—To detect the tissue distribution of DcR3 protein in transgenic mice, tissues were harvested and lysed in cell lysis buffer (27). Total lysate at 30 μg were fractionated by SDS-PAGE and subjected to Western blot analysis, using the anti-DcR3 monoclonal antibody (clone 3H5) (26).

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Isolation of Mature Osteoclasts—Mature osteoclasts were isolated as described previously (29). Long bones were isolated from 6-day-old rabbits (body weight, 70–90 g). After removal of muscle and cartilage, the bones were minced in α-MEM. Cells were dissociated from bone fragments by gentle vortexing, and then bone fragments were allowed to settle under normal gravity. The supernatant was removed and saved, and the mincing and sedimentation were repeated three more times. The supernatants were pooled and centrifuged for 5 min at 60 × g. The cells were plated in an OAAS plate, and after overnight incubation, the adherent cells were washed three times with phosphate-buffered saline and then treated with trypsin/EDTA at 10 min at 37°C to remove contaminating cells. Additionally, treatment with 0.1% collagenase for 5 min at room temperature was performed to remove completely most of the stromal cells. The highly enriched osteoclasts (>90%) were washed three times in phosphate-buffered saline. The resorp-
tion activity of mature osteoclasts was then assayed after 2 days of culture as mentioned above.

**Primary Osteoblast Cultures**—Primary osteoblastic cells were prepared by the method as described previously (30). The calvaria were dissected from fetal rats or mice, divided into small pieces, and then treated with 0.1% type I collagenase solution for 10 min at 37°C. The next two 20-min sequential collagenase digestions were then pooled and filtered through 70-μm nylon filters (Falcon, BD Biosciences). The cells were grown on the plastic cell culture dishes in 95% air, 5% CO2, with α-MEM, which was supplemented with 20 mM HEPES and 10% heat-inactivated fetal bovine serum, 2 mM-glutamine, penicillin (100 units/ml), and streptomycin (100 μg/ml) (pH adjusted to 7.6) and then air-dried. Calcium deposition was determined using alizarin red-S staining. Briefly, the ethanol-fixed cells and matrix were stained for 1 h with 40 mM alizarin red-S (pH 4.2) and extensively rinsed with water. Images of the mineralized matrixes were photographed using an Olympus microscope IX70. After photography, the bound staining was eluted with 10% (w/v) cetylpyridinium chloride, and alizarin red-S in samples was quantified by measuring absorbance at 550 nm and calculated according to a standard curve. 1 mol of alizarin red-S selectively binds about 2 mol of calcium. All protocols complied with institutional guidelines and were approved by Animal Care Committee of Medical College, National Taiwan University.

The characteristics of osteoblasts were confirmed by morphology and the expression of ALP.

**Assay of Cell Proliferation**—Osteoblasts (2 × 10^4 cells/well) were seeded on 24-well plates. Cells were incubated in serum-free medium for 24 h before the addition of DcR3. After incubation with DcR3 for 24 h, bromodeoxyuridine incorporation was assayed according to the protocol of enzyme-linked immunosorbent assay chemiluminescence detection kit (Roche Applied Science) using a luminescence counter (TopCount; Packard Instruments, Meriden, CT). The counts per second correlate directly to the amount of DNA synthesis and the number of proliferating cells.

**Measurement of Alkaline Phosphatase Activity**—Osteoblasts cultured in 24-well plates in the presence or absence of DcR3 were harvested in 0.2% Nonidet P-40, and the cell suspension was disrupted by sonication. After centrifugation at 1500 × g for 5 min, ALP activity in the supernatant was measured as described by Yang et al. (31).

**Measurement of Mineralized Matrix**—The level of mineralized matrix was evaluated by the method described previously (31, 33). Osteoblasts were cultured in α-MEM containing vitamin C (50 μg/ml) and β-glycerophosphate (10 mM) for 2 weeks, and the medium was changed every 3 days. After incubation with DcR3 for 12 days, cells were washed twice with 20 mM Tris-buffered saline containing 0.15 M NaCl (pH 7.4) and fixed in ice-cold 75% ethanol (v/v) for 30 min and then air-dried. Calcium deposition was determined using alizarin red-S staining. Briefly, the ethanol-fixed cells and matrix were stained for 1 h with 40 mM alizarin red-S (pH 4.2) and extensively rinsed with water. Images of the mineralized matrixes were photographed using an Olympus microscope IX70. After photography, the bound staining was eluted with 10% (w/v) cetylpyridinium chloride, and alizarin red-S in samples was quantified by measuring absorbance at 550 nm and calculated according to a standard curve. 1 mol of alizarin red-S selectively binds about 2 mol of calcium. All protocols complied with institutional guidelines and were approved by Animal Care Committee of Medical College, National Taiwan University.
Attenuation of Bone Mass by DcR3

Statistics—The values given are means ± S.E. The significance of difference between the experimental group and control was assessed by the Student’s t test. The difference is significant if the p value is < 0.05.

RESULTS

Attenuation of Bone Mass in DcR3 Transgenic Mice—To understand the function of DcR3 in bone remodeling in vivo, transgenic mice overexpressing DcR3 systemically were generated. The adult mice were used, and the age, sex, and body weight of transgenic mice were matched to the controls. BMD and BMC of total body, as assessed by dual-energy x-ray absorptiometry, were significantly lower (11.9 and 17.1%, respectively) in DcR3 transgenic mice as compared with wild-type controls (Fig. 1, A and B). There is no difference in overall length and thickness of the bones (data not shown). Western blot analysis was performed on several tissues from 2-month-old wild-type controls and DcR3 transgenic mice with DcR3 antibody. DcR3 expression was found in blood, femur, brain, liver, spleen, lung, and heart in transgenic mice (Fig. 1C). The protein expression of DcR3 is higher in blood samples, femur, and spleen tissues. There is no DcR3 expression in these tissues of wild-type mice. The osteoclastic activity was evaluated by measuring the C-terminal telopeptides of type I collagen in blood, and it shows that osteoclast activity increases in DcR3 transgenic mice (Fig. 1D). To evaluate the effect of DcR3 transgene expression on the markers of osteoblastic function, RNA levels of bone markers ALP were quantitated by real-time RT-PCR in calvaria bone. DcR3 transgene expression slightly increased ALP mRNA expression (Fig. 1E). Bone histomorphometric analysis in tibia showed that DcR3 transgenic mice had a 35.7% decrease in trabecular bone volume as compared with wild-type mice and a decrease of about 23.5 and 14.2% in the thickness of cortical and trabecular bone, respectively (Fig. 2 and Table 1). In addition, we also counted the number of osteoclasts and osteoblasts in the region of primary spongiosa. It was found that the number of osteoclasts significantly increased and that of the osteoblasts did not show significant change in DcR3 transgenic mice as compared with wild-type controls (Fig. 2 and Table 1).

Osteoclastogenesis Derived from Bone Marrow Stromal Cell Increases in DcR3 Transgenic Mice—Osteoclasts are specialized monocyte/macrophage family members and differentiate from bone marrow hematopoietic precursors. Formation of large TRAP-positive multinuclei osteoclasts in bone marrow-derived stromal cell cultures in the presence of M-CSF (20 ng/ml) and RANKL (50 ng/ml) increased in DcR3 transgenic mice as compared with wild-type controls (Fig. 3, A and C). The DcR3 protein level was up-regulated in the supernatant of cultured stromal cells obtained from DcR3 transgenic mice (wild-type, not detectable; DcR3 transgenic mice, 3.64 ± 0.49 ng/ml, n = 4). When the stromal cells were seeded in OAAS plates for 8 days in the presence of M-CSF plus RANKL, the resorption pits occurred. It was found that the resorption area increased in osteoclast cultures derived from DcR3 transgenic mice as compared with wild-type mice (Fig. 3, B and D).

Local Administration of DcR3 Decreased Bone Volume of Tibia in Young Rats—To simply look at the effect of exogenous application of DcR3 on trabecular bone, local injection of DcR3 into tibia in young rats was used (27, 28). The tibia of mice are too small to be implanted with needle, so the tibia of rats were thus used in this experiment. Trabecular bone is composed of a lattice or network of branching bone spicules. The spaces between the bone spicules contain bone marrow. DcR3 (30 μg/ml, 10 μl, once/day) was locally administered into tibia through the needle cannula (22 gauge) in young rats weighing 75–90 g, and the rats were sacrificed later on day 14. The vehicle was injected into the contralateral side for comparison. As compared with the vehicle-injected side (Fig. 4A; the arrow shows the hole of injection site), DcR3 significantly decreased the bone volume of the secondary spongiosa (Fig. 4A). Trabecular bone in the secondary spongiosa decreased by 31.5% after local administration of DcR3. In addition, BMD and BMC also decreased after application of DcR3 (Table 2). The TRAP staining in secondary spongiosa also showed that osteoclasts were predominantly localized around the trabecular bone, and long term administration of DcR3 increased the formation of osteoclasts (Fig. 4B and Table 2). It has been previously reported that DcR3 induced formation of TRAP-positive multinucleated cells, probably via the release of TNF-α (25). Here, we show that coadministration of soluble TNFRSF1A (10 ng/ml) but not OPG (100 ng/ml) antagonized the decrease of BMD, BMC, and bone volume and the increase of the osteoclast number by DcR3 (Table 2), sug-
Effect of local administration of DcR3 on the bone turnover

DcR3 (30 µg/ml, 10 µl, once/day) and soluble TNFRSF1A (10 ng/ml) or OPG (100 ng/ml) were locally administered into tibia through the needle cannula in the proximal tibia for 7 consecutive days. Vehicle was injected into the contralateral side for comparison. Rats were sacrificed, and the tibiae were used for analysis 7 days after the last injection. Data are presented as mean ± S.E. (n = 7–10). Definitions are: BV/TV, bone volume/tissue volume; N.Oc/BS, osteoclast number/mm bone surface.

| Control | DcR3 | DcR3 + TNFRSF1A | DcR3 + OPG | TNFRSF1A | OPG |
|---------|------|----------------|------------|----------|-----|
| N.Oc/BS (mm) | 1.81 ± 0.04 | 2.05 ± 0.06a | 1.86 ± 0.03b | 1.93 ± 0.03 | 1.76 ± 0.04 | 1.75 ± 0.04 |
| BMD (g/cm²) | 0.090 ± 0.002 | 0.073 ± 0.003a | 0.085 ± 0.003a | 0.078 ± 0.002 | 0.092 ± 0.003 | 0.093 ± 0.004 |
| BMC (µg) | 0.081 ± 0.002 | 0.071 ± 0.003a | 0.079 ± 0.003b | 0.072 ± 0.002 | 0.082 ± 0.003 | 0.083 ± 0.003 |
| BV/TV (%) | 8.9 ± 0.6 | 6.1 ± 0.8a | 8.3 ± 0.3b | 6.9 ± 0.4 | 8.9 ± 0.6 | 9.0 ± 0.6 |

a p < 0.05 as compared with control groups.
b p < 0.05 as compared with DcR3-treated groups.

Gestating that TNF-α may be the downstream molecules involved in the bone action of DcR3.

Effect of DcR3 on the Resorption Activity of Mature Osteoclasts and Osteoblastic Function—Previously in this section, it was shown that osteoclastogenesis increases in DcR3 transgenic mice. Yang et al. (25) also show that exogenous DcR3 increases osteoclast formation in cultured macrophage or stromal cells. To directly evaluate the effect of DcR3 on the resorption activity of osteoclasts, mature osteoclasts were isolated from long bones of rabbit and cultured on an OAAS plate. Treatment with DcR3 (1–30 µg/ml) for 2 days significantly increased the resorption activity of mature osteoclasts in a concentration-dependent manner (Fig. 5). Two major cell types responsible for bone remodeling are osteoclasts, which resorb bone, and osteoblasts, which form new bone. We then examined the effect of DcR3 on the function of osteoblasts. To examine the effect of DcR3 on osteoblasts, the osteoblastic cells isolated from neonatal rat calvaria were used. Treatment with DcR3 at 30 µg/ml exerted no significant effect on the proliferation rate (Fig. 6A) and RANKL secretion (control, 109 ± 4 pg/ml; DcR3 transgenic mice, 111 ± 6 pg/ml, n = 4) in osteoblastic cultures. However, DcR3 at higher concentrations of 10–30 µg/ml slightly increased ALP activity and formation of mineralized bone nodules (Fig. 6, B–D). We then compared the osteoblastic function using osteoblastic cultures isolated from calvaria of wild-type and DcR3 transgenic mice, respectively. It is found that there is no quantitative difference in the proliferation rate of osteoblasts between DcR3 transgenic mice and wild-type controls (Fig. 6E). As shown in the Western blot of Fig. 6F, osteoblasts isolated from transgenic mice but not wild-type controls expressed DcR3. The mineralized bone nodule formation increased in response to vitamin C (50 µg/ml) plus β-glycerophosphate (10 mM) in transgenic mice. The quantitative data were shown in Fig. 6G. In parallel, the ALP activity of cultured osteoblasts isolated from transgenic mice also slightly increased (Fig. 6H). These data suggest that DcR3 exerted marked effect on osteoclastogenesis and also slightly increases osteoblastic function at higher concentrations.

DISCUSSION

Since DcR3, a decoy receptor belonging to the TNF receptor superfamily, is expressed in a variety of tumor cells and a large increase in bone resorption is associated with bone metastases of many tumors, it is important to determine whether DcR3 is one of the factors that contribute to the osteolytic bone metastases. Previous study has shown that DcR3 increases osteoclastogenesis from monocyte, macrophage, and bone marrow cells (25). In this study, we further demonstrate that bone mass values in DcR3 transgenic mice are significantly decreased as compared with wild-type controls and provide a new insight into the possible mechanism of cancer-associated osteolysis.

To understand the function of DcR3 in bone metabolism in vivo, transgenic mice overexpressing DcR3 were generated (26). It was found that BMD, BMC, and trabecular bone volume in DcR3 transgenic mice were significantly decreased as compared with wild-type controls. These differences were accompanied by a marked increase of the osteoclast numbers, which is consistent with the result of in vitro experiments. Since DcR3 is overexpressed in various cancers, with expression levels being linked to tumor status, it is possible that DcR3 may enable tumor cells to evade immune surveillance by neutralizing FasL- and LIGHT-induced cytotoxicity and blockade of LIGHT- and TL1A-induced T cell costimulation. Moreover, DcR3 has been implicated in the enhancement of tumor growth through neutralizing TL1A, thereby enhancing angiogenesis in human umbilical vein endothelial cells in vitro and inducing angiog-
FIGURE 6. Increase of ALP activity and bone nodule formation in osteoblastic cultures derived from DcR3 transgenic mice. A, primary osteoblasts derived from neonatal rats were treated with DcR3 (30 μg/ml) for the indicated time intervals, and cell proliferation was measured by the bromodeoxyuridine assay as described under “Experimental Procedures.” B, primary osteoblasts were plated in 24-well plates and incubated with DcR3 (3–30 μg/ml) for 48 h. Alkaline phosphatase activity was then determined using an ALP kit. C, primary osteoblasts were cultured in the presence of vitamin C (50 μg/ml) and β-glycerophosphate (10 mM) for 12 days, and mineralized bone nodules were identified using alizarin red staining. D, the bound staining was eluted with a solution of 10% cetylpyridinium chloride and quantified using a microtiter plated reader. E, osteoblasts isolated from neonatal calvaria of wild-type or DcR3 transgenic mice and cell proliferation were measured by the bromodeoxyuridine assay as described under “Experimental Procedures.” F, the expression of DcR3 in osteoblasts of DcR3 transgenic mice was determined by immuno-blotting using an anti-DcR3 monoclonal antibody (upper panel). Osteoblasts isolated from calvaria of wild-type (WT) or DcR3 transgenic mice were cultured in the presence of vitamin C (50 μg/ml) and β-glycerophosphate (10 mM) for 12 days, and mineralized bone nodules were identified using alizarin red staining (lower panel). G, the bound staining was eluted with a solution of 10% cetylpyridinium chloride and quantified using a microtiter plated reader. H, osteoblasts isolated from calvaria of wild-type or DcR3 transgenic mice were plated in 24-well plates for 48 h. Alkaline phosphatase activity was then determined using an ALP kit. Note that DcR3 at higher concentrations or DcR3 transgene expression slightly increased alkaline phosphatase activity and mineralized bone nodule formation of osteoblasts but did not affect cell proliferation. Data are presented as mean ± S.E. *, p < 0.05 as compared with control.
esis in vivo (23). Once cancer cells in bone stimulate osteoclastic bone resorption, they may initiate a vicious cycle in which growth factors released from matrix enhance tumor cell growth and parathyroid hormone-related protein production (32). Therefore, it has been demonstrated that tumor growth is positively correlated with the rate of bone resorption (32). Secretion of DcR3 by cancer cells may probably be associated with the development of bone metastasis.

DcR3 has been reported to be a potent modulator of host immunity (20), as well as to enhance the differentiation of osteoclasts and angiogenesis (25). We further isolated the bone marrow stromal cells to compare the osteoclastogenesis between wild-type and transgenic mice in the presence of M-CSF and RANKL. It was found that more osteoclasts formed in DcR3 transgenic mice. The osteoclastogenesis curves diverged early on days 2 and 4 between wild-type and DcR3 transgenic mice, suggesting that DcR3 increased osteoclast formation instead of decreasing their apoptosis. The increase of bone resorption activity at OAAS plates may result from the increase of osteoclastogenesis and/or resorption activity of osteoclasts. To simply examine the effect of DcR3 on the resorption activity of osteoclasts, the mature osteoclasts were isolated from long bones of rabbits. Treatment with DcR3 concentration-dependently increased resorption activity of mature osteoclasts. Our results suggest that DcR3 is able to affect osteoclastic differentiation as well as the resorption activity of mature osteoclasts. Yang et al. (25) demonstrated that DcR3 induced formation of TRAP-positive multinucleated cells probably via the release of TNF-α. Here we found that the serum level of TNF-α slightly increased in DcR3 transgenic mice (wild-type, 533 ± 12 pg/ml; transgenic mice, 571 ± 11 pg/ml, n = 4, p = 0.049). On the other hand, the osteoblast number in primary spongiosa was not significantly affected in DcR3 transgenic mice in comparison with control. However, the ALP activity slightly increased in DcR3 transgenic mice. Treatment with DcR3 at higher concentrations slightly increased nodule formation and ALP activity of primary cultured osteoblasts. In addition, the serum ALP activity increases in DcR3 transgenic mice in vivo (wild-type, 86.2 ± 4.0 units/liter; DcR3, 102.8 ± 3.7 units/liter, p = 0.02). The increase of serum ALP activity in vivo may also result from the bone remodeling derived from the increase of osteoclastogenesis. Therefore, DcR3 exerts a preferential effect on osteoclasts and also slightly affects osteoblastic function. These results may explain the increase in bone turnover and a decrease of bone mass in DcR3 transgenic mice.

Trabecular bone is composed of a lattice or network of branching bone spicules. The spaces between the bone spicules contain bone marrow. To simply look at the local effect of DcR3 in trabecular bone, DcR3 was locally administered into the tibia of young rats for 7 consecutive days. It was found that local administration of DcR3 decreased the bone volume and increased TRAP-staining osteoclasts in young rats. The decrease of bone volume may result from the increase of osteoclastic activity since DcR3 increases differentiation and resorption activity of osteoclasts. Local injection of DcR3 also decreased BMD and BMC in young rats, indicating that DcR3 may regulate bone remodeling. Previous data show that DcR3-induced osteoclast formation from RAW264.7 cells was inhibited by TNFR1-Fc (25). Here we show that coadministration with TNFRSF1A but not osteoprotegerin antagonized the action of DcR3. The result suggests that TNF-α is also involved in the regulation of osteoclastogenesis by DcR3 in vivo.

Our results suggest that DcR3 antagonist may represent a promising new class of antiresorptive drug for the treatment of osteoporosis and other bone diseases associated with DcR3-mediated increase of osteoclast activity. In conclusion, using an in vivo study of DcR3 transgenic mice, we provide evidences to demonstrate that the decoy receptor for TNF family cytokines, DcR3, is a novel effector molecule to enhance the formation of osteoclasts and their resorption activity. DcR3 thus has a hitherto unrecognized role in the regulation of bone mass and bone turnover. The novel function of DcR3 demonstrated in this study indicates a new role of DcR3 in osteolytic bone metastases of cancer cells and will be helpful in developing better strategies for the treatment of cancer metastasis in bone.

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Attenuation of Bone Mass by DcR3

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