Brief Definitive Report

TRANCE Is Necessary and Sufficient for Osteoblast-mediated Activation of Bone Resorption in Osteoclasts

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Summary

TRANCE (tumor necrosis factor–related activation-induced cytokine) is a recently described member of the tumor necrosis factor superfamily that stimulates dendritic cell survival and has also been found to induce osteoclastic differentiation from hemopoietic precursors. However, its effects on mature osteoclasts have not been defined. It has long been recognized that stimulation of osteoclasts by agents such as parathyroid hormone (PTH) occurs through a hormonal interaction with osteoblastic cells, which are thereby induced to activate osteoclasts. To determine whether TRANCE accounts for this activity, we tested its effects on mature osteoclasts. TRANCE rapidly induced a dramatic change in osteoclast motility and spreading and inhibited apoptosis. In populations of osteoclasts that were unresponsive to PTH, TRANCE caused activation of bone resorption equivalent to that induced by PTH in the presence of osteoblastic cells. Moreover, osteoblast-mediated stimulation of bone resorption was abrogated by soluble TRANCE receptor and by the soluble decoy receptor osteoprotegerin (OPG), and stimulation of isolated osteoclasts by TRANCE was neutralized by OPG. Thus, TRANCE expression by osteoblasts appears to be both necessary and sufficient for hormone-mediated activation of mature osteoclasts, and TRANCE-R is likely to be a receptor for signal transduction for activation of the osteoclast and its survival.

Key words: osteoblast • osteoclast • TRANCE • bone resorption • parathyroid hormone

Bone resorption, a function unique to the osteoclast, is regulated at several levels. Although supply from hemopoietic precursors sustains osteoclast numbers, regulation of mature cells also occurs, as evidenced by the rapid morphologic and calcemic responses that precede changes in osteoclast numbers when calciotropic hormones such as parathyroid hormone (PTH) and calcitonin are administered systemically. It has been known for many years that cells of the osteoblastic lineage play a crucial role in this process, such that agents like PTH, which stimulate resorption, do so by inducing osteoblastic cells to stimulate osteoclasts (1, 2). The molecular basis for this stimulation has remained elusive.

Recently, TRANCE (TNF-related activation induced cytokine) and its receptor (TRANCE-R; also called RANK) were identified in T and dendritic cells, respectively, as novel members of the TNF/TNFR superfamily (3–5). The same ligand was independently identified in bone marrow stromal cells and shown to induce the differentiation of hemopoietic progenitors into osteoclasts (6). Consistent with a role in osteoclastogenesis, the ligand was upregulated in osteoblasts incubated in calciotropic hormones. Lacey et al. (7) also identified TRANCE as an osteoblast-expressed ligand that stimulates osteoclastic differentiation and bone resorption. TRANCE-induced osteoclast formation and resorption were shown to be inhibited by osteoprotegerin (OPG), a soluble member of the TNFR superfamily encoded by a different gene from that for TRANCE-R.

TRANCE thus clearly induces osteoclastic differentiation from immature precursors. However, the effects of TRANCE on mature osteoclasts, and in particular the question whether TRANCE can by itself account for the action of osteoblastic cells on mature osteoclasts, or whether osteoblastic cofactors are also required, are questions that have not been addressed. We therefore assessed the effects of TRANCE on the motility, cytoplasmic spreading, survival, and bone resorptive activity of mature osteoclasts isolated from neonatal rat bone.

Materials and Methods

Recombinant soluble TRANCE (hCD8-TRANCE), in which the extracellular domain of murine TRANCE (amino acids 245–

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316) was fused to human CD8α (amino acids 1–182), was prepared as previously described (3, 4). hCD8-TRANCE was purified on cyanogen bromide–activated Sepharose gel conjugated to OKT8 following the manufacturer's protocol (Amersham Pharmacia Biotech, Piscataway, NJ). TRANCE receptor-Fc, a recombinant protein of the extracellular domain of TRANCE-R (also called RANK; reference 5) fused to the constant region of human IgG1, was prepared in a similar way using a baculovirus system. Recombinant human M-CSF was provided by Dr. J. Wozney (Genetics Institute, Cambridge, MA). Bovine PTH (1-34), used at 0.1 U/ml throughout, was purchased from Sigma Chemical Co. (Poole, UK). $1,25(OH)_2$vitamin D_3 (used at 10^{-8} M), was provided by Solvay Duphar (Weesp, The Netherlands). OPG was provided by Dr. C. Dunstan (Amgen, Thousand Oaks, CA). Osteoblast-like UMR 106 cells were from Dr. T.J. Martin (St. Vincents Institute for Medical Research, Melbourne, Australia).

Slices of bovine cortical bone were employed as substrates for osteoclastic resorption. Bone slices ($4 \times 3 \times 0.1$ mm) were prepared from adult bovine femora by using a low-speed saw (Isomet Corp., Springfield, VA), as previously described (8), cleaned by ultrasonication, washed, and stored dry at room temperature.

Assessment of Bone Resorption by Mature Osteoclasts. Osteoclasts were disaggregated, as previously described, from neonatal rat long bones (8). Wistar rats from the St. George's Medical School colony were killed by cervical dislocation within 72 h of birth. The femora, tibiae, and humeri were removed and dissected free of adherent tissue. Bones were then cut across their epiphyses and curetted in medium 199, and the curettings were vigorously agitated with a plastic pipette. Large fragments were allowed to sediment for 10 s. The cell suspension was then transferred to a 100×18 mm multiwell dish (Sterilin, Teddington, UK) containing bone slices. After 15 min of sedimentation, the bone slices were removed, washed in medium 199, and incubated overnight in a 96-well tissue culture plate (Sterilin) in the presence or absence of hCD8-TRANCE, TRANCE-R, OPG, and/or calciotropic hormones. For some experiments osteoclasts were co-cultured with either UMR 106 (5×10^4 cells/well) or calvarial cells (10^4 cells/well). To prepare calvarial cells, calvarias were removed from rats within 5 d of birth, dissected free of soft tissues and periosteum, and incubated for 2 h at 37°C in collagenase (type II; Sigma Chemical Co.; 1 mg/ml in medium 199). The bones were then vigorously agitated with a plastic pasteur pipette and the cell suspension was harvested. The cells were washed and resuspended in Hanks' MEM for addition to osteoclasts on bone slices. For all experiments, the final volume of incubation medium per well was 200 μl.

Bone resorption was assessed after 18 h of incubation, as previously described (9). Bone slices were removed from wells, immersed in 10% NaCl (BDH, Poole, UK) for 10 min to remove cells, washed in distilled water, dried, and sputter coated with gold. The entire surface of each bone slice was then examined in a scanning electron microscope (S90; Cambridge Instruments, Cambridge, UK).

Survival Studies. Bone cell suspensions were added to the wells of a 96-well plate (Sterilin) containing Thermanox coverslips (Gibco, Uxbridge, UK) and incubated for 15 min. Coverslips were then removed, washed in PBS, and placed into individual wells of a 96-well plate. Cells were incubated for 1 or 24 h in a total volume of 200 μl MEM/BSA in the presence of hCD8-TRANCE, M-CSF, or vehicle. Cells were then fixed for 2 min in formalin and stained for tartrate-resistant acid phosphatase (TRAP) (10). The substrate used was naphthol AS-B1 phosphate. Tartrate resistance was assessed in the presence of 0.05 M sodium tartrate. TRAP-positive multinuclear cells were counted by light microscopy.

Time-Lapse Observations. The bone cell suspension, obtained as above, was added to 25-cm^2 tissue culture flasks (Sterilin) and incubated for 15 min to allow osteoclast sedimentation and attachment. Nonadherent and loosely adherent cells were removed by washing with PBS. 10 ml of MEM/BSA were placed into the flasks and the cells were incubated for 30 min. Flasks were then sealed and placed in the incubation chamber of an Olympus 1MT-373 inverted microscope (Gallenkamp & Co. Ltd., London, UK). A suitable osteoclast-containing field was chosen and recorded for 30 min on a time-lapse video recorder at 1/60 normal speed. Vehicle or hCD8-TRANCE was then added in 0.5 ml prewarmed MEM/BSA and recording continued.

Measurement of Osteoclast Spread Area. Osteoclasts were incubated on Thermanox coverslips as above in the presence of M-CSF, hCD8-TRANCE, or vehicle for 1 h. Then, they were fixed and stained for TRAP activity. Osteoclast spread area was determined with a light microscope linked to a computer-assisted image analyzer (Perceptive Instruments, Cambridge, UK).

Results and Discussion

Addition of recombinant soluble TRANCE (hCD8-TRANCE) to isolated osteoclasts caused a dramatic change in their behavior. Within minutes the osteoclasts showed increased pseudopodial motility, associated with increased cell spreading (Fig. 1). Unlike M-CSF, which induces extension of cytoplasm around much or all of the periphery (9), osteoclastic motility and spreading remained localized in broad focal pseudopods, and was associated with rhythmic contractions of the cell body, similar to those we have occasionally observed to occur "spontaneously" in previous time-lapse studies of these cells (our unpublished data). Osteoclastic spreading has previously been noted in response to PTH, and was found to be dependent upon the presence of osteoblastic cells (11).

Isolated osteoclasts rapidly undergo apoptosis in vitro, which can be suppressed by M-CSF (9). However, the existence of alternative osteoblast-derived survival factors was suggested by the ability of osteoblastic cells from *op/op* mice, which lack M-CSF, to support survival of osteoclasts (our unpublished data), and by the prolonged resorption of bone that occurs after even a single administration of M-CSF to these mutants (12). hCD8-TRANCE stimulated osteoclastic survival at 100 and 1,000 ng/ml, similar to the concentrations that support dendritic cell survival (4) (Fig. 1). The relatively high concentration of the soluble ligand needed might reflect a physiological presentation of the ligand as a cell surface molecule (3, 5, 6). A previous study (7) found no effect of TRANCE in the survival of mature osteoclasts. However, no zero time point was included, so that the possibility was not excluded that sufficient levels of TRANCE or M-CSF, an alternative osteoclast survival factor (9), were generated by contaminant bone cells, for survival to occur in the absence of exogenous TRANCE.

We then tested the ability of hCD8-TRANCE to stimulate bone resorption by isolated osteoclasts. The con-

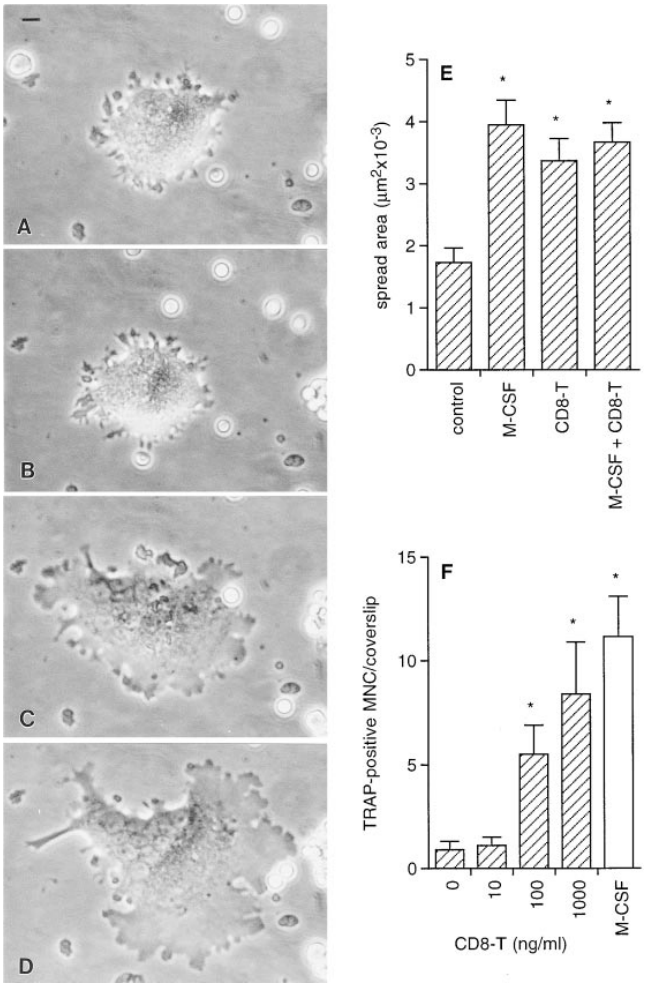

Figure 1. An osteoclast photographed 30 (*A*) and 1 (*B*) min before, and 30 (*C*) and 60 (*D*) min after addition of hCD8-TRANCE to culture (100 ng/ml). Bar = 2 μm. *E*, spread area of osteoclasts after incubation for 1 h with or without hM-CSF (5 ng/ml) or hCD8-TRANCE (*CD8-T*; 100 ng/ml). *F*, number of TRAP-positive multinuclear cells (*MNC*) present on coverslips in cultures of osteoclasts disaggregated from rat bone and incubated for 24 h in M-CSF (5 ng/ml) or hCD8-TRANCE (12 cultures per variable). Number of osteoclasts remaining after 24 h in hCD8-TRANCE (1,000 ng/ml) and M-CSF did not differ significantly from number present on coverslips incubated for 1 h. $*P < 0.05$ versus control (Student's *t* test).

founding influence of TRANCE on osteoclast survival was avoided by adding M-CSF to all cultures. We found that in populations of osteoclasts isolated from neonatal rat bone under conditions that render them unresponsive to PTH (i.e., in cultures lacking the ability to generate detectable levels of osteoclast resorption stimulating activity), hCD8-TRANCE induced dose-responsive stimulation of bone resorption (Fig. 2). TRANCE is the first agent found to be capable of stimulating bone resorption in this system, without added osteoblasts, in cultures that are unresponsive to indirect acting agents, such as PTH. Similar results were reported by Lacey et al. (7). However, in that report no control cultures incubated with an indirect-acting calciotropic hormone were included, so that the extent to which contaminant osteoblasts contributed to or mediated this response could not be determined. The very rapid motile responses seen in isolated osteoclasts, and the increase in bone resorption by hCD8-TRANCE in cultures unable to generate endogenous osteoclast-stimulating activity, suggest that hCD8-TRANCE directly stimulates osteoclasts. Like the response of osteoclasts to stimulation by osteoblastic cells, increased resorption was accomplished predominantly by an increase in the number of excavations formed without significant change in the area of individual excavations (data not shown), consistent with an increase in the proportion of osteoclasts that were resorptive (9, 13). Moreover, stimulation of bone resorption by hCD8-TRANCE was quantitatively similar to that induced by the maximally active concentration of PTH in cocultures of mature osteoclasts with osteoblastic UMR 106 cells (Fig. 2); and PTH treatment induced TRANCE mRNA expression in these cells (data not shown). Moreover, PTH and hCD8-TRANCE showed no additive effect, suggesting that TRANCE alone is sufficient to fully account for the stimulation of bone resorption induced in mature osteoclasts by PTH.

The osteoblast-dependent ability of PTH to stimulate osteoclastic bone resorption was abrogated by soluble TRANCE-R–Fc fusion protein (TR-Fc) (Fig. 3). In addition, a high level of TRANCE-R mRNA was detected in mature osteoclasts (data not shown). This not only supports the view that TRANCE stimulates the differentiation (6) and activation of osteoclasts, but also provides evidence that, similar to its action on dendritic cells (3, 4), TRANCE-R is likely to be a receptor for signal transduction for activation of the osteoclast and its survival. In neither of the two coculture systems did TR-Fc suppress bone resorption below the basal levels of resorptive activity demonstrated by osteoclasts in unstimulated cultures. This suggests that stimulated bone resorption was inhibited through neutralization

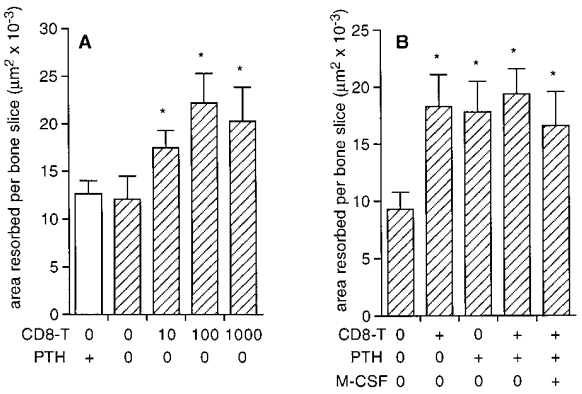

Figure 2. Bone resorption by osteoclasts freshly isolated from neonatal rat bones and incubated for 24 h on bone slices without (*A*) or with (*B*) UMR 106 cells. *A*, osteoclasts incubated with M-CSF (5 ng/ml) with or without PTH (0.1 U/ml) or hCD8-TRANCE. *B*, osteoclasts incubated with hCD8-TRANCE (100 ng/ml) and/or PTH (0.1 U/ml) and/or M-CSF (5 ng/ml). $n = 12$ cultures per variable. $*P < 0.05$ versus control. Number of TRAP-positive cells present after incubation did not differ significantly between groups (range of means: *A*, 11.5–14.7; *B*, 17.8–20.8). *CD8-T*, hCD8-TRANCE.

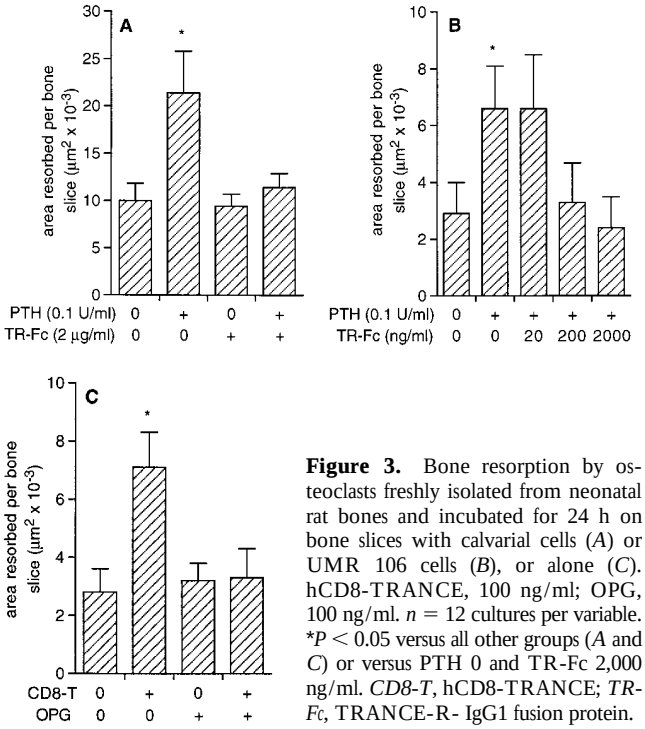

Figure 3. Bone resorption by osteoclasts freshly isolated from neonatal rat bones and incubated for 24 h on bone slices with calvarial cells (*A*) or UMR 106 cells (*B*), or alone (*C*). hCD8-TRANCE, 100 ng/ml; OPG, 100 ng/ml. $n = 12$ cultures per variable. $^*P < 0.05$ versus all other groups (*A* and *C*) or versus PTH 0 and TR-Fc 2,000 ng/ml. *CD8-T*, hCD8-TRANCE; *TR-Fc*, TRANCE-R- IgG1 fusion protein.

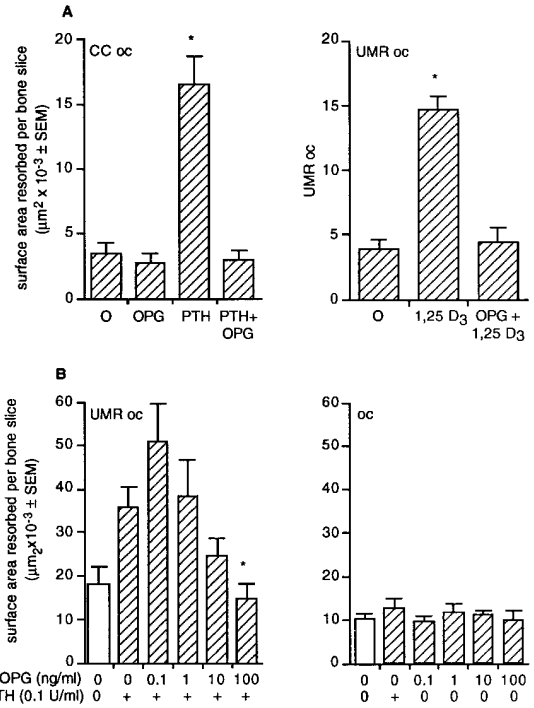

Figure 4. (*A*) Bone resorption by mature osteoclasts incubated for 18 h on bone slices with calvarial cells (*CC oc*) or UMR 106 cells (*UMR oc*) in the presence and absence of PTH (0.1 U/ml), 1,25$(OH)_2$vitamin D_3 (10^{-8} M), and/or OPG (100 ng/ml). $n = 12$ cultures per variable. $^*P < 0.01$ versus all other groups (Student's *t* test). (*B*) Bone resorption by mature osteoclasts disaggregated from neonatal rat long bones and incubated for 18 h on bone slices alone (*oc*) or with added osteoblastic UMR 10^6 cells (*UMR oc*). At no concentration did OPG significantly stimulate bone resorption. Significant suppression of bone resorption by OPG ($^*P < 0.01$ versus O+PTH group) occurred at 100 ng/ml. $n = 12$ cultures per variable.

of TRANCE, rather than through induction (e.g., via reverse signaling by receptor-bound TRANCE, as can occur in some members of this ligand family; references 14–17) of osteoclast-inhibitory activity in osteoblastic cells. OPG also neutralized the ability of both hCD8-TRANCE (Fig. 3) and osteoblastic cells (Fig. 4) to stimulate bone resorption by mature osteoclasts. Thus, OPG inhibits bone resorption not only through inhibition of osteoclast formation (6, 7, 18), but also through suppression of osteoblast-stimulated bone resorption by mature osteoclasts.

TRANCE thus appears to be both necessary and sufficient to account for the ability of osteoblastic cells to stimulate mature osteoclasts. Although osteoclast-stimulating activity has on occasion been detected in culture supernatants from osteoblastic cells (19–23), it has also frequently been detectable only when osteoblasts are cocultured with osteoclasts (11, 24–26). It may be that, like some other members of this family of ligands, TRANCE exists physiologically as membrane-expressed and soluble forms (27, 28). However, the relatively high concentrations of hCD8-TRANCE that were required for activity suggests that, like M-CSF (29), the ligand is normally presented to osteoclasts on the surface of osteoblastic cells. It is also likely that under some circumstances any TRANCE that is released by osteoblastic cells will be neutralized by OPG, which is also expressed by bone cells (6, 18). These and other potential interactions are now amenable to analysis. The actions of TRANCE on mature osteoclasts make this system of ligand, receptor, and decoy receptor seem likely to be crucial to the local regulation of osteoclastic localization, survival, and resorptive activity on bone surfaces, and thus to our understanding of the mechanisms by which the intricate and dynamic patterns of resorptive activity that occur during skeletal morphogenesis and restructuring are regulated.

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