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TNFα Potently Activates Osteoclasts, through a Direct Action Independent of and Strongly Synergistic with RANKL

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TNFα is pivotal to the pathogenesis of inflammatory and possibly postmenopausal osteolysis. Much recent work has clarified mechanisms by which TNFα promotes osteoclastogenesis, but the means by which it activates osteoclasts to resorb bone remain uncertain. We found that very low concentrations of TNFα promoted actin ring formation, which correlates with functional activation in osteoclasts, both in osteoclasts formed *in vitro* and extracted from newborn rats. TNFα was equipotent with RANKL for this action. Activation by TNFα was unaffected by blockade of RANKL by OPG, its soluble decoy receptor, suggesting that this was due to a direct action on osteoclasts. Bone resorption was similarly directly and potently stimulated, in a RANKL-independent manner in osteoclasts, whether these were formed *in vitro* or *in vivo*. Interestingly, TNFα promoted actin ring formation at concentrations an order of magnitude below those required for osteoclastic differentiation. Moreover, TNFα strongly synergized with RANKL, such that miniscule concentrations of TNFα were sufficient to substantially augment osteoclast activation. The extreme sensitivity of osteoclasts to activation by TNFα suggests that the most sensitive osteolytic response of bone to TNFα is through activation of existing osteoclasts; and the strong synergy with RANKL provides a mechanism whereby increased osteolysis can be achieved without disturbance to the underlying pattern of osteoclastic localization. (*Endocrinology* 143: 1108–1118, 2002)

BONE REMODELING, WHEREBY osteoclasts resorb and osteoblasts reform bone, is an essential function that maintains skeletal integrity throughout life. Excessive activity by osteoclasts is responsible for the development of postmenopausal osteoporosis and for the destruction of joints and generalized bone loss that accompanies inflammatory diseases such as rheumatoid arthritis. Recently, it has become clear that the formation and activity of osteoclasts *in vivo* is dependent on the expression of RANKL by osteoblastic or bone marrow stromal cells (see Refs. 1 and 2 for reviews). Transgenic experiments have shown that deletion of the gene for RANKL or RANK, or overexpression of OPG, a soluble decoy receptor for RANKL, causes failure of osteoclast formation and osteopetrosis. It has been shown that the bone resorption that is caused by agents such as PTH, $1,25(OH)_2D_3$ and IL-1 is associated with and dependent upon increased expression of RANKL by osteoblastic/marrow stromal cells (see Refs. 1 and 2 for reviews).

TNFα is crucial to the pathogenesis of bone and joint destruction that occurs in rheumatoid arthritis and has been implicated in the bone loss of periodontitis, orthopedic implant loosening, and other forms of chronic inflammatory osteolysis (3–7). Moreover, it has been reported that TNFα mediates bone loss in estrogen deficiency (8, 9). Like many other resorptive agents, TNFα induces expression of RANKL in osteoblastic cells (10, 11). However, recent reports have claimed that TNFα, like RANKL, directly induces osteoclastic differentiation in bone marrow macrophages *in vitro* (12, 13). This direct action may be dependent on priming of the precursors by RANKL (14).

However, while the ability of TNFα to induce osteoclast formation has been established, its effects on osteoclast function have been much less studied. Ultimately, bone loss depends on the latter. For example, injection of PTH leads to an increase in the extent of ruffled borders within 15 min, with rapid onset of hypercalcemia (15–17). Thus, the resorption rate is controlled not only through changes in osteoclast numbers but also by activation of preexisting osteoclasts. The distinct nature of the regulation of these two processes is emphasized by the ability of PGE_2 (18) and TGFβ (19, 20) to stimulate osteoclast formation but not activity. There is evidence that TNFα does not directly stimulate resorption by osteoclasts (12, 21).

We have therefore elected to test the ability of TNFα to directly activate osteoclasts, in an attempt to clarify its role in osteoclast regulation. For most of this work, actin ring formation, rather than bone resorption, was used as an indicator of the activated status of the cells. This is because, in the time scale needed to detect bone resorption, changes in osteoclast differentiation and apoptosis can also occur that may contribute to the result. In contrast, actin rings form rapidly when osteoclasts are activated. These distinctive rings represent the circumferential zone of polymerized actin at the lateral limit to the resorptive hemivacuole and are characteristic of osteoclasts that are actively resorbing bone (22–27). We found that TNFα directly activated osteoclasts, whether these were formed *in vivo* or *in vitro*. TNFα was as potent as RANKL in osteoclast activation and appeared even more potent for osteoclast-activation than for osteoclast formation. These actions

Abbreviations: EMEM, MEM with Earle's salts; HMEM, MEM with Hanks' salts; hTNF, human TNF; M-CSF, macrophage colony-stimulating factor; MNCs, multinucleate cells.

were independent of but strongly synergistic with RANKL.

Materials and Methods

Media and reagents

Nonadherent, macrophage colony-stimulating factor (M-CSF)-dependent bone marrow cells were incubated with MEM with Earle's salts (EMEM) (Sigma, Poole, Dorset, UK), supplemented with 10% FCS (Autogen Bioclear, Calne, Wiltshire, UK). HEPES-buffered medium 199 (Sigma) was used for isolation and sedimentation of osteoclasts *ex vivo*. MEM with Hanks' salts (HMEM) was used for subsequent incubation of osteoclasts for assessment of actin ring formation and bone resorption. EMEM was used in cell spreading experiments. All media were supplemented 2 mM glutamine, 100 IU/ml benzylpenicillin, and 100 μg/ml streptomycin (Sigma). Incubations were performed at 37 C in 5% CO_2 in humidified air. Recombinant human M-CSF was provided by Chiron Corp. (Emeryville, CA); soluble recombinant murine RANKL and human recombinant OPG were gifts from Amgen, Inc. (Thousand Oaks, CA). Murine TNFα was used throughout unless otherwise stated. Recombinant murine or human (h) TNFα and IL1 receptor antagonist (RA) were purchased from R&D (Abingdon, Oxon, UK); bovine PTH (1–34) was obtained from Sigma.

Slices of bovine cortical bone were prepared as previously described (28).

Isolation and culture of bone marrow precursors

Osteoclast-like cells were induced from nonadherent, M-CSF-dependent bone marrow cells as previously described (18). Briefly, MF1 mice (5–8 wk old) were killed by cervical dislocation. Femora and tibiae were aseptically removed and dissected free of adherent soft tissue. The bone ends were cut, and the marrow cavity was flushed out into a Petri dish by slowly injecting PBS at one end of the bone using a sterile 21-gauge needle. The bone marrow suspension was carefully agitated through a 21-gauge needle to obtain a single cell suspension. Bone marrow cells were then washed, resuspended in EMEM/FCS, and incubated at a density of 3×10^5 cells/ml for 24 h in a 75-cm^2 flask (Helena Biosciences, Sunderland, Tyne & Wear, UK) with M-CSF (5 ng/ml) in the presence or absence of OPG (500 ng/ml) or RANKL (30 ng/ml). This incubation of bone marrow cells at low density in M-CSF for 24 h efficiently depletes the cell preparations of stroma: stromal cells were not detectable in the cell preparations and no cells were present in cultures subsequently incubated without M-CSF. After 24 h, nonadherent cells were harvested, washed, and resuspended in EMEM/FCS. A total of 2×10^5 cells were added to the wells of 24-well plates (Helena Biosciences) containing a Thermanox coverslip (Life Technologies, Inc., Paisley, UK) or a slice of bovine cortical bone in a total volume of 1 ml EMEM/FCS containing M-CSF (50 ng/ml) with or without cytokines. Cultures were fed every 2–3 d by replacing 600 μl of culture medium with an equal volume of fresh medium and cytokines. Coverslips and bone slices were assessed for TRAP positivity, bone resorption, or actin ring formation as described below.

Isolation and culture of spleen cells

MF1 mice (5 d old) were killed by cervical dislocation and their spleens aseptically removed. The capsule was cut open at the ends and hemopoietic spleen cells were carefully squeezed out into suspension. The resulting cell suspension was repeatedly passed through a 21-gauge needle to obtain a single cell suspension. Spleen cells were then washed, resuspended in EMEM/FCS, and incubated at a density of 3×10^5 cells/ml for 24 h in a 75-cm^2 flask with M-CSF (5 ng/ml). After 24 h, nonadherent cells were harvested, washed and resuspended in EMEM/FCS. A total of 3×10^4 cells were added to the wells of 96-well plates (Helena Biosciences) containing a Thermanox coverslip or a slice of bovine cortical bone and incubated in a total volume of 200 μl EMEM/FCS containing M-CSF (50 ng/ml) in the presence or absence of TNFα. Cultures were fed every 2–3 d by replacing 120 μl of culture medium with an equal volume of fresh medium and cytokines. After incubation, coverslips and bone slices were assessed for TRAP positivity or bone resorption as described below.

Isolation of osteoclasts ex vivo

Osteoclasts were disaggregated from the long bones of 2- to 3-d-old Wistar rats, as previously described (28). The femora, tibiae, and humeri were removed and dissected free of adherent tissue. Bones were then cut across their epiphyses and curetted into medium 199, and the curettings were vigorously agitated with a plastic pipette. Large fragments were allowed to sediment for 10 sec. The cell suspension was then transferred either to a 100 × 18-mm multiwell dish (Sterilin, Teddington, Middlesex, UK) containing bone slices or to wells of a 96-well plate containing Thermanox coverslips and incubated for 10 min. Bone slices and coverslips were then removed, washed in PBS, and placed into wells of a 96-well plate. Cells were incubated in a total volume of 200 μl MEM in the presence or absence of cytokines, before assessment of actin ring formation or bone resorption, or measurement of osteoclast spread area as described below.

TRAP cytochemistry

Osteoclast-like cell formation in liquid cultures of M-CSF-dependent bone marrow cells, evaluated by quantification of TRAP-positive cells with three or more nuclei, osteoclast spread area, and cellular constitution of colonies formed in semisolid medium, were assessed using a modification of the method of Burstone (29). After incubation, cells on microscope slides, coverslips, or bone slices were fixed in formalin for 10 min, washed, permeabilized in acetone for 10 min, washed, and stained for acid phosphatase in the presence of 0.05 M sodium tartrate (Sigma). The substrate used was napthol AS-BI phosphate (Sigma). Cells were counterstained with hematoxylin.

Assessment of bone resorption

After incubation, bone slices were immersed in 10% (vol/vol) sodium hypochlorite (British Drug Houses, Lutterworth, Leicestershire, UK) for 10 min to remove cells, washed, and dried. After drying, the bone slices were either mounted onto stubs for scanning electron microscopy or glass slides, and sputter coated with gold. The entire surface of each bone slice from experiments with osteoclasts formed *in vivo* was examined in a scanning electron microscope (S90; Cambridge Instruments, Cambridge, UK) (30). Bone slices from experiments using *in vitro*-generated osteoclast-like cells were examined by reflected light microscopy and bone resorption quantified using an eyepiece graticule.

Assessment of actin ring formation

Osteoclast-like cells generated *in vitro* and *ex vivo* osteoclasts on bone slices were washed in PBS. The bone slices were then transferred to wells of a 96-well plate containing 100 μl of HMEM/FCS with or without M-CSF (0.5 ng/ml) and cells incubated for 1 h at 37 C. One hundred microliters of medium, with or without cytokines, were added and incubation continued for 2 h. After incubation, bone slices were washed in PBS, fixed for 5 min in 10% formalin, and cells permeabilized with 0.1% Triton X-100 for 5 min. Bone slices were then incubated in 1 μg/ml FITC-conjugated phalloidin (Sigma) for 45 min at 37 C, washed three times in PBS, once in sterile water, and mounted onto glass slides in fade-resistant mounting medium (DAKO Corp., Ely, Cambridgeshire, UK). Actin rings were visualized using a Carl Zeiss (Jena, Germany) fluorescent microscope fitted with narrow band FITC filters and a video camera (Panasonic WV CL-700) linked to a monitor. The number of actin rings and the total area of bone surface contained within actin rings was quantified using a grid superimposed over the monitor screen by an individual blinded to the nature of the samples measured. Only those structures that consisted of complete and broad actin bands were counted.

Measurement of osteoclast spread area

Osteoclasts, isolated from neonatal rat long bones and sedimented onto Thermanox coverslips as above, were incubated for 30 min in 100 μl EMEM containing 1 mg/ml BSA (Sigma). One hundred microliters of medium with or without TNFα was then added and incubation continued for a further 1 h. Coverslips were fixed and stained for TRAP activity. The total area contained within TRAP-positive multinuclear osteoclasts was quantified using a grid superimposed over a monitor linked to a Carl Zeiss light microscope.

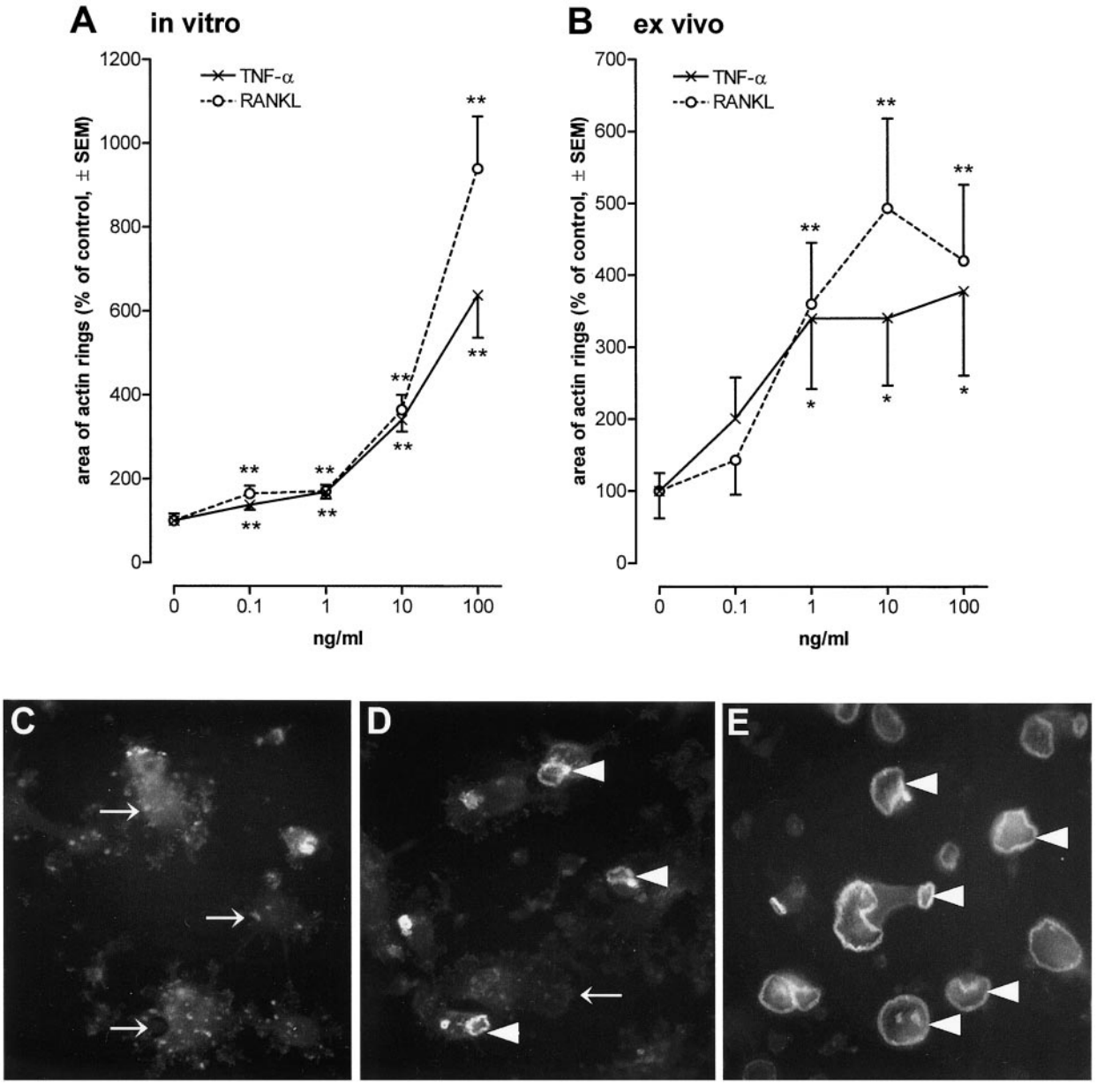

FIG. 1. TNFα induces actin ring formation in osteoclasts. Osteoclast-like cells (A, C, D, E) were formed by incubation of M-CSF-dependent bone marrow cells on bone slices in RANKL (30 ng/ml) and M-CSF (50 ng/ml) for 5 d. Bone slices were washed extensively and transferred to new wells containing HMEM/FCS containing 0.5 ng/ml M-CSF, which is sufficient for survival (30). Cells were incubated for 1 h. RANKL or TNFα was then added and incubation was continued for a further 2 h. Cells were fixed and stained with FITC-phalloidin and evaluated for actin ring formation. Actin ring formation in osteoclasts isolated from neonatal rat long bones (*ex vivo* osteoclasts) was also evaluated (B). For this, cells curetted from long bones were allowed to adhere to bone slices for 10 min, washed, and treated as above. The area of actin rings ($\mu m^2 \times 10^{-3} \pm$ SEM) per cm^2 in control cultures was: A: TNFα control 131.2 ± 12.3; RANKL control 213.2 ± 36.9; B: TNFα 3.0 ± 0.7; RANKL 2.2 ± 0.8. A, n = 8 (RANKL) or 16 (TNFα) bone slices per variable; B, n = 22 bone slices per variable. *, $P < 0.05$; **, $P < 0.01$ *vs.* controls. C–E, Photomicrographs of osteoclasts formed *in vitro* and incubated without TNFα (C), with 1 ng/ml TNFα (D), or with 100 ng/ml TNFα (E). *Arrows* denote osteoclasts without actin rings; *arrowheads* denote some of the actin rings. Magnification, ×90.

Colony formation in semisolid medium

Murine bone marrow cells were incubated (2×10^4/ml) in 1 ml EMEM with 1.2% methyl cellulose (Sigma) and 20% FCS in the presence or absence of M-CSF (50 ng/ml), RANKL (100 ng/ml), and TNFα (100 ng/ml) in 35-mm Petri dishes (Helena Bioscience) for 8 d. Discrete colonies were picked using a 3-μl pipette then smeared onto glass slides, fixed in formalin, and stained for TRAP to allow assessment of cellular constitution.

Statistical analysis

Differences between groups were analyzed using unpaired *t* test.

Results

To resorb bone, osteoclasts must become polarized and form a circumferential seal with the substrate. Proteases and protons are released into the specialized extracellular re-

sorptive compartment circumscribed within this sealing ring. Numerous laboratories have described the specialized actin ring structure that overlies this zone of tight sealing (*e.g.* 22, 23, 25, 26). It corresponds to the clear zone identified by microscopy because the actin network excludes organelles from this region. There is an excellent correlation between actin ring formation and bone resorption (24), and it appears that the actin ring is required for bone resorption (27).

We found that TNFα strongly stimulated actin ring formation in both osteoclast-like cells formed *in vitro* and in osteoclasts formed *in vivo* (Fig. 1). TNFα was equipotent with RANKL for this action and induced significant increases in the area of actin rings at concentrations as low as 0.1 ng/ml. Experiments performed in the absence of M-CSF (an osteoclast survival factor) produced the same results; and osteoclast numbers on bone slices incubated in parallel to those assessed for actin ring formation did not differ significantly between treatments (data not shown). The increase in total area of actin rings induced by TNFα and RANKL was achieved by an approximately equal contribution from an increase in the number of actin rings, and in the average area of individual rings (data not shown).

Previously, we have shown that activation of osteoclasts by osteoblastic cells is associated with the induction of increased cell spreading in osteoclasts (31, 32). This effect is mimicked by RANKL (33). We found (Fig. 2) that TNFα has the ability to similarly increase osteoclast cell spreading. The short incubation time used for this assay adds further support to the notion that osteoclastic cells can respond directly to TNFα.

The possibility that TNFα activates osteoclasts directly, independent of RANKL-RANK interactions, was further investigated by assessing TNFα-mediated stimulation of actin ring formation in the presence of OPG, the soluble decoy receptor for RANKL. We found no inhibition of TNFα-induced actin ring formation by OPG (Fig. 3). Furthermore, we also found no inhibition of TNFα-induced actin ring formation by IL-1RA (data not shown).

It has been reported that TNFα synergizes with RANKL in the induction of osteoclastic differentiation (14, 34). To explore whether TNFα also has the capacity to synergize with RANKL in osteoclastic activation, we incubated osteoclast-like cells in TNFα and/or RANKL at concentrations that alone would be expected to induce a minimal activation response. As seen in Fig. 4, simultaneous culture in even minimal concentrations of both ligands elicited a dramatic activation response, equivalent to the response achieved by incubation in RANKL alone at a concentration an order of magnitude greater. The potency of this synergy prompted us to test the ability of PTH, another osteolytic agent believed to act primarily through induction of RANKL in osteoblastic cells, to similarly synergize with RANKL for actin ring formation. In contrast, we saw no evidence for synergy between PTH and RANKL (Fig. 4).

The ability of TNFα to act directly on osteoclasts was surprising, in view of previous experiments, in which we found that TNFα had no effect on bone resorption by osteoclasts isolated from rodent bone (21). We therefore further tested the ability of TNFα to activate bone resorption by osteoclasts isolated from neonatal rats. We found (Fig. 5) that

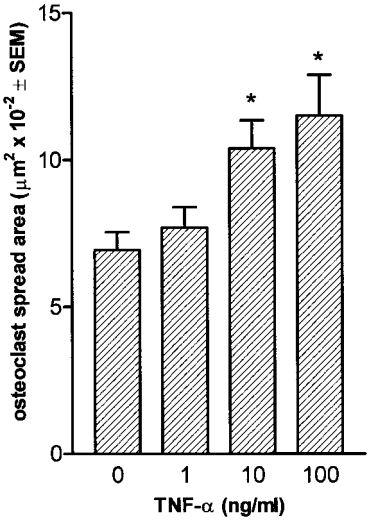

FIG. 2. TNFα directly stimulates osteoclast spread area. Osteoclasts were isolated from neonatal rat long bones and sedimented on to plastic coverslips. Coverslips were washed after 10 min and the cells were preincubated in EMEM containing BSA (1 mg/ml) for 30 min before addition of TNFα or vehicle. Incubation was continued for 1 h before cells were fixed for evaluation of spread area. *, $P < 0.01$ *vs.* control. Sixty osteoclasts were measured for each variable.

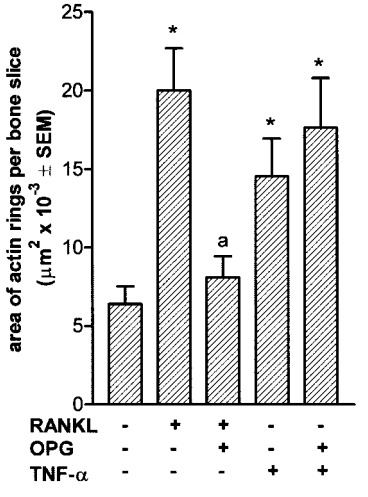

FIG. 3. OPG does not inhibit the induction of actin rings by TNFα. Osteoclasts were disaggregated from neonatal rat long bones and sedimented on to bone slices. After a 1-h preincubation in MEM/FCS, RANKL (30 ng/ml), TNFα (100 ng/ml), and OPG (500 ng/ml) were added and incubation was continued for a further 2 h before assessment of actin rings. *, $P < 0.01$ *vs.* controls; a, $P < 0.01$ *vs.* RANKL. n = 30 bone slices per variable.

TNFα promoted bone resorption over a similar dose range to that noted using actin rings. Also, as found with actin rings, the response appeared to be a direct effect, independent of RANKL, since it was completely unaffected by OPG at a concentration (500 ng/ml) substantially higher than that capable of completely abolishing osteoblast-mediated stimulation of osteoclasts isolated *ex vivo* (10 ng/ml; Ref. 33) (Fig. 5).

In those previous experiments (21), we used human TNFα. It has since been found that human TNFα is much less potent on rodent cells than is murine TNFα. The lack of detectable

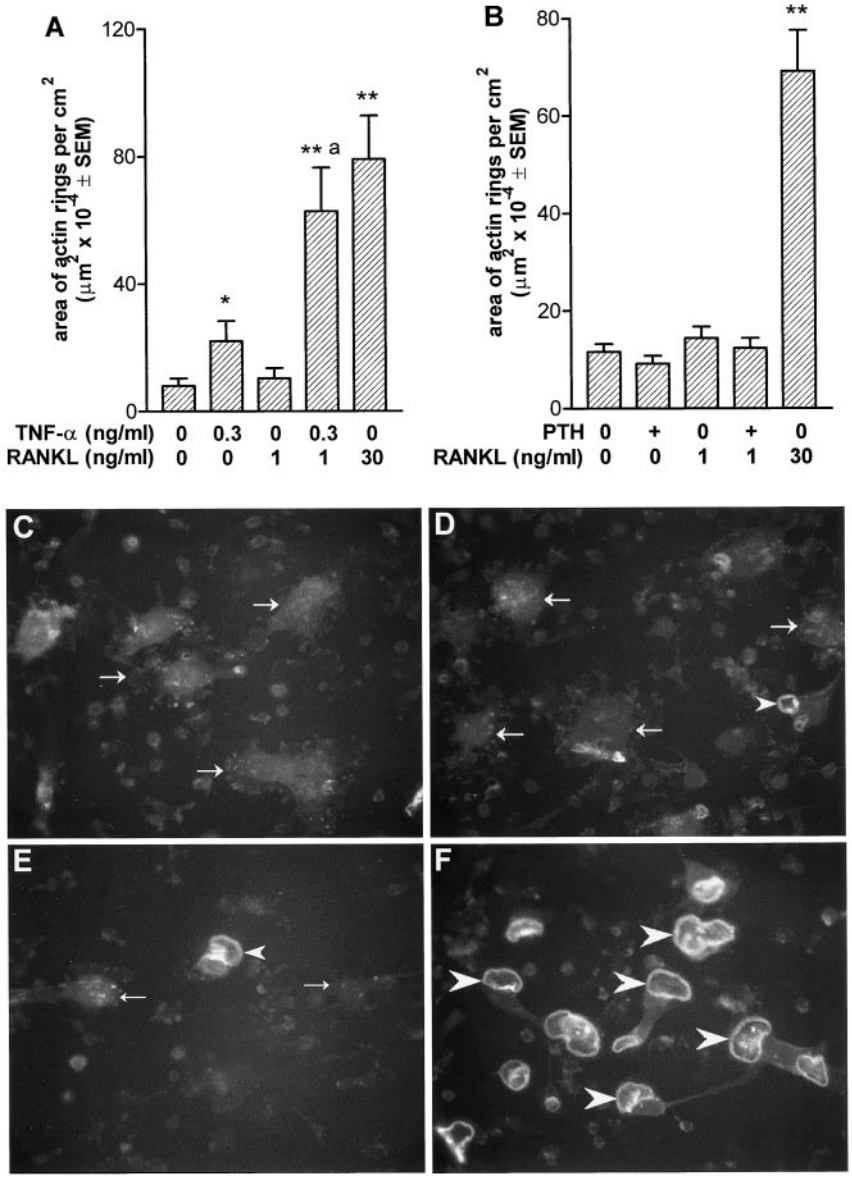

FIG. 4. TNFα potently synergizes with RANKL for the induction of actin rings in osteoclasts. A, Osteoclast-like cells were generated *in vitro* on bone slices by incubation of M-CSF-dependent bone marrow precursors in M-CSF and RANKL for 4 d. Bone slices were washed extensively and transferred to wells containing HMEM/FCS and preincubated for 1 h. TNF and/or RANKL was then added and incubation continued for a further 2 h, before fixation and staining for F-actin. Results derived from four cultures per variable. *, $P < 0.05$; **, $P < 0.01$ *vs.* control; a, $P < 0.01$ *vs.* TNFα alone and RANKL (1 ng/ml) alone. B, Osteoclast-like cells generated and treated as in A but tested for synergy between RANKL and PTH. **, $P < 0.01$ *vs.* all other groups. Results derived from mean of four bone slices per group. C–F, Photomicrographs of osteoclasts after incubation in control medium (C), TNFα 0.3 ng/ml (D); RANKL 1 ng/ml (E); or TNFα plus RANKL at the same concentrations as used in D and E (F). *Arrows* denote osteoclasts without actin rings; *arrowheads* denote some of the actin rings. Magnification, ×90.

stimulation of osteoclasts isolated from rat long bones by hTNFα at the concentration originally used (~16 ng/ml) was confirmed (area μm^2) resorbed per bone slice, ± SEM, in hTNFα (ng/ml): 0: 9230 ± 2420; 0.1: 8730 ± 1270; 1: 8940 ± 1910; 10: 9080 ± 2810; 100: 13890 ± 3180; none of these values differed significantly from controls).

In the present experiments, osteoclast-like cells were generated in RANKL for assessment of TNFα responsiveness. This allows us to conclude that TNFα is capable of activating osteoclastic cells generated by RANKL. To determine whether osteoclastic cells induced in TNFα are similarly capable of activation by TNFα, we assessed the ability of osteoclast-like cells generated in TNFα to resorb bone. We found that TNFα showed a potency very similar to that of RANKL, the primary resorptive cytokine, for the induction of actively resorptive osteoclasts from M-CSF-dependent bone marrow cells (Fig. 6). We also noted in these experiments that the concentration of either cytokine required to induce osteoclast formation was approximately an order of magnitude greater than that we previously observed to be capable of promoting activation of osteoclasts. Therefore, osteoclasts appear to be activated by lower concentrations of TNFα than are needed for the induction of differentiation.

We detected no stromal cell growth in control cultures that were incubated for 5 or 12 d without M-CSF (to facilitate their detection). Thus, like activation, the action of TNFα on osteoclast differentiation seemed likely to be independent of the known ability of TNFα to augment RANKL expression in stromal or osteoblastic cells. However, it has been suggested that osteoclastogenesis by TNFα is dependent upon exposure of precursors to RANKL (14). To further clarify whether the response of bone marrow cells was independent of RANKL, we compared the ability of TNFα to generate osteoclastic cells in our cultures in the presence *vs.* the absence of OPG. We found no difference (Fig. 7). Moreover, OPG did not influence osteoclast-like cell formation even

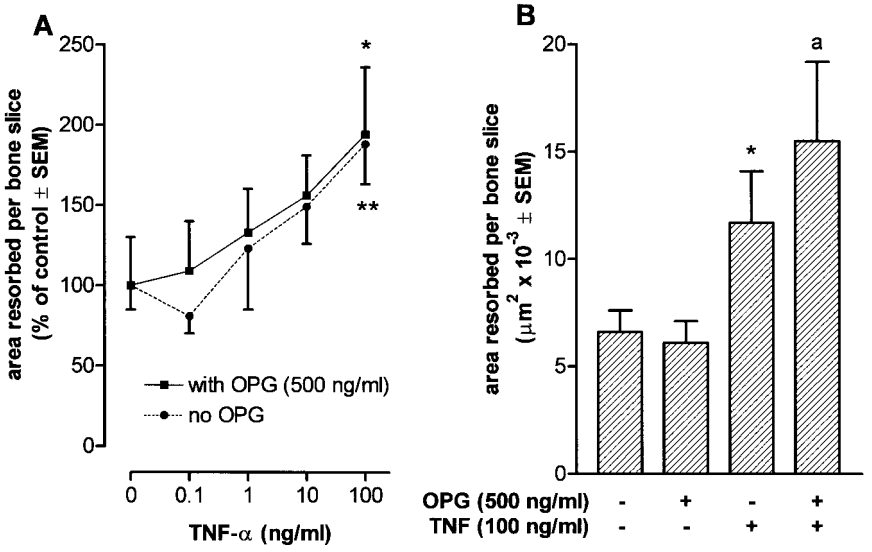

FIG. 5. TNFα stimulates bone resorption by osteoclasts disaggregated from neonatal rat bone, independent of RANKL. Osteoclasts were obtained from neonatal rat long bones and sedimented on to bone slices for 10 min. Bone slices were then vigorously washed and incubated with/without TNFα and OPG for 18 h. Bone resorption was quantified by scanning electron microscopy. A, Bone resorption expressed as percentage of control bone slices incubated without TNFα. **, $P < 0.01$ *vs.* control; *, $P < 0.05$ *vs.* control. Each *point* is derived from 12 bone slices. Resorption of control bone slices was: 5,460 ± 1,670 μm^2/bone slice in the experiment with OPG (500 ng/ml) (■); and 21,410 ± 3,200 μm^2/bone slice in the experiments without OPG (●). B, Mean ± SEM of bone resorbed on 12 bone slices per variable. *, $P < 0.05$ *vs.* control; a, $P < 0.05$ *vs.* OPG and control. PTH had no effect on bone resorption in parallel cultures (data not shown).

when it was added to the bone marrow cells immediately after extraction, before removal of stromal cells, and at very high concentrations (10 ng/ml of OPG is sufficient to abolish stromal-cell-mediated osteoclast-induction; Ref. 35). In fact, we found that addition of RANKL during preparation of the M-CSF-dependent precursors did not did not noticeably enhance their potential for subsequent osteoclastogenesis (Fig. 7). Furthermore, we noted robust generation of TRAP-positive multinucleate cells in semisolid cultures, in which hemopoietic cells are very widely dispersed and free from detectable influence by stromal cells (Fig. 8). Lastly, we noted induction of TRAP-positive multinucleate cells, and bone resorption, by TNFα in hemopoietic spleen cell cultures (Fig. 8).

We then tested whether we could detect synergy in our cultures, between TNFα and RANKL, for osteoclast formation. We found (Fig. 9) that, as has been previously reported (14, 34), the cytokines were strongly synergistic for osteoclastogenesis, as they were for activation. These results suggest that TNFα is not dependent upon past or present exposure to RANKL for its ability to induce osteoclastogenesis, but rather is strongly synergistic with the cytokine.

Discussion

TNFα stimulates bone resorption *in vivo* and *in vitro* (21, 36–38) and has been implicated in the bone loss that accompanies many inflammatory diseases such as rheumatoid arthritis, and even estrogen deficiency (8, 9). The mechanisms by which it causes bone loss are unclear. It has been shown that, in addition to its ability to induce expression of RANKL in osteoblastic/stromal cells, it stimulates precursors to differentiate *in vitro* into osteoclasts (12–14, 34, 39). Yet TNFα fails to induce a significant osteoclastogenic response in RANK-deficient mice (40); and OPG, the soluble decoy receptor for RANK, abrogates bone erosion in experimental arthritis (41). One suggested explanation is that TNFα-induced osteoclastogenesis requires that the precursors be primed by RANKL (14).

Information concerning the effect of TNFα on the function of mature osteoclasts is more sparse. Because TNFα is an important mediator of bone loss, we elected to test the ability of TNFα to activate osteoclasts. We used actin ring formation as an indicator of the activation status of osteoclasts for most of the work because actin rings correlate strongly with resorptive activity (24). Also, because actin rings are formed rapidly upon stimulation, this measure of activation is less distorted by changes in differentiation and apoptosis than assessments that quantify bone resorption. We found that TNFα directly activates actin ring formation in osteoclasts formed *in vivo* or *in vitro*. Moreover, osteoclasts are as sensitive to activation by TNFα as by RANKL. Activation was independent of the presence of RANKL, and occurred at concentrations of TNFα substantially below those that induce osteoclast differentiation. There was, moreover, very strong synergy between TNFα and RANKL for osteoclast activation, such that activation occurred at extremely low concentrations of either ligand. We found that, similar to activation, osteoclast formation by TNFα was independent of but strongly synergistic with RANKL.

The potency of TNFα for activation and differentiation *in vitro* was very similar to that of RANKL. This makes it even more surprising that while soluble RANKL can induce osteoclast formation and hypercalcemia in mice deleted for RANKL (Dr. C. R Dunstan, Amgen, October 2, 2001, personal communication), TNFα fails to induce significant osteoclastogenesis in mice deleted for the gene for RANK (40). Moreover, OPG abolishes bone erosion with-

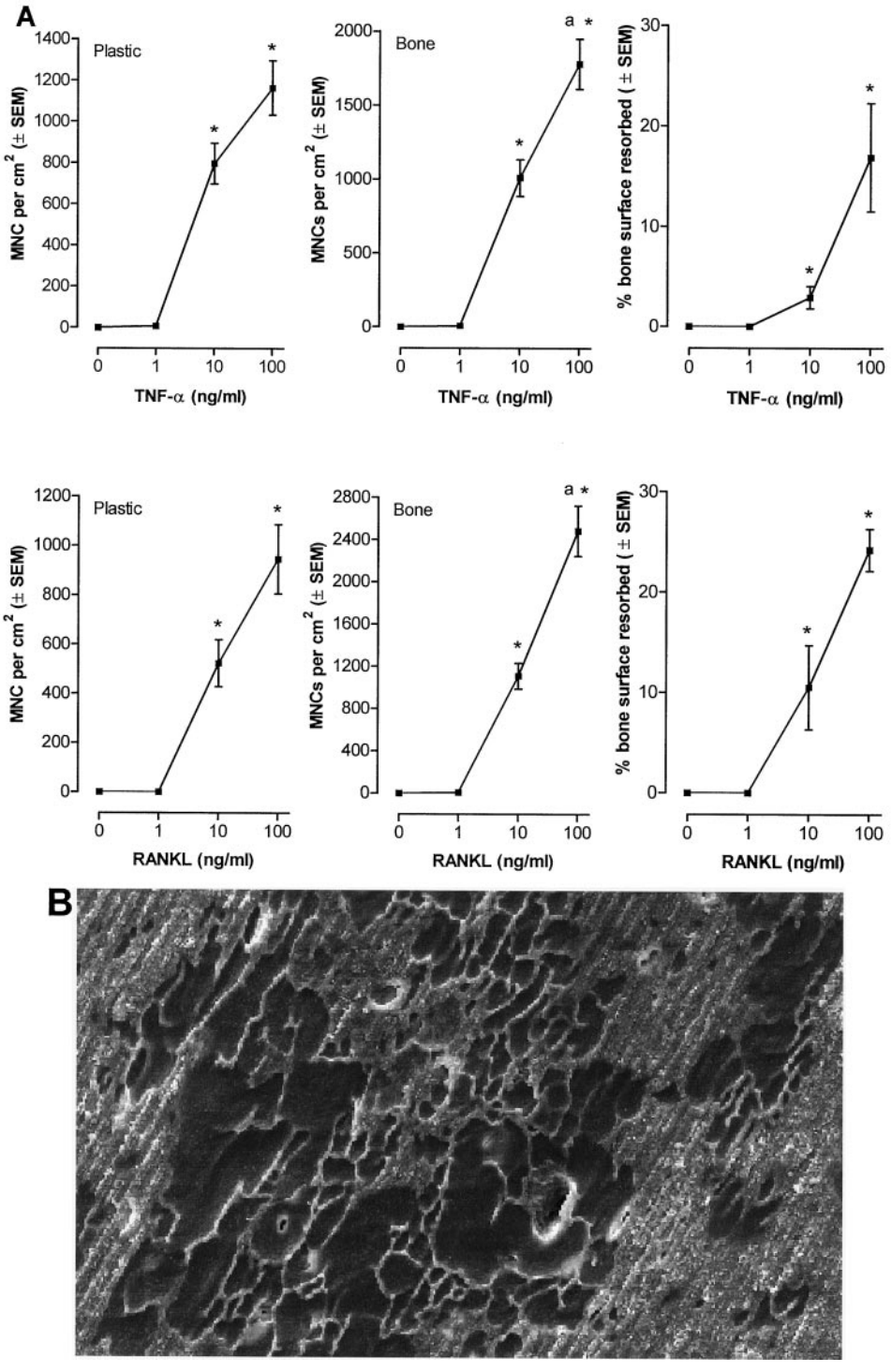

FIG. 6. Induction of osteoclastic differentiation and function by TNFα and RANKL. M-CSF-dependent bone marrow cells were incubated in M-CSF (50 ng/ml) with TNFα or RANKL for 5 d on plastic coverslips or bone slices (to assess the number of osteoclasts formed) or for 12 d on bone slices (to evaluate bone resorption). A, Osteoclastic differentiation was assessed by counting the number of TRAP-positive cells with three or more nuclei per cell (multinucleate cells, MNCs), and resorption was assessed by calculating the percentage of bone surface that showed excavation. n = 4 cultures per variable. *, $P < 0.01$ *vs.* control; a, $P < 0.01$ *vs.* plastic coverslip. B, Scanning electron micrograph of bone slice after incubation of bone marrow cells for 12 days in M-CSF and TNFα (100 ng/ml). Cells have been removed to enable visualization of the bone surface. Magnification, ×250.

out affecting inflammation in experimental arthritis (41), implying that even abundant local TNFα does not contribute to the destruction of bone that occurs in this model. One explanation for the discrepant effects of TNFα and RANKL *in vivo* would be that TNFα induces osteoclastic differentiation only in precursors primed by RANKL (14). We, however, could find no evidence that osteoclastogenesis by TNFα in our experimental systems was dependent upon prior exposure to RANKL *in vitro*. In particular, stromal cells were morphologically undetectable in our cultures even after 12 d of incubation, and we could not detect any effect of even a very high concentration (500 ng/ml) of OPG on TNFα-induced osteoclastogenesis *in vitro* (Fig. 7), despite the ability of OPG (10 ng/ml) to completely abolish stromal cell-induced osteoclast formation *in vitro* (Ref. 35, and Fuller. K., unpublished observations), and to suppress experimental arthritis *in vivo*; and TNFα was the equal of RANKL in the induction of

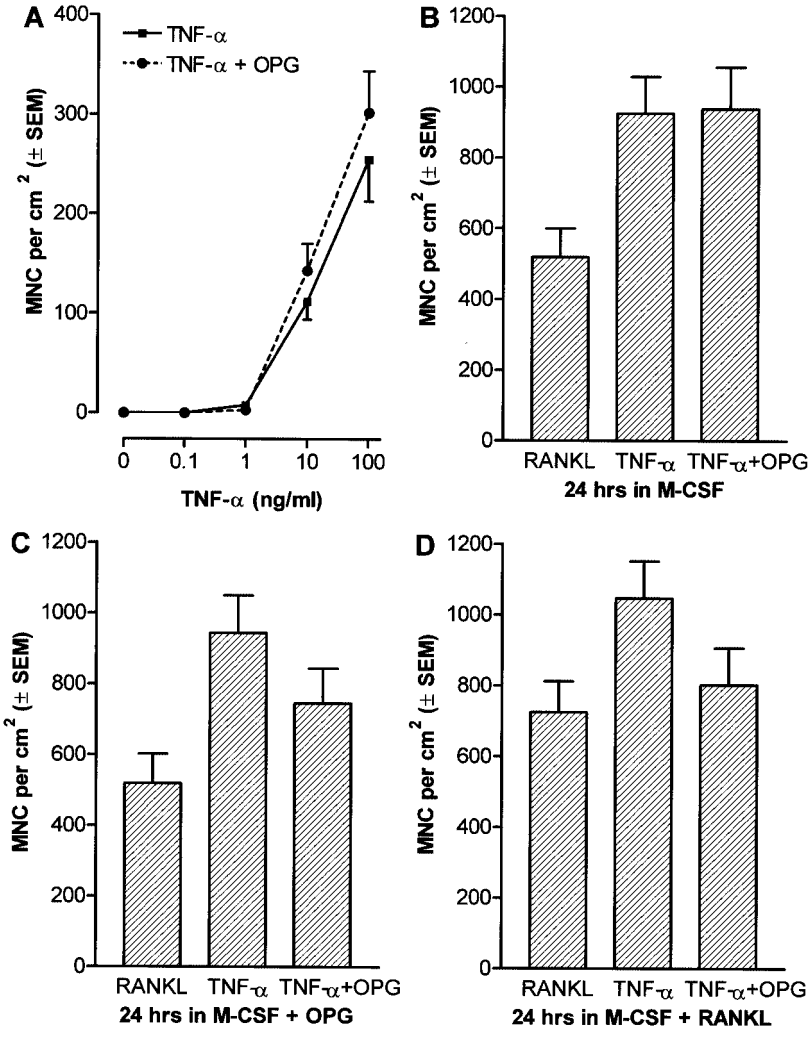

FIG. 7. Induction of osteoclastic differentiation by TNFα is not dependent upon RANKL. A, Bone marrow cells were incubated at low density in M-CSF (5 ng/ml) for 24 h to remove stromal cells. The nonadherent cells were then incubated for 5 d in TNFα with/without OPG (500 ng/ml) before enumeration of TRAP-positive MNCs. Osteoclastic differentiation was unaffected by OPG. n = 4 cultures per variable. B–D, To assess the possibility that the induction of osteoclastic differentiation might be due to exposure of precursors to priming doses of RANKL during the preincubation phase during which stromal cells are removed, bone marrow cells were incubated for 24 h in M-CSF (5 ng/ml) alone, or with OPG (500 ng/ml) or RANKL (30 ng/ml). Nonadherent cells were then removed and incubated in RANKL (30 ng/ml) or TNFα (100 ng/ml) with or without OPG (500 ng/ml) for 6 d before enumeration of MNC formation. The different preincubation conditions did not significantly affect subsequent osteoclastic differentiation. n = 4 cultures per variable.

abundant osteoclast-like cells in semisolid medium, in which stromal cells are at very low density, and dispersed from contact with osteoclastic precursors. Moreover, osteoclastic cells were generated from nonosseous sources [spleen cells; and peritoneal macrophages (18)]. However, while our results suggest that TNFα can induce osteoclastic differentiation, at least *in vitro*, in the absence of RANKL, definitive resolution of this question awaits experiments using hemopoietic cells from animals deleted for the gene for RANKL or RANK.

These considerations suggest that the response of the mononuclear phagocyte precursors of osteoclasts and macrophages to TNFα is modified *in vivo* by other factors. Indeed, it would be surprising if this were not so because TNFα is ubiquitous in inflammatory sites, yet osteoclasts are essentially seen only in bone. Presumably, TNFα coexists in inflammatory exudates with other inflammatory cytokines such as GM-CSF, IL4, and IFN-γ, which inhibit osteoclast formation (33, 39, 42–45). Because TNFα is proinflammatory, wherever TNFα is present, so will be such inflammatory cytokines. The factors that enable osteoclasts to form in inflammation in bone but not elsewhere remain uncertain.

There is nevertheless clear evidence that injection of TNFα into wild-type animals induces a brisk osteoclastogenesis (14). It therefore seems likely that the main mechanism by which TNFα promotes osteoclastogenesis *in vivo* is through synergy with RANKL because this might occur at concentrations below those that induce proinflammatory changes in tissues. This might be further assisted by the presence in bone of TGF-β, which suppresses the proinflammatory actions of TNFα, and synergizes with RANKL and TNFα in osteoclastogenesis (13, 20, 39, 46, 47). Thus, while TNFα in inflammatory sites might not be osteoclast-inductive (see above), this synergistic mode of action would provide a mechanism whereby the much lower levels of systemic TNFα are insufficient to provide an inflammatory, antiosteoclastic environment, but sufficient, due to synergy with RANKL, to promote skeletal catabolism. Because of the need for synergy with RANKL in this model, low levels of systemic TNFα could promote bone loss without disturbing the underlying patterns of osteoclastic localization. Thus, osteoclastic localization remains as directed by osteoblastic cells, in patterns appropriate for the needs of bone morphogenesis and restructuring.

This is the first report that TNFα itself can stimulate re-

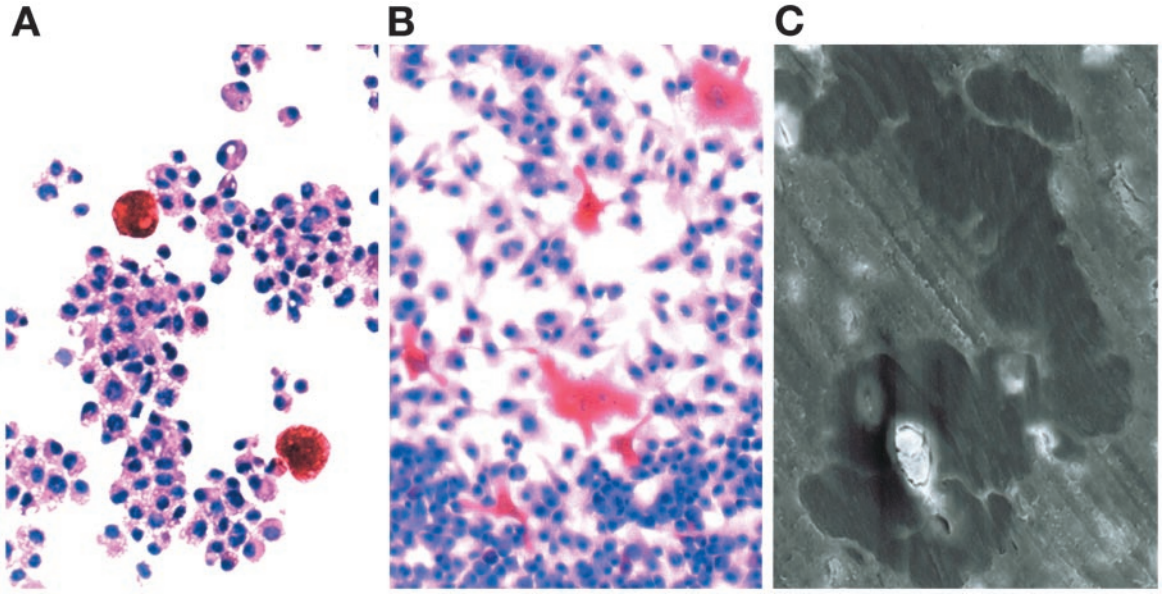

FIG. 8. A, TNFα induces osteoclastic differentiation in bone marrow cells incubated in semisolid medium. Bone marrow cells were incubated in methylcellulose at low cell density in TNFα (100 ng/ml) and M-CSF (50 ng/ml) for 8 d. Colonies were then picked or smeared onto glass slides, and stained for TRAP. Two large TRAP-positive cells can be seen among TRAP-negative cells. B and C, TNFα induces TRAP-positive multinuclear cells that resorb bone from nonadherent M-CSF-dependent spleen cells. Spleen cells were incubated on plastic coverslips or bone slices for 5 or 7 d, respectively, in TNFα (100 ng/ml) and M-CSF (50 ng/ml). The cultures formed TRAP-positive multinuclear cells (B) and showed evidence of excavation of the surface of the bone slices (C).

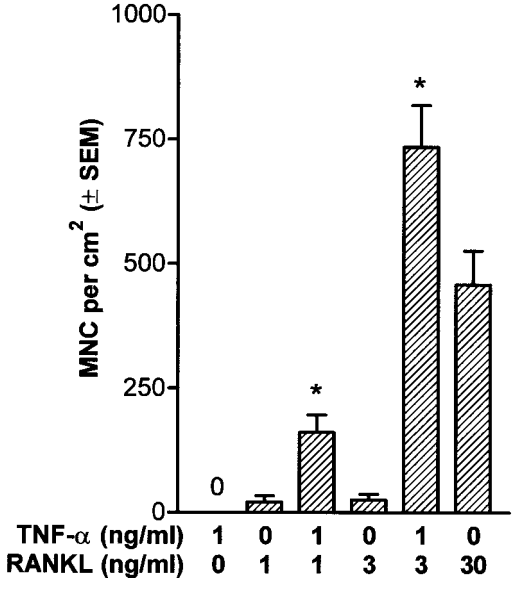

FIG. 9. TNFα and RANKL are synergistic for the induction of osteoclastic differentiation. M-CSF-dependent bone marrow cells were incubated for 5 d in M-CSF (50 ng/ml) and TNFα with/without RANKL. TRAP-positive multinucleate cells were enumerated after culture. n = 6 coverslips per variable. *, $P < 0.01$ *vs.* RANKL (1 ng/ml) alone.

sorptive activity in osteoclasts independent of RANKL. Our results suggest that TNFα promotes bone resorption not only by augmenting osteoclast formation but also by direct activation of existing osteoclasts. Indeed, the sensitivity of osteoclasts to activation by TNFα suggests that increased resorption need not always be associated with increased osteoclast numbers.

Previous reports (12, 13, 21, 34) have not demonstrated direct activation of osteoclastic function by TNFα independent of RANKL. Previously, we found (21) that TNFα stimulates bone resorption by osteoclasts disaggregated from rat long bones only when osteoblastic cells were also present. However, in those early experiments human TNFα was used, which in repeat experiments was also unable to directly activate osteoclasts *ex vivo* even at 100 ng/ml. It has previously been shown that human TNFα is less active than murine TNFα as a direct stimulus of osteoclast formation in a related model system (12). This may occur because human TNF activates murine TNF receptor 1 but not murine TNF receptor 2, whereas murine TNF activates both murine TNF receptors (12, 48). Therefore, it seems likely that in the previous experiments (21) the activity of the cytokine was sufficient to activate osteoclasts only in synergy with RANKL, provided by coculture with RANKL-expressing osteoblastic cells. It may be that other agents that appear not to act directly on osteoclasts, might do so in synergy with RANKL. However, we found that for one such agent at least (PTH) this appears not to be so.

We have identified for the first time a direct effect of TNFα on osteoclastic activation. Clearly, the osteoclastogenic actions and interactions of TNFα are complex: in addition to the induction of RANKL in osteoblastic cells, TNFα directly promotes osteoclastic differentiation and activation, and synergizes with RANKL for both. This emphasizes the potency of the cytokine as an osteolytic agent. Nevertheless, the strength of its synergistic action for osteoclast activation provides the opportunity for systemic TNFα to promote bone resorption with minimal disturbance to the underlying, physiologic patterns of os-

teoclastic localization. Perhaps the most compelling message of this study is the fact that this synergy is so potent for osteoclast activation, that miniscule circulating levels of TNFα, below even those needed for osteoclastogenesis, are likely to promote osteolysis, through a direct action on existing osteoclasts. Thus, for the systemic osteolysis that accompanies chronic inflammatory conditions, cachexia and perhaps postmenopausal osteoporosis, osteoclast activation is likely to be the major target mechanism for bone loss.

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References

1. **Suda T, Takahashi N, Udagawa N, Jimi E, Gillespie MT, Martin TJ** 1999 Modulation of osteoclast differentiation and function by the new member of the tumor necrosis factor receptor and ligand families. Endocr Rev 20:345–357
2. **Chambers TJ** 2000 Regulation of the differentiation and function of osteoclasts. J Pathol 192:4–13
3. **Lannigan FJ, O'Higgins P, McPhie P** 1993 The cellular mechanism of ossicular erosion in chronic suppurative otitis media. J Laryngol Otol 107:12–16
4. **Baroukh B, Saffar JL** 1991 Identification of osteoclasts and their mononuclear precursors. A comparative histological and histochemical study in hamster periodontitis. J Periodont Res 26:161–166
5. **Nair SP, Meghji s, Wilson M, Reddi K, White P, Henderson B** 1996 Bacterially induced bone destruction: mechanisms and misconceptions. Infect Immun 64:2371–2380
6. **Bom-van Noorloos AA, van der Meer JW, van de Gevel JS, Schepens E, van Steenbergen TJ, Burger EH** 1990 Bacteriodes gingivalis stimulates bone resorption via interleukin-1 production by mononuclear cells. The relative role for B. gingivalis endotoxin. J Clin Periodontol 17:409–413
7. **Birkedal-Hansen H** 1993 Role of cytokines and inflammatory mediators in tissue destruction. J Periodont Res 28:500–510
8. **Kimble RB, Srivastava S, Ross FP, Matayoshi A, Pacifici R** 1996 Estrogen deficiency increases the ability of stromal cells to support murine osteoclastogenesis via an interleukin-1- and tumor necrosis factor-mediated stimulation of macrophage colony-stimulating factor production. J Biol Chem 271:28890–28897
9. **Cenci S, Weitzmann MN, Roggia C, Namba N, Novack D, Woodring J, Pacifici R** 2000 Estrogen deficiency induces bone loss by enhancing T-cell production of TNF-α. J Clin Invest 106:1229–1237
10. **Hofbauer LC, Lacey DL, Dunstan CR, Spelsberg TC, Riggs BL, Khosla S** 1999 Interleukin-1β and tumor necrosis factor-α, but not interleukin-6, stimulate osteoprotegerin ligand gene expression in human osteoblastic cells. Bone 25: 255–259
11. **Horwood NJ, Eliott J, Martin TJ, Gillespie MT** 1998 Osteotropic agents regulate the expression of osteoclast differentiation factor and osteoprotegerin in osteoblastic stromal cells. Endocrinology 139:4743–4746
12. **Kobayashi K, Takahashi N, Jimi E, Udagawa N, Takami M, Kotake S, Nakagawa N, Kinosaki M, Yamaguchi K, Shima N, Yasuda H, Morinaga T, Higashio K, Martin TJ, Suda T** 2000 Tumor necrosis factor α stimulates osteoclastic differentiation by a mechanism independent of the ODF/RANKL-RANK interaction. J Exp Med 191:275–285
13. **Azuma Y, Kaji K, Katogi R, Takeshita S, Kudo A** 2000 Tumor necrosis factor-α induces differentiation of and bone resorption by osteoclasts. J Biol Chem 275:4858–4864
14. **Lam J, Takeshita S, Barker JE, Kanagawa O, Ross FP, Teitelbaum SL** 2000 TNF-α induces osteoclastogenesis by direct stimulation of macrophages exposed to permissive levels of RANK ligand. J Clin Invest 106:1481–1488
15. **Tatevossian A** 1973 Effect of parathyroid extract on blood calcium and osteoclast count in mice. Calcif Tissue Res 11:251–257
16. **Miller SC** 1978 Rapid activation of the medullary bone osteoclast cell suface by parathyroid hormone. J Cell Biol 76:615–618
17. **Miller SC, Bowman BM, Myers RL** 1984 Morphological and ultrastructural aspects of the activation of avian medullary bone osteoclasts by parathyroid hormone. Anat Rec 208:223–231
18. **Wani MR, Fuller K, Kim NS, Choi Y, Chambers T** 1999 Prostaglandin E_2 cooperates with TRANCE in osteoclast induction from hemopoietic precursors: synergistic activation of differentiation, cell spreading, and fusion. Endocrinology 140:1927–1935
19. **Hattersley G, Chambers TJ** 1991 Effects of transforming growth factor β_1 on the regulation of osteoclastic development and function. J Bone Miner Res 6:165–172
20. **Fuller K, Lean JM, Bayley KE, Wani MR, Chambers TJ** 2000 A role for TGF-β_1 in osteoclast differentiation and survival. J Cell Sci 113:2445–2453
21. **Thomson BM, Mundy GR, Chambers TJ** 1987 Tumor necrosis factors α and β induce osteoblastic cells to stimulate osteoclastic bone resorption. J Immunol 138:775–779
22. **Turksen K, Kanehisa J, Opas M, Heersche JNM, Aubin JE** 1988 Adhesion patterns and cytoskeleton of rabbit osteoclasts on bone slices and glass. J Bone Miner Res 3:389–400
23. **Lakkakorpi P, Tuukkanen J, Hentunen T, Järvelin K, Väänänen K** 1989 Organization of osteoclast microfilaments during the attachment to bone surface *in vitro*. J Bone Miner Res 4:817–825
24. **Lakkakorpi PT, Väänänen HK** 1991 Kinetics of the osteoclast cytoskeleton during the resorption cycle *in vitro*. J Bone Miner Res 6:817–826
25. **Lakkakorpi P, Helfrich MH, Horton MA, Väänänen HK** 1993 Spatial organization of microfilaments and vitronectin receptor, $\alpha_v\beta_3$, in osteoclasts. J Cell Sci 104:663–670
26. **Nakamura I, Takahashi N, Sasaki T, Jimi E, Kurokawa T, Suda T** 1996 Chemical and physical properties of the extracellular matrix are required for the actin ring formation in osteoclasts. J Bone Miner Res 11: 1873–1879
27. **Teti A, Marchisio PC, Zallone AZ** 1991 Clear zone in osteoclast function: role of podosomes in regulation of bone-resorbing activity. Am J Physiol 261:C1–C7
28. **Chambers TJ, McSheehy PMJ, Thomson BM, Fuller K** 1985 The effect of calcium-regulating hormones and prostaglandins on bone resorption by osteoclasts disaggregated from neonatal rabbit bones. Endocrinology 116:234–239
29. **Burstone MS** 1958 Histochemical demonstration of acid phosphatases with naphthol AS-phosphate. J Natl Cancer Inst 21:423–539
30. **Fuller K, Owens JM, Jagger CJ, Wilson A, Moss R, Chambers TJ** 1993 Macrophage colony-stimulating factor stimulates survival and chemotactic behavior in isolated osteoclasts. J Exp Med 178:1733–1744
31. **Chambers TJ** 1982 Osteoblasts release osteoclasts from calcitonin-induced quiescence. J Cell Sci 57:247–260
32. **Chambers TJ, Athanasou NA, Fuller K** 1984 The effect of parathyroid hormone and calcitonin on the cytoplasmic spreading of isolated osteoclasts. J Endocrinol 102:281–286
33. **Fuller K, Wong B, Fox S, Choi Y, Chambers TJ** 1998 TRANCE is necessary and sufficient for osteoblast-mediated activation of bone resorption in osteoclasts. J Exp Med 188:997–1001
34. **Komine M, Kukita A, Kukita T, Ogata Y, Hotokebuchi T, Kohashi O** 2001 Tumor necrosis factor-α cooperates with receptor activator of nuclear factor κB ligand in generation of osteoclasts in stromal cell-depleted rat bone marrow cell culture. Bone 28:474–483
35. **Simonet WS, Lacey DL, Dunstan CR, Kelley M, Chang MS, Luthy R, Nguyen HQ, Wooden S, Bennett L, Boone T, Shimamoto G, DeRose M, Elliott R, Colombero A, Tan HL, Trail G, Sullivan J, Davy E, Bucay N, Renshaw-Gegg L, Hughes TM, Hill D, Pattison W, Campbell P, Sander S, Van G, Tarplay J, Derby P, Lee R, Amgen EST Program, Boyle WJ** 1997 Osteoprotegerin: a novel secreted protein involved in the regulation of bone density. Cell 89: 309–319
36. **Kitazawa R, Kimble RB, Vannice JL, Kung VT, Pacifici R** 1994 Interleukin-1 receptor antagonist and tumour necrosis factor binding protein decrease osteoclast formation and bone resorption in ovariectomized mice. J Clin Invest 94:2397–2406
37. **Lerner UH, Ohlin A** 1993 Tumor necrosis factors α and β can stimulate bone resorption in cultured mouse calvariae by a prostaglandin-independent mechanism. J Bone Miner Res 8:147–155
38. **van der Pluijm G, Most W, van der Wee-Pals L, de Groot H, Papapoulos S, Lowick C** 1991 Two distinct effects of recombinant human tumor necrosis factor-alpha on osteoclast development and subsequent resorption of mineralized matrix. Endocrinology 129:1596–1604
39. **Fox SW, Fuller K, Bayley KE, Lean JM, Chambers TJ** 2000 TGF-β_1 and IFN-γ direct macrophage activation by TNF-α to osteoclastic or cytocidal phenotype. J Immunol 165:4957–4963
40. **Li J, Sarosi I, Yan XQ, Morony S, Capparelli C, Tan HL, McCabe S, Elliott R, Scully S, Van G, Kaufman S, Juan SC, Sun Y, Tarpley J, Martin L, Christensen K, McCabe J, Kostenuik P, Hsu H, Fletcher F, Dunstan CR, Lacey DL, Boyle WJ** 2000 RANK is the intrinsic hematopoietic cell surface receptor that controls osteoclastogenesis and regulation of bone mass and calcium metabolism. Proc Natl Acad Sci USA 97:1566–1571
41. **Kong Y-Y, Felge U, Sarosi I, Bolon B, Tafuri A, Morony S, Capparelli C, Li J, Elliott R, McCabe S, Wong T, Campagnuolo G, Moran E, Bogoch ER, Van G, Nguyen LT, Ohashi PS, Lacey DL, Fish E, Boyle WJ, Penninger JM** 1999

Activated T cells regulate bone loss and joint destruction in adjuvant arthritis through osteoprotegerin ligand. Nature 402:304–309

42. **Bucay N, Sarosi I, Dunstan CR, Morony S, Tarpley J, Capparelli C, Scully S, Tan HL, Xu W, Lacey DL, Boyle WJ, Simonet WS** 1998 Osteoprotegerin-deficient mice develop early onset osteoporosis and arterial calcification. Genes Dev 12:1260–1268
43. **Mizuno A, Amizuka N, Irie K, Murakami A, Fujise N, Kanno T, Sato Y, Nakagawa N, Yasuda H, Mochizuki S, Gomibuchi T, Yano K, Shima N, Washida N, Tsuda E, Morinaga T, Higashio K, Ozawa H** 1998 Severe osteoporosis in mice lacking osteoclastogenesis inhibitory factor/osteoprotegerin. Biochem Biophys Res Commun 247:610–615
44. **Yamamoto M, Murakami T, Nishikawa M, Tsuda E, Mochizuki S, Higashio K, Akatsu T, Motoyoshi K, Nagata N** 1998 Hypocalcemic effect of osteoclastogenesis inhibitory factor/osteoprotegerin in the thyroparathyroidectomized rat. Endocrinology 139:4012–4015
45. **Takayanagi H, Ogasawara K, Hida S, Chiba T, Murata S, Sato K, Takaoka A, Yokochi T, Oda H, Tanaka K, Nakamura K, Taniguchi T** 2000 T-cell-mediated regulation of osteoclastogenesis by signalling cross-talk between RANKL and IFN-γ. Nature 408:600–605
46. **Sells Galvin RJ, Gatlin CL, Horn JW, Fuson TR** 1999 TGF-beta enhances osteoclast differentiation in hematopoietic cell cultures stimulated with RANKL and M-CSF. Biochem Biophys Res Commun 265:233–239
47. **Kaneda T, Nojima T, Nakagawa M, Ogasawara A, Kaneko H, Sato T, Mano H, Kumegawa M, Hakeda Y** 2000 Endogenous production of TGF-β is essential for osteoclastogenesis induced by a combination of receptor activator of NF-κB ligand and macrophage-colony-stimulating factor. J Immunol 165: 4254–4263
48. **Lewis M, Tartaglia LA, Lee A, Bennett GL, Rice GC, Wong GH, Chen EY, Goeddel DV** 1991 Cloning and expression of cDNAs for two distinct murine tumor necrosis factor receptors demonstrate one receptor is species specific. Proc Natl Acad Sci USA 88:2830–2834