A role for TGFβ1 in osteoclast differentiation and survival

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SUMMARY

Recently, tumour necrosis factor-related activation-induced cytokine (TRANCE) was shown to be necessary for osteoclast formation. We now report that TGFβ, a cytokine enriched in bone matrix, is also required. TGFβ not only powerfully synergized with TRANCE for induction of osteoclast-like cells (OCL) from bone marrow precursors and monocytes, but OCL formation was abolished by recombinant soluble TGFβ receptor II (TGFβsRII). Preincubation in TGFβ was as effective as simultaneous incubation with TRANCE. TGFβ-preincubation enhanced OCL formation at least partly by preventing the development of resistance to OCL-induction that otherwise occurs when precursors are incubated in M-CSF. OCL formed in TRANCE also showed more rapid apoptosis than OCL in TRANCE plus TGFβ. Like TGFβ, incubation on bone matrix prolonged and enhanced the sensitivity of precursors to OCL-induction by TRANCE, and this was reversed by TGFβsRII. Taken together, this data is compelling evidence for a model in which TGFβ in matrix or released from bone-lining or other cells maintains and enhances the osteoclast-forming potential of precursors as they migrate towards sites of cell-bound TRANCE. Thus, the specific circumstances necessary for osteoclast formation and survival are TRANCE expression on osteoblastic cells and TGFβ in bone.

Key words: RANKL, Osteoclast, TGFβ, Bone resorption, Bone matrix

INTRODUCTION

The osteoclast is the cell that resorbs bone. Excessive activity by this cell is responsible for the bone loss that causes osteoporosis and other diseases of clinical importance. It has been known for some time that osteoclasts derive from a macrophage colony-stimulating factor (M-CSF)-dependent precursor shared with the macrophage, which differentiates into osteoclasts when precursors are incubated with osteoblastic or bone marrow stromal cells (Chambers, 1992; Suda et al., 1992). It was recently found that tumour necrosis factor (TNF)-related activation-induced cytokine (TRANCE) (also called RANKL, ODF and OPGL), originally identified as a member of the TNF family that stimulates dendritic cells (Anderson et al., 1997; Wong et al., 1997a,b), is expressed by osteoblastic and bone marrow stromal cells, and that soluble recombinant TRANCE, with M-CSF, substitutes for stromal cells in osteoclast formation and activation (Fuller et al., 1998; Jimi et al., 1999; Lacey et al., 1998; Yasuda et al., 1998). The ligand is regulated, as predicted in osteoblastic cells, by calciotropic hormones (Fuller et al., 1998; Jimi et al., 1999; Lacey et al., 1998; Yasuda et al., 1998). Transgenic experiments have shown that deletion of the gene for TRANCE or overexpression of osteoprotegerin (OPG), a soluble decoy receptor for TRANCE, are associated with failure of osteoclast formation and osteopetrosis (Bucay et al., 1998; Hsu et al., 1999; Kong et al., 1999).

However, while TRANCE is necessary for osteoclastogenesis, only a proportion of precursors incubated in TRANCE develop into osteoclasts (Wani et al., 1999). When haemopoietic cells are incubated in TRANCE with M-CSF in semisolid cultures, osteoclasts develop in colonies mixed with macrophages (our personal observation), suggesting that TRANCE-expression alone might not be sufficient to ensure that precursors become osteoclasts at sites destined for resorption. Responses to stimuli from the TNF superfamily are characteristically associated with critical inputs from cofactors (see Smith et al., 1994).

There is much evidence that transforming growth factor β (TGFβ) is involved in skeletal cell physiology (see Bonewald and Dallas, 1994; Centrella et al., 1994). Not only is TGFβ synthesised by osteoblasts and osteoclasts in vivo, but TGFβ1, TGFβ2 and TGFβ3 can all be extracted from bone matrix, where TGFβ is the most abundant cytokine, and is present at concentrations substantially greater than in other tissues (Hauschka et al., 1986; Massaque, 1990; Pelton et al., 1991; Robey et al., 1987; Sandberg et al., 1988; Seyedin et al., 1986; Sporn and Roberts, 1989). TGFβ has potent effects on bone cells in vitro and in vivo. Perioistal injection of TGFβ stimulates bone resorption and bone formation (Joyce et al., 1990; Noda and Camilliere, 1989). In vitro, TGFβ induces extracellular matrix secretion by osteoblasts, inhibits matrix mineralization, and modulates osteoprogenitor cell proliferation and the expression of osteoblastic differentiation markers (see Bonewald and Dallas, 1994; Centrella et al., 1994).

TGFβ also has powerful effects on bone resorption. Stimulation and inhibition of resorption have both been
were performed at 37°C in a humidified atmosphere of 5% CO2 in air.

The bone marrow suspension was carefully agitated with a plastic 199 (Imperial) at one end of the bone using a sterile 21-gauge needle. Free of adherent soft tissue. The bone ends were cut, and the marrow dislocation. Femora and tibiae were aseptically removed and dissected. Recombinant human M-CSF was provided by Genetics Institute (Cambridge, MA); soluble recombinant human TRANCE and TRAIL were from Insight Biotechnology (Wembley, Middlesex, UK); purified human TGFβ1, recombinant TGFβ2 and soluble recombinant type II TGFβ receptors (TGFβRII) were from R & D (Abingdon, Oxon, UK). All incubations were performed at 37°C in a humidified atmosphere of 5% CO2 in air.

MATERIALS AND METHODS

Media and reagents

Cells were incubated in minimum essential medium (MEM) with Earle's salts, supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, 100 i.u./ml benzylpenicillin, and 100 μg/ml streptomycin (all Imperial Laboratories, Andover, Hants, UK). Recombinant human M-CSF was provided by Genetics Institute (Cambridge, MA); soluble recombinant human TRANCE and TRAIL were from Insight Biotechnology (Wembley, Middlesex, UK); purified human TGFβ1, recombinant TGFβ2 and soluble recombinant type II TGFβ receptors (TGFβRII) were from R & D (Abingdon, Oxon, UK). All incubations were performed at 37°C in a humidified atmosphere of 5% CO2 in air.

Isolation and culture of bone marrow precursors

Bone marrow cells were isolated from 5- to 8-week-old MF1 mice as previously described (Wani et al., 1999). Mice 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 medium 199 (Imperial) at one end of the bone using a sterile 21-gauge needle. The bone marrow suspension was carefully agitated with a plastic Pasteur pipette to obtain a single cell suspension. The bone marrow cells were washed twice, resuspended in MEM containing 10% FBS, and incubated for 24 hours in M-CSF (5 ng/ml) at a density of 3×10^5 cells/ml in a 75 cm² flask (Helena Biosciences, Sunderland, Tyne and Weir, UK). After 24 hours, nonadherent cells were harvested, washed and resuspended (3×10^5/ml) in MEM-FBS. This suspension was added (100 μl/well) to the wells of 96-well plates (Helena Biosciences) containing a 6 mm Thermox coverslip (Helena Biosciences) or a slice of bovine cortical bone. To each of these wells is an additional 100 μl medium containing M-CSF, TRANCE, and/or cytokines or soluble receptors were added. Cultures were fed every 3-4 days by replacing 100 μl of culture medium with an equal quantity of fresh medium and reagents. Absence of contaminating stromal cells was confirmed in cultures in which M-CSF was omitted. Such cultures showed no cell growth. Coverslips and bone slices were assessed for TRAP-positive cells containing at least three nuclei. In some experiments the total number of nuclei present was counted (including those in OCL) using an eyepiece graticule.

TRAP cytochemistry

Osteoclast formation was also evaluated by quantification of TRAP-positive osteoclast-like multinuclear cell number (OCL) using a modification of the method of Burstone (1958). After incubation, cells on coverslips or bone slices were washed in PBS, fixed in 10% formalin for 10 minutes, and stained for acid phosphatase in the presence of 0.05 M sodium tartrate (Sigma Chemical Co). The substrate used was naphthol AS-BI phosphate (Sigma Chemical Co). Osteoclasts were counted after counterstaining (with Haematoxylin) as TRAP-positive cells containing at least three nuclei. In some experiments the total number of nuclei present was counted (including those in OCL) using an eyepiece graticule and a 40× objective lens.

Northern analysis

After incubation for 3-9 days in 75 cm² flasks, total RNA was prepared from bone marrow cells according to an established method (Chomczynski and Sacchi, 1987). For northern blot analysis, 25 μg total RNA were separated on a 1.2% agarose-formaldehyde gel, transferred to a nylon membrane, and hybridised with 32P-labeled cDNA probes for murine TRAP, RANK, OPG, TGFβ1, and β-actin prepared by the random primer method (Amersham). The probe for murine TGFβ1 was from ATCC (Rockville, MD, USA) and was cut with SmaI. For other species, cDNA probes were prepared (all murine) by RT-PCR with the following sense (S) and antisense (AS) primers: TRAP, S, 5'-gTTACCAACTggCACgA TA Tgg-3', AS, 5'-TTTCTTTTTgTCgCTgTTTTACgA-3'; OPG, S, 5'-gTCCCA-CCTACCTAAAPCAGCA-3', OPG, AS, 5'-gTTACACTCTGgCATCGCATCg-3'; β-actin S, 5'-gTTACACTCTGgCATCGATATgg-3', β-actin AS, 5'-gATCTTGgATCATgTgC-3'. These primers were designed to span exon-intron boundaries, and yielded predicted products of 690, 820, 578 and 760 bp, respectively, using RNA obtained from neonatal mouse long bone. PCR products were subcloned into pGEMTeasy (Promega, Southampton, Hants, UK). Inserts were then excised and 32P-labeled.

Assessment of osteoclast apoptosis

Nonadherent M-CSF-dependent bone marrow cells were incubated...
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in 24-well plates (Helena Biosciences) (10^5/well) containing a coverslip, in TRANCE + M-CSF ± TGFβ for 7 days. At this time, osteoclast numbers are greatest, and fall, by apoptosis, most rapidly (Wani et al., 1999). Osteoclast apoptosis is associated with reduced adhesion, cell rounding and nuclear condensation and fragmentation, and apoptotic osteoclasts are readily removed from culture substrates by pipetting (Fuller et al., 1993). Nonadherent and loosely adherent cells were therefore removed from cultures after 7 days of incubation, and cytocentrifuged. The great majority (>95%) of multinuclear cells in such cytospin preparations showed nuclear morphology characteristic of apoptosis. Apoptosis in such cells was confirmed by TUNEL staining (Promega) of coverslips and cytospin preparations, and apoptotic osteoclasts were expressed as a percentage of the total number of osteoclasts (apoptotic plus viable) in each culture well.

**Statistical analysis**

Differences between groups were analysed using unpaired Student’s t-test. P<0.05 was considered significant.

**RESULTS**

TGFβ1 induced a substantial increase in the number of tartrate-resistant acid phosphatase (TRAP)-positive osteoclast-like multinuclear cells (OCL) formed in response to soluble recombinant TRANCE in nonadherent M-CSF-dependent bone marrow cells (Fig. 1). In fact, assessment of osteoclastic differentiation by enumerating OCL probably represents a substantial underestimate of the augmentation of osteoclastic differentiation by TGFβ1, because not only were OCL more numerous, but they were also larger (Fig. 1B). This is consistent with the similar synergy recently reported by Sells Galvin et al. (1999). Approximately 90% of these OCL also expressed calcitonin receptors, another marker for osteoclasts, by autoradiography (data not shown and see Wani et al., 1999). TGFβ2 exerted a similar effect, maximal at 10 ng/ml (data not shown). TGFβ1 did not itself induce OCL, but substantially augmented the ability of TRANCE to do so (Fig. 1), by at least

**Fig. 1.** TGFβ acts as a costimulator for osteoclast formation. (A) Nonadherent M-CSF-dependent bone marrow cells were incubated on plastic coverslips in soluble TRANCE (10 ng/ml), M-CSF (30 ng/ml) and TGFβ1 for 10 days. n=24 cultures per variable. The numbers of OCL per unit area were counted. (B) Photomicrographs of similar cultures incubated for 8 days without (left) and with (right) TGFβ1 (1 ng/ml), reacted cytochemically for TRAP. Note many more and larger (TRAP-stained) OCL with TGFβ1 ×180. (C) Dose-response effect on OCL formation and bone resorption of addition of TRANCE to bone marrow cells together with M-CSF (30 ng/ml) and TGFβ1 (1 ng/ml). Few OCL, and no bone resorption was seen in the set of cultures incubated for 5 days. n=12 cultures. Values are means ± s.e.m. *P<0.05 versus control cultures.
TGFβsRII (ng/ml) versus control (no TGF-β murine blood mononuclear cells by M-CSF (30 ng/ml).

Fig. 3. Effect of TGFβ1 (1 ng/ml) on the number of OCL and total cell numbers formed from murine blood mononuclear cells by M-CSF (30 ng/ml). n=12 cultures per variable. *P<0.05 versus control (no TGF-β1).

Since the effect of soluble receptors on OCL formation was reversed by adding back TGFβ1, moreover, the soluble receptors increased the total number of nuclei in the same cultures, an effect more consistent with neutralisation of the known action of TGFβ as a suppressor of macrophage proliferation (Bogdan and Nathan, 1993) than with toxicity.

The effects of TGFβ do not seem to be mediated by stroma, since stroma was not detectable in the cell preparations used: no cells were present in cultures incubated without M-CSF. Moreover, murine monocytes showed a similar augmentation of OCL formation by TGFβ1 (Fig. 3). As in bone marrow cells, TGFβ1 simultaneously suppressed total cell number while inducing an absolute increase in OCL formation. We previously found that PGE2 can synergise with TRANCE in OCL formation (Wani et al., 1999), but the ability of TGFβ1 to augment OCL formation was not significantly affected by indomethacin (10^{-6} M) (data not shown). As expected, TGFβ1 potentiated the TRANCE-induced expression of mRNA for TRAP. TGFβ1 also upregulated mRNA for receptor activator of NF-kB (RANK), the receptor for TRANCE (Anderson et al., 1997; Fuller et al., 1998; Hsu et al., 1999) in 3-day cultures, and may have slightly reduced the level of mRNA for osteoprotegerin (OPG), the decoy receptor for TRANCE (Lacey et al., 1998), at least in early cultures (Fig. 4). However, TRAIL, which neutralises TRANCE-binding by OPG but not RANK (Emery et al., 1998), did not significantly affect OCL formation (data not shown) further suggesting that TGFβ does not augment OCL formation through suppression of OPG expression alone in M-CSF-dependent bone marrow cells or their progeny in our cultures. RANK itself appeared to be increased by TRANCE (Fig. 4).

It seemed possible that TGFβ, a known macrophage deactivator, might facilitate OCL formation by suppressing the maturation of precursors to committed macrophages. We noted previously that bone marrow cells incubated in M-CSF became less responsive to OCL-induction by TRANCE during culture (Wani et al., 1999). Maturation of macrophages to an unresponsive state occurs for other cytokines also. We therefore compared the ability of TRANCE to induce OCL from precursors preincubated with and without TGFβ1. While precursors incubated in M-CSF alone were able to produce very few OCL, precursors preincubated with M-CSF and TGFβ1 fully maintained their OCL-potential.
Osteoclast differentiation requires TGFβ (Table 1). TGFβ1 did not significantly affect OCL number when added for the last 7 days. This suggests that TGFβ can make a contribution to the osteoclast phenotype, independent of the presence of TRANCE, which is effective before exposure of precursors to TRANCE.

We previously noted that when M-CSF-dependent precursors are incubated in TRANCE a dramatic decline in the number of OCL occurs around day 8 of culture, associated with apoptosis (Wani et al., 1999). Similar cells showing the morphology characteristic of apoptosis in osteoclasts (Fuller et al., 1993; Hughes et al., 1996), with chromatin condensation and multiple nuclear fragments, were observed in the present experiments (Fig. 5). Many more such cells were observed in cultures incubated without than with TGFβ1, but because apoptotic osteoclasts detach from the substrate (Hughes et al., 1996) their number on coverslips is an unreliable guide to the proportion of osteoclasts undergoing apoptosis. Therefore, the cultures were vigorously washed after incubation, and the nonadherent cells were collected (Hughes et al., 1996). OCL remaining adherent to coverslips were nonapoptotic by TUNEL staining, while nonadherent OCL showed the morphologic features of apoptosis in osteoclasts (Fuller et al., 1998; Hughes et al., 1996), and were apoptotic by TUNEL staining (Fig. 5). We found a substantially greater proportion of OCL showed the morphologic changes of apoptosis in cultures incubated with TRANCE alone than with TRANCE plus TGFβ1 (Fig. 5). Apoptosis was rarely seen in accompanying mononuclear cells.

We noted that while TGFβ strongly synergized with TRANCE for OCL formation on plastic, its effect on bone

**Fig. 4.** Northern analysis of mRNA from cultures of bone marrow cells incubated for 3, 6 or 9 days with TRANCE (30 ng/ml), M-CSF (30 ng/ml) and/or TGFβ1 (0.1 ng/ml). TRANCE-induced mRNA for TRAP is augmented by TGFβ1. mRNA levels for RANK, OPG and TGFβ1 are not significantly changed by TGFβ1.

**Fig. 5.** TGFβ1 suppresses apoptosis in OCL. (A) TRAP-stained cytospin of cells derived from precursors incubated for 7 days in M-CSF and TRANCE, showing chromatin condensation and nuclear fragmentation characteristic of apoptosis in a multinuclear TRAP-positive cell. (B) Cytospin from culture incubated in M-CSF and TRANCE for 7 days, showing TUNEL-positive nuclei in a multinuclear cell. (C) Bone marrow cells were incubated for 7 days in M-CSF (30 ng/ml) and TRANCE (10 ng/ml) with/without TGFβ1. Nonadherent and loosely adherent cells were suspended, cytocentrifuged and stained for TRAP. The remaining adherent cells were stained for TRAP and OCL, were counted. Results are expressed as the total number of cells showing nuclear changes observed in A, as a percentage of the total number of apoptotic plus nonapoptotic OCL. OCL showing apoptosis were the great majority of multinuclear cells in cytospins. *P<0.05 versus control. ×600 (A); ×1140 (B).
resorption was much less dramatic (Fig. 1). This was surprising, because TGFβ does not affect bone resorption by osteoclasts ex vivo (Hattersley and Chambers, 1991). We addressed the discrepancy suggested by Fig. 1, by comparing the effect of TGFβ on the number of OCL formed by precursors incubated on bone versus plastic substrates.

We found that the number of OCL formed on bone slices by TRANCE alone was an order of magnitude greater than that on plastic coverslips under the same conditions, and was not increased by exogenous TGFβ1 (bone marrow floaters incubated in M-CSF (30 ng/ml) and TRANCE (10 ng/ml) on plastic for 6 days without exogenous TGFβ1: 121±23 OCL/cm² (mean ± s.e.m.); with TGFβ1 (0.1 ng/ml) added: 520±44 OCL/cm²; on bone, in M-CSF + TRANCE, 791±86 OCL/cm²; with TGFβ1 added, 633±74 OCL/cm². The increased number of OCL/cm² on plastic with TGFβ1 is significant (P<0.01 versus no TGFβ). The number of OCL/cm² on bone ± TGFβ1 is significantly greater than plastic without TGFβ). Because bone is an extremely rich source of TGFβ, we hypothesised that the precursors might be exposed to TGFβ in, or released from, the bone slices. To test this, precursors were incubated on plastic coverslips or bone slices for 7 days, with or without TGFβsRII. TRANCE was then added. We found that bone, like TGFβ, substantially increased OCL formation by TRANCE, and OCL formation and bone resorption were both suppressed by TGFβsRII (Table 2, Fig. 6).

### DISCUSSION

Recently, TRANCE has been found to be essential for osteoclast formation. Thus, if M-CSF-dependent bone marrow cells are incubated in M-CSF alone, only macrophages form; in the presence of TRANCE, a proportion of the cells become TRAP-positive multinuclear osteoclast-like cells (OCL). We have found that not only does TGFβ synergise with TRANCE for induction of OCL from bone marrow precursors (Sells Galvin et al., 1999) and also from monocytes, but OCL formation was abolished by recombinant soluble TGFβ receptor II (TGFβsRII). Preincubation in TGFβ was as effective as simultaneous incubation with TRANCE, and preincubation in TGFβ enhanced OCL formation at least partly by preventing the resistance to OCL-induction that otherwise occurs when precursors are incubated in M-CSF. We also found that TGFβ1 suppressed apoptosis in OCL. Like TGFβ, bone matrix prolonged and enhanced the sensitivity of precursors to OCL-induction by TRANCE; and this was reversed by TGFβsRII.

TGFβ has been known for some time to have powerful effects on bone cell physiology, but its effects on osteoclast precursors are unclear. This is because analysis has depended
Osteoclast differentiation requires TGFβ

Table 2. Bone matrix mimics effects of TGFβ3 on OCL formation

<table>
<thead>
<tr>
<th>Incubation conditions</th>
<th>OCL per cm² (mean ± s.e.m.)</th>
<th>Surface area of bone resorbed (%) (mean ± s.e.m.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>7 days in:</td>
<td>7 days in:</td>
<td></td>
</tr>
<tr>
<td>M-CSF</td>
<td>TRANCE (10 ng/ml) M-CSF</td>
<td>6±4</td>
</tr>
<tr>
<td>M-CSF</td>
<td>TRANCE (30 ng/ml) M-CSF</td>
<td>51±15</td>
</tr>
<tr>
<td>M-CSF TGFβsRII</td>
<td>TRANCE (10 ng/ml) M-CSF</td>
<td>0</td>
</tr>
<tr>
<td>M-CSF TGFβsRII</td>
<td>TRANCE (30 ng/ml) M-CSF</td>
<td>8±5‡</td>
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</tbody>
</table>

Bone marrow cells were incubated on plastic coverslips or bone slices in M-CSF (30 ng/ml) with or without soluble TGFβsRII (1000 ng/ml) for 7 days. Medium was then removed and replaced with TRANCE and M-CSF for 7 days. n=12 cultures per variable. *P<0.05 versus plastic; ‡P<0.05 versus no soluble receptors.

on experimental systems in which TRANCE-expressing cells are present, introducing the potential for indirect or confounding influences. These might account for the oft-contradictory responses reported. In vivo, while TGFβ injection close to bone increases bone resorption and bone formation (Marcelli et al., 1990), transgenic overexpression by osteoblasts causes osteopenia (Erlebacher and Derynck, 1996).

In vitro, using systems that contain both precursors and accessory cells, TGFβ inhibits and/or stimulates bone resorption (see Bonewald and Mundy, 1990; Chenu et al., 1988; Dieudonné et al., 1991; Hattersley and Chambers, 1991; Pfeilschifter et al., 1988; Tashjian et al., 1985) and induces apoptosis in osteoclasts (Hughes et al., 1996). The osteoclast-inhibitory effects, including the induction of apoptosis, might reflect a homeostatic signal from sites of active bone resorption, since it has recently been shown that TGFβ can induce OPG production in bone marrow stromal and osteoblastic cells (Murakami et al., 1998; Takai et al., 1998).

How might TGFβ facilitate osteoclast formation? We have previously noted that bone marrow cells incubated in M-CSF become resistant to OCL-induction by TRANCE during culture (Wani et al., 1999). This is consistent with the ability of M-CSF to induce terminal macrophage differentiation in mononuclear phagocytes (Munn et al., 1995; Tushinski and Stanley, 1985; Wijffels et al., 1993). Maturation of mononuclear phagocytes to an unresponsive state occurs for other cytokines as well. TGFβ is known to suppress several macrophage characteristics (Bogdan and Nathan, 1993; Letterio and Roberts, 1998). Therefore, the costimulatory activity, synergy and the prolongation of osteoclast-forming potential we have observed might all be explained through suppression by TGFβ of macrophage commitment.

While TGFβ deactivates some macrophagic functions, however, particularly the generation of reactive oxygen and nitrogen intermediates, other macrophage functions, including phagocytosis and chemotaxis, are enhanced (Goerd and Orf anos, 1999; Noble et al., 1993; Parent and Stankova, 1993; Tsunawaki et al., 1988; Wahl et al., 1993; Welch et al., 1990). TGFβ therefore does not merely suppress, but redirects macrophage differentiation: it favours the expression of degrading and scavenging over inflammatory functions in macrophages (Fadok et al., 1998; Welch et al., 1990), perhaps analogous to its crucial role in lineage regulation in T helper 1 and 2 lymphocytes (Letterio and Roberts, 1998; Sad and Mosmann, 1994; Sallusto et al., 1998). From this perspective, the essential role of TGFβ in osteoclast formation might be not only to suppress macrophage differentiation, but to facilitate mononuclear phagocyte differentiation towards that of the professional scavenger of bone matrix, the osteoclast. This is supported by the effectiveness of TGFβ in osteoclast-induction even when precursors were incubated consecutively in TGFβ and TRANCE: TGFβ is not merely a synergist for TRANCE, but makes a distinct contribution to osteoclast lineage-induction.

The view that TGFβ is actively pro-osteoclastic is also favoured by its anti-apoptotic action in osteoclasts, and by its ability to enhance RANK mRNA expression, at least in early cultures, in precursors incubated in M-CSF. Indeed, enhanced RANK levels or changes in RANK signalling patterns might underlie the prolonged responsiveness we observed in precursors incubated in TGFβ1. Suppression of apoptosis by TGFβ might also contribute to osteoclast formation. Against this, we noted that TGFβ1 increased the number of osteoclasts and reduced the total number of nuclei in our cultures, while soluble receptors did the reverse. This pattern is not explicable through apoptotic effects alone, but suggests that TGFβ is participating actively in the lineage switch to osteoclasts.

Although TGFβ strongly potentiated osteoclast formation on plastic substrates, we noticed that its effects were much less marked when bone resorption was used as an assay end-point. We previously found that TGFβ has no effect on bone resorption by isolated mature osteoclasts (Hattersley and Chambers, 1991). We also noted that the number of osteoclasts formed on bone was substantially greater than on plastic, and was unaffected by exogenous TGFβ. Because TGFβ is enriched in bone matrix, it seemed possible that the bone slices were substituting as a source of TGFβ in these cultures. This interpretation is consistent with abrogation of the ability of bone to enhance osteoclastic differentiation by TGFβsRII.

A second possible role for TGFβ arises from the particular
mode of expression of TRANCE. TRANCE is expressed by osteoblastic cells as a membrane-inserted ligand, providing the potential for osteoblastic cells to direct the exquisitely complex temporospatial patterns of resorption required for bone morphogenesis and remodelling. The corollary is that haemopoietic precursors might not detect the osteoclast-inductive ligand until they reach the resorptive site. TGFβ might therefore serve to maintain TRANCE-responsiveness of precursors during this migration and facilitate migration. The TGFβ might be released from bone matrix during resorption, or from osteoclasts, their precursors, or osteoblastic cells, which have all been shown to secrete TGFβ (see Bonewald and Mundy, 1990). There is also evidence that osteoclast precursors release growth factors from extracellular matrix during migration (Vu et al., 1998).

While TGFβ seems to be essential for osteoclast formation, its widespread expression and presence in connective tissues outside bone make it questionable whether the effects of TGFβ we have observed in precursors, in the absence of TRANCE, are specific for osteoclastic differentiation. It may be that the mononuclear phagocyte precursors are exposed to much greater quantities of TGFβ in bone matrix, which contains abundant TGFβ1, TGFβ2 and TGFβ3 (Pelton et al., 1991), than elsewhere. Alternatively, TGFβ may play a more general role in the physiology of the mononuclear phagocyte system, to maintain precursor responsiveness, to facilitate migration, and to both deactivate inflammatory signals and prepare precursors for differentiation-inductive signals specific for noninflammatory macrophage microniches or functions. Whether or not this is so, our results show that in bone, TRANCE expression by osteoblastic cells may not be sufficient to induce cells of the mononuclear phagocyte system to become osteoclasts: TGFβ is a costimulator of the differentiation and survival of osteoclastic cells.

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REFERENCES


Osteoclast differentiation requires TGFβ