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ARTICLES

Tumor Necrosis Factor-α Mediates RANK Ligand Stimulation of Osteoclast Differentiation by an Autocrine Mechanism

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Abstract Osteoblasts or bone marrow stromal cells are required as supporting cells for the in vitro differentiation of osteoclasts from their progenitor cells. Soluble receptor activator of nuclear factor-κB ligand (RANKL) in the presence of macrophage colony-stimulating factor (M-CSF) is capable of replacing the supporting cells in promoting osteoclastogenesis. In the present study, using Balb/c-derived cultures, osteoclast formation in both systems — osteoblast/bone-marrow cell co-cultures and in RANKL-induced osteoclastogenesis — was inhibited by antibody to tumor necrosis factor-α (TNF-α), and was enhanced by the addition of this cytokine. TNF-α itself promoted osteoclastogenesis in the presence of M-CSF. However, even at high concentrations of TNF-α the efficiency of this activity was much lower than the osteoclastogenic activity of RANKL. RANKL increased the level of TNF-α mRNA and induced TNF-α release from osteoclast progenitors. Furthermore, antibody to p55 TNF-α receptors (TNF receptors-1) (but not to p75 TNF-α receptors (TNF receptors-2) inhibited effectively RANKL- (and TNF-α() induced osteoclastogenesis. Anti-TNF receptors-1 antibody failed to inhibit osteoclastogenesis in C57BL/6-derived cultures. Taken together, our data support the hypothesis that in Balb/c, but not in C57BL/6 (strains known to differ in inflammatory responses and cytokine modulation), TNF-α is an autocrine factor in osteoclasts, promoting their differentiation, and mediates, at least in part, RANKL's induction of osteoclastogenesis. J. Cell. Biochem. 83: 70–83, 2001.

Key words: bone resorption; cytokines; macrophage; bone marrow; osteoblast

The bone-resorbing cell, the multinucleated osteoclast, is formed by fusion of its precursor cells belonging to the monocyte/macrophage lineage [Kahn et al., 1982; Baron et al., 1986; Suda et al., 1992, 1996; Reddy and Roodman, 1998]. In vitro experiments have shown that cell–cell contact between osteoclast progenitors and osteoblasts or bone marrow stromal cells is required for osteoclastogenesis [Takahashi et al., 1988; Udagawa et al., 1989; Martin and Ng, 1994; Jimi et al., 1996]. It was found recently that a cell surface molecule on the osteoblast/stromal cell surface has a crucial role in the osteoclastogenic activity of these cells. This molecule is named by different investigators osteoclast differentiation factor (ODF) [Yasuda et al., 1998], osteoprotegerin ligand (OPGL) [Lacey et al., 1998], TNF-related activation-induced cytokine (TRANCE) [Wong et al., 1997], or receptor activator of nuclear factor-κB ligand (RANKL; the term used herein) [Anderson et al., 1997; Suda et al., 1999; Takahashi et al., 1999]. The receptor protein, RANK is expressed on the surface of osteoclast progenitors [Hsu et al., 1999; Nakagawa et al., 1998]. A soluble fragment containing part of the extracellular domain of RANKL (the carboxyterminal half of the protein, amino acids 158–316), is capable of promoting osteoclastogenesis in the presence of M-CSF. Most importantly, the supporting cells

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(osteoblasts or stromal cells) are not required for this activity [Lacey et al., 1998; Quinn et al., 1998; Burgess et al., 1999].

The role of immune system-derived cytokines (including TNF-α) in bone metabolism, and particularly in resorption, is well documented [Manolagas and Jilka, 1995]. It is likely that TNF-α also plays important roles in pathological situations where bone metabolism is affected. For example, periodontal disease is characterized by loss of alveolar bone, a process mediated by osteoclasts [Carson et al., 1978]. In this disease the high local abundance of bacteria-derived lipopolysaccharide (LPS, known as a potent inflammatory cytokine inducer) is responsible, at least in part, for the increased resorption of bone. Cytokine modulation by estrogen may play a crucial role in post-menopausal osteoporosis [Kitazawa et al., 1994; Manolagas and Jilka, 1995] which is caused by the reduced circulating levels of the female sex hormone. TNF-α is produced abundantly in rheumatoid arthritis [Ng et al., 1984] and probably plays a central role in the pathogenesis of rheumatoid arthritis-associated osteoporosis one of the most devastating complications of this disease [Maini et al., 1993]. TNF-α has been also shown recently to mediate via its p55 receptor the LPS stimulated osteoclastogenesis [Abu-Amer et al., 1997], as well as osteolysis induced by implant-derived particles in orthopedic disease [Merkel et al., 1999].

In the present study we examine the hypothesis that TNF-α is an autocrine factor in osteoclastogenesis, and that RANKL exerts its activity, at least in part, by promoting the osteoclast progenitor to synthesize and release TNF-α.

METHODS

Mice

Newborn Balb/c mice and 7–9 week old male Balb/c and C57BL/6 mice were obtained from Harlan Laboratories Ltd. (Jerusalem, Israel).

Reagents

Recombinant mouse soluble RANKL/OPGL (158–316) (△RANKL(158–316)) was a gift from Amgen Inc. (Thousand Oaks, CA) and prepared as described [Lacey et al., 1998]. Rat monoclonal anti-mouse TNF-α, hamster monoclonal anti-mouse p55 (TNF-α receptor-1 [TNFR-1]) neutralizing antibody, hamster monoclonal anti-mouse p75 (TNF-α receptor-2 [TNFR-2]) neutralizing antibody, hamster monoclonal anti-mouse CD51 (αV), and rat monoclonal anti-mouse CD14 were purchased from Pharmingen (San Digeo, CA). Rabbit polyclonal anti-mouse TNF-α was purchased from Genzyme (Cambridge, MA). Alkaline phosphatase-conjugated goat anti-hamster IgG, horseradish peroxidase-conjugated goat anti-hamster IgG and horseradish peroxidase-conjugated goat anti-rat IgG were purchased from Jackson ImmunoResearch (West Grove, PA). Recombinant mouse TNF-α, OPG-Fc (OPG/Fc chimera containing OPG amino acids 1–398 residues) and M-CSF were purchased from Research and Development systems Inc. (Minneapolis, MN). 1,25-dihydoxyvitamin D_3 [$1,25(OH)_2D_3$] was purchased from Biomol (Plymouth Meeting, PA). Endotoxin testing (LAL kit from Biowhittaker Inc. Walkersville, MD) confirmed the absence of LPS contamination in our reagents. LPS was purchased from Difco laboratories (Detroit, MI). Dexamethasone was purchased from Sigma Chemical Co. (St Louis, MO). Media and sera were purchased from Biological Industries (Beth Haemek, Israel). All chemicals and reagents were of analytical grade.

In Vitro Osteoclast Formation Assay

Mouse bone marrow mononuclear cells (prepared as described) [Tamura et al., 1993] were incubated with calvarial osteoblasts [Udagawa et al., 1996]. In short, osteoblasts (7×103/well) and bone marrow mononuclear cells (1.3×10^5/well) were co-cultured in 96-well plates in 0.2 ml of α-MEM (modified Eagle's minimum essential medium) containing 10% charcoal-treated FCS (FCS-medium), with $1,25(OH)_2D_3$ (10 nM), dexamethasone (100 nM) and LPS (20 ng/ml,). Cells were incubated in quadruplicate and were replenished on Day 4 with fresh medium. Osteoclast formation was evaluated on Day 7, following removal of the osteoblasts by collagenase [Shioi et al., 1994].

To assay osteoclast formation induced by RANKL or TNF-α bone marrow mononuclear cells (1.3×10^5/well) were plated in 96-well plates in 0.2 ml of FCS-medium in the presence of mouse M-CSF (30 ng/ml) and the indicated dose of RANKL or TNF-α. On Day 3, medium was changed and on Day 5, osteoclast formation was evaluated. In all studies in this com-

munication when bone marrow cells (alone) were incubated with M-CSF in the absence of RANKL almost all adherent cells express macrophage markers.

Tartrate-Resistant Acid Phosphatase (TRAP) Staining

A commercial kit (Cat. No. 387-A, Sigma) was used according to the manufacturer instructions, omitting counter stain with hematoxylin. TRAP-positive cells containing three or more nuclei were scored as osteoclasts. In some experiments, TRAP-positive mononuclear cells were also counted.

TRAP Activity

The enzyme activity [Simonet et al., 1997] was examined by the conversion of *p*-nitro phenylphosphate (20 nM) to p-nitrophenol in the presence of 80 mM sodium tartrate. Absorbence was measured at 405 nm using a plate reader (Dynatech).

Methylene Blue Staining

The cell number was estimated by the methylene blue staining assay [Goldman and Bar-Shavit, 1979] using a plate reader (Dynatech).

Western Blot Analysis for CD14

Western analysis was performed as described previously [Abu-Amer and Bar-Shavit, 1994], with the exception that the enzyme used for detection was horseradish peroxidase. Bands were quantified by densitometry.

Northern Blot Analysis

Total cellular RNA was extracted using TRI REAGENT, fractionated by electrophoresis on 1.2% agarose formaldehyde gels (10 μg/lane), and transferred to nylon membranes (Hybond-N, Amersham International, Little Chalfont, UK). [^{32}P]-labeled 1.1 Kb mouse TNF-α cDNA (in one experiment [^{32}P]-labeled 0.5 Kb mouse RANKL cDNA) and [^{32}P]-labeled mouse ribosomal protein L32 cDNA (as an internal house keeping gene control) were used for simultaneous hybridization. The hybridized membrane was then subjected to autoradiography and the density of each TNF-α and L32 mRNA bands were quantified using a Fluor-STM MultiImager and Multi-Analyst/PC software (Bio-Rad Laboratories, Hercules, CA) [Sambrook et al., 1989].

Pit Formation

Calcium phosphate ceramic thin-film-coated quartz 16-well multitest slides (BD Biosciences, Bedford, MA) were used, in a similar manner to previous studies [Kurihara et al., 1998; Loomer et al., 1998]. Bone marrow mononuclear cells (0.3×105/well) were plated in 0.3 ml of FCS-medium in the presence of mouse M-CSF (30 ng/ml) and RANKL (20 ng/ml) with or without anti-TNF-α antibody (1:100, 20 μg/ml) or rat IgG (20 μg/ml). Cells were removed according to the manufacturer's instructions, pits were counted and resorbed area was measured by image analyzer. In parallel, cells were plated, stained for TRAP and counted.

Statistical Analysis

Student's *t*-test was used to determine of significance of differences. MultiImager and Multi-Analyst/PC software (Bio-Rad Laboratories, Hercules, CA). Values are presented as mean±SD of $n = 4–6$.

RESULTS

We first examined the involvement of TNF-α in osteoclast differentiation in an in vitro co-culture system containing bone marrow cells and calvaria-derived osteoblasts. As shown in Figure 1, anti-TNF-α antibody inhibited in a dose dependent manner TRAP positive multi-

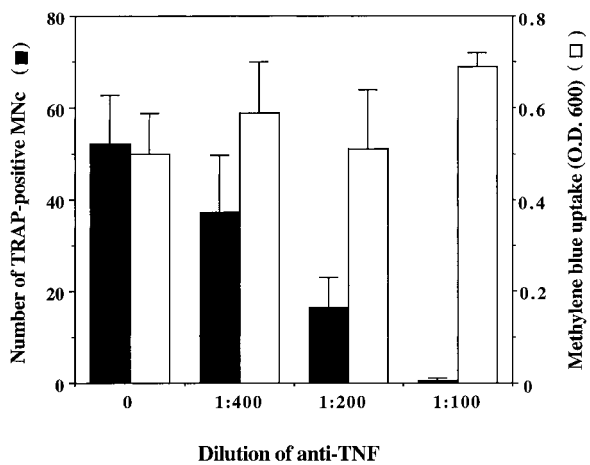

Fig. 1. Effect of anti-TNF-α antibody on osteoclastogenesis in osteoblast/bone marrow cell co-cultures. Balb/c mouse bone marrow cells were co-cultured for 7 days with primary osteoblasts in the presence of $1,25(OH)_2D_3$, dexamethasone and LPS (10 nM, 100 nM, and 20 ng/ml, respectively), with or without anti-TNF-α (1:400 to 1:100). Number of TRAP positive multinucleated cells/well (>2 nuclei/cell) (close bars) and methylene blue staining values (open bars) are presented.

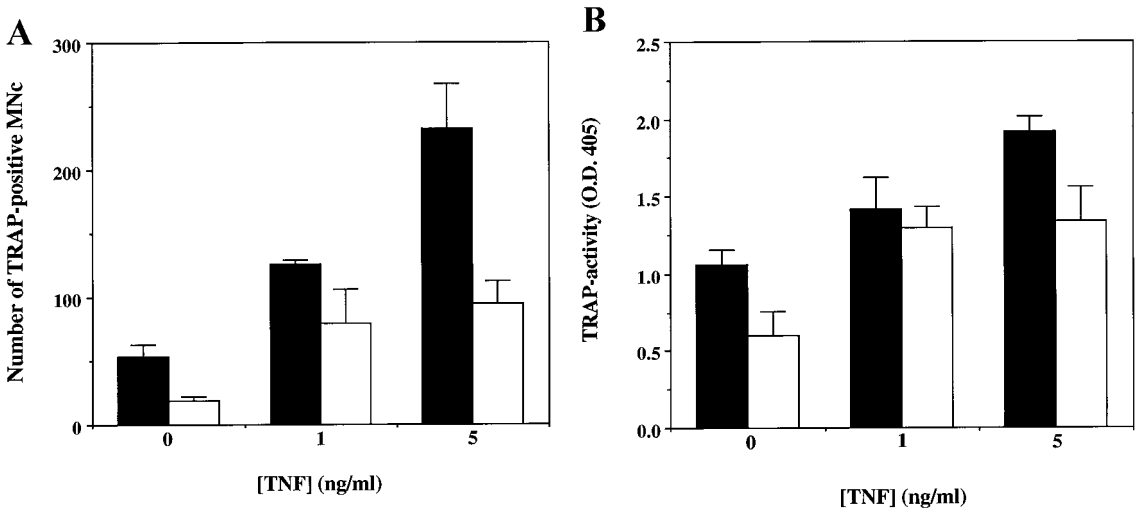

Fig. 2. Effect of TNF-α on osteoclastogenesis in osteoblast/bone marrow cell co-cultures. The non-adherent bone marrow cells were co-cultured with primary osteoblast in the presence of $1,25(OH)_2D_3$ and dexamethasone (10 and 100 nM, respectively). Mouse TNF-α was added in the presence (open bars) or absence (closed bars) of TNF-α antibody (1:100). **A:** Number of TRAP positive multinucleated cells/well (> 2 nuclei/cell). **B:** TRAP enzymatic activity.

nucleated cell formation in the co-cultures: a slight reduction in osteoclast numbers is obtained at 1:400 antibody dilution (not significant statistically); reductions of 70% ($P < 0.002$) and of 99% ($P < 0.001$) were observed at 1:200 and 1:100 antibody dilutions, respectively. No effect was observed by rat IgG, (20 μg/ml, the highest concentration of anti-TNF-α used). Cell proliferation was unaffected by anti-TNF-α antibody as assessed by methylene blue staining. Thus, the reduction in osteoclast numbers in the culture is not the result of a global inhibition of proliferation.

Next, we tested the effect of TNF-α addition on osteoclast formation. TNF-α increases osteoclastogenesis in the co-culture system, in a dose dependent manner (Fig. 2A). TNF-α at 1 and 5 ng/ml increased osteoclast numbers by 132 ($P < 0.001$) and 337% ($P < 0.001$), respectively. This effect was inhibited by anti-TNF-α antibody. We obtained similar results when measuring the effect by TRAP activity (Fig. 2B). The effects using the latter method were less pronounced, because it detects also TRAP-positive mononuclear cells. The findings indicate that TNF-α plays a necessary role in the differentiation of osteoclasts from their precursors under these conditions. Since both bone marrow cells and osteoblasts synthesize and release TNF-α, the co-culture system is not suitable to examine whether the cytokine exerts its action in an autocrine fashion.

Therefore, we examined the role of TNF-α in a system containing bone marrow cells, RANKL and M-CSF. First, we found that osteoclasts are formed from their bone marrow derived progenitors in response to RANKL: we observed the appearance of TRAP-positive multinucleated bone resorbing (pit forming) cells, the acquisition of calcitonin receptors, the increased expression of α chain of vitronectin-receptor (CD51), as well as disappearance of CD14 [Quinn et al., 1991; Akatsu et al., 1992; Athanasou et al., 1992] (not shown).

Addition of TNF-α increased RANKL-induced osteoclast formation. The increase was more pronounced using a lower dose of RANKL (93% increase in cells treated with 5 ng/ml vs. 35% increase in cells treated with 10 ng/ml) (Fig. 3A). Addition of anti-TNF-α antibodies inhibited RANKL-induced formation of TRAP positive multinucleated cells, in a dose dependent manner, without affecting cellular contents (Fig 3B). Inhibition of 20 ($P < 0.05$), 27 ($P < 0.005$), and 54% ($P < 0.001$) in osteoclast numbers were obtained by antibody dilutions of 1:400, 1:200, and 1:100, respectively. Anti-TNF-α-antibody inhibited both the RANKL and the RANKL + TNF-α induced osteoclast formation (Fig. 3C) ($P < 0.001$ for RANKL alone and for

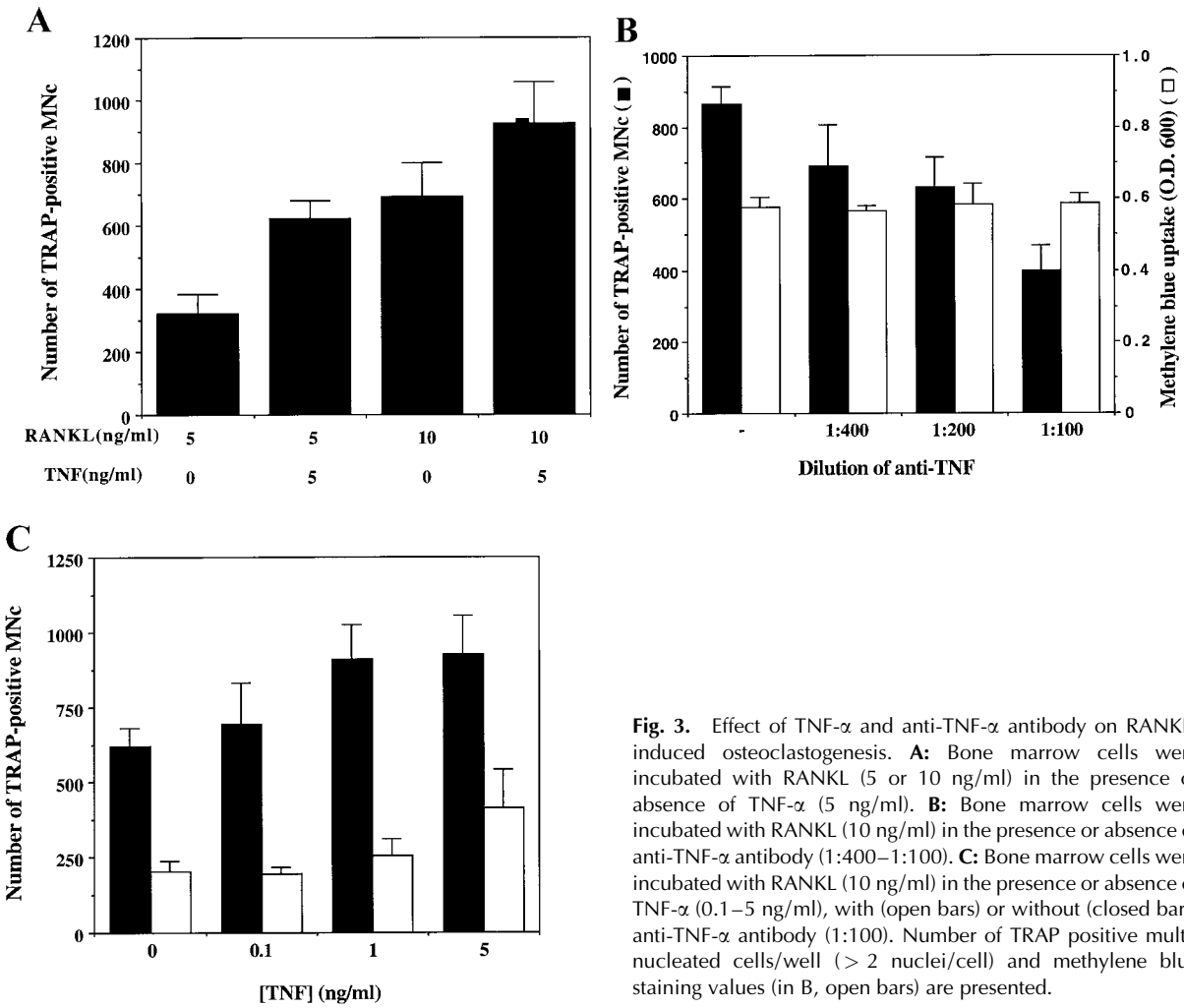

Fig. 3. Effect of TNF-α and anti-TNF-α antibody on RANKL-induced osteoclastogenesis. **A:** Bone marrow cells were incubated with RANKL (5 or 10 ng/ml) in the presence or absence of TNF-α (5 ng/ml). **B:** Bone marrow cells were incubated with RANKL (10 ng/ml) in the presence or absence of anti-TNF-α antibody (1:400–1:100). **C:** Bone marrow cells were incubated with RANKL (10 ng/ml) in the presence or absence of TNF-α (0.1–5 ng/ml), with (open bars) or without (closed bars) anti-TNF-α antibody (1:100). Number of TRAP positive multinucleated cells/well (> 2 nuclei/cell) and methylene blue staining values (in B, open bars) are presented.

RANKL with 0.1 and 1 ng/ml TNF-α, and $P < 0.002$ for RANKL with 5 ng/ml TNF-α). As control we have used rat IgG, (20 μg/ml, the anti TNF-α concentration in 1:100 dilution), and found no effect.

As seen in Table I, RANKL-treated bone marrow cells were also capable of forming pits, and the addition of anti-TNF-α antibodies, but not of rat IgG, inhibited the RANKL-induced bone resorption (pit formation). In cultures where the antibodies were present, in addition to the reduced TRAP positive multinucleated cells, these cells were less efficient (0.66 vs. 0.51 pits/multinucleated cell were observed in the absence and presence of the antibodies, respectively). Moreover, the average area of a pit was almost two-fold in the absence of the antibodies.

One must take into account, however, that RANKL is a TNF-related molecule. To examine the possibility that the anti-TNF-α antibodies exert their inhibitory activity via direct interaction with RANKL, we measured their cross-reactivity with RANKL. We used the inhibitory antibodies in an ELISA with a detection limit of 5 pg of TNF-α. No cross-reactivity could be observed even at 2000 pg of RANKL (not shown). Therefore we conclude that TNF-α, produced by osteoclast progenitors, plays a role in RANKL-induced osteoclastogenesis.

TNF-α alone (in the presence of M-CSF, but in the absence of RANKL) exhibited a low osteoclastogenic activity as assessed by TRAP-positive multinucleated cell formation, and this only in a high concentration (20 ng/ml) (Fig. 4). However, a large number of mononuclear TRAP positive cells were observed. To appreciate the difference using 20 ng/ml of TNF-α and 10 ng/ml of RANKL one should note that in a TNF-α-containing well, ~110 multinucleated and ~3,200 mononuclear TRAP positive (Fig. 4) as well as ~10,000 mononuclear TRAP negative

TABLE I. Effect of Anti-TNF-α on RANKL-Induced Pit Formation

Treatment					
RANKL	Anti-TNF-α	IgG	TRAP+/MNs[a]	Pits[b]	Resorbed area[c]
None	None	None	0	0	0
20 ng/ml	None	None	218±36	144±22	9.87±1.22
20 ng/ml	20 μg/ml	None	63±11	32±11	1.18±0.17
20 ng/ml	None	20 μg/ml	207±21	149±16	9.39±1.14

Bone marrow cells were incubated in the presence of M-CSF and the indicated proteins. In each condition, cells were plated in parallel slides. In one, the numbers of TRAP positive cells containing more than two nuclei (first column), and in the other the number of pits (second column) and the resorbed area (third column) were assessed.
[a]-TRAP positive multinucleated cells/well.
[b]-Pits/well.
[c]-Percent well area resorbed.

cells (not shown) were detected, while in the RANKL-containing wells ~1,100 multinucleated and ~9,000 mononuclear TRAP positive (Fig. 4) and few TRAP-negative mononuclear cells were observed. Furthermore, the average number of nuclei per cell of the TRAP positive multinucleated cells in the RANKL-containing wells is higher than that of cells in TNF-α-containing wells.

If TNF-α mediates RANKL's osteoclastogenic activity, one would expect that RANKL induces TNF-α expression. We found that RANKL addition to osteoclast progenitors increased TNF-α mRNA abundance. Figure 5A shows a representative autoradiogram of an experiment in which RANKL (20 ng/ml) was added to bone marrow cells for 5 to 30 min. Total RNA was then extracted and Northern blotting analysis was performed using probes for TNF-α and L32. Figure 5B represents the mean of TNF-α to L32 ratio of five experiments. A significant increase in TNF-α mRNA abundance is evident already after 5 min incubation with RANKL, reaching a maximum after 30 min (~4.5-fold). Between 6–8 h (not shown) the mRNA abundance was lowered back to control levels. The dose dependency is shown in Figure 5C (a representative autoradiogram) and Figure 5D (mean of two independent experiments). Furthermore, addition of 5, 10, or 20 ng/ml of RANKL for 4 h, induced 0.6, 0.7, and 1.1 ng/ml of TNF in the bone marrow cells culture medium. The specific RANKL/OPGL binding protein, OPG, prevents the increase in TNF-α mRNA abundance (Fig. 5E). This indicates that the effect observed is mediated by RANKL. We have studied the effects of LPS in our system. Low LPS doses (1–10 pg/ml) do not affect TNF-α production in the absence or presence RANKL. A higher dose of LPS (100 pg/ml), increases TNF-α mRNA levels, but with a different kinetics than RANKL. Furthermore, LPS (1, 10, and 100 pg/ml) does not induce osteoclast differentiation and does not enhance the ability of RANKL to promote osteoclast formation (data not shown). Thus, it is impossible that under our culture conditions TNF was generated by endotoxin contamination.

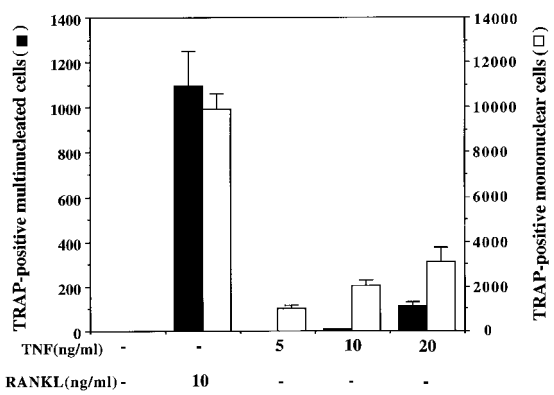

Fig. 4. Comparison of TNF-α and RANKL in induction multinucleated and mononuclear TRAP positive cells. Effects on multinucleated cells (> 2 nuclei/cell) (closed bars), and on mononuclear cells (open bars).

If indeed the effect of RANKL is mediated via TNF-α, it is expected that the p55 TNF-α receptor (TNFR-1) is involved [Abu-Amer et al., 1997]. We used neutralizing antibody against TNFR-1 and against TNFR-2. The anti TNFR-1 antibody inhibited both-the lower osteoclastogenic effect of TNF-α (Fig. 6, open bars) as well as the marked effect of RANKL (Fig. 6, closed bars). The anti-TNFR-1 antibody, at dilutions of 1:400, 1:200, and 1:100 caused inhibition of osteoclast formation of 50, 85 (not shown), and > 95% ($P < 0.001$) (Fig. 6), respectively. (Note that different scales are used for measuring the effects of RANKL and TNF-α). The antibodies also blocked the appearance of mononuclear

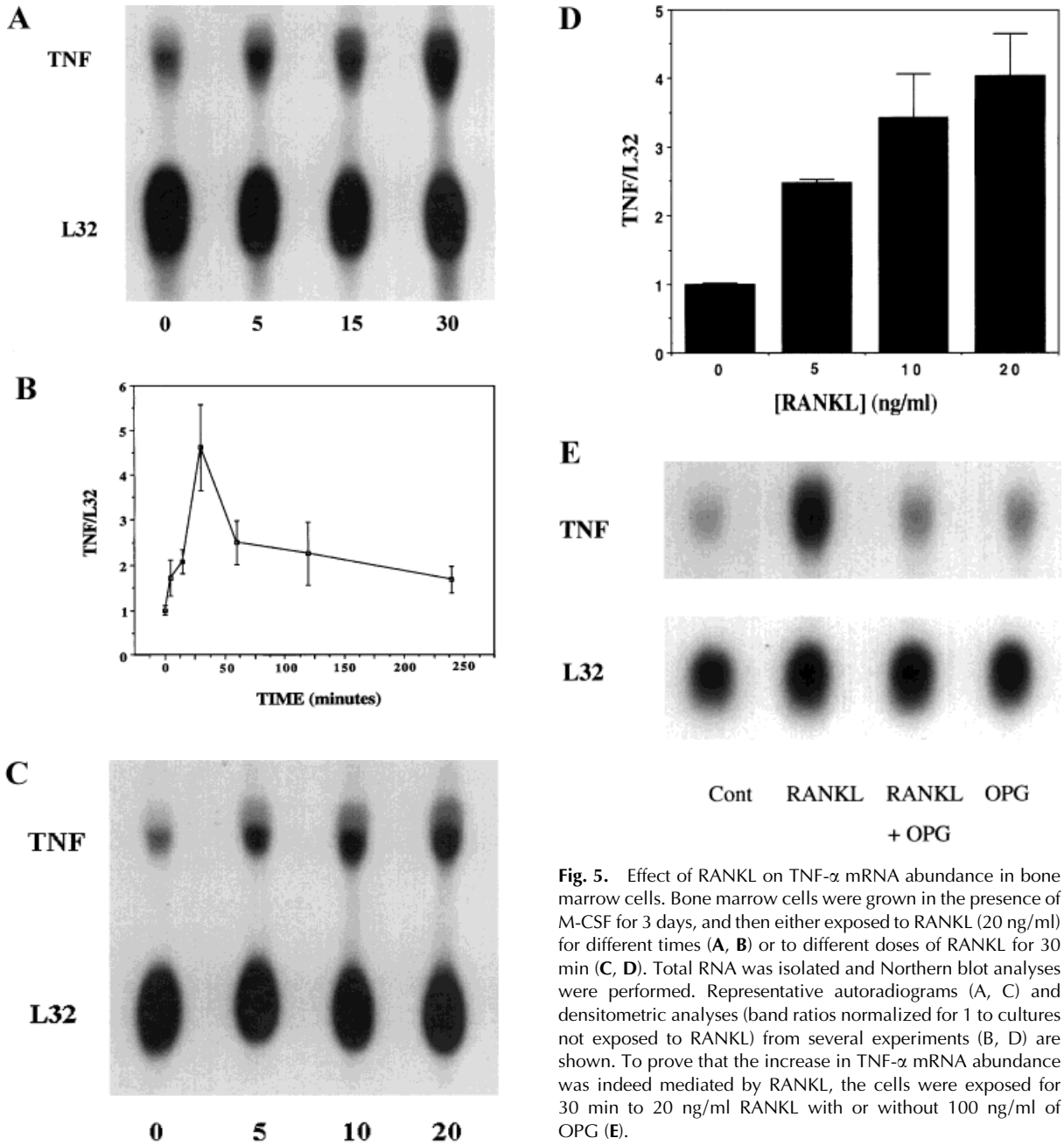

Fig. 5. Effect of RANKL on TNF-α mRNA abundance in bone marrow cells. Bone marrow cells were grown in the presence of M-CSF for 3 days, and then either exposed to RANKL (20 ng/ml) for different times (**A**, **B**) or to different doses of RANKL for 30 min (**C**, **D**). Total RNA was isolated and Northern blot analyses were performed. Representative autoradiograms (A, C) and densitometric analyses (band ratios normalized for 1 to cultures not exposed to RANKL) from several experiments (B, D) are shown. To prove that the increase in TNF-α mRNA abundance was indeed mediated by RANKL, the cells were exposed for 30 min to 20 ng/ml RANKL with or without 100 ng/ml of OPG (**E**).

TRAP positive cells (not shown). In contrast, anti TNFR-2 antibody did not inhibit RANKL-induced osteoclastogenesis (1004.3 TRAP positive multinucleated cells were scored in the presence of anti TNFR-2 antibody). No effects were observed by rat IgG (20 μg/ml), and by hamster IgG (10 μg/ml). These concentrations are similar to those of the corresponding antibodies.

The possibility that TNF-α-induced osteoclastogenesis involves modulation of RANKL synthesis was excluded by the inability of OPG to affect significantly TNF-α activity (Fig. 7A) (osteoclast numbers of 86.3 ± 4.1 and 82.8 ± 6.4 in the absence or presence of OPG, respectively). In the same experiment OPG blocked completely RANKL-induced osteoclastogenesis. Furthermore, Northern blot analysis (Fig. 7B) showed that the precursor cells do not express RANKL (3rd lane) and that TNF-α (4 days, 4th lane or 6 h, 5th lane) does not induce such an expression. As a control for the quality of RANKL probe, we confirm RANKL mRNA abundant expression in osteoblasts (1st lane)

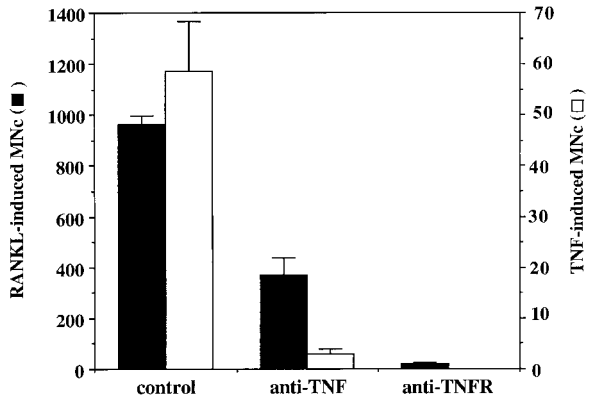

Fig. 6. Effect of anti-TNFR-1 and anti-TNF-α- antibody on TNF-α- and RANKL-induced osteoclastogenesis Bone marrow cells were grown with TNF-α (20 ng/ml) (open bars), or RANKL (10 ng/ml) (closed bars) in the presence of either anti-TNF-α-antibody (1:100, 20 μg/ml) or anti-TNFR-1 antibody (1:100, 10 μg/ml) or in their absence.

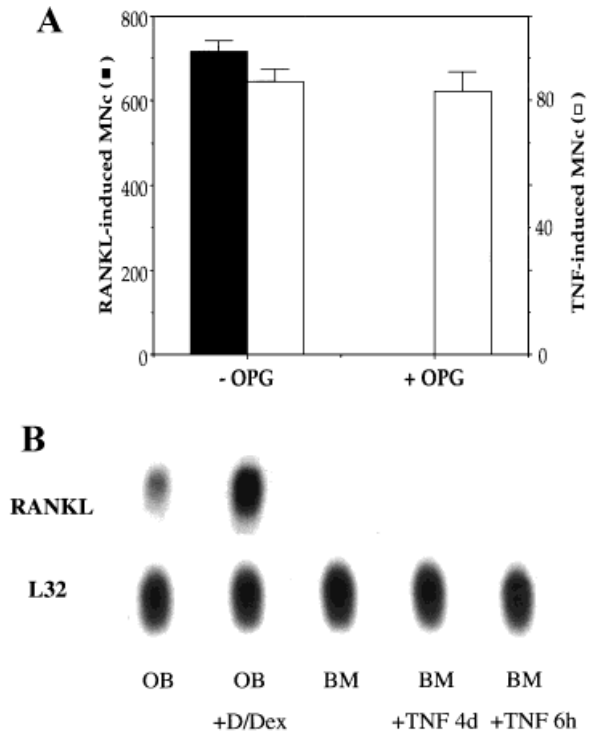

Fig. 7. Involvement of RANKL in TNF-α induced osteoclastogenesis **A:** Bone marrow cells were grown with TNF-α (20 ng/ml) (open bars), or RANKL (10 ng/ml) (closed bars) in the presence or absence of OPG (100 ng/ml). Number of TRAP positive multinucleated cells/well (>2 nuclei/cell) were counted. **B:** Bone marrow cells were grown in the presence of M-CSF for 4 days without (3rd lane) or with 20 ng/ml of TNF-α (4th lane). Fifth lane represents cells which were exposed to TNF-α for the last 6 h. As a positive control, we also analyzed calvarial osteoblasts grown in the absence (first lane) or presence (second lane) of dexamethasone and $1,25(OH)_2D_3$ (100 and 10nM, respectively). Total RNA was isolated and Northern blot analyses were performed to measure RANKL and L32 mRNAs abundance.

and up-regulation of this expression by dexamethasone and $1,25(OH)_2D_3$ (2nd lane) [Yasuda et al., 1998]. The lack of RANKL expression in the bone marrow derived cultures suggests they do not contain stromal cells.

To examine the relative role played by TNF-α in various stages of RANKL-induced osteoclast differentiation, we divided the 5-day experiment into an early (first 3 days, in which no cell fusion is observed) and late (last 2 days, in which cell fusion takes place) stages. We compared the inhibitory activity of anti-TNFR-1 when present only at the early or only at the late stage (Table II). When the antibody was present in the first stage the inhibitory effect was almost similar to that obtained when it was present throughout the experiment. On the other hand, when the antibody was present only in the second stage, a slight inhibition of RANKL osteoclastogenic activity was observed. However, the antibodies present only at the later stage (Table II), inhibited the appearance of larger cells (>10 nuclei/cell) more effectively than that of the smaller ones (>2 nuclei/

TABLE II. Effect of Duration of Anti-TNFR-1 Presence on RANKL-Induced Osteoclast Formation

Antibody treatment				
Days 0–3	Days 3–5	TRAP+/MN[a] (>2 nuclei/cell)	TRAP+/MN[a] (>10 nuclei/cell) (% of control)	TRAP+/Mono[b]
None	None	100	100	100
Anti-TNFR	Anti-TNFR	3	0	2
Anti-TNFR	None	5	0	2
None	Anti-TNFR	87	48	73

Bone marrow cells were incubated in the presence of RANKL (10 ng/ml) and M-CSF without or with anti-TNFR-1 antibody for the time indicated. In each condition, the numbers of TRAP positive cells containing more than 2 (first column) or more than 10 (second column) as well as mononuclear TRAP positive cells (third column) were scored. Data are presented as percentage of cultures grown in the absence of the antibody.
[a]-TRAP positive multinucleated cells.
[b]-TRAP positive mononuclear cells.

TABLE III. Effect of Anti-TNFR-1 on RANKL- and TNF-α-Induced Osteoclast Formation in C57BL/6- Derived Cells

Treatment		TRAP+/MN[a]	TRAP+/MONO[b]
Cytokine	Anti-TNFR-1	(cell number/well)	(cell number/well)
RANKL	—	1013±52	Not counted
RANKL	1:100	876±31 (13%)	Not counted
TNF-α (10 ng/ml)	—	2±1	708±113
TNF-α (10 ng/ml)	1:100	0	23±4 (97%)
TNF-α (20 ng/ml)	—	16±2	2133±125
TNF-α (20 ng/ml)	1:100	0 (100%)	47±5 (98%)

Bone marrow cells were incubated in the presence of M-CSF with either RANKL (10 ng/ml) or TNF-α (10 or 20 ng/ml, as indicated) without or with anti-TNFR-1. In each condition, the numbers of TRAP positive cells containing more than 2 nuclei (first column) or mononuclear TRAP positive cells (second column) were scored. Data are presented as number of cells/well±SD. In wells containing the antibody percent inhibition is given in parentheses.
[a]-TRAP positive multinucleated cells.
[b]-TRAP positive mononuclear cells.

cell) (52 vs. 13% inhibition, respectively). No effect was observed by control hamster IgG (10 μg/ml).

Our results (using mice with Balb/c genetic background) are not consistent with previous studies (using mice with C57BL/6 genetic background) (detailed in Discussion). To examine if the role played by TNFR-1 in RANKL-induced osteoclastogenesis is affected by the genetic background of the mice, osteoclastogenesis assay in the presence or absence of anti-TNFR-1 antibody was also performed in C57BL/6-derived bone marrow cells. In contrast to the almost complete block of RANKL-mediated osteoclastogenesis by the antibody in Balb/c-derived cells (Fig. 6), only a slight inhibition (although statistically significant, $P < 0.05$) (Table III) was observed in C57BL/6-derived cultures. The low osteoclastogenic activity of TNF-α and the induction of mononuclear TRAP positive cells by the cytokine were blocked completely by anti-TNFR-1 antibody in C57BL/6-derived cultures, similar to the findings with Balb/c-derived cells. It is of note that TNF-α activity was significantly lower using C57BL/6 than using Balb/c-derived cells (e.g., at 20 ng/ml of TNF-α induction of 16 vs. 60−110 multinucleated cells/well; data in Table III compared to Figs. 4 and 6).

A possible mechanism to the different behavior of cells derived from C57BL/6 and Balb/c is a differential modulation of TNF-α expression by RANKL in cells from the two mouse strains. Northern analyses, however, showed that TNF-α is modulated by RANKL in the same manner in bone marrow cells derived from (not shown).

We next examined if cells from the two strains respond to the same extent to TNF-α. As we showed above, TNF-α exhibits a low osteoclastogenic effect. On the other hand, the cytokine synergises with RANKL. We wondered if RANKL "primes" the bone marrow cells to respond to TNF-α. To this end, bone marrow cells derived from either Balb/c or C57BL/6 were incubated for 3 days with RANKL, and then washed and incubated with TNF-α in the absence of RANKL. We see (Table IV), that indeed TNF-α is a very effective osteoclasto-

TABLE IV. Effect of RANKL Priming on Osteoclastogenic Activity of TNF-α in Balb/c and C57BL/6

Treatment (last 30 h)				
RANKL (ng/ml)	TNF-α	Balb/c	C57BL/6	*P*
None	None	47.5±6.8	37.5±10.5	>0.1
None	0.2	83.3±25.2	41.5±8.2	<0.05
None	1.0	143.5±27.9	91.3±12.7	<0.02
None	5.0	793.5±144.7	312.3±49.8	<0.002
None	10.0	1044.5±83.9	638.8±42.1	<0.002
2.5	None	224.3±26.5	239.5±58.3	>0.5

Bone marrow cells were incubated in the presence of M-CSF (30 ng/ml) with RANKL (2.5 ng/ml) for 3 days. Monolayers washed, and incubated for additional 30 h in the presence of M-CSF with the indicated doses of RANKL or TNF-α. In each condition, the numbers of TRAP positive cells containing more than 2 nuclei (first column) or mononuclear TRAP positive cells (second column) were scored. Data are presented as number of cells/well±SD.

genic agent in RANKL "primed" bone marrow cells. While no difference between the strains observed in the negative controls (only M-CSF), and in controls containing RANKL, TNF-α was significantly more effective in Balb/c than in C57BL/6 derived RANKL-pre-treated cultures. We observed 60–150% more TRAP positive multinucleated cells in Balb/c at the different TNF-α doses.

DISCUSSION

The demonstration that osteoclasts differentiate from bone marrow cells when co-cultured with stromal cells [Takahashi et al., 1988; Udagawa et al., 1989; Martin and Ng, 1994; Jimi et al., 1996] has contributed to our ability to study these bone resorbing cells. It has been shown later that osteoblasts can be removed from those co-cultures permitting analyses of relatively pure in-vitro generated osteoclasts [Takahashi et al., 1988]. An important step forward was the identification of a factor promoting osteoclast differentiation from its bone-marrow precursor in the absence of supportive cells [Jimi et al., 1996; Anderson et al., 1997; Lacey et al., 1998; Kong et al., 1999; Suda et al., 1999; Takahashi et al., 1999]. This factor was identified as the ligand for RANK. Thus in the last four years much information has accumulated regarding the involvement of cytokines, including TNF-α, in bone physiology and pathology [Brandstrom et al., 1998, 1998a; Horwood et al., 1998; Takai et al., 1998; Tsukii et al., 1998, Vidal et al., 1998; Hofbauer et al., 1999; Morony et al., 1999]. TNF-α has been shown to exert its osteoclastogenic activity through TNF-α made by osteoblasts [Thomson et al., 1987]. The discovery of RANKL, and especially its ability to promote osteoclast differentiation in the absence of osteoblasts or stromal cells, enable us to study a possible role of TNF-α in direct osteoclast/osteoclast precursor interactions.

In the present study we examined the hypothesis that: (1) TNF-α is an autocrine factor in osteoclasts modulating their differentiation and (2) RANKL induction of osteoclastogenesis is mediated in part by TNF-α.

We show here that: (1) antibodies to TNF-α and to its type 1 receptors (but not to type 2 receptors) inhibit RANKL induction of osteoclastogenesis, (2) antibodies are most effective when present throughout the differentiation, (3) TNF-α enhances osteoclastogenesis in both, co-cultures and RANKL-treated bone marrow cells, (4) TNF-α osteoclastogenic activity is less efficient than RANKL's, (5) RANKL increases the abundance of TNF-α mRNA in bone marrow cells and induces the secretion of the cytokine while TNF-α does not modulate RANKL expression, and (6) OPG inhibits RANKL-induced osteoclastogenesis and TNF-α expression, but not TNF-α activity.

It is of note, that in addition to the lower number of pits in wells of RANKL-treated bone marrow cells containing antibodies to TNF-α (as expected from the lower osteoclast number in those wells), those resorption pits are smaller. A careful analysis of this issue is required.

Taken together, our data strongly support our hypothesis regarding the autocrine mode of TNF-α action. Based on a co-culture system [Tani-Ishii et al., 1999] a similar mode of action for TNF-α was proposed. The limitations of the model system did not enable them to exclude the possibility that the cytokine was derived from the osteoblastic line. Furthermore, from the experiment in which anti-TNFR-1 was present in the different stages of the experiment TNF-α seems to play a role in bone marrow cells commitment to the osteoclastogenic lineage, and in the later stages of fusion. Although our cultures contained less than 0.5% alkaline positive cells, and no RANKL expression could be detected, it is impossible to exclude a role for these cells in the effects we see. We have repeated our experiments using the macrophage-like mouse line, RAW 264.7, and confirmed that RANKL is capable of inducing osteoclast differentiation in these cells [Hsu et al., 1999]. Anti-TNF-α and anti-TNFR-1 antibodies inhibited the RANKL osteoclastogenic effect. Furthermore, RANKL increased TNF-α mRNA abundance in similar kinetic and dose response as observed with bone marrow cells (data not shown). The results using RAW 264.7 cells strengthen the conclusion regarding the autocrine manner of TNF-α in RANKL osteoclastogenic activity.

It was shown recently that TNF-α stimulates osteoclast differentiation by a mechanism independent of RANKL–RANK interaction [Kobayashi et al., 2000]. Some findings in this publication are in disagreement with ours. For example, while similar to our findings here, antagonistic antibodies to TNFR-1 inhibit the osteoclastogenic activity of TNF-α, unlike our

findings, in Kobayashi's studies these antibodies do not inhibit the osteoclastogenic activity of RANKL. Another inconsistency is that Kobayashi's study showed a normal effect of RANKL on bone marrow cells derived from TNFR-1 (−/−) mice. To resolve the apparent contradiction, we have examined the involvement of TNFR-1 in RANKL osteoclastogenic activity in C57BL/6-derived cells. We found that unlike in cells from Balb/c, in C57BL/6-derived cells anti-TNFR-1 antibodies do not affect markedly RANKL-induced osteoclastogenesis. Thus, our findings demonstrate a different RANKL mode of action in the two strains. RANKL induced TNF-α in the two strains to the same extent, but TNF-α is more efficient in cells derived from Balb/c. This was true in the absence of RANKL (Table III compared to Figs. 4 and 6), but more evident in RANKL "primed" cells (Table IV).

Several studies demonstrated that Balb/c and C57BL/6 respond differently to inflammatory stimuli. Differential regulation of cytokines (including TNF-α) has been also noted between these strains [Karupiah, 1998; Sapru et al., 1999; Uzonna et al., 1999; Horai et al., 2000; Nicklin et al., 2000; Ulett et al., 2000].

Analyses of polymorphism in the TNF-α and TNFRs genes revealed differential expression of those genes' alleles in C57BL/6 and Balb/c [Takao et al., 1993; Iraqi and Teale, 1999]. Polymorphisms were also identified in human in TNF-α and TNFR-1 genes, and associations have been found between TNF-α polymorphism and human disease [Hajeer and Hutchinson, 2000].

The role of TNF-α in mediating RANKL osteoclastogenic activity in vivo is suggested by a recent study [Li et al., 2000] examining the ability of RANKL, PTHrP, $1,25(OH)_2D_3$, IL-1β, and TNF-α to induce hypercalcemia and appearance of osteoclasts in RANK(−/−) mice. While each of these agents induced in normal mice hypercalcemia, and appearance of TRAP positive and cathepsin K positive osteoclasts, they failed to do so in the RANK deficient mice. TNF-α was an exception and rare appearance of TRAP positive and cathepsin K positive osteoclasts were observed. In this in vivo study, as well as in our in vitro experiments, TNF-α does not mimic quantitatively RANKL activity, indicating that TNF-α modulation cannot be the sole mechanism of RANKL activity.

Our conclusions are also consistent with a recent publication [Zhang et al., 2001] showing impairment of RANKL-induced osteoclastogenesis in TNFR-1 deficient mice, while enhanced RANKL-induced osteoclastogenesis is observed in TNFR-2 deficient mice. Moreover, various RANKL-induced intracellular events, considered as part of its mechanism of action were found to be TNFR-1 dependent.

We could demonstrate that RANKL is capable of priming the osteoclast precursors to respond to TNF-α. Recently [Lam et al., 2000], using immunopurified myeloid cells, it was shown that TNF-α induces osteoclastic differentiation only in RANKL primed cells. It seems to us, that the TNF-α capability to induce osteoclastogenesis depends on the genetic background of the mice tested (in the present study in Balb/c and in C57BL/6, TNF-α exhibited ∼10 and 1.5% osteoclastogenic capability, respectively, as compared to RANKL, while in the study of Lam et al. [2000] in which TNF-α failed to induce osteoclasts in the absence of RANKL C3H/HeN mice were used).

The involvement of various cytokines in osteoclast differentiation, longevity, and resorption activity has been established [Thomson et al., 1987; Jimi et al., 1999]. Involvement of the role played by cytokines in the activity of RANKL is currently investigated. Studies with different inbred strains showing polymorphism in TNF-α, TNFRs, and possibly RANKL and RANK will shed light on alternative pathways participating in RANKL's osteoclastogenic activity.

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