Tumor Necrosis Factor-α Supports the Survival of Osteoclasts through the Activation of Akt and ERK*

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Differentiated osteoclasts have a short life span. We tested various cytokines and growth factors for the effects on the survival of purified mature osteoclasts. In the absence of any added factors, osteoclasts exhibited the survival rate of less than 25% after a 24-h incubation. Among the tested factors, tumor necrosis factor-α (TNF-α) was found to increase the survival rate to ~80%. The TNF-α-enhanced survival of osteoclasts appeared to be associated with reduction in apoptosis and suppression of caspase activation. The antiapoptotic signaling pathways involved in the TNF-α-induced osteoclast survival were investigated. TNF-α treatment increased the phosphorylation of Akt in osteoclasts, which was suppressed by a phosphatidylinositol 3-kinase inhibitor LY294002 and an Src family kinase-selective inhibitor PP1. These inhibitors also attenuated the TNF-α stimulation of osteoclast survival. In addition an increase in the phosphorylation of ERK was observed upon TNF-α stimulation. PD98059, a specific inhibitor of the ERK-activating kinase MEK-1, abolished the TNF-α-induced ERK phosphorylation and osteoclast survival, and in these responses the involvement of Grb2 and ceramide was observed. These results suggest that TNF-α promotes the survival of osteoclasts by engaging the phosphatidylinositol 3-kinase Akt and MEK/ERK signaling pathways.

Osteoclasts are multinucleated cells responsible for bone resorption, which occurs throughout the life as well as during bone development. These cells are continuously formed from the monocyte/macrophage lineage of hematopoietic cells to replace dying cells. The formation and activation of osteoclasts are regulated by numerous cytokines, hormones, and growth factors. Recently, a tumor necrosis factor (TNF) family molecule, receptor activator of nuclear factor κB ligand (RANKL; also called TRANCE, ODF, and OPGL), was found to play essential roles for osteoclast differentiation and activation (1–4). Subsequently, many osteotrophic factors were shown to induce the expression of RANKL on supporting osteoblastic/stromal cells explaining the necessity of cell-to-cell contact between the supporting cells and osteoclast progenitor cells for differentiation of the latter (1, 5). Binding of RANKL to its receptor RANK, a TNF receptor (TNFR) family protein present on osteoclast lineage and dendritic cells, induces a strong activation of NF-κB and c-Jun N-terminal kinase through the membrane-proximal adaptor molecules TNFR-associated factors (TRAFs) (3, 4, 6–10). The crucial roles of RANK and RANKL were clearly shown in knock-out mice that displayed osteopetrosis that results from the lack of osteoclast formation (11, 12).

Once terminally differentiated, osteoclasts have a short life span and undergo apoptotic cell death. Survival of osteoclasts appears to be dependent on osteotropic factors presented by osteoblast/stromal cells, and in the absence of the supporting cells, osteoclasts readily undergo apoptosis (13). Some studies have also reported that the survival of osteoclasts is regulated by certain cytokines and hormones. Estrogen and transforming growth factor-β were shown to inhibit osteoclast survival (14, 15), whereas macrophage-colony-stimulating factor (M-CSF), interleukin-1 (IL-1), and calcitonin were demonstrated to increase osteoclast viability (13, 16, 17). RANKL has also been shown to promote osteoclast survival in the absence of support from osteoblast/stromal cells (18–20). However, current understanding of the molecular mechanism by which these survival factors prevent osteoclast death is limited.

Several signaling pathways are known to be involved in the regulation of cell death. Phosphatidylinositol (PI) 3-kinase (PI 3-K) produces D3-phosphorylated phosphoinositides that recruit Akt, through binding to the pleckstrin homology domain, to the plasma membrane where an Akt-activating kinase phosphoinositide-dependent kinase-1 (PDK-1) is also translocated. The binding of D3-phosphorylated inositol to the pleckstrin homology domain is believed to cause conformational changes that allow access of PDK-1, which results in the phosphorylation of Akt on Thr-308 (21). The PI 3-K/Akt pathway has been demonstrated to transduce antiapoptotic signals from various tumor necrosis factor receptor; RANKL, receptor activator of nuclear factor kappa B ligand; TRAF, TNF receptor-associated factor; M-CSF, macrophage colony-stimulating factor; IL, interleukin; PI 3-K, phosphatidylinositol 3-kinase; ERK, extracellular signal-regulated kinase; MEK, MAPK/ERK kinase; MAPK, mitogen-activated protein kinase; DAPI, 4,6-diamidino-2-phenylindole; h, human; m, mouse; pNA, p-nitroanilide; FBS, fetal bovine serum; DTT, dithiothreitol; PMSF, phenylmethylsulfonyl fluoride.
stimuli including growth factors, cytokines, matrix-cell interaction, and neuronal cell differentiation (22). Mitogen-activated protein kinase (MAPK)/ERK is activated by phosphorylation on Thr and Tyr by a dual specificity kinase MEK, which can be activated by Raf-1. Raf-1 activation can be achieved by the GTP-bound activated form of Ras. The Ras/ERK pathway has also been shown to be an important component for survival of neuronal and hematopoietic cells (23–25). However, the roles played by Akt and MAPK/ERK for osteoclast survival have not been studied in detail.

In the course of searching for new factors that regulate osteoclast survival, we found that TNF-α promotes the survival of differentiated mature osteoclasts. We show that the TNF-α stimulation of osteoclast survival is mediated by the Akt and ERK pathways.

**EXPERIMENTAL PROCEDURES**

**Reagents**—1a,25-Dihydroxyvitamin D₃, prostaglandin E₂, C₆-ceramide, 4,6-diamidino-2-phenylindole (DAPI), and LY294002 were purchased from Sigma. PD98059 was from Calbiochem. Recombinant human sRANKL was obtained from PeproTech EC (London, UK). TNF-α and interleukin 1-α (IL-1α) were from R & D Systems (Minneapolis, MN). Neutralizing antibodies against TNF receptor type I and type II and an isotype-matching hamster IgG were purchased from BD Pharmingen (San Diego, CA). A functional peptide (DRQIKIWFQPGRPMWK) for interfering Grb2 binding to TNFRI and a mutated peptide (DRQIKIWFQNLKWKKLAP) were synthesized by AnyGen (Gwangju, Korea).

**Osteoclast Preparation**—Osteoclasts were obtained by the co-culture of mouse bone marrow cells and primary osteoblasts as described (26). Briefly, primary osteoblasts were obtained by growing calvarial cells from ICR newborn mice for 3 days in α-minimum essential medium (Life Technologies, Inc.) supplemented with 10% (v/v) fetal bovine serum (FBS; Life Technologies, Inc.), 100 units/ml penicillin, and 100 μg/ml streptomycin. Bone marrow cells were obtained by flushing tibiae from 6- to 8-week-old ICR mice. 1×10⁷ bone marrow cells and 1×10⁶ osteoblasts were seeded on a 90-mm dish coated with collagen gel and cultured for 6 days in the presence of 8M 1,25-dihydroxyvitamin D₃ and 6M prostaglandin E₂. The complete culture medium was changed every 3 days. After 6 days of culture, cells were harvested by treating with 0.2% collagenase (Life Technologies, Inc.) at 37°C for 10 min, replated on a plain 6- or 48-well culture plate, and cultured for another day. The plate was then treated with 0.1% collagenase at 37°C for 30 min and intensely pipetted to remove osteoblasts. The remaining cells were more than 95% pure osteoclasts when determined by tartrate-resistant acid phosphatase (TRAP) cytochemistry as described below.

**Osteoclast Survival Assay**—Purified osteoclasts prepared as above were incubated in the presence or absence of TNF-α or other cytokines for 24 h. After washing twice with medium, cells remaining attached were stained for TRAP enzyme with the Leukocyte Acid Phosphatase Assay Kit (Sigma) as per the manufacturer's instructions. TRAP-positive cells with five or more nuclei were counted. Cells at the beginning (t = 0) of cytokine addition were analyzed in the same way, and the

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**Fig. 1. Effects of TNF-α on the osteoclast survival.** Purified osteoclasts were treated with the control (Cont) vehicle, 20 ng/ml mouse TNF-α, 1 μg/ml human soluble RANKL, or 20 ng/ml mouse IL-1α for 24 h. A, photographs of TRAP-stained osteoclasts before (0 h) and after the cytokine treatment. B, survival of osteoclasts is expressed as the percentage of TRAP-positive multinuclear cells remaining after cytokine treatment (mean ± S.D.). C, TNF-α-mediated osteoclast survival is inhibited by neutralizing TNFRI and TNFRII antibodies. Purified osteoclasts were pretreated with 20 μg/ml of the isotype control IgG or a neutralizing anti-mouse TNFRI or TNFRII antibody for 30 min and cultured in the presence of 20 ng/ml TNF-α for 24 h. The percentage of surviving osteoclasts is shown (mean ± S.D.).
survival rate was calculated as the percentage of TRAP-positive multinuclear cells remaining after 24-h treatments.

**DAPI Staining**—Purified osteoclasts were incubated with or without TNF-α for 9 h. After rinsing once in phosphate-buffered saline, cells were fixed with 10% formaldehyde for 5 min at 20 °C and then incubated with 1 μg/ml DAPI in phosphate-buffered saline for 20 min. Cells showing fragmented chromatin morphology were considered apoptotic.

**Caspase Assays**—The purified mature osteoclasts were seeded in 6-well plates and treated with or without TNF-α for 9 h. After brief rinsing in phosphate-buffered saline, cells from three wells of a 6-well plate were lysed in 150 μl of the Cell Lysis Buffer included in the Caspase-9 Colorimetric Assay Kit (R & D Systems). The cell lysates were concentrated with Centricon (molecular weight cut-off 3000; Millipore, Bedford, MA) to achieve protein concentrations of 2–4 mg/ml. The enzymatic reaction for caspase-9 activity was carried out with the p-nitroanilide-conjugated LEHD peptide (LEHD-pNA) substrate as described by the manufacturer. The caspase-3 activity was measured similarly using the DEVD-pNA substrate contained in the Caspase-3 Colorimetric Assay Kit (R & D Systems).

**Western Blotting**—Western blotting analyses were performed as described (27). Briefly, cells were lysed in a buffer containing 20 mM Tris-HCl, 150 mM NaCl, 1% Triton X-100, and protease and phosphatase inhibitors. Protein concentrations of cell lysates were determined with the DC Protein Assay Kit (Bio-Rad). The NF-κB protein 5 (2 ng/ml), mIL-11 (0.3 ng/ml), mIL-17 (6 ng/ml), mouse macrophage colony-stimulating factor (0.8 ng/ml), human nerve growth factor (3 ng/ml), mIL-6 (0.12 ng/ml) plus hIL-6R (30 ng/ml), mIL-3 (0.2 ng/ml), mIL-11 (0.3 ng/ml), mIL-17 (6 ng/ml), mouse macrophage-derived chemokine (40 ng/ml), mouse stromal cell-derived factor 1 alpha (18 ng/ml), human bone macrophagic protein 5 (2 μg/ml), or human pleiotrophin (16 μg/ml) did not increase osteoclast survival at concentrations more than 2-fold of ED_{50} (data not shown). Mouse vascular endothelial growth factor (8 ng/ml) showed a very weak stimulation of the survival (data not shown). In contrast, RANKL and IL-1α significantly increased the number of surviving osteoclasts as reported previously (16, 18). TNF-α was also found to stimulate osteoclast survival (Fig. 1A). In fact, TNF-α could elicit a stronger effect than RANKL or IL-1α (Fig. 1B). The TNF-α-induced osteoclast survival was attenuated by neutralizing antibodies to either TNF receptor type I or type II (Fig. 1C).

**RESULTS**

**Effect of TNF-α on Mature Osteoclast Survival**—To find a new factor involved in osteoclast survival, we incubated mature osteoclasts in the presence of various cytokines, chemokines, and growth factors and assessed the survival rate. The purified mature osteoclasts that were obtained by removing the supporting osteoblasts from co-cultures of mouse bone marrow cells and primary osteoblasts (see “Experimental Procedures”) showed ~80% death rate in the absence of any added factors within 24 h (Fig. 1, A and B). Addition of human hepatocyte growth factor (80 ng/ml), mouse stem cell factor (20 ng/ml), human keratinocyte growth factor (50 ng/ml), human epidermal growth factor (0.8 ng/ml), human nerve growth factor (3 ng/ml), mIL-6 (0.12 ng/ml) plus hIL-6R (30 ng/ml), mIL-3 (0.2 ng/ml), mIL-11 (0.3 ng/ml), mIL-17 (6 ng/ml), mouse macrophage-derived chemokine (40 ng/ml), mouse stromal cell-derived factor 1 alpha (18 ng/ml), human bone macrophagic protein 5 (2 μg/ml), or human pleiotrophin (16 μg/ml) did not increase osteoclast survival at concentrations more than 2-fold of ED_{50} (data not shown). Mouse vascular endothelial growth factor (8 ng/ml) showed a very weak stimulation of the survival (data not shown). In contrast, RANKL and IL-1α significantly increased the number of surviving osteoclasts as reported previously (16, 18). TNF-α was also found to stimulate osteoclast survival (Fig. 1A). In fact, TNF-α could elicit a stronger effect than RANKL or IL-1α (Fig. 1B). The TNF-α-induced osteoclast survival was attenuated by neutralizing antibodies to either TNF receptor type I or type II (Fig. 1C).

**Inhibition of Osteoclast Apoptosis by TNF-α**—Mature osteoclasts die by apoptosis (13). As TNF-α increased the survival of purified osteoclasts, we next investigated the effect of TNF-α on apoptosis of these cells. When DAPI staining was performed, apoptotic osteoclasts with condensed chromatin and fragmented nuclei were evident in the absence of a survival factor (Fig. 2A). TNF-α greatly reduced the percentage of cells with apoptotic nuclei from 53.4 ± 1.7 to 26.6 ± 2.2% (Fig. 2B). Caspases play an important role in apoptosis. Apoptotic stimuli

![Figure 2. TNF-α inhibits apoptosis of purified osteoclasts.](image-url)
mediate Akt activation in osteoclasts in response to RANKL-induced Akt Activation and Osteoclast Survival—The Src protein, the PI 3-K inhibitor greatly occlast survival, LY294002, known to specifically inhibit PI 3-K, analyses.

Involvement of Akt in the TNF-α-induced Osteoclast Survival—The PI 3-K/Akt signaling pathway has been demonstrated to have an antiapoptotic role in various cell types. To determine whether this pathway is involved in the TNF-α-mediated osteoclast survival, LY294002, known to specifically inhibit PI 3-K, was used. As shown in Fig. 3A, the PI 3-K inhibitor greatly attenuated the effect of TNF-α on survival of mature osteoclasts. We next examined the extent of Akt phosphorylation, which reflects the activation of this PI 3-K downstream target. The treatment of TNF-α increased the phosphorylation of Akt by 15 min, which was reduced to the basal level by 60 min (Fig. 3B). Furthermore, treatment with LY294002 blocked the increase in Akt phosphorylation by TNF-α (Fig. 3C). The neutralizing TNF receptor antibodies that prevented the TNF-α-dependent survival of osteoclasts also inhibited the TNF-α-induction of Akt phosphorylation (data not shown).

Effect of an Src Family Kinase Inhibitor on the TNF-α-induced Akt Activation and Osteoclast Survival—The Src protein tyrosine kinase or an Src family member was suggested to mediate Akt activation in osteoclasts in response to RANKL.

release cytochrome c from mitochondria that together with Apaf-1 activate caspase-9. Active caspase-9 cleaves pro-caspase-3 into active subunits that cleave a number of substrates to execute cell death (28). When the activity of caspase-9 was measured in purified osteoclasts, an increase (2.35 ± 0.57-fold) was observed in cells incubated in the control medium by 9 h (Fig. 2C). This increase in caspase-9 activity was attenuated to 1.35 ± 0.12-fold by TNF-α (Fig. 2C). An increase (2.02 ± 0.16-fold) in caspase-3 activity was also detected in osteoclasts incubated for 9 h in the absence of TNF-α (Fig. 2D). In the presence of TNF-α, the stimulation of caspase-3 activity was reduced to 1.45 ± 0.05-fold (Fig. 2D).

![Graph A](image1.png)  
**FIG. 3.** TNF-α-induced osteoclast survival is mediated by the PI 3-K/Akt pathway. A, purified osteoclasts were pretreated with 500 nM LY294002 and cultured in the presence of 20 ng/ml TNF-α for 24 h. Survival is shown as a percentage of starting TRAP-positive multinuclear cells (mean ± S.D.). B, osteoclasts were deprived of serum in the medium containing 0.5% FBS for 5 h and stimulated with 100 ng/ml TNF-α for the indicated times. The cell lysates were subjected to Western blotting with anti- phospho-Akt (top). The same membrane was stripped and reprobed with anti-Akt (bottom). C, purified osteoclasts were pretreated with or without LY294002 (20 μM) and stimulated with 100 ng/ml TNF-α for 30 min. The cell lysates were subjected to Western blotting with anti-phospho-Akt (top) and then anti-Akt (bottom) Western blotting analyses.

![Graph B](image2.png)  
**FIG. 4.** Inhibition of Src family kinases suppresses the TNF-α induction of Akt activation and survival of osteoclasts. A, purified osteoclasts were pretreated with 0.1 or 1.0 μM PP1 and cultured in the presence of 20 ng/ml TNF-α for 24 h. The percentages of surviving osteoclasts are presented as mean ± S.D. *, p < 0.05, statistical significances between the groups. B, osteoclasts were deprived of serum in the medium containing 0.5% FBS for 5 h and pretreated with 10 μg/ml PP1 followed by stimulation with 100 ng/ml TNF-α for 30 min. The cell lysates were subjected to Western blotting with anti-phospho-Akt (top). The same membrane was stripped and reprobed with anti-Akt (bottom).

![Graph C](image3.png)  
**FIG. 5.** The ERK pathway participates in the TNF-α-induced osteoclast survival. A, purified osteoclasts were pretreated with 10 μM (+) or 40 μM (+ +) PD98059 and cultured in the presence of 20 ng/ml TNF-α for 24 h. The percentages of surviving osteoclasts are presented. B, osteoclasts were serum-deprived for 5 h in the medium containing 0.5% FBS and stimulated with 100 ng/ml TNF-α for the indicated times. Western blotting analyses were performed with anti-phospho-ERK1/ERK2 (top). The same membrane was reprobed with anti-ERK2 (bottom). C, mature osteoclasts were pretreated with 40 μM PD98059 and stimulated with 100 ng/ml TNF-α for 15 min. An anti-phospho-ERK1/ERK2 blot (top) and the reprobed anti-ERK2 (bottom) blot are shown.
containing 20 purified osteoclasts were incubated in the medium alone or the medium containing 0.5% FBS for 5 h and stimulated with 100 ng/ml TNF-α for 15 min. The cell lysates were subjected to Western blotting with anti-phospho-ERK (top). The same membrane was stripped and reprobed with anti-ERK (bottom).

B, osteoclasts were deprived of serum in the medium containing 0.5% FBS for 5 h and treated with 50 μM PD98059 for 30 min followed by stimulation with 100 ng/ml TNF-α for 24 h. The percentages of surviving osteoclasts are presented (mean ± S.D.). *, p < 0.05, statistical significance between the groups.

C, cells were pretreated with 0.2 μM C₈- ceramide and incubated in the presence of 20 ng/ml TNF-α for 24 h. The percentages of surviving osteoclasts were shown (mean ± S.D.). **, p < 0.01, statistical significance between the groups.

D, osteoclasts were deprived of serum in the medium containing 0.5% FBS for 5 h and treated with 50 μM C₈-ceramide in the presence or absence of 100 ng/ml TNF-α for 20 min. Western blotting analyses with anti-phospho-ERK (top) and then with anti-ERK (bottom) were performed.

Role of Grb2 and Ceramide in the ERK Activation and Osteoclast Survival Stimulation by TNF-α—TNF-α has been shown to activate c-Raf-1 in a manner requiring both Grb2 and ceramide-activated protein kinase in the embryonic kidney cell line 293 (29). In addition, the PLAP sequences of TNFRI were mapped as the binding site for Grb2 that links SOS/Ras/Raf1 pathway to the TNF receptor (29). Activated Raf-1 can stimulate MEK-1, which in turn activates ERKs (30). Therefore, we investigated whether Grb2 and ceramide play roles for the ERK pathway-mediated survival of osteoclasts in response to TNF-α. Purified osteoclasts were incubated with TNF-α in the presence of a cell-permeable peptide (FP) that consisted of membrane-localizing sequences of the third helix of the antenapedia homeodomain protein and the PLAP motif of TNFRI (29). A mutated peptide (MP) containing a lysine substitution for the first proline residue of the PXXP motif was used as a control. FP significantly reduced the TNF-α-induced survival (Fig. 6A) and ERK phosphorylation (Fig. 6B). Treatment with cell-permeable C₈-ceramide synergistically enhanced the osteoclast survival (Fig. 6C) and ERK phosphorylation (Fig. 6D) stimulated by TNF-α. These results suggest that Grb2 binding and ceramide generation are involved in the TNF-α signaling to ERK for survival of osteoclasts.

Dissociation of Antiapoptotic Function of the PI 3-K/Akt Pathway from NF-κB Activation in the TNF-α-induced Osteoclast Survival—The PI 3-K/Akt pathway has been shown to activate NF-κB in several cell types (31, 32), and NF-κB activation can suppress apoptosis (33, 34). Therefore, the antiapoptotic effect of PI 3-K/Akt pathway may involve NF-κB activation. We tested whether the antiapoptotic function of the PI 3-K/Akt pathway in the TNF-α-induced osteoclast survival would involve NF-κB activation. Mature osteoclasts were treated with the PI 3-K inhibitor, LY294002, which blocked TNF-α enhancement of osteoclast survival, and effects on NF-κB activation were assessed by an electrophoretic mobility shift assay. TNF-α stimulated the DNA binding activity of NF-κB in mature osteoclasts at 15–60 min (Fig. 7A). Treatment of the cells with LY294002 (20 μM) did not affect TNF-α-stimulated NF-κB activation (Fig. 7B, lane 3). Also blocking the ERK pathway with PD98059 (40 μM) did not have any effect on the NF-κB stimulation by TNF-α (Fig. 7B, lane 5).
DISCUSSION

Differentiated osteoclasts readily undergo apoptosis in the absence of supporting stromal/osteoblastic cells or ectopic survival factors. A few molecules have been reported to support osteoclast survival. M-CSF was the first one shown to promote the survival of isolated rat osteoclasts by inhibiting apoptosis (13). In addition to M-CSF, IL-1 was also found to stimulate mouse osteoclasts in vitro (16). The recently cloned membrane protein RANKL, which plays an essential role in osteoclast differentiation, has also been demonstrated to increase survival of mouse osteoclasts generated from bone marrow cells either by co-culturing with calvarial osteoblasts (18) or by incubating with soluble RANKL plus M-CSF (20). In the present study, we found rather unexpectedly that TNF-α decreases apoptosis and increases survival of mature mouse osteoclasts (Figs. 1 and 2).

The TNF-α-stimulated survival of osteoclasts was found to be dependent on the PI 3-K/Akt signaling pathway and Src family kinases (Figs. 3 and 4). The Src family kinase and Akt dependence of TNF-α-stimulated survival is reminiscent of the RANKL-induced osteoclast survival (19). RANKL was shown to activate Akt through TRAF6, which appears capable of binding to and activating c-Src (19). Activated c-Src can then recruit PI 3-K, which generates D3-phosphoinositides for Akt activation. An analogous mechanism may operate in the TNF-α-induced Akt activation in osteoclasts. TNF-α has been shown to activate PI 3-K and Akt in established cell lines and to stimulate c-Src kinase activity in murine bone marrow macrophages (35). However, the mechanism by which the triggering of TNF receptors is linked to c-Src and PI 3-K activation remains elusive, and the involvement of TRAF6 in the TNFR signaling has not been demonstrated to date. One speculation about the mechanism of PI 3-K Akt activation assumes formation of a heterocomplex between TRAF2, which has been shown to be recruited to TNF receptors (36), and TRAF6. The C-terminal TRAF domain of TRAF family proteins has been demonstrated to be capable of forming homo- and hetero-oligomers (37). Alternatively, the PXXP motif in the cytoplasmic domain of TNFR1 that has been shown to be the binding site for the SH3 domain of Grb2 (29) may also interact with the SH3 domain of c-Src. As TNF receptors form a trimer upon ligand binding, the receptor complexes have the potential to contain both Grb2 and Src family proteins at the same time.

In various cell types, evidence that TNF-α activates both PI 3-K/Akt and NF-κB, which both have antiapoptotic function, and that the PI 3-K/Akt pathway can activate NF-κB has been reported. Therefore, whether NF-κB is downstream of PI 3-K/Akt in antiapoptotic signal transduction pathways for TNF-α-stimulated osteoclast survival was an intriguing question. Our results suggest that NF-κB is unlikely to mediate the antiapoptotic function of PI 3-K/Akt in the TNF-α-stimulated osteoclast survival (Fig. 7). Similarly, the antiapoptotic effect of PI 3-K/Akt activated by TNF-α was shown to be independent of NF-κB activation in primary endothelial cell cultures (38). However, we could not exclude the possibility that the PI 3-K/Akt pathway might activate NF-κB through a mechanism independent of nuclear translocation and DNA binding, which could not be tested by electrophoretic mobility shift assays. Madrid et al. (39) reported that overexpression of the activated form of Akt stimulated NF-κB transactivation potential without nuclear translocation of NF-κB in 293T cells. The ultimate way to test NF-κB activation would involve assessment of an NF-κB-responsive promoter activity by using a reporter system, but we were unsuccessful in transfecting a reporter plasmid into mature osteoclasts. The uncoupling of NF-κB activity from osteoclast survival is also supported by a study of Miyazaki et al. (40). In this study, the manipulation of NF-κB activity with constitutively active or dominant-negative IKK using an adenosviral system did not affect the survival of purified mouse osteoclasts (40).

The antiapoptotic property of ERK has been described in neurons deprived of neuronal growth factors (24) and in IL-3-dependent hematopoietic cells after IL-3 withdrawal (25). A study using an adenoviral system to overexpress dominant-negative and constitutively active forms of Ras and MEK-1 implicated the ERK signaling pathway in osteoclast survival (40). However, whether this pathway mediates the survival induced by an osteoclast survival factor has not been addressed. The present study clearly demonstrates that the ERK pathway operates in the TNF-α-stimulated survival of osteoclasts (Fig. 5). The transduction mechanism for TNF-α signaling to ERK activation appears to involve Grb2 and ceramide (Fig. 6) that have been shown to mediate the TNF-α activation of c-Raf-1 (29).

TNF-α has long been known to stimulate bone resorption (41). This cytokine has been implicated in bone loss associated with osteoporosis, orthopedic implant, and periodontitis (42–44). The stimulatory effect of TNF-α on bone resorption could be achieved in part by increasing the formation of multinuclear osteoclasts (45). The TNF-α induction of osteoclastogenesis has been ascribed to an indirect effect via osteoblasts, perhaps through induction of other osteoclastogenic cytokines (46). However, recent studies (47, 48) demonstrated the presence of both types of receptors for TNF-α on osteoclasts and a direct action of this cytokine on the committed precursor cells to induce differentiation. TNF-α also showed a potent synergy with RANKL in osteoclastogenesis and activation of NF-κB and c-Jun N-terminal kinase (49, 50). In this study, we identified another effect of TNF-α on osteoclasts, the stimulation of the survival of differentiated osteoclasts. The combined effects of TNF-α on formation and survival of osteoclasts might be responsible for bone loss in inflammatory bone diseases.

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