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Curcumin (Diferuloylmethane) Inhibits Receptor Activator of NF- κ B Ligand-Induced NF- κ B Activation in Osteoclast Precursors and Suppresses Osteoclastogenesis¹

Alok C. Bharti, Yasunari Takada, and Bharat B. Aggarwal²

Numerous studies have indicated that inflammatory cytokines play a major role in osteoclastogenesis, leading to the bone resorption that is frequently associated with cancers and other diseases. Gene deletion studies have shown that receptor activator of NF- κ B ligand (RANKL) is one of the critical mediators of osteoclastogenesis. How RANKL mediates osteoclastogenesis is not fully understood, but an agent that suppresses RANKL signaling has potential to inhibit osteoclastogenesis. In this report, we examine the ability of curcumin (diferuloylmethane), a pigment derived from turmeric, to suppress RANKL signaling and osteoclastogenesis in RAW 264.7 cells, a murine monocytic cell line. Treatment of these cells with RANKL activated NF- κ B, and preexposure of the cells to curcumin completely suppressed RANKL-induced NF- κ B activation. Curcumin inhibited the pathway leading from activation of I κ B α kinase and I κ B α phosphorylation to I κ B α degradation. RANKL induced osteoclastogenesis in these monocytic cells, and curcumin inhibited both RANKL- and TNF-induced osteoclastogenesis and pit formation. Curcumin suppressed osteoclastogenesis maximally when added together with RANKL and minimally when it was added 2 days after RANKL. Whether curcumin inhibits RANKL-induced osteoclastogenesis through suppression of NF- κ B was also confirmed independently, as RANKL failed to activate NF- κ B in cells stably transfected with a dominant-negative form of I κ B α and concurrently failed to induce osteoclastogenesis. Thus overall these results indicate that RANKL induces osteoclastogenesis through the activation of NF- κ B, and treatment with curcumin inhibits both the NF- κ B activation and osteoclastogenesis induced by RANKL. *The Journal of Immunology*, 2004, 172: 5940–5947.

Osteoclasts are multinucleated cells formed by the fusion of mononuclear progenitors of the monocyte/macrophage family and are the major resorptive cell of bone (1). In vitro maturation of macrophages into osteoclasts requires the presence of stromal cells or their osteoblast progeny (2). Extensive research in the last few years has indicated that these accessory cells express M-CSF and receptor for activation of NF- κ B (RANK)³ ligand (RANKL) and these are essential and sufficient to promote osteoclastogenesis. Besides M-CSF and RANKL, several other inflammatory cytokines and osteotropic agents including TNF and IL-1 β have been implicated in osteoclastogenesis, most likely through the osteoblastic modulation of RANKL, its decoy receptor osteoprotegerin (OPG), and M-CSF (3, 4). The effects of

parathyroid hormone and lipopolysaccharides on osteoclastogenesis are also mediated through expression of RANKL.

RANKL is a member of the TNF superfamily (5) that interacts with the cell surface receptor RANK, which in turn recruits TNFR-associated factors (TRAF)1, 2, 3, 5, and 6 (6, 7). By receptor deletion analysis, we and others have shown that sequential recruitment of TRAF6 and NF- κ B-inducing kinase by RANK leads to NF- κ B activation, and recruitment of TRAF2 leads to c-Jun N-terminal kinase (JNK) activation (8, 9).

That RANK can mediate osteoclastogenesis was first demonstrated by Hsu et al. (10). Further gene-deletion analysis of RANK, RANKL, and TRAF6 showed that these genes are positive regulators of osteoclastogenesis (11–13), whereas OPG, a decoy receptor for RANKL, was found to be a negative regulator of this process (14, 15). Gene-deletion analysis also suggested a critical role of M-CSF, c-fms (M-CSF receptor), and Src in osteoclastogenesis (16–18).

Although RANKL is known to activate NF- κ B, JNK, and p38 and p44/p42 mitogen-activated protein kinase (5, 9, 19, 20), how this cytokine mediates osteoclastogenesis is not fully understood. Furthermore agents that can suppress RANKL signaling can suppress osteoclastogenesis-induced bone loss.

Curcumin, a diferuloylmethane derived from turmeric (*Curcuma longa*), has been shown to suppress proliferation of a wide variety of tumor cells; to down-regulate transcription factors NF- κ B, AP-1, and early growth response gene-1; to suppress the expression of cyclooxygenase-2, lipoxygenase, NO synthase, matrix metalloproteinase-9, urokinase-type plasminogen activator, TNF, chemokines, cell surface adhesion molecules and cyclin D1; to inhibit the expression of growth factor receptors (such as epidermal growth factor receptor and human epidermal growth factor receptor 2); and to inhibit the activity of JNK, protein tyrosine kinases, and several other protein serine/threonine kinases (21).

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³ Abbreviations used in this paper: RANK, receptor activator of NF- κ B; RANKL, RANK ligand; OPG, osteoprotegerin; TRAF, TNFR-associated factor; IKK, I κ B kinase; TRAP, tartrate resistance acid phosphatase; I κ B α -DN, dominant negative I κ B α mutant; JNK, c-Jun N-terminal kinase.

This polyphenol has antioxidant and anti-inflammatory activity and found to suppress tumor initiation, promotion, and metastasis.

The effect of curcumin has been shown by us and others to suppress NF- κ B activation induced by various inflammatory stimuli (22–24), inhibit the activation of I κ B kinase (IKK) needed for NF- κ B activation (25–27), and found to be safe in humans even at 8 g per day (28), and we examined the effect of curcumin on RANKL-induced NF- κ B activation and on osteoclastogenesis in osteoclast precursor cells. We demonstrate that RANKL induces NF- κ B activation through activation of IKK and I κ B α phosphorylation and degradation, and curcumin inhibits RANKL-induced NF- κ B activation and osteoclastogenesis. The critical role of NF- κ B in this process was further confirmed by using a dominant-negative form of I κ B α .

Materials and Methods

Materials

The rabbit polyclonal Abs to I κ B α , p50, p65, cyclin D1, were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Ab against phospho-I κ B α was purchased from Cell Signaling Technology (Beverly, MA). Anti-IKK- α and anti-IKK- β Abs were kindly provided by Imgenex (San Diego, CA). Goat anti-rabbit HRP conjugate was purchased from Bio-Rad (Hercules, CA), goat anti-mouse HRP and BioCoat Osteologic Bone Cell Culture System from BD Biosciences (San Jose, CA), and MTT from Sigma-Aldrich (St. Louis, MO). Curcumin with a purity >98% was purchased from LKT Laboratories (St. Paul, MN) and was prepared as a 20 mM solution in DMSO and then further diluted in cell culture medium. DMEM-F12, FBS, 0.4% trypan blue vital stain, and antibiotic-antimycotic mixture were obtained from Invitrogen (Carlsbad, CA). Protein A/G-Sepharose beads were obtained from Pierce (Rockford, IL). [γ - 32 P]ATP was from ICN Pharmaceuticals (Costa Mesa, CA). Highly purified recombinant murine TNF- α was provided by Genentech (South San Francisco, CA).

Cells

The mouse macrophage cell line RAW 264.7 was obtained from American Type Culture Collection (Manassas, VA). RAW 264.7 cells were cultured in DMEM-F12 medium supplemented with 10% FBS and antibiotics. This cell line has been shown to express RANK and differentiate into tartrate resistance acid phosphatase (TRAP)-positive, functional osteoclasts when cocultured with bone slices or soluble RANKL (10). Moreover, RANKL has been shown to activate NF- κ B in these cells (29). TRAP staining was performed using a leukocyte acid phosphatase kit (387-A) from Sigma-Aldrich.

Plasmids

The plasmid pCMV4-Flag-I κ B α -DN (lacking residues 1–36) was kindly provided by Dr. D. W. Ballard (Vanderbilt University School of Medicine, Nashville, TN) (30, 31). The tetracycline inducible expression vector pEC 1214A was generously provided by Dr. H.-J. Xu (University of Texas M.D. Anderson Cancer Center, Houston, TX) (32). We constructed a tetracycline inducible FLAG-tagged I κ B α -dominant negative (DN) by inserting a *Hind*III-*Bam*HI fragment from pCW4-Flag-I κ B α -DN into the *Hind*III-*Bam*HI site of pEC 1214A, and the resulting plasmid was named pTet-Flag-I κ B α -DN. The expression vector of full-length murine RANKL with a TNF-related activation-induced cytokine (pcDNA 3.1-TRANCE) was generously provided by Y. Choi (Rockefeller University, New York, NY). To generate a bacterial expression vector for RANKL, we used specific 5' and 3' primers with *Hind*III and *Not*I sites, respectively, to amplify the DNA, which encodes residues 157–316 of RANKL from the pcDNA3.1-TRANCE template. The PCR product was digested with *Hind*III-*Not*I and ligated in-frame with a hemagglutinin tag (N-terminal) and a histidine tag (C-terminal) into the expression vector pHB6. Soluble RANKL was expressed and purified using Ni-agarose.

RAW 264.7 cells stably expressing Flag-I κ B α -DN

RAW 264.7 cells were plated at 0.5×10^6 cells/well on six-well plates and transfected the next day with pTet-Flag-I κ B α -DN (2.5 μ g total DNA) using 9 μ l of Eugene 6. After 48 h, cells were trypsinized and plated in 100-cm dishes in the presence of G418 (600 μ g/ml) and tetracycline (1 μ g/ml). Single colonies were isolated after 2 wk of G418 selection, expanded, and examined for expression of Flag-tagged I κ B α -DN in the absence of tetracycline.

Osteoclast differentiation assay

RAW 234.7 cells were cultured in 24-well dishes at a density of 1×10^4 cells per well and were allowed to adhere overnight. Medium was then replaced and the cells were treated with 5 nM (\sim 100 ng/ml) RANKL. At day 5, cultures were stained for TRAP expression as described (33) using an acid phosphatase kit, and the total number of TRAP-positive multinucleated osteoclasts (>3 nuclei) per well were counted.

To determine whether RANKL induces osteoclastogenesis in bone marrow-derived macrophages, cells were isolated from the femur bone of the mice. These bone marrow-derived macrophages (1×10^4 cells/well) were pretreated with curcumin (10 μ M) for 2 h and then exposed to M-CSF (100 ng/ml) either alone or in the presence of indicated concentrations of RANKL. After 5 days, cells were stained for TRAP expression.

Resorption pit assay

This assay was performed as described (34). To determine the effect of curcumin on pit formation, RAW 264.7 cells were seeded into each well of calcium phosphate apatite-coated plates (BioCoat Osteologic Bone Cell Culture System; BD Biosciences) in culture medium, and then pretreated with curcumin (10 μ M) for 2 h, before adding either RANKL (5 nM) or TNF (1 nM). After 5 days incubation, all remaining cells were lysed using 1 N NaOH. The images of the resorbed pits were obtained under light microscopy.

Preparation of nuclear extracts for NF- κ B assay

Nuclear extracts were prepared as described earlier (24). Briefly, 2×10^6 RAW 264.7 cells were washed with cold PBS, scraped, and suspended in 0.1 ml of hypotonic lysis buffer containing protease inhibitors for 30 min. The cells were then lysed with 3.2 μ l of 10% Nonidet P-40. The homogenate was centrifuged, and supernatant containing the cytoplasmic extracts was stored frozen at -80°C . The nuclear pellet was resuspended in 25 μ l ice-cold nuclear extraction buffer. After 30 min of intermittent mixing, the extract was centrifuged, and supernatants containing nuclear extracts were secured. The protein content was measured by the Bradford method. If the extracts were not used immediately, they were stored at -80°C .

EMSA for NF- κ B

NF- κ B activation was analyzed by EMSA as previously described (35). In brief, 8 μ g of nuclear extracts prepared from curcumin-treated or untreated cells were incubated with 32 P end-labeled 45-mer double-stranded NF- κ B oligonucleotide from HIV-1 long-terminal repeat (5'-TTGTTACAAGG GACTTCCGCT GGGGACTTCCAG GGAGGCGTGG-3') for 30 min at 37°C , and the DNA-protein complex resolved in a 6.6% native polyacrylamide gel. The radioactive bands from the dried gels were visualized and quantitated by the PhosphorImager (Molecular Dynamics, Sunnyvale, CA) using ImageQuant software.

Western blot analysis

Cytoplasmic protein extracts (30–40 μ g) were resolved on 10% SDS-PAGE. After electrophoresis, the proteins were electrotransferred to a nitrocellulose membrane, blocked with 5% nonfat milk, and probed with Abs against either I κ B α , phospho-I κ B α , IKK- α , IKK- β , or β -actin (1:3000) for 1 h. Thereafter, the blot was washed, exposed to HRP-conjugated secondary Abs for 1 h, and finally detected by ECL (Amersham Pharmacia Biotech, Arlington Heights, IL).

I κ B α kinase assay

The IKK assay was performed by a modified method as described earlier (36). Briefly, 200 μ g of cytoplasmic extracts were immunoprecipitated with 1 μ g of anti-IKK- α and anti-IKK- β Abs each, and the immune complexes so formed were precipitated with 0.01 ml of protein A/G-Sepharose beads for 2 h. The beads were washed first with lysis buffer and then with the kinase assay buffer (50 mM HEPES pH 7.4, 20 mM MgCl₂, and 2 mM DTT). The immune complex was then assayed for kinase activity using kinase assay buffer containing 20 μ Ci [γ - 32 P]ATP, 10 μ M unlabeled ATP, and 2 μ g/sample GST I κ B α (aa 1–54). After incubation at 30°C for 30 min, the mixture was boiled with $6\times$ SDS sample buffer to stop the reaction. The reaction mixture was resolved on 12% SDS-PAGE. The radioactive bands of the dried gel were visualized and quantitated by PhosphorImager. To determine the total amount of IKK- α and IKK- β in each sample, 60 μ g of the cytoplasmic protein was resolved on a 7.5% acrylamide gel, and Western blot analysis was performed.

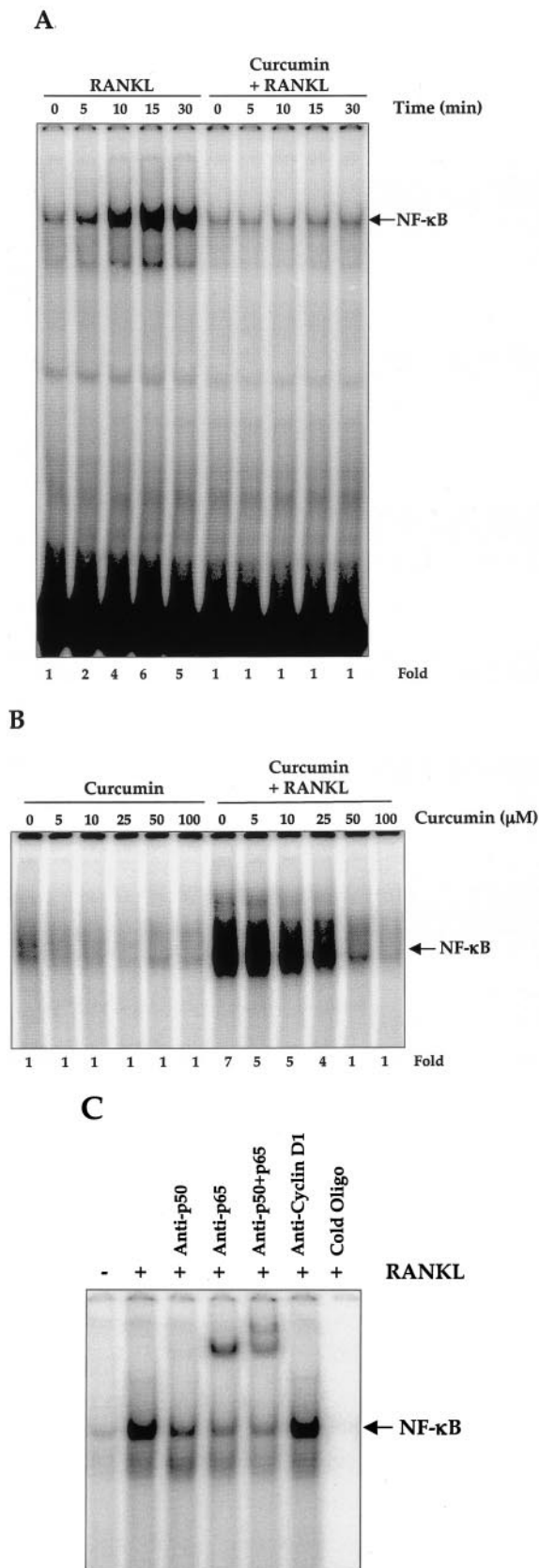


FIGURE 1. RANKL induces NF- κ B activation and curcumin inhibits it in dose- and time-dependent manner. *A*, RAW 264.7 cells (1×10^6 cells) were either incubated alone or in the presence of curcumin ($50 \mu\text{M}$) for 2 h, treated with RANKL (10 nM) for the indicated times, and then tested for nuclear NF- κ B by EMSA as described. *B*, RAW 264.7 cells (1×10^6 cells) were incubated without or with the indicated concentrations of curcumin for 2 h and then treated with RANKL (10 nM) and tested for nuclear

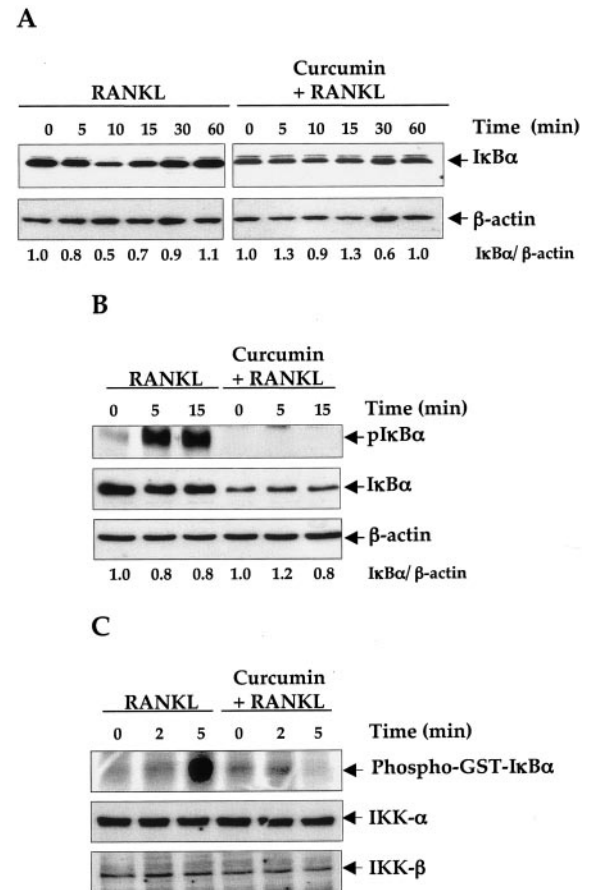


FIGURE 2. Curcumin inhibits RANKL-induced I κ B α phosphorylation and degradation through inhibition of IKK activity. RAW 264.7 cells (1×10^6 cells) were either incubated alone or in the presence of curcumin ($50 \mu\text{M}$) for 2 h and then treated with RANKL (10 nM) for the indicated times; cytoplasmic extracts were prepared to check the following: the level of I κ B α (*A*); the level of phosphorylated I κ B α by Western blot analysis (*B*); IKK activity (*C*, upper); immunoprecipitated IKK and performed the kinase assay, and total IKK- α and IKK- β proteins by Western blot analysis (*middle* and *lower*) in cytoplasmic extracts. Quantitation of I κ B α after normalization with β -actin (*A* and *B*) is presented.

Results

We investigated the effect of curcumin on RANKL-induced NF- κ B activation and on osteoclastogenesis in the murine monocytic cell line RAW 264.7.

Curcumin inhibits RANKL-induced NF- κ B activation

To determine the effect of curcumin on RANKL-induced NF- κ B activation in RAW 264.7 cells, we first incubated these cells with curcumin for 2 h and then treated them with RANKL, prepared nuclear extracts, and assayed NF- κ B activation by EMSA. RANKL activated NF- κ B maximally within 15 min, and curcumin completely abrogated the RANKL-induced NF- κ B activation (Fig. 1*A*). The inhibition of NF- κ B by curcumin increased with dose. Complete inhibition was observed at a $50 \mu\text{M}$ concentration of curcumin (Fig. 1*B*). Supershift assay of NF- κ B-DNA probe binding

NF- κ B by EMSA as described. *C*, The binding of NF- κ B is specific and consists of p50 and p65 subunits. Nuclear extracts were prepared from untreated RAW 264.7 cell or the cells treated with RANKL, incubated for 15 min with different Abs or unlabeled oligonucleotide probe, and then assayed for NF- κ B by EMSA in 5% gel.

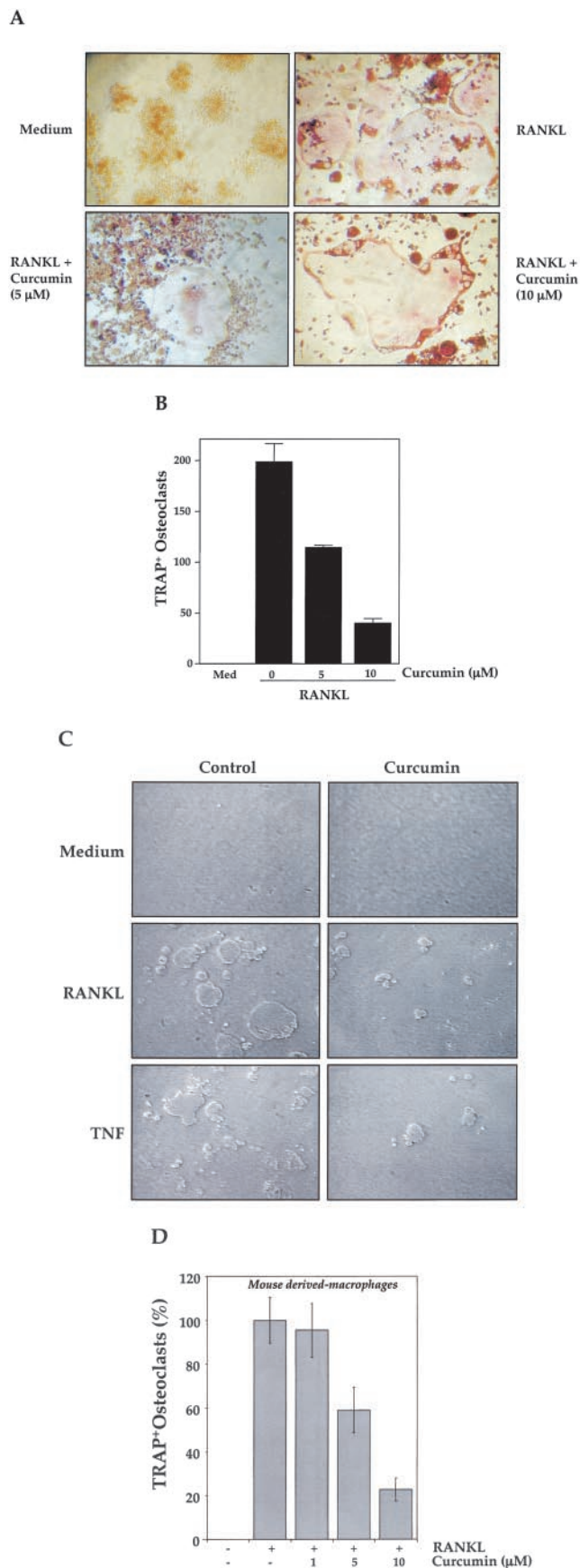


FIGURE 3. Curcumin inhibits RANKL-induced osteoclastogenesis. RAW 264.7 cells (1×10^4 cells) were incubated either alone or in the presence of RANKL (5 nM) without or with curcumin for 5 days and stained for TRAP expression. *A*, TRAP-positive cells were photographed

showed that RANKL-activated NF- κ B consisted of p65 and p50 subunits (Fig. 1C). Reaction mixtures containing Abs to p50 or p65 showed either lesser NF- κ B-DNA complex (with anti-p50) or a further shift in the NF- κ B-DNA complex band (with anti-p65). The specificity of the RANKL-induced NF- κ B-DNA complex was further confirmed by demonstrating that the binding was unaffected by irrelevant Ab (anti-cyclin D1) and was abolished by the presence of a 100-fold excess of unlabeled κ B-oligonucleotides.

Curcumin inhibits RANKL-induced I κ B α phosphorylation and degradation through inhibition of IKK activity

Activation of NF- κ B by most agents requires phosphorylation and degradation of its inhibitory subunit I κ B α . To investigate the mechanism involved in the inhibition of NF- κ B activation by curcumin, we first checked the effects of curcumin treatment on the levels of I κ B α by Western blot analysis. The I κ B α level dropped down within 10 min in the cells treated with RANKL, and returned to normal levels within 60 min of treatment (Fig. 2A, left). The upper minor band is probably due to overwhelming signals from I κ B α seen in the lower band. In contrast, cells pretreated with curcumin suppressed RANKL-induced I κ B α degradation (Fig. 2A, right). Next we investigated the effect of curcumin on the RANKL-induced phosphorylation of I κ B α , which occurs before its dissociation, ubiquitination, and degradation (37). Western blot analysis for phospho-I κ B α in Fig. 2B clearly indicates that RANKL induced I κ B α phosphorylation in RAW 264.7 cells and that curcumin eliminated the RANKL-induced phosphorylation. Treatment of cells with curcumin alone did not result in phosphorylation of I κ B α . It is noticeable that the content of I κ B α in curcumin-treated sample was lesser than the control; quantitating of I κ B α to β -actin ratio indicates that curcumin treatment down-regulates the expression of I κ B α and inhibits the RANKL-induced degradation of I κ B α .

Because IKK phosphorylates I κ B α (38), we next checked whether curcumin alters the activity or the levels of IKK. In vitro IKK assay, cells treated with RANKL showed a sharp rise in IKK activity as indicated by the phosphorylation of GST-I κ B α within 5 min. In contrast, cells pretreated with curcumin could not phosphorylate GST-I κ B α upon RANKL treatment (Fig. 2C, upper panel). To check whether the apparent loss of IKK activity was due to the loss of IKK protein expression, the expression levels of the IKK subunits IKK- α and IKK- β were tested by Western blot analysis. Results in Fig. 2C (middle and lower panels) clearly showed that curcumin treatment did not alter the expression of IKK- α or IKK- β .

Curcumin inhibits RANKL-induced osteoclastogenesis in RAW 264.7 cells

Next we investigated the effect of curcumin on osteoclastogenesis. RAW 264.7 cells were incubated with different concentrations of

(original magnification, $\times 100$). *B*, Multinucleated (>3 nuclei) osteoclasts were counted (error bar indicates SD). *C*, Curcumin inhibits RANKL-induced and TNF-induced pit formation. RAW 264.7 (3×10^3 cells) cells were seeded into calcium phosphate apatite-coated plates, treated with 10 μ M curcumin for 2 h, and then with either 5 nM RANKL or 1 nM murine TNF. After 5 day incubation, cells were lysed, and images obtained under light microscopy. *D*, Curcumin inhibits RANKL-induced osteoclastogenesis in bone marrow-derived macrophages. Bone marrow-derived macrophages (1×10^4 cells) were incubated with M-CSF (100 ng/ml) either alone or in the presence of indicated concentrations of RANKL either with or without curcumin for 5 days and then stained for TRAP expression. Multinucleated (>3 nuclei) osteoclasts were counted (error bar indicates SD).

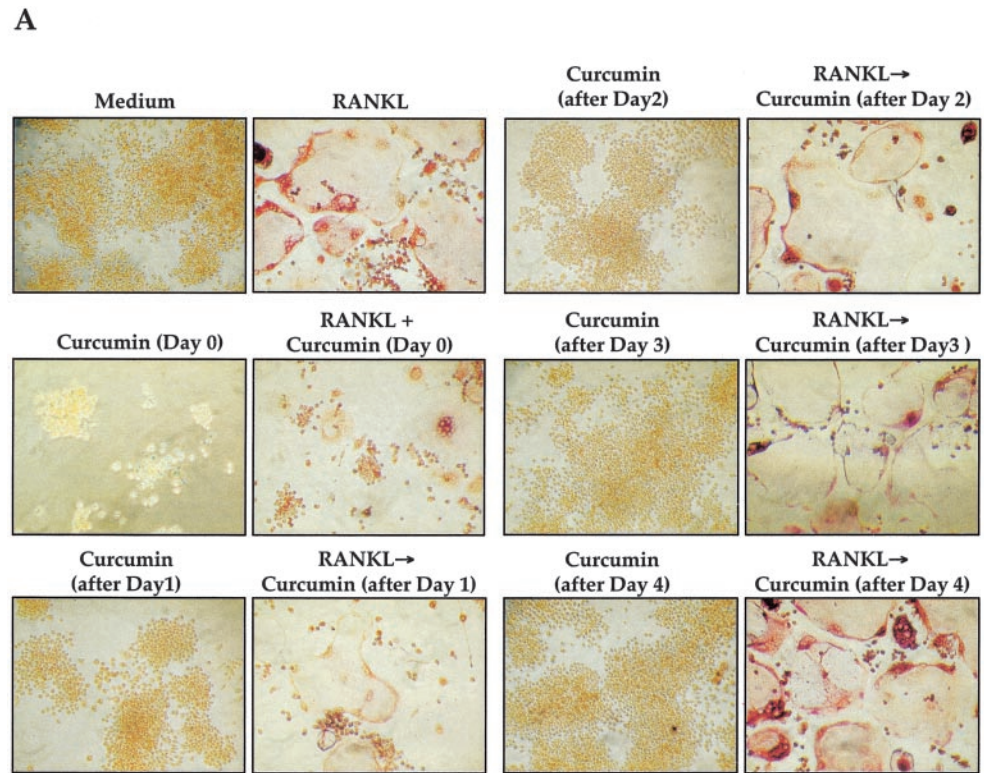
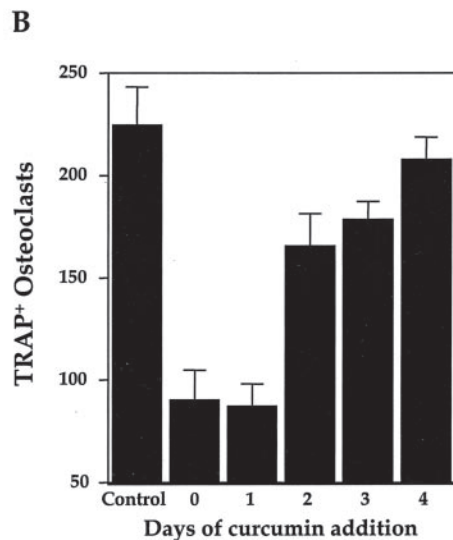


FIGURE 4. Curcumin effectively inhibits RANKL-induced osteoclastogenesis 24 h after stimulation. RAW 264.7 cells (1×10^4 cells) were incubated either alone or in the presence of RANKL (5 nM), and curcumin (10 μ M) was added at the same time or after indicated time periods. Cells were cultured for 5 days after RANKL treatment and stained for TRAP expression. **A**, Photographs of cells (original magnification, $\times 100$). **B**, Multinucleated (>3 nuclei) osteoclasts were counted. Values indicate mean of total osteoclasts in triplicate cultures (error bar indicates SD).



curcumin in the presence of RANKL and allowed to grow and differentiate into osteoclasts. Fig. 3A illustrates that RANKL induced osteoclasts both in the presence and absence of curcumin. However, the number of osteoclasts decreased with increasing concentration of curcumin (Fig. 3B).

Curcumin inhibits RANKL-induced pit formation

Whether inhibition of RANKL-induced osteoclastogenesis by curcumin leads to inhibition of pit formation was investigated. To determine this, RAW 264.7 cells were seeded into calcium phosphate apatite-coated plates, treated with curcumin, along with RANKL, and then the pit formation was analyzed after 5 days. Result showed that RANKL induced pit formation and this was significantly suppressed by curcumin (Fig. 3C, middle panels).

Besides RANKL, TNF has also been shown to induce osteoclastogenesis (1). Whether TNF-induced pit formation is inhibited by

curcumin, was also investigated. Like RANKL, result showed that murine TNF induced pit formation and this was significantly suppressed by curcumin (Fig. 3C, bottom panels). Thus these results suggest that curcumin suppressed not only osteoclastogenesis, but also pit formation.

Curcumin inhibits RANKL-induced osteoclastogenesis in mouse bone marrow-derived macrophages

Whether curcumin inhibits the RANKL-induced osteoclastogenesis in mouse bone marrow-derived macrophages was also investigated. Fresh mouse bone marrow-derived macrophages were incubated with curcumin in the presence of different concentrations of RANKL and allowed to grow and differentiate into osteoclasts. Fig. 3D shows that RANKL induced osteoclast formation, and curcumin suppressed it in a dose-dependent manner.

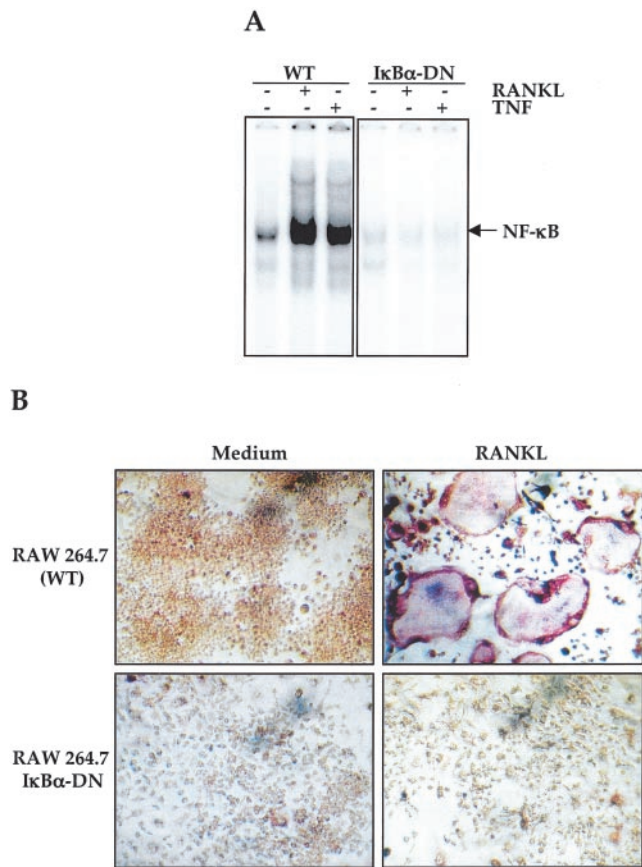


FIGURE 5. RANKL-induced NF- κ B activation is essential for RANKL-induced osteoclastogenesis. RAW 264.7 wild-type (WT) or I κ B α -dominant negative (I κ B α -DN) stably transfected cells are shown. *A*, A total of 1×10^6 cells were incubated alone or in the presence of RANKL (10 nM) or TNF (1 nM) for 30 min and tested for nuclear NF- κ B by EMSA as described. *B*, A total of 1×10^4 cells were treated in the absence or presence of RANKL (5 nM) for 5 days, TRAP-stained, and checked for osteoclastogenesis.

Curcumin acts early in the pathway leading to RANKL-induced osteoclastogenesis

It normally takes up to 5 days for RAW 264.7 cells to differentiate into osteoclasts in response to RANKL. To determine how early in this pathway curcumin acts, we treated the RAW 264.7 cells with RANKL, added curcumin on different days, and then checked its effect on osteoclast formation. Curcumin inhibited osteoclastogenesis even when the cells were exposed 24 h after the RANKL treatment (Fig. 4). However the inhibitory effect decreased significantly when cells were treated with curcumin 2 days after RANKL treatment.

Activation of NF- κ B is critical for RANKL-induced osteoclastogenesis

Besides NF- κ B activation, RANKL is known to activate several other signals in the cell. It is possible that curcumin inhibits RANKL-induced osteoclastogenesis by suppressing signals other than NF- κ B. To establish that curcumin suppressed osteoclastogenesis by inhibiting NF- κ B activation, we generated RAW 264.7 cells stably transfected with plasmid construct containing I κ B α -DN. RANKL activated NF- κ B in wild-type cells but not in I κ B α -DN expressing cells (Fig. 5*A*). RANKL also failed to induce osteoclastogenesis in I κ B α -DN expressing RAW cells (Fig. 5*B*), i.e., in cells that did not respond to NF- κ B activation. These results

suggest the critical role of NF- κ B activation in RANKL-induced osteoclastogenesis.

Discussion

In the present study we used a homogeneous, clonal population of murine monocytic cells RAW 264.7 to define the direct effect of curcumin on osteoclast development induced by RANKL. The advantage of this system is that it does not contain any osteoblast/bone marrow stromal cells or cytokine like M-CSF and allows us to focus on RANK signaling in preosteoclast cells. In our study curcumin inhibited RANKL-mediated NF- κ B activation by inhibiting IKK activity and it prevented osteoclast formation. We also found that curcumin inhibited the initial phase of cell growth by RANKL that is required for cell fusion and formation of a multinucleated cell. The critical role of NF- κ B in RANKL-induced osteoclastogenesis was further confirmed by the use of I κ B α -DN.

Our results indicate that RANKL activates NF- κ B in osteoclastic precursor cells through the activation of IKK and subsequent I κ B α phosphorylation and degradation. These results are in agreement with those of Wei et al. (29). We also showed that curcumin inhibits RANKL-induced IKK activation, leading to the suppression of NF- κ B activation. The mechanism of NF- κ B activation induced by RANKL differs from that of TNF. For instance NF- κ B-inducing kinase, although required for RANKL-induced NF- κ B activation (39), is dispensable for TNF-induced NF- κ B activation (40). Although curcumin has been shown to suppress TNF-induced IKK activation (25, 26), ours is the first report to suggest that curcumin can also suppress RANKL-induced NF- κ B activation. This is in agreement with our recent results that curcumin inhibits NF- κ B activation, not by inhibiting upstream kinase to IKK but by inhibiting IKK directly (24).

We also found that suppression of NF- κ B activation by curcumin correlated with inhibition of osteoclastogenesis. Whether NF- κ B activation is needed for osteoclastogenesis is controversial. Although not all the cytokines that activate NF- κ B induce osteoclastogenesis, other evidence suggests that activation of NF- κ B is essential for osteoclast development (29, 41–45). p50 and p52 Double knockout mice showed defects in osteoclastogenesis and severe osteopetrosis (41). Results presented in this study show that NF- κ B activation is critical for RANKL-induced osteoclastogenesis. It is possible that the inhibitory effect of curcumin on osteoclastogenesis is not mediated through suppression of NF- κ B. This is unlikely, however, as we also found that RAW 264.7 cells transfected with a dominant-negative form of I κ B α , which as a result could not activate NF- κ B in response to RANKL, did not differentiate into multinucleated osteoclasts.

We show in this study for the first time that curcumin can inhibit the formation of osteoclasts. Curcumin has been shown to induce apoptosis in osteoclasts (46). It is possible that apoptotic effects of curcumin are responsible for suppression of osteoclastogenesis. We think this is unlikely, however, because we found that growth inhibitory effects of curcumin were reversed by RANKL (our unpublished observation). Besides, RAW 264.7 cells transfected with I κ B α -DN failed to activate NF- κ B and were also unable to differentiate to osteoclasts in response to RANKL.

Our group showed that stimulation of RANK also results in activation of JNK activity along with NF- κ B (6). Recently, JNK has also been implicated in osteoclastogenesis (47). Curcumin can effectively inhibit JNK activity (48), so it is possible that JNK activity is also affected by curcumin in osteoclast precursors and may synergize with inhibition of NF- κ B activation. That RAW 264.7 cells, which lacked RANKL-induced NF- κ B activation, failed to differentiate to osteoclasts suggests that NF- κ B plays a major role.

Recently several cytokines have been reported that can suppress RANKL-induced osteoclastogenesis. These include IFN- β , IFN- γ , and IL-4, (29, 49–52). All these cytokines mediate their effects through different mechanisms; for instance, IFN- γ induces the degradation of TRAF6 through ubiquitination-dependent pathway; IFN- β down-regulates *c-fos* expression; and IL-4 down-regulates NF- κ B activation (53) through a STAT6-dependent mechanism (50). Our results show that curcumin inhibits osteoclastogenesis through inhibition of NF- κ B.

As indicated by epidemiologic evidence, and by several phase I clinical trials, curcumin is pharmacologically safe in humans (28). Curcumin is being investigated for its anticancer activity in breast cancer and multiple myeloma and has provided encouraging results both in vitro and in vivo (24, 54, 55). That curcumin could be used in the treatment of secondary bone lesions associated with breast cancer and multiple myeloma and those associated with nonmalignant diseases like postmenopausal osteoporosis, Paget's disease and rheumatoid arthritis in which severe osteolytic activity is observed (56) has great promise.

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