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SHORT COMMUNICATION

Stimulatory Effect of Curcumin on Osteoclast Apoptosis

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ABSTRACT. Curcumin is a potent inhibitor of the transcriptional factors activator protein-1 and nuclear factor-κB. Since transcriptional factors may play a functional role in the survival of osteoclasts, it was of interest to us to examine the effect of curcumin on osteoclast apoptosis. We observed that curcumin is a potent stimulator of this process in rabbit osteoclasts, as evidenced by morphological changes in nuclei and DNA fragmentation as criteria of apoptosis. The curcumin stimulation of the osteoclast apoptosis was dose-and treatment time-dependent. In addition, curcumin dramatically inhibited osteoclastic bone resorption, supporting our data that curcumin is a potent stimulator of osteoclast apoptosis. BIOCHEM PHARMACOL **59**;12:1577–1581, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. curcumin; osteoclasts; apoptosis; transcriptional factor; bone resorption

Osteoclasts play a crucial role in bone remodeling, given that they are the predominant cells involved in bone resorption. The osteoclastic cell functions are regulated negatively or positively by several kinds of cytokine (e.g., growth factors), by vitamins, and by inflammatory factors [1, 2]. Several recent studies [3–5] have demonstrated an important role for the *c-Src* gene in osteoclastic bone resorption, though the precise role of the oncogene is not well understood. On the other hand, it has also been shown that transcriptional factors such as AP-1† and NF-κB are actively induced by cytokines [6, 7]. Therefore, it is of interest to understand the molecular mechanism of these transcriptional factors in the regulation of osteoclastic bone resorption.

Curcumin exhibits anticarcinogenic and anti-inflammatory properties [8–12]. Firstly, this pigment found in turmeric powder has been demonstrated to be a powerful inhibitor of AP-1 [13, 14]. However, a recent study [15] has shown that it is also able to inhibit NF-κB. Therefore, curcumin should prove useful for examining the role of these transcriptional factors in osteoclastic bone resorption.

In the present study, we examined the effect of curcumin on rabbit osteoclast apoptosis and demonstrated that curcumin drastically inhibits bone resorption in parallel with its stimulation of apoptosis in the cells.

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† *Abbreviations:* AP-1, activator protein-1; NF-κB, nuclear factor-κB; α-MEM, α-modification of Eagle's minimum essential medium; and TRAP, tartrate-resistant acid phosphatase.

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MATERIALS AND METHODS

Preparation of Rabbit Osteoclasts

Rabbit osteoclasts were prepared and isolated by a slight modification of the method described by Tezuka *et al.* [16]. Briefly, rabbit femoral bones were minced in α-MEM (Flow Laboratories), and then the cell suspension was overlaid on a Percoll (Pharmacia) solution having a specific gravity of 1.07. Thereafter, the cells were centrifuged for 20 min at 350 *g*. Cells having this specific gravity were harvested and washed with α-MEM and then seeded into 30-mm plastic dishes (Falcon, Becton Dickinson) or Lab-Tek chambers (Nunc). Five hours later, the purified osteoclasts were prepared by removing stromal cells with 0.002% EDTA and 0.02% pronase, and used in subsequent experiments. The purified cells were judged to be more 95% osteoclasts from the criteria of TRAP staining and multinucleation.

Fluorescence Microscopy

Morphological changes in the nuclear chromatin of apoptotic cells were detected by staining with Hoechst 33258. After treatment with curcumin, cells were fixed with 3% paraformaldehyde for 10 min, washed in PBS, and stained with 16 μg/mL of Hoechst 33258 for 15 min. Thereafter, the cells were observed by fluorescence microscopy (Olympus). More than 200 osteoclastic cells were scored for the incidence of apoptotic chromatin changes.

Agarose Gel Electrophoresis for DNA Fragmentation

To assess DNA fragmentation, we prepared DNA and analyzed it by a slight modification of the electrophoretic method described previously [17]. Isolated osteoclasts were

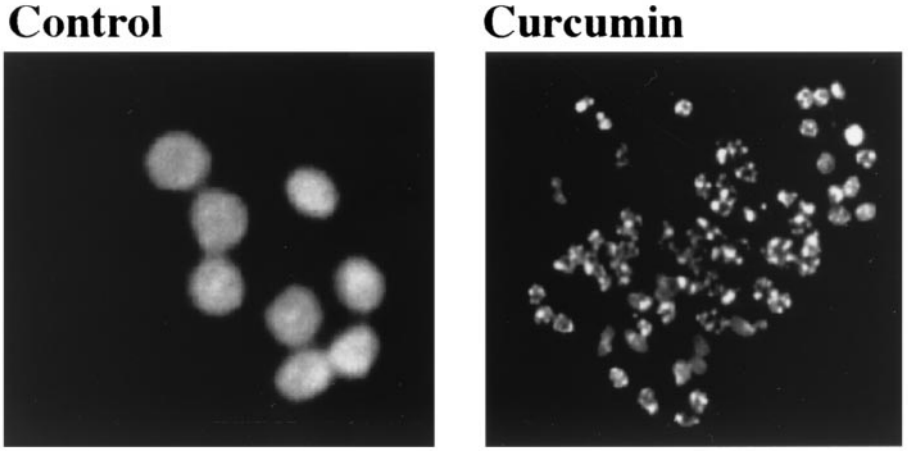

FIG. 1. Characterization of apoptosis in mature rabbit osteoclasts. TRAP-positive cells (1×10^4) in a femoral bone cell suspension were inoculated into each well of a Lab-Tek chamber. Stromal cells were then removed as described in Materials and Methods and the isolated osteoclasts treated or not with curcumin at 10 μM. After 12 hr, the cells were fixed and stained with Hoechst 33258.

treated or not with curcumin at 10 μM and then lysed by incubation in digestion buffer (150 mM NaCl, 25 mM EDTA, 100 μg/mL of proteinase K, and 0.2% SDS). The DNA was extracted with phenol/chloroform, precipitated with 0.5 M NaCl and ethanol, and electrophoresed on 3% agarose gel containing ethidium bromide, and DNA fragments were then visualized under UV light.

Bone Resorption Assay

Bone resorption assay was determined by measuring bone resorption pits as follows. TRAP-positive cells were measured as described previously [18]. The TRAP-positive cells (1×10^3) in a cell suspension of rabbit femoral bones were incubated for 60 min on a dentin slice (4 × 4 mm) in each well of a 24-well flat-type Falcon plastic plate containing α-MEM supplemented with 10% fetal bovine serum (Flow Laboratories). The non-adherent cells were then removed by washing with α-MEM. Thereafter, the cells on the dentin slices were incubated for 24 hr in α-MEM with or without curcumin. The bone resorption pits were measured as described previously [18]. The results were expressed as the means ± SD of quadruplicate cultures. Significance of differences was analyzed by Student's *t*-test.

Gel Mobility Shift Assay

The gel mobility shift assay was carried out as described previously [19]. Briefly, binding reactions were performed with 15 μg of the sample protein in a mixture of 2 mM Tris (pH 7.5), 8 mM NaCl, 0.2 mM EDTA, 0.8% (v/v) glycerol, 0.2 mM dithiothreitol, and 20,000 cpm of a ^{32}P-labeled AP-1 or NF-κB oligonucleotide containing a tandem repeat of the consensus sequence for the binding site for AP-1, the -TGACTCA-, or that for NF-κB, -GGGGACTTTCC-, which was end-labeled by the T4 polynucleotide kinase–[γ-^{32}P]ATP method. Unlabeled double-stranded oligonucleotide was used as a competitor. DNA–protein complexes were electrophoresed on native 5% polyacrylamide gels in 0.25 × TBE buffer (22 mM Tris, pH 8.0), 22 mM boric acid, 0.6 mM EDTA). Gels were vacuumed, dried, and exposed for 18 hr to Kodak x-ray film at −70°.

RESULTS

Since curcumin is a potent inhibitor of transcriptional factors AP-1 and NF-κB, we assumed that this reagent might be a regulator of osteoclast apoptosis, because many studies [20–24] have demonstrated that these transcriptional factors are associated with apoptosis in several kinds of cells. Therefore, we first looked for apoptotic chromatin changes in curcumin-treated osteoclasts by fluorescence microscopy. As shown in Fig. 1, we observed that curcumin-treated multinucleate osteoclasts exhibited morphological changes indicative of apoptosis, including chromatin condensation and nuclear fragmentation. Since these results suggested that curcumin may be a stimulator of osteoclast apoptosis, we also analyzed DNA from these cells by agarose gel electrophoresis for evidence of fragmentation, another well-documented characteristic of apoptosis. The typical ladder pattern of DNA cleavage resulting from apoptosis when DNA from curcumin-treated osteoclasts was electrophoresed was observed (data not shown). Figure 2A shows that curcumin stimulated osteoclast apoptosis in a dose-dependent fashion. Furthermore, this stimulatory action was also treatment time-dependent (Fig. 2B). These results demonstrate that curcumin is a potent stimulator of rabbit osteoclast apoptosis.

On the other hand, the curcumin stimulation of osteoclast apoptosis suggested to us that this natural pigment may be able to inhibit bone resorption by the osteoclasts. Therefore, we examined this point, and as shown in Fig. 3, found that curcumin inhibited bone resorption in a dose-dependent manner.

Finally, we examined by the gel mobility shift assay whether AP-1 or NF-κB binding activity in osteoclasts is

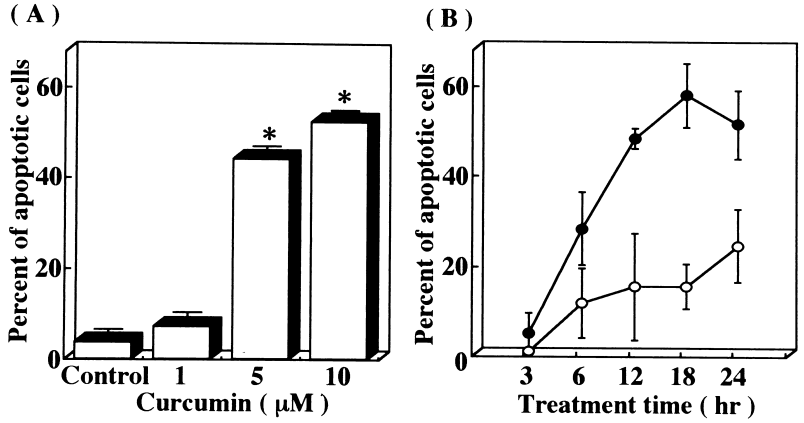

FIG. 2. Curcumin induced nuclear morphological changes in mature osteoclasts from rabbits. (A) Isolated osteoclasts prepared as described in Fig. 1 were treated or not for 12 hr with curcumin at the indicated doses. (B) Isolated osteoclasts prepared as described in Fig. 1 were treated (●) or not (○) with curcumin at 10 μM in α-MEM. More than 200 osteoclastic cells were scored for the incidence of apoptotic chromatin changes. The results are expressed as the means ± SD of triplicate cultures. *P < 0.05, control versus curcumin treatment.

inhibited by curcumin. As shown in Fig. 4A, although the appearance of AP-1 in the cells was not detected in curcumin-untreated or -treated cells, the binding of nuclear extracts from the cells to the consensus sequence of the binding site for NF-κB was observed, and the NF-κB binding clearly disappeared following treatment with the inhibitor. The NF-κB binding was completely inhibited by its competitor (Fig. 4B). These results suggest that curcumin inhibits the constitutive appearance of NF-κB. Our observations also suggest that NF-κB, but not AP-1, may function as an important transcriptional factor in the survival of rabbit osteoclasts.

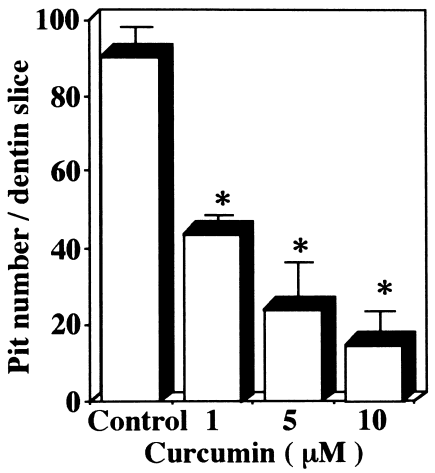

FIG. 3. Effect of curcumin on osteoclast-mediated bone resorption. TRAP-positive cells (1×10^3) in a femoral bone cell suspension were inoculated onto a dentin slice and rinsed to remove the non-adherent stromal cells after 1 hr. Then, the cells were treated or not with curcumin at the indicated doses. The number of bone resorption pits was measured after 24 hr. The results are expressed as means ± SD of quadruplicate cultures. *P < 0.05, control versus curcumin treatment.

DISCUSSION

We observed here the stimulatory action of curcumin toward rabbit osteoclast apoptosis. Interestingly, curcumin also clearly inhibited the osteoclastic bone resorption. To our knowledge, this is the first observation that curcumin inhibits osteoclastic bone resorption in relation to promotion of its cell apoptosis in osteoclasts.

The transcriptional factors AP-1 and NF-κB activate the transcription of certain genes by binding to specific DNA consensus sequences. Although several investigators [6, 7] have shown that both transcriptional factors are induced by estrogen and interleukin-1, it is unknown whether these factors actually stimulate transcription of genes involved in the survival and bone-resorbing activity of osteoclasts. The precise mechanism whereby curcumin stimulates apoptosis in mature rabbit osteoclasts was not addressed in the present study. However, since curcumin is a potent inhibitor of AP-1 and NF-κB, both of these factors may function in the survival of osteoclasts. In this regard, recent studies [25, 26] have suggested that interleukin-1 and macrophage colony-stimulating factor may be involved in the survival of osteoclast-like cells. Therefore, it is of interest to examine whether these cytokines promote the survival via NF-κB and AP-1 activation. In fact, a recent study [27] has shown that pyrrolidine dithiocarbamate (PDTC), a potent inhibitor of NF-κB [28], inhibits osteoclastic bone resorption. Furthermore, we observed previously that PDTC also stimulated apoptosis in rabbit osteoclasts [19], again suggesting the possible involvement of NF-κB in osteoclast survival. In contrast, as shown in this study, the constitutive presence of AP-1 was not detected at all in the cells.

Curcumin is a potent inhibitor of tumor promotion and the inflammatory response. Since cancer and bone inflammation are diseases that increase bone resorption, curcumin may be useful in the therapy of these diseases by serving as an inhibitor of bone resorption.

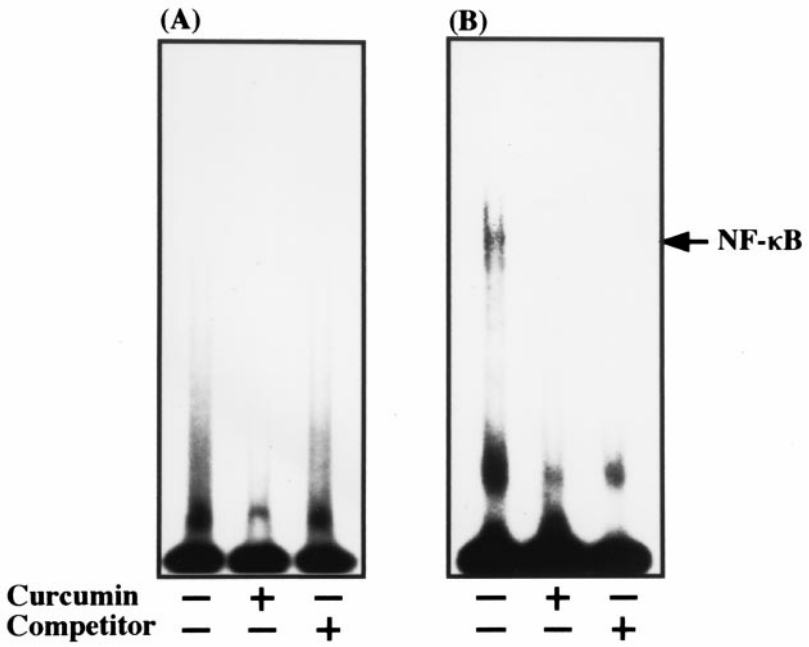

FIG. 4. Curcumin inhibits NF-κB binding in mature rabbit osteoclasts. Osteoclasts were treated or not with curcumin at 10 μM, and after 6 hr the nuclear proteins were prepared. The gel mobility shift assay was performed with ^{32}P-labeled oligonucleotide or unlabeled oligonucleotide as a competitor containing the AP-1 (A) or NF-κB (B) consensus sequence in the presence of the nuclear proteins. An identical experiment independently performed gave similar results.

In conclusion, we showed herein that curcumin is a potent stimulator of osteoclast apoptosis and also an inhibitor of bone resorption caused by these cells. Further experiments are needed to determine the functional role of transcriptional factors in osteoclast apoptosis.

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