# NF-κB inhibitors stimulate apoptosis of rabbit mature osteoclasts and inhibit bone resorption by these cells

Ken Ozaki, Hiroyuki Takeda, Hiroyoshi Iwahashi, Shigeo Kitano, Shigemasa Hanazawa*

*Department of Oral Microbiology, Meikai University School of Dentistry, Keyakidai, Sakado, Saitama 350-02, Japan*

Received 12 May 1997

**Abstract Interesting, recent studies have suggested a possibility that transcriptional factor NF-κB may play a functional role in the survival of mouse osteoclasts. However, it has not been known whether NF-κB is involved in apoptosis of and bone resorption by mature osteoclasts. Thus, using NF-κB inhibitors, we examined the functional role of NF-κB in the induction of apoptosis in rabbit mature osteoclasts. PDTC, a potent inhibitor of NF-κB, stimulated markedly apoptosis of the osteoclasts and inhibited bone resorption by these cells. These effects also was observed when three other inhibitors of NF-κB were used. And a gel mobility shift assay showed that PDTC also inhibited NF-κB binding to its consensus sequence in the cells. These results suggest a regulatory role for NF-κB in apoptosis in and bone resorption by rabbit mature osteoclasts.**



*Key words:* Apoptosis; NF-κB; Bone resorption; Rabbit osteoclast

## 1. Introduction

Osteoclasts play a crucial role in bone remodeling, because these cells are the predominant type involved in bone resorption. The osteoclastic cell functions are regulated negatively or positively by several kinds of cytokine such as growth factors, vitamins, and inflammatory factors [1,2]. Although these factors may regulate the functions of the osteoclasts via some transcriptional factor(s), the precise role of such a factor(s) in osteoclastic function has not been demonstrated in detail. Recent studies [3,4] have suggested the possible involvement of NF-κB in osteoclast activation. Therefore, we carried out experiments to examine the possible role of this transcriptional factor in apoptosis in and bone resorption by rabbit mature osteoclasts.

In the present study, using several inhibitors of NF-κB activity, we examined the functional role of NF-κB in apoptosis and bone resorption by rabbit osteoclasts and demonstrated that the NF-κB inhibitors clearly inhibited bone resorption in parallel with their stimulation of apoptosis in the cells.

## 2. Materials and methods

### 2.1. Preparation of rabbit osteoclasts

Rabbit osteoclasts were prepared and isolated by a slight modification of the method described by Tezuka et al. [5]. Briefly, rabbit femoral bones were minced in α-MEM (Flow Lab., McLean, VA), and then the cell suspension was overlaid on a Percoll (Pharmacia Japan, Tokyo, Japan) solution having a specific gravity of 1.07. Thereafter, the cells were centrifuged for 20 min at 350×*g*. The cells having the specific gravity 1.07 were harvested and washed with α-MEM, and then seeded into 30-mm plastic dishes (Falcon, Becton Dickinson Labware, Oxnard, CA) or Lab-Tek chambers (Nunc, Naperville, IL). 5 h later, the purified osteoclasts were prepared by removing stromal cells with 0.002% EDTA and 0.02% pronase, and used in subsequent experiments.

### 2.2. NF-κB inhibitors

Pyrrolidine dithiocarbamate (PDTC), *N*-tosyl-L-phenylalanine chloromethyl keton (TPCK), and gliotoxin were purchased from Sigma Chemical Co. (St. Louis, MO). Curcumin was obtained from Nakarai Tesque (Kyoto, Japan). The characterizations of each inhibitor for NF-κB were made previously [6–9].

### 2.3. Cytochemical staining

Morphological changes in the nuclear chromatin of apoptotic cells were detected by staining with Hoechst 33258 (Sigma) as described previously [10]. After treatment with each inhibitor, cells were fixed with 3% paraformaldehyde for 10 min, washed in PBS, and stained with Hoechst 33258 (16 μg/ml) for 15 min. Thereafter, the cells were observed by fluorescence microscopy (Olympus BX60; Tokyo, Japan). More than 200 osteoclasts were scored for the incidence of apoptotic chromatin changes. The results were expressed as the mean ± S.D. of triplicate cultures.

### 2.4. Agarose gel electrophoresis for DNA fragmentation

To assess DNA fragmentation, we prepared DNA from the osteoclasts and analyzed it by a slight modification of the electrophoretic method described previously [11]. The osteoclasts were treated or not with each inhibitor and then lysed by incubation in digestion buffer (150 mM NaCl, 25 mM EDTA, 100 μg/ml 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 then the DNA fragments were visualized under UV light.

### 2.5. Bone resorption assay

Bone resorption assay was determined by counting bone resorption pits as described previously [12], tartrate-resistant acid phosphatase (TRAP)-positive cells ($1\times10^3$ cells) 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 Labs, McLean, VA). Then, the nonadherent cells were removed by washing with α-MEM. Thereafter, the cells on the dentin slices were incubated for the selected times in α-MEM with or without each inhibitor. The bone resorption pits were counted, and the results were expressed as the mean ± S.D. of quadruplicate cultures.

### 2.6. Gel mobility shift assay

Preparation of cell extracts and the assay were carried out as described previously [13,14]. 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 DTT, and 20 000 cpm of a $^{32}$P-labeled NF-κB oligonucleotide containing a tandem repeat of the consensus sequence for the binding site 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

*Corresponding author. Fax: 81-492-85-1035.


*PII* S0014-5793(97)00653-4

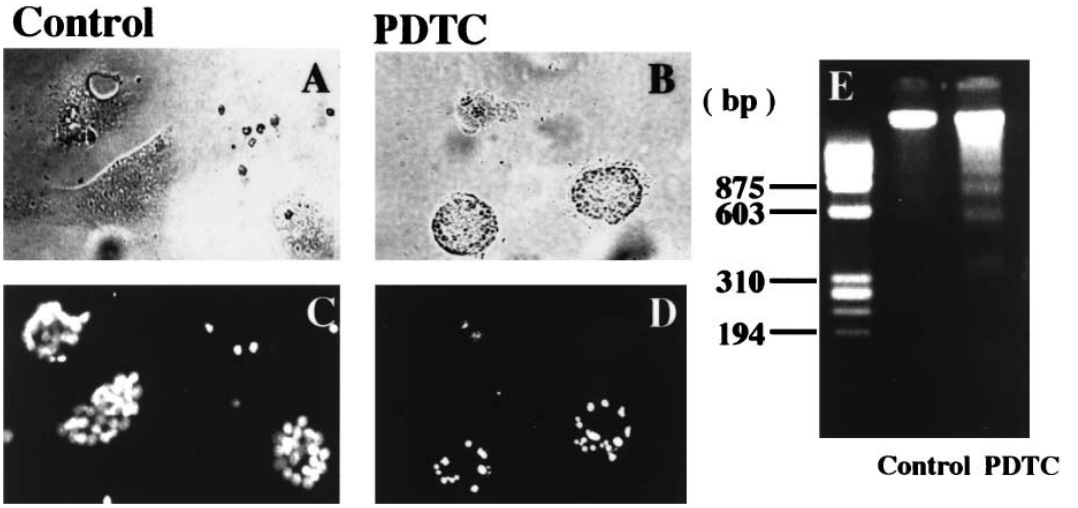


Fig. 1. PDTC stimulates apoptosis of rabbit mature osteoclasts. (A–D): TRAP-positive cells ($1\times10^4$ cells) in a femoral bone cell suspension were inoculated into each well of a Lab-Tek chamber, and then isolated osteoclasts prepared as described in Section 2 were treated (B, D) or not (A, C) with PDTC at 10 μM. After 12 h, the cells were fixed. Phase-contrast photomicrographs and corresponding fluorescence photomicrographs taken after nuclear staining with Hoechst 33258 are shown. (E) Osteoclasts prepared as described above were treated or not with PDTC at 10 μM, and lysed in digestion buffer 12 h later. Then, their genomic DNA was extracted and subjected to agarose gel electrophoresis. φX174 RF DNA/*Hae*III Fragments were used as molecular weight markers (left lane).

vacuumed, dried, and exposed for 18 h to Kodak X-ray film at −70°C.

## 3. Results

### 3.1. PDTC stimulates apoptosis of rabbit osteoclasts

Many studies [11,15–17] have demonstrated that NF-κB is associated with apoptosis in several kinds of cells. Therefore, firstly, using PDTC, a potent inhibitor of NF-κB, we looked by phase contrast and fluorescence microscopy for apoptotic chromatin changes in PDTC-untreated or -treated osteoclasts. As shown in Fig. 1 (B and D), we observed that PDTC-treated multinucleate osteoclasts exhibited morphological changes indicative of apoptosis, including chromatin condensation and nuclear fragmentation. However, such morphological changes were not observed in the control, untreated osteoclasts (Fig. 1A and C). We also analyzed DNA from these cells by agarose gel electrophoresis for evidence of fragmentation (Fig. 1E). By 12 h of treatment with PDTC, a substantial DNA ladder was detected in the treated cells.

Fig. 2 shows that PDTC at 10 μM stimulated apoptosis in the osteoclasts in a treatment time-dependent fashion. This apoptotic effect was also observed with 1 μM PDTC (data not shown). These results suggest that NF-κB is an important transcriptional factor in the regulation of apoptosis of rabbit mature osteoclasts.

### 3.2. PDTC inhibits NF-κB factor binding in rabbit osteoclasts

Next, we examined by the gel mobility shift assay whether the presence of NF-κB could be detected in the osteoclasts, and if so, also whether NF-κB binding activity in the cells is inhibited by PDTC. As shown in Fig. 3, although the binding of nuclear extracts from control osteoclasts to the consensus sequence of the binding site for NF-κB was observed, the NF-κB binding clearly disappeared by treatment with PDTC at 10 μM. Since the NF-κB binding was completely inhibited by its competitor, these results suggest that PDTC inhibits the constitutive appearance of NF-κB in rabbit osteoclasts.

### 3.3. PDTC inhibits osteoclastic bone resorption

The PDTC stimulation of osteoclast apoptosis suggested to us the possibility that PDTC treatment may also cause inhibition of bone resorption by the osteoclasts. Therefore, we examined this point. As shown in Fig. 4, PDTC treatment inhibited the bone resorption of the osteoclasts in a treatment time-dependent manner.

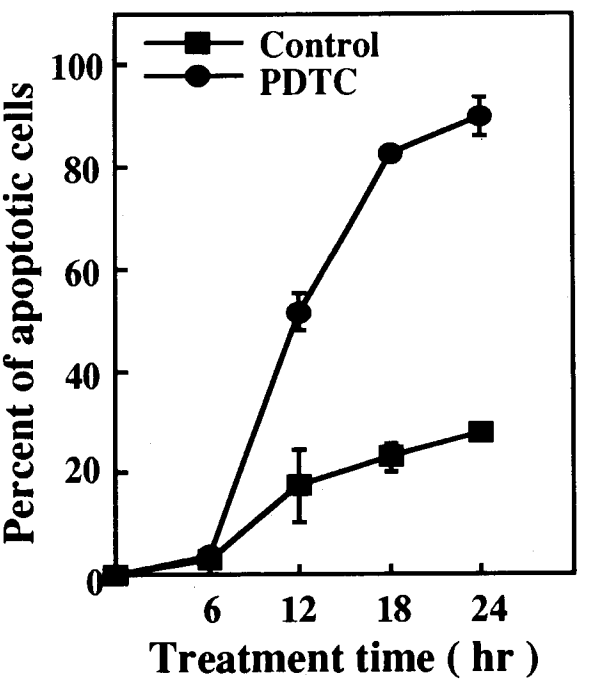


Fig. 2. Kinetics of PDTC stimulation of apoptosis of rabbit mature osteoclasts. Isolated osteoclasts prepared as described in Section 2 were treated or not with PDTC at 10 μM in α-MEM. More than 200 osteoclasts were scored for the incidence of apoptotic chromatin changes. Three identical experiments independently performed gave similar results.

### 3.4. Effect of several NF-κB inhibitors on apoptosis in and bone resorption by osteoclasts

As shown above, NF-κB may play regulatory role in bone resorption by osteoclasts through regulation of apoptosis of its cells. Therefore, to confirm this possibility, we also examined several additional inhibitors of NF-κB for their effect on apoptosis and bone resorption of rabbit osteoclasts. As shown in Fig. 5 (A and B), we observed a significant increase in the number of apoptotic cells, which became apparent 12 h after the addition of each inhibitor. Also, each inhibitor dramatically inhibited osteoclastic bone resorption of the osteoclasts. And the inhibitory action of each reagent was dose dependent (data not shown).

## 4. Discussion

Transcriptional factor NF-κB activates the transcription of certain genes via its binding to specific DNA consensus sequences on these genes. The involvement of NF-κB in apoptosis has been suggested previously [11,15–17], and more recent studies [18–21] have suggested that activation of NF-κB is able to protect against cell killing by apoptotic stimuli in several kinds of cells. However, the actual role of this transcriptional factor in apoptosis in and bone resorption by mature osteoclasts has not been demonstrated in detail.

We observed here the stimulatory action of PDTC, a NF-κB inhibitor, toward rabbit osteoclast apoptosis. Interestingly, this chemical also clearly inhibited osteoclastic bone resorption. Furthermore, we showed that three other NF-κB inhibitors, gliotoxin, curcumin, and TPCK, exhibited the same mode of action as PDTC toward rabbit osteoclasts. To our knowledge, this is the first observation that NF-κB is a potent regulator of apoptosis in and bone resorption by mature osteoclasts.

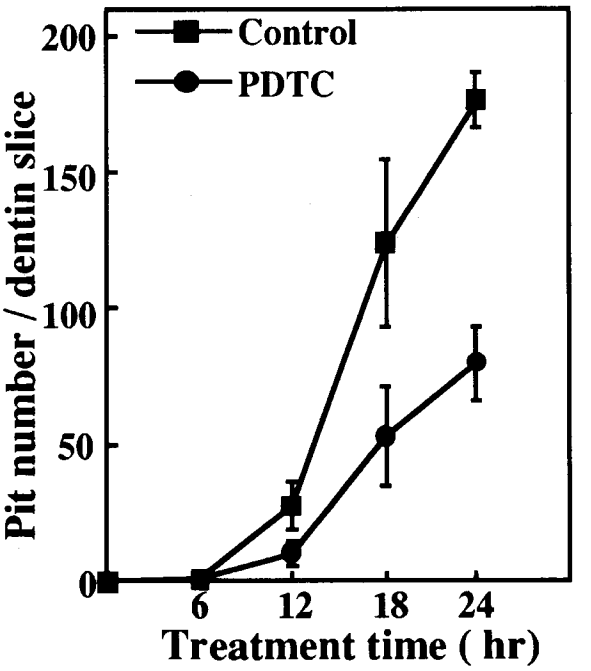


Fig. 4. Inhibitory effect of PDTC on osteoclast-mediated bone resorption. TRAP-positive cells ($1\times10^3$ cells) in a femoral bone cell suspension were inoculated onto each of several dentin slices and rinsed 1 h later to remove the nonadherent stromal cells. Then the cells were treated or not with PDTC at 10 μM. After the selected times, the resorption pit number was counted. The results are expressed as the mean ± S.D. of quadruplicate cultures. Three identical experiments independently performed gave similar results.

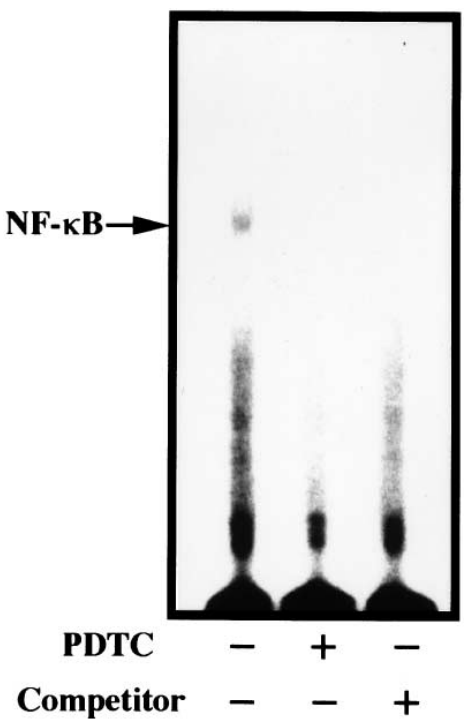


Fig. 3. PDTC inhibits NF-κB binding in rabbit mature osteoclasts. Osteoclasts were treated or not with PDTC at 10 μM; and after 6 h, the nuclear proteins were prepared. Gel mobility shift assay was performed with $^{32}$P-labeled oligonucleotide or unlabeled oligonucleotide as a competitor containing the NF-κB consensus sequence in the presence of the nuclear proteins. An identical experiment independently performed gave similar results.

Recent studies [22,23] have shown that NF-κB in mouse osteoclasts is stimulated by interleukin-1 (IL-1). On the other hand, although we showed that the constitutive binding of NF-κB to its consensus sequence in rabbit osteoclasts was inhibited by PDTC with high specificity, this inhibition of NF-κB binding does not directly prove the functional role of this transcriptional factor in bone resorption by osteoclasts in relation to stimulation of apoptosis in these cells. However, NF-κB may be an important transcriptional factor in these processes in view of the fact that the other three NF-κB inhibitors tested also showed the same mode of action as PDTC.

As described above, since IL-1 may involved in the survival of osteoclast-like cells and since this cytokine is able to induce the appearance of NF-κB in the cells, these facts together with our present study, results suggest that NF-κB plays a functional role in the survival of osteoclasts.

In conclusion, we showed herein that NF-κB inhibition potently stimulates osteoclast apoptosis and inhibits bone resorption by these cells. Stimulation of osteoclast apoptosis may be proposed as a useful strategy for the therapy of diseases involving increased osteoclastic bone resorption. In further experiments, it will be very important for us to analyze the molecular mechanisms for the precise action of NF-κB in survival or apoptosis of osteoclasts.

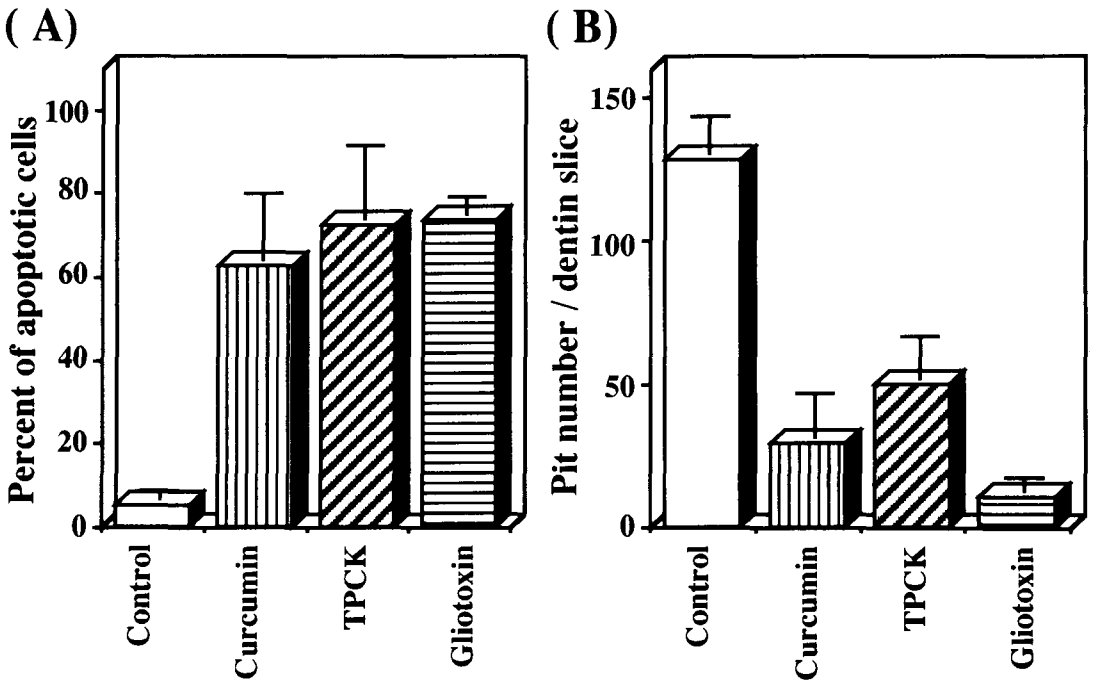


Fig. 5. Other inhibitors also induce apoptosis of osteoclasts and block osteoclastic bone resorption. (A) Isolated osteoclasts prepared as described in Section 2 were treated or not with 10 μM curcumin, 10 μM TPCK, or 1 μg/ml gliotoxin in α-MEM. After a 12-h incubation, more than 200 osteoclastic cells were scored for the incidence of apoptotic chromatin changes. (B) TRAP-positive cells ($1 \times 10^3$ cells) were prepared as described in Section 2. Then, the cells were treated or not with curcumin at 10 μM, TPCK at 10 μM, or gliotoxin at 1 μg/ml in α-MEM. After an 18-h incubation, the resorption pit number was counted. The results are expressed as the mean ± S.D. of quadruplicate cultures. Three identical experiments independently performed gave similar results.

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