Communication

Interleukin-1α Activates an NF-κB-like Factor in Osteoclast-like Cells*

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We investigated the NF-κB transcription factor in osteoclast-like cells. Osteoclast-like cells were differentiated from mouse bone marrow cells in co-culture with mouse calvaria-derived primary osteoblasts in the presence of 1α,25-dihydroxyvitamin D₃ and prostaglandin E₂ in collagen gel-coated dishes. We enriched osteoclast-like cells from the co-cultures by Pronase treatment. When the enriched osteoclast-like cells were treated with phorbol 12-myristate 13-acetate, interleukin-1 (IL-1), calcitonin, or macrophage colony-stimulating factor, only IL-1 activated an NF-κB-like factor, which specifically bound to a κB motif DNA sequence, as detected by an electrophoretic mobility shift assay. IL-1 also activated NF-κB induction in osteoblasts. However, the NF-κB-like factor induced by IL-1-stimulated osteoclast-like cells is of smaller molecular size than the factor in osteoblasts, as shown by an electrophoretic mobility shift assay. The NF-κB activity of osteoclast-like cells was recognized completely by antibodies against the p50 subunit and only partially by antibodies against the p65 subunit of NF-κB. Antibodies against c-Rel, Rel B, and p52 did not recognize the NF-κB-like factor. These results suggest that IL-1 activates an NF-κB-like factor in osteoclast-like cells, which contains p50 and p65-related proteins.

Osteoclasts play a crucial role in the resorption of bone physiologically and pathologically. Extraordinary induction and activation of osteoclasts results in excess bone resorption, which overtakes the rate of bone formation, which in turn gives rise to the pathological loss of bone such as in osteoporosis and periodontitis. Thus, it is important to investigate the biochemical events occurring in osteoclasts in order to understand the pathology of bone destruction.

It had been difficult to study osteoclasts because it is very difficult to culture and amplify enough primary osteoclasts for biochemical studies. Recently, a method where osteoclast-like cells were induced from cultured bone marrow cells under a certain set of conditions was developed (1–3). The resulting osteoclast-like cells have a similar phenotype to osteoclasts, including their morphology as giant and multinucleated cells (MNC), their expression of tartrate-resistant acid phosphatase (TRAP) and calcitonin (CT) receptors, and their ability to resorb dentine slices (1–3). Therefore, the use of these induced osteoclast-like cells enables scientists to examine osteoclasts biochemically. Because of this, information about the intracellular events occurring in osteoclasts has accumulated rapidly, but little information on the intracellular signaling molecules of osteoclasts, especially transcription factors, exists.

NF-κB is a ubiquitous transcription factor which can activate a number of genes including the IL-6, tumor necrosis factor-α, major histocompatibility complex class I, and adhesion molecule genes (4). The target genes of NF-κB are involved in inflammatory reactions where bone is degraded. Conventional NF-κB is a heterodimer composed of a p50 and a p65 subunit, both of which show a high degree of amino acid sequence homology to the Rel family of proteins, which includes c-Rel, Rel B, and p52 (5–7). Latent NF-κB, which complexes with an inhibitor IκB, resides in the cytoplasm (8). After stimulation, NF-κB is released from IκB, and translocates into the nucleus to bind to a specific DNA sequence. NF-κB seems to be one of the central intracellular signals for interleukin-1 (IL-1), which is one of the most potent osteoclast activating factors (9). Many genes activated by IL-1 are the target genes of NF-κB (4, 10, 11).

In this study, we investigated NF-κB activity in osteoclast-like cells treated with IL-1. The NF-κB-like factor of osteoclast-like cells appears to be different from the NF-κB in osteoblasts. Our results suggest that osteoclasts possess an NF-κB-like factor, which can be induced by IL-1.

EXPERIMENTAL PROCEDURES

Culture of Osteoclast-like Cells—Bone marrow cells were obtained from the tibiae and femora of 6–8-week-old male ddY mice (Seiwa Experimental Animal, Fukuoka, Japan) as reported previously (12). Primary osteoblasts were prepared from the calvaria of newborn ddY mice (13). Bone marrow cells (5 × 10⁶ cells) were co-cultured with osteoblasts (1 × 10⁶ cells) in a minimal essential medium (Life Technologies, Inc.) containing 10% fetal bovine serum, 1α,25-dihydroxyvitamin D₃ (1α,25(OH)₂ D₃) (10⁻⁸ M), and prostaglandin E₂ (PGE₂) (10⁻⁶ M) in 100 mm-diameter dishes coated with collagen gel (Nitta Gelatin Co., Osaka, Japan) according to the method described by Akatsu et al. (13). Seven days after inoculation, osteoclast-like cell formations had reached a maximum and all adherent cells were detached from the dishes by treatment with 0.2% collagenase (Wako Pure Chemical Co., Osaka, Japan). The cell suspension from each dish was inoculated again into a 100-mm diameter dish. Ten hours later the adherent cells were treated with phosphate-buffered saline (PBS) containing 0.01% Pronase E (Sigma) and 0.02% EDTA for 10 min at 37°C to remove the osteoblasts and other bone marrow cells (14). More than 95% of the adherent cells remaining, which were resistant to Pronase E, were TRAP-positive MNCs and mononuclear cells (15).

Preparation of Nuclear Extracts—Cell extracts were prepared according to methods described by Dignam et al. (16) and Osborn et al. (17). For samples of enriched osteoclast-like cells, cells were scraped off each dish and put in ice-cold PBS. The cells were washed in ice-cold PBS

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1 The abbreviations used are: MNC, multinucleated cell(s); TRAP, tartrate-resistant acid phosphatase; CT, calcitonin; NF-κB, nuclear factor κB; IL, interleukin; PG, prostaglandin; PMA, phorbol 12-myristate 13-acetate; PMSG, phenylmethanesulfonyl fluoride; EMSA, electrophoretic mobility shift assay; OCL, osteoclast-like cell(s); POB, primary osteoblasts; DTT, dithiothreitol; MMP, matrix metalloproteinase; 1α,25(OH)₂ D₃; 1α,25-dihydroxyvitamin D₃.
and then in ice-cold hypotonic buffer (buffer A: 10 mM HEPES, pH 7.9, 1.5 mM MgCl2, 10 mM KCl, 0.5 mM PMSF, 0.5 mM DTT), then lysed for 10 min on ice in buffer A containing 0.1% Nonidet P-40. The lysates were centrifuged for 10 min at 10,000 g and the resulting supernatants, which are referred to as nuclear extracts, were supplemented with five volumes of storage buffer (buffer D: 20 mM HEPES, pH 7.9, 420 mM NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, 25% glycerol, 0.5 mM PMSF, 0.5 mM DTT) and incubated at 4°C for 15 min. Lysed nuclei were then centrifuged for 10 min at 10,000 g and the resulting supernatants, which are referred to as nuclear extracts, were supplemented with five volumes of storage buffer (buffer D: 20 mM HEPES, pH 7.9, 100 mM KCl, 0.2 mM EDTA, 20% glycerol, 0.5 mM PMSF, 0.5 mM DTT). One dish of osteoclast-like cells, yielded 30 μg of nuclear extract.

Electrophoretic Mobility Shift Assays (EMSA)—The sequence of the NF-κB binding oligonucleotide used as a radioactive DNA probe was 5'-AGCTTTGACCCAGTTCGAG-3'. The mutated oligonucleotide was 5'-AGGTTCCTAGCTTCCGAG-3'. The AP-1-binding oligonucleotide was 5'-GGCTTATGACTGACCGGAAA-3'. For use in the EMSA, the oligonucleotides were labeled with [-32P]ATP by T4 polynucleotide kinase and purified by precipitation with ethanol as described (10). The DNA binding reaction was performed at room temperature in a volume of 20 μl, which contained the binding buffer (10 mM Tris/HCl, pH 7.5, 1 mM EDTA, 4% glycerol, 100 mM NaCl, 5 mM DTT, 100 μM bovine serum albumin), 3 μg of poly(dI-dC), 1 × 10^5 cpm 32P-labeled probe, and 5–10 μg of nuclear proteins. After 15 min of incubation, samples were electrophoresed on native 5% acrylamide/0.25 TBE gels. Unlabeled oligonucleotides were added as 50-fold excess of unlabeled wild type oligonucleotides as labeled (data not shown). Macrophage-colony stimulating factor, which is essential for osteoclast formation in op/op mice (19), also had no effect on NF-κB induction in osteoclast-like cells (data not shown).

We treated these osteoclast-like cells with 10 ng/ml IL-1α, 10^{-7} M CT, or 1 μg/ml PMA for 30 min, and prepared nuclear extracts from osteoclast-like cells from each dish separately. NF-κB activity in the nuclear extracts was measured by EMSA. Fig. 2A shows that IL-1α, but not PMA or CT, induced an NF-κB-like activity in the nucleus of osteoclast-like cells. This IL-1-induced NF-κB activity appeared as a single band in the EMSA. Excess amounts of the unlabeled wild type NF-κB motif oligonucleotide, but not the mutated NF-κB motif oligonucleotide, inhibited NF-κB-like activity completely as shown in the EMSA (Fig. 2B). This means that the NF-κB-like factor in osteoclast-like cells can recognize and bind to the specific DNA sequence of the NF-κB motif.

NF-κB is a well known potent NF-κB activator, as it activates protein kinase C to phosphorylate IκB, which in turn releases active NF-κB (18). Interestingly, in osteoclast-like cells PMA did not activate the NF-κB-like factor. AP-1 activity, another transcription factor known to be activated by PMA, was also examined in osteoclast-like cells. Both PMA and IL-1 greatly induced AP-1 activity in primary osteoblasts (POB) but not in osteoclast-like cells (OCL) in the experimental conditions used in this study (Fig. 2C). The same nuclear preparations of POB treated with PMA and IL-1 and OCL treated with IL-1 contained an activated NF-κB-like factor (data not shown). Macrophage-colony stimulating factor, which is essential for osteoclast formation in op/op mice (19), also had no effect on NF-κB induction in osteoclast-like cells (data not shown).

As for the origin of NF-κB-like activity, we were worried about the possibility of contamination from other cell types, such as osteoblasts and bone marrow cells, even though the purity of our osteoclast-like cell preparation was more than 95%. In order to confirm whether this IL-1-induced NF-κB-like factor is derived from osteoclast-like cells, we compared the mobility of the NF-κB-like factor bands in the enriched osteoclast-like cell population (purified OCL preparation) with bands from osteoblasts alone, and mixed populations of osteoblasts and osteoclast-like cells (crude OCL preparation) in an

RESULTS

Mouse bone marrow cells were cocultured with the primary osteoblasts, which were derived from mouse calvaria, in the presence of 10^{-8} M 1α,25(OH)2D3 and 10^{-6} M PGE2. Seven days after inoculation, all cells including MNCs were detached from the dishes by treatment with 0.2% collagenase. The cell suspension was inoculated again into plastic dishes and cultured for 10 h (A). Then, osteoblasts and other cells were removed by Pronase E treatment (B). The remaining adherent MNCs were stained for TRAP and observed under the microscope.
osteoclast-like cells contained the p50 or p65 subunits of NF-κB shown in Fig. 4, anti-p50 antibodies bound and supershifted EMSA. IL-1 induced two NF-κB-like factors in primary osteoblasts, crude OCL preparation, and purified OCL, respectively. On the heterodimers and p50-p50 homodimers, respectively. On the other hand, anti-p50 antibodies, but not anti-c-Rel, Rel B, or p52 antibodies, recognized the NF-κB-like factor of osteoclast-like cells in the EMSA (Fig. 4). The NF-κB-like factor band was partially reduced in the presence of anti-p50 antibodies. It remains unknown whether the partial effect of anti-p50 antibodies is due to the weak affinity of the antibodies or to the presence of a p56-related subunit. It seems likely that the NF-κB-like factor in osteoclast-like cells is a heterodimer composed of a p50 and a p65-related subunit.

**DISCUSSION**

This study indicates that IL-1 can induce an NF-κB-like factor in the nuclei of osteoclast-like cells. It is unknown whether this NF-κB-like factor can actually activate the transcription of certain genes, but it can bind to specific DNA sequences in the nucleus. The molecular size of this NF-κB factor is smaller than the p50 homodimer in osteoblasts in view of its mobility in an EMSA. From the supershift assay, we assume that this factor is a heterodimer composed of a p50 and a p65 subunit, whose size could be larger than a p50 homodimer. We speculate that the partner of the p50 subunit may be a p65-related protein, to which anti-p65 antibodies can bind only weakly (21). However, the function of this factor is not clear.

There have been few studies on transcription factors in osteoclasts. In situ hybridization showed the levels of NF-IL-6 expression in osteoclasts of patients with osteoarthritic and Paget's bone (22). Roodman et al. (23) reported that IL-6 might be an autocrine/paracrine factor that regulates osteoclastic bone resorption, and NF-κB and NF-IL-6 were involved in the expression of IL-6 induced by inflammatory cytokines, such as IL-1 and tumor necrosis factor-α (24, 25). In addition, the 5′-flanking region of the TRAP gene, whose expression is a useful marker for osteoclasts, was reported to contain an AP-1 binding sequence (26). Furthermore, it is known that c-Fos, which is a component of AP-1, is essential for the differentiation of osteoclast precursors, probably from mononuclear phagocytes into osteoclasts (27). These findings indicate that AP-1 may be present and functioning in osteoclasts, although we could not detect AP-1 in osteoclast-like cells stimulated with PMA. Recently, Tezuka et al. (28) showed that the matrix metalloproteinase (MMP)-9 gene, whose product can degrade some extracellular matrices, was expressed in isolated rabbit osteoclasts (28). NF-κB may be involved in the expression of the MMP-9 gene since the promoter region of the MMP-9 gene has an NF-κB binding sequence (29, 30).

Whether or not osteoclasts can respond to IL-1 directly is still controversial (i.e. do osteoclasts express IL-1 receptors or not?), even though IL-1 is the most potent osteoclast-activating factor which promotes bone resorption. We previously demonstrated that IL-1 can increase the survival rate of osteoclast-like cells, as osteoclast-like cells could adhere to plastic dishes for longer periods in the presence of IL-1 (31). When measuring survival rates, osteoclast-like cells must be cultured in the presence of IL-1 for 24 h. Culturing for 24 h may be long enough for any contaminating cells, even if they are present in very small numbers, to synthesize and release unknown factor(s), which in turn could act on osteoclasts. In contrast, the NF-κB-like factor in osteoclast-like cells was induced within 5 min of the addition of IL-1 (data not shown). Even if other cells such as osteoblasts were present in our enriched osteoclast-like cell

**Fig. 4. Subunit composition of the NF-κB-like factor of osteoclast-like cells.** Nuclear extracts were prepared from IL-1-stimulated primary osteoblasts (POB, lanes 1–3) and from IL-1-stimulated osteoclast-like cells (OCL, lanes 4–9). The nuclear extracts (5 µg of protein) were pretreated with vehicle (lanes 1 and 3), or 1 µl of one of the following polyclonal antibodies: anti-p50 (lanes 2 and 5), anti-p65 (lanes 3 and 6), anti-c-Rel (lane 7), anti-Rel B (lane 8), or anti-p52 (lane 9) at 4°C for 60 min. EMSAs were performed on these pretreated nuclear extracts. Arrowheads show the DNA binding complexes supershifted by antibodies. Arrows identify the two NF-κB activity bands from primary osteoblasts and the NF-κB-like factor band from osteoclast-like cells.
cultures, the number of the contaminating cells would be too small to exert any influences on the osteoclast-like cells that rapidly. Moreover, PMA activated AP-1 in osteoblasts, but not in osteoclast-like cells. Thus, from our results, we conclude that IL-1 binds to putative IL-1 receptors on osteoclast-like cells, resulting in the induction of an NF-κB-like factor in the nucleus of these cells.

The osteoclast-like cells seem to be differentiated from mononuclear phagocytes in bone marrow, in the presence of osteoblasts (32). Recently, Grigoriadis et al. reported that c-Fos is a key regulator in osteoclast-macrophage lineage determination (27). Therefore, it will be interesting to see if differences in NF-κB expression between macrophages and osteoclasts depends on the stage of differentiation of the osteoclasts. Further experiments need to be done to determine this.

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