Activation of NF-κB in human osteoblasts by stimulators of bone resorption

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Abstract Several bone resorptive stimuli affect osteoclasts indirectly by modulating the production and release of osteoblastic factors. Using electrophoretic mobility shift assays, we found that not only tumour necrosis factor-α (TNF-α) but also interleukin-1β and parathyroid hormone (PTH) caused dose and time-related increases in nuclear factor κB (NF-κB)-DNA binding in Saos-2 human osteoblastic (hOB) cells. Activation of NF-κB by TNF-α was reproduced in primary hOBs. In contrast, consistent with their previously reported lack of response to steroid hormones, Saos-2 cells did not respond to 1,25-dihydroxyvitamin D_3. We suggest that NF-κB activation in osteoblastic cells constitutes an important pathway in osteoblast-mediated resorptive signalling.

Key words: Osteoblast; Resorptive signalling; Parathyroid hormone; Interleukin-1; Tumour necrosis factor; Nuclear factor κB

1. Introduction

Osteoclasts are responsible for bone removal during resorption. However, their generation from haemopoietic precursors and the rate of their activity are regulated by various immune and haemopoietic factors produced locally from other cells present in the bone microenvironment [1].

It is now well established that several bone resorptive stimuli, such as parathyroid hormone (PTH), thyroid hormone (T3), 1,25-dihydroxyvitamin D_3 (1,25-$(OH)_2$ vitamin D_3), tumour necrosis factor-α (TNF-α), interleukin-1 (IL-1) and prostaglandin (PG) E_2 (PGE_2), affect osteoclast formation and activity indirectly by modulating the production and/or release of a variety of marrow stromal/osteoblastic factors. These include (a) various cytokines and growth factors, such as IL-1α and β, IL-6, IL-11, TNF-α, differentiation inducing factor/leukemia inhibitory factor TGF-β, M-CSF and GM-CSF (reviewed in [2]), the membrane associated TNF-related activation-induced cytokine (TRANCE) and its decoy receptor osteoprotegerin (OPG)/osteoclastogenesis inhibitory factor (OCIF) [3], (b) PGs (such as PGE_2, [4]) and nitric oxide (NO, [5]), which are the products of cyclooxygenase-2 (COX-2) and inducible NO synthase (iNOS), respectively, and (c) collagenase, other metalloproteinases and adhesion molecules, such as osteopontin and VCAM-1 [2].

Among these factors, TRANCE, which is a newly discovered member of the membrane-associated TNF ligand family (also called receptor activator of nuclear factor κB (NF-κB) (RANK) ligand, OPG ligand or osteoclast differentiation factor (ODF) (reviewed in [6])), has recently been identified as the long-sought osteoblast-derived osteoclastogenic factor that mediates the resorptive activity of various systemic and local stimuli via cell-to-cell contact [3,6]. By binding to its receptor RANK on osteoclast progenitors and mature osteoclasts, TRANCE induces osteoclast differentiation and activation via the NF-κB signal transduction pathway [6].

NF-κB is a ubiquitous transcription factor found in many different cell types. It regulates the transcription of many genes involved in inflammation and, depending on the cell type, it either prevents or promotes apoptosis (reviewed in [7]). In its most common form, NF-κB is a heterodimer of one p50 subunit and one p65 subunit. In unstimulated cells, NF-κB exists in an inactive, cytosolic form, bound to its inhibitor IκB. Upon stimulation of cells with a large variety of pathophysiological agents, including cytokines and reactive oxygen intermediates (such as hydrogen peroxide), the phosphorylation and ubiquitin-regulated proteolysis of IκB releases the active form of NF-κB, facilitating translocation of active NF-κB into the nucleus where it binds to DNA and controls gene expression.

There is no doubt that NF-κB activation in osteoclastic cells plays a critical role in the differentiation and activation of these cells. However, there is also indirect evidence that NF-κB may be involved in regulating the expression of important genes in osteoblasts. Decameric binding sites for NF-κB have been demonstrated within the promoter regions of genes encoding many stromal/osteoblastic factors involved in bone resorption, such as cytokines (reviewed in [7]), as well as COX-2 [8], iNOS [9] and matrix metalloproteinases-1 (MMP-1; collagenase [10]) and -9 (MMP-9; gelatinase 95 kDa) [11].

Therefore, we used a human osteoblastic (hOB) cell line (Saos-2) to screen a panel of structurally different, local and systemic resorptive stimuli (namely TNF-α, IL-1β, PTH and 1,25-$(OH)_2$ vitamin D_3) for their ability to activate NF-κB in hOBs. To show that any response was not a unique property of the Saos-2 cell line, we also tested the TNF-α effect on normal hOBs derived from trabecular bone surfaces.

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Abbreviations: NF-κB, nuclear factor κB; TRANCE, TNF-related activation-induced cytokine; RANK, receptor activator of NF-κB; ODF, osteoclast differentiation factor; OPG, osteoprotegerin; OCIF, osteoclastogenesis inhibitory factor; IL-, interleukin-; TNF-α, tumour necrosis factor-α; PTH, parathyroid hormone; PGE_2, prostaglandin E_2; COX-2, cyclooxygenase-2; NO, nitric oxide; iNOS, inducible nitric oxide synthase; 1,25-$(OH)_2$ vitamin D_3, 1,25-dihydroxyvitamin D_3; TCM, tissue culture medium; FCS, foetal calf serum; NE, nuclear extract; EMSA, electrophoretic mobility shift assay

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2. Materials and methods

2.1. Tissue culture materials and hormones

All tissue culture materials were obtained from Gibco BRL (Paisley, UK) unless otherwise stated. Tissue culture medium (TCM) was RPMI 1640 with HEPES (25 mM), supplemented with foetal calf serum (FCS) (10% v/v), L-glutamine (2 mM) and antibiotics penicillin (100 IU/ml) and streptomycin (100 μg/ml). Synthetic bovine PTH (1–34) fragment (bPTH (1–34); National Institute of Biological Standards and Control, NIBSC, South Mimms, UK) was dissolved in 1 mM acetic acid containing protease free 0.1% bovine serum albumin (Sigma, Poole, UK). The snap-frozen aliquots of bPTH (1–34), recombinant human TNF-α (rhTNF-α) and rhIL-1β (both from Boehringer-Mannheim, East Sussex, UK) were diluted in TCM immediately prior to addition to cultures. Ten μM stock of 1,25-$(OH)_2$ vitamin D_3 in isopropanol:α-MEM/10% FCS (25:100; v/v) (kindly provided by Dr. Martin Calverley, Leo Labs, UK) was serially diluted further in TCM prior to addition to cultures. The final concentration of isopropanol in samples with the highest 1,25-$(OH)_2$ vitamin D_3 concentration (10 nM) was 0.00025%.

2.2. Cell cultures

Saos-2 cells (passage numbers 18 and 19) were cultured with a seeding density of 1.5×10^6 cells/90 mm dish (Falcon) in TCM. When confluent, cells were treated with either rhTNF-α (2.5 pg/ml–25 ng/ml) or rhIL-1β (1 pg/ml–10 ng/ml) or synthetic bPTH (1–34) fragment (0.001–10 IU/ml) or 1,25-$(OH)_2$ vitamin D_3 (1 pM–10 nM) or their respective vehicles for various time periods ranging from 30 min to 6 h as stated in the figure legends.

Adult hOBs were obtained from trabecular bone chips of a 38 year old male with no metabolic bone disease. The bone sample was obtained during corrective orthopedic surgery. Osteoblasts, isolated and cultured as described previously [5], were removed from dishes by trypsinisation at confluency (approximately 1 month later), counted and used for experiments. With enzyme histochemistry, at least 60% of the isolated cells were found to be positive for alkaline phosphatase, a marker of osteoblastic cells. For experiments, primary osteoblastic cells were cultured at a seeding density of 3×10^6 cells/90 mm dish in TCM. At confluency (usually 24–48 h post cell seeding), TNF-α (10 ng/ml) was added to cultures for 1 and 4 h. Cultures were terminated as described below and nuclear proteins were extracted and analysed by electrophoretic mobility shift assays (EMSA).

2.3. Extraction of nuclear proteins

Cultures were terminated by washing cells (three times) in ice-cold phosphate-buffered saline and lysing them with 1 ml of RNA lysis buffer (0.6% NP-40, 150 mM NaCl, 10 mM Tris pH 7.9, 1 mM EDTA) directly in the tissue culture dish while standing on ice. Nuclear extracts (NE) were prepared by the method of Hoppe-Seyler et al. [12] and the aliquots of NE were kept at −70°C until assayed. The concentration of protein in NE was measured using a dye binding assay kit (Bio-Rad). By this method, a 90 mm dish of confluent Saos-2 cells would typically yield 25–30 μg protein in the NE fraction.

2.4. EMSA

Ten μg of NE was used per binding reaction (total volume of 20 μl) and was incubated with a ^{32}P-labelled synthetic double-stranded oli-

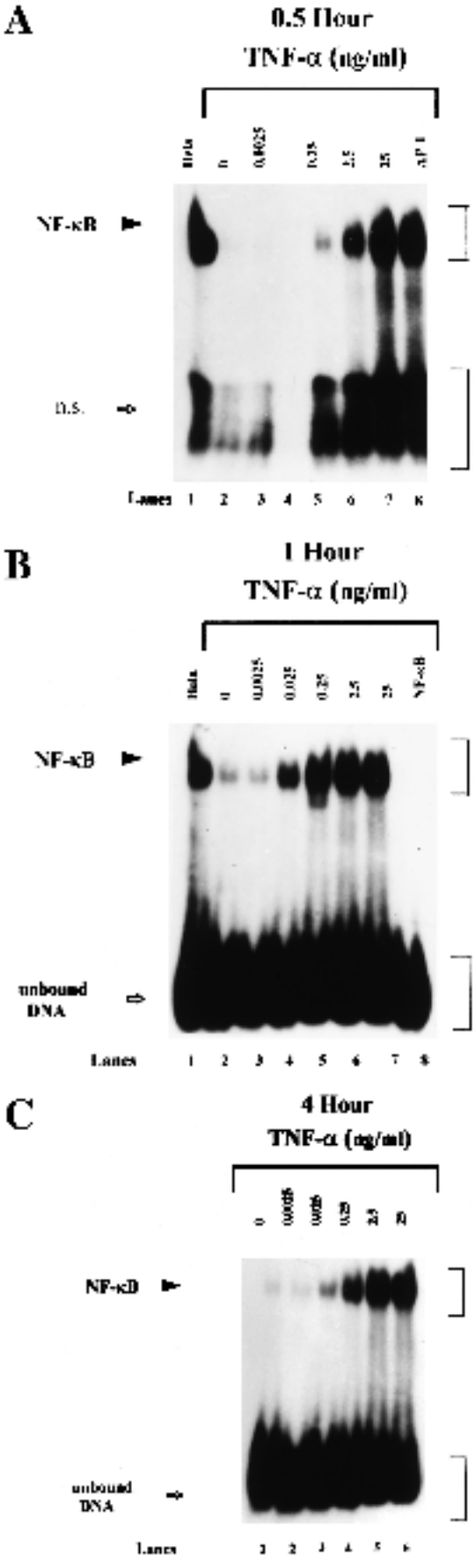

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Fig. 1. The dose-response and time-course effects of TNF-α on the activation of NF-κB in hOB cells in culture. A–C: EMSA of NE from Saos-2 cells cultured in the absence or presence of rhTNF-α (0.0025–25 ng/ml) for 30 min (A), 1 h (B) and 4 h (C). Lanes in A: 1: Hela nuclear protein extract (Hela NE); 2, 3, 5–7: TNF-α (ng/ml) at 0 (control), 0.025, 0.25, 2.5, 25, respectively; 8: 100-fold excess synthetic oligonucleotide containing the AP-1 DNA binding site incubated with NE from 25 ng/ml TNF-α. Lanes in B: 1: Hela NE; 2–7: TNF-α (ng/ml) at 0 (control), 0.0025, 0.025, 0.25, 2.5 and 25, respectively; 8: 100-fold excess of synthetic oligonucleotide containing the NF-κB DNA binding site incubated with NE from 25 ng/ml TNF-α. Lanes in C: 1–6: TNF-α (ng/ml) at 0 (control), 0.0025, 0.025, 0.25, 2.5 and 25, respectively. The dose-response relationship was reproduced in a total of eight separate experiments, each carried out at a time point of either 30 min, 1, 2, 4 or 6 h.

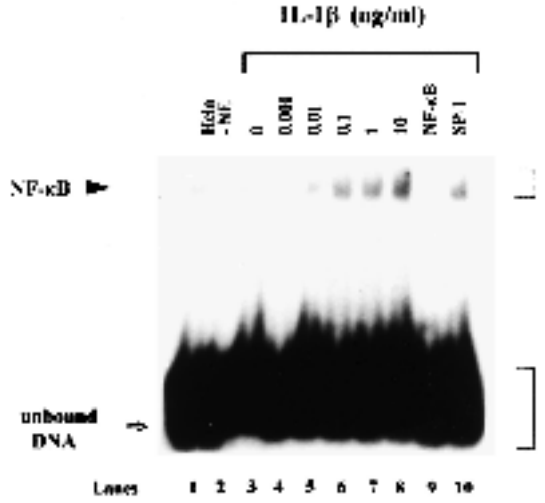

Fig. 2. The dose-response effect of IL-1β on the activation of NF-κB in hOB cells in culture. NE from cells cultured in the absence or presence of rhIL-1β (0.001–10 ng/ml) for 1 h were analysed by EMSA. Lane 1: Hela NE; lane 2: ^{32}P-labelled probe (synthetic oligonucleotide containing NF-κB binding site) without NE; lanes 3–8: IL-1β (ng/ml) at 0 (control), 0.001, 0.01, 0.1, 1.0 and 10, respectively; lane 9: 100-fold excess synthetic oligonucleotide containing the NF-κB binding site incubated with NE from 10 ng/ml IL-1β; lane 10: 100-fold excess of synthetic oligonucleotide containing the SP-1 binding site incubated with NE from 10 ng/ml IL-1β. The dose-response relationship was reproduced in two separate experiments.

gonucleotide having the consensus sequence specific for NF-κB (Promega, Southampton, UK). Binding reactions were carried out at room temperature and samples were electrophoresed on native 4% polyacrylamide/0.5×TBE gels as described (by Promega). Following electrophoresis, the gels were dried under vacuum for 45 min and exposed to films (GRI, Essex, UK) in the dark at −70°C for time periods between 7 h and 3 days.

As a positive control, Hela cell nuclear protein extract was used instead of the sample. The synthetic oligonucleotide with the complete NF-κB motif or oligonucleotide with SP-1 or AP-1 binding sites (Promega) were incubated with the NE for 10 min prior to the addition of the labelled probe in cold competition experiments. Other control reactions included those where the nuclear protein extracts were omitted from the binding reactions.

3. Results

The activation of NF-κB in osteoblastic cells in response to various resorptive stimuli was assessed by EMSA of NE prepared from Saos-2 cells. EMSA detects nuclear factor binding to a specific consensus sequence on double-stranded oligonucleotides. As shown in Figs. 1–3, treatment of Saos-2 cells

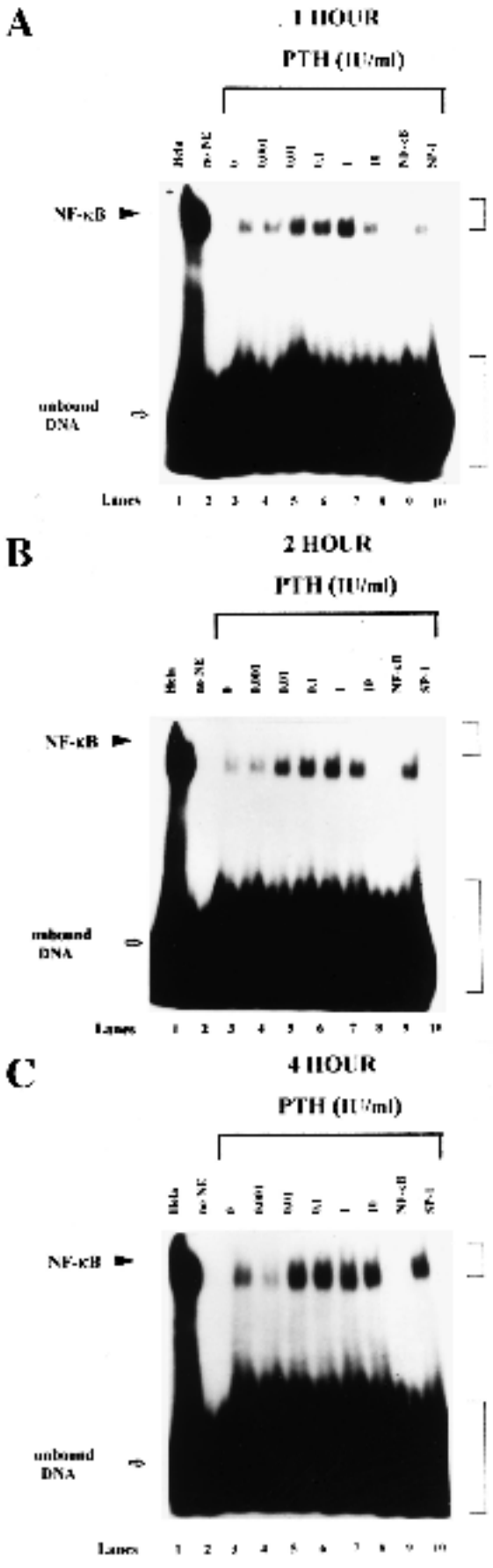

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Fig. 3. The dose-response and time-course effects of PTH on the activation of NF-κB in human Saos-2 osteoblastic cells in culture. A–C: EMSA of NE from Saos-2 cells cultured in the absence or presence of synthetic bPTH (0.001–10 IU/ml) for 1 h (A), 2 h (B) and 4 h (C). Lanes in A–C: 1: Hela NE; 2: ^{32}P-labelled NF-κB (synthetic oligonucleotide probe) without NE; 3, 5–8: PTH at 0 (control), 0.01, 0.1, 1.0 and 10 IU/ml, respectively; 9: 100-fold excess of unlabelled synthetic oligonucleotide containing the NF-κB binding site; 10: 100-fold excess of unlabelled synthetic oligonucleotide containing the SP-1 binding site. The dose-response relationship was reproduced in a total of five separate experiments, each carried out at a time point of either 1, 2 or 4 h.

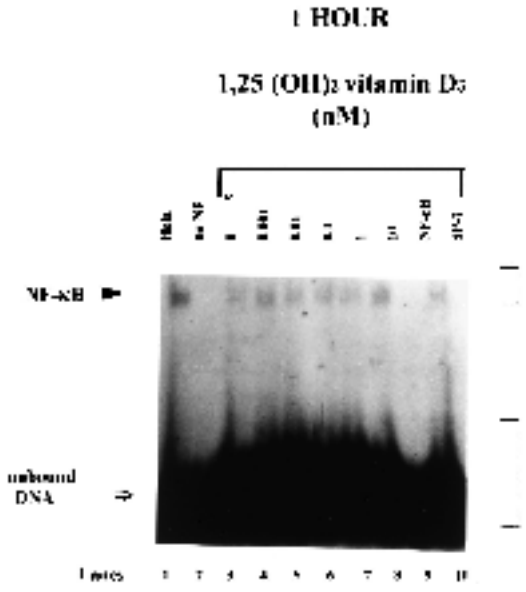

Fig. 4. The dose-response effect of 1,25-$(OH)_2$ vitamin D_3 on the activation of NF-κB in hOB cells in culture. NE from Saos-2 cells cultured in the absence or presence of 1,25-$(OH)_2$ vitamin D_3 (0.001–10 nM) for 1 h were analysed by EMSA. Lane 1: Hela NE; lane 2: ^{32}P-labelled NF-κB (synthetic oligonucleotide probe) without NE; lanes 3–8: 1,25-dihydroxyvitamin D_3 at 0 (control), 0.001, 0.01, 0.1, 1.0 and 10 nM, respectively; lane 9: 100-fold excess of unlabelled synthetic oligonucleotide containing the NF-κB binding site; lane 10: 100-fold excess of unlabelled synthetic oligonucleotide containing the SP-1 binding site. The absence of a dose-response relationship was reproduced in a total of four separate experiments, each carried out at a time point of either 1, 2 or 4 h.

with either rhTNF-α (0.025–25 ng/ml; Fig. 1A–C) or rhIL-1β (0.01–10 ng/ml; Fig. 2) or synthetic bPTH (1–34) fragment (0.01–10 IU/ml; Fig. 3A–C) resulted in time and dose-related increases in NF-κB-DNA binding. Competition experiments revealed the DNA binding to be specific for the NF-κB, since complexes were effectively competed off by excess of unlabelled NF-κB consensus oligonucleotide (Fig. 1B, lane 8) but not by non-competitive oligonucleotides containing binding sites for unrelated nuclear factors, such as AP-1 (Fig. 1A, lane 8) or SP-1 (Fig. 2, lane 10).

Activation of NF-κB was readily observed at the pro-resorptive concentrations of both cytokines, TNF-α, IL-1β, and PTH. NF-κB activation in response to TNF-α treatment was observed within 30 min of treatment (the shortest time examined) (Fig. 1A). It persisted for up to 6 h (the longest time tested), at which time slight reductions in NF-κB-DNA binding were detected (results not shown). Compared with untreated cultures, there was no visible change in the morphology of the TNF-α-treated cells, as examined by phase contrast light microscopy. This suggested that TNF-α was not cytotoxic to the cells at any of the concentrations tested. As shown in Fig. 2, the IL-1β-induced NF-κB activation was detectable at 0.01 ng/ml. The response to IL-1β was observed within 1 h (the shortest time examined) and persisted for over 4 h (the longest time tested, results not shown). Similar observations were made for PTH (Fig. 3A–C).

In contrast, 1,25-$(OH)_2$ vitamin D_3 (1 pM–10 nM) produced no effect on NF-κB activation at any time period studied (1–4 h; Fig. 4). This is consistent with the previously reported lack of effect of steroid hormones, including 1,25-$(OH)_2$ vitamin D_3, on Saos-2 cells [13].

In order to demonstrate that activation of NF-κB was not unique to the transformed nature of the Saos-2 osteosarcoma-derived osteoblastic cells, we tested the effect of TNF-α on primary cultures of trabecular bone surface-derived normal hOBs. Fig. 5 shows a time-dependent activation of NF-κB in normal hOBs treated with 10 ng/ml TNF-α for 1 and 4 h. In both the Saos-2 and primary hOB cell systems, TNF-α caused activation of NF-κB at similar doses and time periods (compare Fig. 1B,C to Fig. 5). We noted that, in contrast to primary hOBs, the unstimulated Saos-2 cells had detectable levels of activated NF-κB. Despite this background, we obtained dose and time-related responses from this cell line.

4. Discussion

In this study, we looked at NF-κB activation in osteoblasts by examining NF-κB-DNA binding in the NE obtained from Saos-2 cells and in normal hOB cells after they were treated with various resorptive agents. The tested range of concentrations represented those which induce bone resorption in vitro in murine and rat test systems (there are no human organ culture models of resorption).

We found that TNF-α, IL-1β and PTH, three structurally unrelated bone resorptive agents, activated NF-κB in hOBs. Although it is well recognised that TNF, IL-1 and PTH act through different receptors and intracellular signalling pathways, our results suggest that the activation of NF-κB in

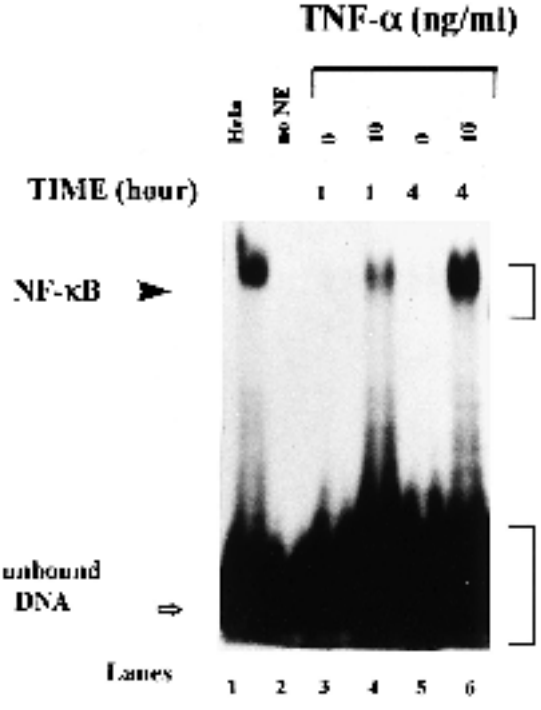

Fig. 5. The time-course effect of rhTNF-α on the activation of NF-κB in trabecular bone surface-derived hOB cells in culture. NE from cells cultured in a single experiment in the absence or presence of rhTNF-α (10 ng/ml) for 1 and 4 h were analysed by EMSA. Lane 1: Hela NE; lane 2: ^{32}P-labelled probe (synthetic oligonucleotide containing NF-κB site) without NE; lanes 3 and 5: 0 TNF-α (controls for 1 and 4 h TNF-α treatments, respectively); lanes 4 and 6: TNF-α at 10 ng/ml cultured for 1 and 4 h, respectively.

osteoblasts could be one common pathway for the activation of genes that will signal to osteoclasts, leading to resorption of bone. Of particular significance is our finding for PTH, which is known to bind to a single receptor on the osteoblast and exert its biological action via dual signal transduction pathways (reviewed in [14]), involving protein kinase A and protein kinase C. Short time-course (5 min–1 h) experiments will help to determine whether NF-κB activation is a primary event in the PTH signal transduction pathway.

The removal of the thin layer of osteoid from the bone surface by means of a collagenase-dependent process is thought to be crucial for osteoclast attachment and initiation of osteoclastic bone resorption [15]. The osteoblast mediation of PTH-induced resorptive activity is thought to involve several mechanisms. One of these is the up-regulation of collagenase (MMP-1) [16], the transcription of which may be regulated directly by NF-κB binding to its consensus sequence on the gene [10]. It is also noteworthy that an AP-1 binding site is present within the promoter of the collagenase gene [17]. Since an interaction between NF-κB and AP-1 may result in enhancement of the AP-1 response element function [18], it is possible that the PTH effect on the collagenase gene may also be via this interaction. PTH is also known to induce cytokine synthesis by the osteoblast [19,20].

Both IL-1 and TNF-α stimulate bone resorption and inhibit bone formation and they have been implicated in the pathogenesis of osteoporosis [2]. Using a mouse osteoblastic cell line, Kitajima et al. [21] have recently linked the induction of NF-κB activation by high levels of TNF-α (exceeding 100 U/ml) with the apoptosis of these cells. The authors suggested that this mechanism may explain bone thinning due to inhibition of bone formation seen in severe bone inflammation. In the present study, however, the activation of NF-κB in hOB cells occurred at much lower concentrations of TNF-α than those required to induce apoptosis.

Several studies have demonstrated that IL-1 and TNF-α induce both COX-2 and iNOS in osteoblasts, with the release of PGs and NO, respectively ([22] and references therein). Lader and Flanagan [23] have reported that PGs are essential for human osteoclast formation under most conditions. Furthermore, formation of human osteoclasts in vitro, stimulated by IL-1α and TNF-α, is mediated by PGE_2, which causes osteoclast differentiation via its effect on late osteoclast precursors [23]. PGE_2 was also found to stimulate the expression of messenger RNA for TRANCE in osteoblastic cells [3]. Recently, Chambers and co-workers [24] have shown that PGE_2 synergises with TRANCE to induce osteoclastic differentiation from haemopoietic precursors. There is evidence from murine osteoblastic cells that NF-κB is involved in the induction of COX-2 gene expression by TNF-α [25]. NF-κB also plays a role in the expression of the iNOS gene [9]. Furthermore, an interaction between COX-2 and iNOS pathways in murine osteoblastic cells has been reported [22]. IL-6 and intercellular adhesion protein-1 (ICAM-1) are also among the TNF-induced genes shown to be directly activated by NF-κB in rat osteoblastic cells (ROS17/2.8) [26]. Future work will identify which other 'bone resorptive genes' are directly activated by NF-κB in response to the above mentioned agents in hOBs, especially in primary cells.

Bax et al. [27] first reported that addition of hydrogen peroxide to the bone slice assay caused an increase in bone resorption by isolated osteoclasts and discussed the possible involvement of NF-κB in osteoclast activation. Thus, several recent studies into the role of NF-κB in bone resorption have focused on the activation of NF-κB in osteoclasts [28–30]. These included the demonstration that the targetted disruption of both p50 and p52 NF-κB subunits in mice resulted in osteopetrosis [29,30]. However, although the osteoclast plays a crucial role in the resorptive process, its generation and activity is controlled by cells of the osteoblastic lineage via the factors which they produce in response to bone resorption stimulators.

We suggest that, in addition to the proposed roles of NF-κB in the osteoclast, the activation of NF-κB in the osteoblast may also play an important role in bone resorption. This hypothesis is supported by (i) the results of our present study in which we show that three structurally unrelated bone resorptive agents, TNF-α, IL-1β and PTH, activate NF-κB in hOBs, (ii) recent observations in p50, p52 double gene knockout mice [29,30], in which the osteopetrotic phenotype was only partially rescued by bone marrow transplantation. This indicated that an additional contribution of the bone marrow microenvironment, especially of osteoblasts, could not be ruled out and (iii) the information discussed earlier that NF-κB is involved in the regulation of genes involved in bone cell signalling.

The activation of NF-κB is a multi-step process involving the generation of reactive oxygen intermediates, together with the phosphorylation, ubiquitination and proteolytic degradation of IκB. Specific inhibitors of each of these steps are available and new drugs targeted at NF-κB activation pathways are under development [31]. Such drugs could be useful in the treatment of pathological bone loss.

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