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Decoy oligodeoxynucleotides targeting NF-kappaB transcription factors: induction of apoptosis in human primary osteoclasts

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Abstract

Proteins belonging to the nuclear factor kappaB (NF-kappaB) superfamily are involved in osteoclast formation, playing a very important role for both differentiation of osteoclast precursors and survival of mature osteoclasts. Several drugs used to fight bone loss in a variety of human pathologies, including osteoporosis, act by increasing the frequency of osteoclast apoptosis, since it was demonstrated that small changes in osteoclast apoptosis can result in large changes in bone formation. In this respect, targeting of NF-kappaB transcription factor could be of great interest. Among nonviral gene therapy strategies recently proposed to inhibit or even block NF-kappaB activity, the transcription factor decoy (TFD) should be taken in great consideration. The main issue of the present study was to examine the effects of decoy DNA/DNA molecules targeting NF-kappaB on apoptosis of human osteoclasts (OCs), with the aim to interfere with the pathway regulating osteoclast differentiation and programmed cell death. To this aim, we used a mixture of receptor activator of NF-kappaB ligand (RANKL), macrophage colony-stimulating factor (M-CSF) and parathyroid hormone (PTH) to prepare human OCs from peripheral blood cells. Then, transfection with the decoy molecules targeting NF-kappaB was performed. The results obtained demonstrate that in primary cells expressing typical osteoclast markers such as TRAP and MMP9, NF-kappaB decoy significantly stimulated apoptosis. Inhibition of IL-6 expression and induction of Caspase 3 were found in OCs treated with NF-kappaB DNA/DNA decoys. We consider these data as the basis for setting up experimental conditions allowing nonviral gene therapy of several bone disorders.

Keywords: Human osteoclasts; Apoptosis; Nuclear factor kappaB; Transcription factor decoy; Nonviral gene therapy

1. Introduction

Osteoclasts are multinucleated cells of hematopoietic origin responsible for bone resorption [1]. The process governing both differentiation and activation of osteoclasts involves supporting cells as well as several growth factors and hormones able to regulate the activity of transcription factors; however, the detailed molecular interplay implicated in these processes still remain elusive [2–4]. Recently, several genes, including cathepsin K, macrophage colony-stimulating factor (M-CSF) , osteoclast differentiation factor, c-fos, NF-kappaB and c-src, have been reported to be essential for osteoclasts differentiation and activation, even if, at present, the role of their interactions is not clear [1–4]. In this respect, accumulating evidences indicate that NF-kappaB is associated with activation of osteoclasts and is important for both differentiation of osteoclasts precursors and survival of mature osteoclasts [5–8].

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Abbreviations: OCs, osteoclasts; MITF, microphthalmia transcription factor; NF-kappaB, nuclear factor kappaB; TNF-α, tumour necrosis factor alpha; EMSA, electrophoretic mobility shift assay; TFD, transcription factor decoy; ODN, oligodeoxyribonucleotide; RANKL, receptor activator of nuclear factor kappaB ligand; M-CSF, macrophage colony-stimulating factor; PTH, parathyroid hormone; PBMC, peripheral blood mononuclear cells; TRAP, Tartrate-resistant acid phosphatase; PC, phosphatidylcholine; DOTAP, *N*[-1-(2,3-dioleyloxy)propyl]-*N*,*N*,*N*-trimethylammonium methylsulfate.

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Several drugs used to fight bone loss in a variety of disorders, such as osteoporosis, act by increasing the frequency of apoptosis of osteoclasts, since it was demonstrated that small changes in osteoclasts apoptosis can result in large changes in bone formation [7–10]. In this respect, targeting of NF-kappaB transcription factors could be of great interest. Several recent studies indicate indeed that NF-kappaB activation can block cell-death pathways [11,12]. For instance, it was shown that NF-kappaB activation is required to protect cells from the apoptotic cascade induced by TNF and other stimuli [13,14]. Therefore, experimental strategies aimed at the inhibition of NF-kappaB activity could greatly facilitate therapeutical approaches to osteopenic disorders.

Among nonviral gene therapy strategies able to inhibit or even block NF-kappaB activity, the transcription factor decoy (TFD) approach [15,16] should be taken in great consideration, since NF-kappaB decoys have been recently used in several preclinical studies and proven to be active in several experimental systems [17–21]. For instance, Nakamura *et al.* recently demonstrated prevention and regression of atopic dermatitis by ointment containing NF-kappaB decoy oligodeoxynucleotides in the NC/Nga atopic mouse model [22]; in addition, decoy ODN were demonstrated to be useful to inhibit tumour cell growth and invasion [23,24], as anti-inflammatory agents [25,26], in myocardial preservation [27–30], in cerebral angiopathy [31]. Accordingly, TFD molecules targeting NF-kappaB transcription factors might bring opportunities for a specific and controlled bone formation with interrupted bone resorption.

The main issue of the present study was to examine the effects of decoy ODNs against NF-kappaB on apoptosis of primary human osteoclasts (OCs), in order to find experimental conditions leading to alteration of the pathway regulating osteoclast differentiation, activation and programmed cell death. To this aim, we used a mixture of receptor activator of nuclear factor kappaB ligand (RANKL), M-CSF and parathyroid hormone (PTH), to develope a method, slightly modified from that reported by Matsuzaki *et al.* [32], for preparation of human OCs from peripheral blood mononuclear cells. Then, transfection with the decoy molecules and analysis of the biological effects of the DNA treatment were performed.

2. Materials and methods

2.1. Culture of human primary OCs and OCs cell line

Human OCs were prepared as reported by Matsuzaki *et al.* [32] with slight modifications. Briefly, peripheral blood was collected from healthy normal volunteers after informed consent. Mononuclear cells (PBMCs) were prepared from diluted peripheral blood (1:2 in Hank's Balanced Salt Solution), which was layered over Histopaque 1077 (Sigma) solution, centrifuged (400 *g*), washed and resuspended in D-MEM/10% FCS. 3×10^6 PBMCs/cm^2 were plated in 24-well plates or in chamber slides and allowed to settled for 2 hr; wells were then rinsed to remove nonadherent cells. Monocytes were maintained at 37°, in 5% CO_2, in medium supplemented with 10% FCS and cultured for 14 days in the presence of human M-CSF (25 ng/mL), RANKL (30 ng/mL) and 10^{-7} M PTH. Culture media were replenished with fresh media every 3–4 days. Cells were used for the described experiments when mature multinuclear cells were predominant in the cultures. GCT23 osteoclast-like cell line [33] were maintained in Iscove's medium supplemented with 10% FCS, 100 U/mL penicillin, 100 μg/mL streptomycin and 2.5 μg/mL amphotericin at 37°, in 5% CO_2.

2.2. Tartrate-resistant acid phosphatase (TRAP) staining

TRAP staining of the cells was performed as reported by Villanova *et al.* [34]. Cells were fixed in 3% paraformaldehyde with 0.1 M cacodilic buffer, pH 7.2 (0.1 M sodium cacodilate, 0.0025% $CaCl_2$) for 15 min, extensively washed in the same buffer, and stained for TRAP (Acid Phosphatase Kit n. 386; Sigma). After washing with distilled water and drying, mature TRAP positive multinucleated cells containing more than three nuclei were counted as osteoclasts.

2.3. Immunocytochemistry analysis

Immunocytochemistry analysis was performed employing the streptavidin–biotin method using Ultraystain Polyvalent-HRP Immunostaining Kit. OCs cells grown in multichamber slides were fixed in 100% cold methanol, and permeabilized with (v/v) Triton X-100 (Sigma) in TBS (Tris-buffered saline). Cells were incubated in 3% H_2O_2 and blocked with Super Block reagent (Ultraystain Polyvalent-HRP Immunostaining Kit). After the reaction with the primary antibodies, rabbit polyclonal antibodies of human origin (Santa Cruz Biotech) against MMP9, NF-kappaB, IL-6 [35] and Caspase 3 [36] (200 μg/mL) were used accordingly to the manufacturer's protocols, at 1:500 (MMP9), 1:250 (NF-kappaB), 1:800 (IL-6) and 1:800 (Caspase 3) dilutions; incubation was carried out at 4° for 16 hr. Cells were then incubated at room temperature with anti-polyvalent Biotinylated Antibody (Ultraystain Polyvalent-HRP Immunostaining Kit). After rinsing in TBS, Streptavidin HRP (Ultraystain Polyvalent-HRP Immunostaining Kit) was applied, followed by the addition of Substrate-chromogen mix (AEC Cromogeno kit). After washing, cells were mounted in glycerol/TBS 9:1 and observed using a Leitz microscope.

2.4. Preparation of nuclear extracts from human OCs

Nuclear extracts were prepared from GCT23 cells [33] essentially as described by Dignam *et al.* [37]. The cells

were washed twice with PBS by scraping. After homogeneization with a Dounce B homogeneizer, nuclear proteins were obtained and protein concentration was determined using a Bio-Rad protein assay. Nuclear extracts were brought to a concentration of 0.5 μg/μL for electrophoretic mobility shift assay (EMSA) experiments.

2.5. Electrophoretic mobility shift assay

The EMSA [38] was performed by using the double-stranded synthetic oligonucleotides mimicking the NF-kappaB binding sites present within the promoters of microphthalmia transcription factor (MITF) (sense strand 5′-TGG ATT GGA GTT TCC AGG G-3′) and tumour necrosis factor alpha (TNF-α) (sense strand 5′-CGC TGG GGA CTT TCC ACG G-3′) genes (comparison between these oligonucleotide sequences and the NF-kappaB decoy ODN is shown in Fig. 2). The synthetic oligonucleotides were 5′-end labeled using [γ-^{32}P]ATP and T4 polynucleotide kinase (MBI Fermentas). Binding reactions were set up as described elsewhere [34–36] in a total volume of 25 μL containing buffer TF plus 5% glycerol, 1 mM DTT, 10 ng of human NF-kappaB p50 protein (Promega Corporation) and 0.25 ng of ^{32}P-labeled oligonucleotides. In the case of EMSA employing nuclear factors from human OCs, they were used at a concentration of 1 μg per reaction and poly(dI:dC) (1 μg per reaction) was also added to decrease nonspecific binding. After 5 min binding at room temperature, the samples were electrophoresed at constant voltage (200 V) under low ionic strength conditions (0.25× TBE buffer = 22 mM Tris–borate, 0.4 mM EDTA) on 6% polyacrylamide gels. Gels were dried and subjected to standard autoradiographic procedures [38,39]. In competition experiments, the competitor double-stranded DNA molecules were preincubated for 20 min with purified NF-kappaB p50 factor, before the addition of labeled target DNA. The sequences of the competitor double-stranded target DNAs used were 5′-CGC TGG GGA CTT TCC ACG G-3′ (sense strand, HIV-1 NF-kappaB binding site), 5′-TAA TAT GTA AAA ACA TT-3′ (sense strand, NF-IL-2A), 5′-CAC TTG ATA ACA GAA AGT GAT AAC TCT-3′ (sense trand, GATA-1) and 5′-CAT GTT ATG CAT ATT CCT GTA AGT G-3′ (sense strand, STAT-1) [38,39].

2.6. DNA transfection and decoy experiments

OCs cells, plated at the density of 3×10^6 cm^{-2} in multichamber slides, as indicated, were transiently transfected with 0.5, 1 and 2 μg/mL of DNA complexed with 2.5, 5 and 10 μg of PC:DOTAP, respectively, for 24, 48 and 72 hr, performing the decoy experiments with the protocol previously described [40,41]. For NF-kappaB decoy experiments, double-stranded oligonucleotides mimicking nonsymmetric HIV-1 NF-kappaB binding site were used (5′-CGC TGG GGA CTT TCC ACG G-3′).

2.7. Cytotoxicity studies

The cytoxicity effect of PC:DOTAP cationic liposomes complexed with scrambled oligonucleotide was determined on *in vitro* cultured human OCs cells. 3×10^6 cm^{-2} PBMCs were plated in 96-well plates and, after 14 days, OCs were incubated with PC:DOTAP/DNA complex at different concentration (0.15, 0.3, 1 and 2 μg/mL) for 3 days. Determinations of viable cells after DNA treatment were performed after colorimetric assay with MTT (thiazolyl blue). The assay, based on the conversion of the yellow tetrazolium salt MTT to purple formazan crystals by metabolically active cells [43], provides a quantitative determination of viable cells. After 72 hr of treatment with DNA decoy molecules in triplicate, 200 μL of MTT was added to each well of cells, and the plate was incubated for 2 hr at 37°. The medium was removed, and the MTT crystals were solubilized with 50% DMF: spectrophotometric absorbance of each sample was then measured at 570 nm.

2.8. Measurement of apoptosis

After 14 days of cell culture and 2–4 days of decoy experiments, the cells were rinsed two times with PBS solution and fixed for 25 min in 4% paraformaldehyde at room temperature. Apoptotic cells were detected by the DeadEnd Colorimetric Apoptosis Detection System (Promega) according to the manufacturer's instructions. Measurement of apoptosis was calculated as a percentage of apoptotic nuclei (dark brown nuclei) versus total nuclei of multinucleated TRAP positive cells, evaluated in three independent experiments.

2.9. Statistical analysis

Data are presented as the mean ± SEM from at least three independent experiments. Statistical analysis was performed by one-way analysis of variance followed by the Student's *t*-test. A *P*-value <0.001 was considered statistically significant.

3. Results and discussion

3.1. Two-phase culture of human primary osteoclasts

After 14 days of cell culture in the presence of a combined treatment with RANKL, M-CSF and PTH, the cells were analysed for the expression of the osteoclast-associated enzyme TRAP. Fig. 1 shows the proportion of TRAP-positive cells in four independent experiments, demonstrating that OCs are reproducibly obtained in these experimental conditions. Most of them were multinucleated and likely represent prefusion osteoclasts, since they also express the MMP9 osteoclast-associated antigen [43], being on the contrary negative to immunostaining

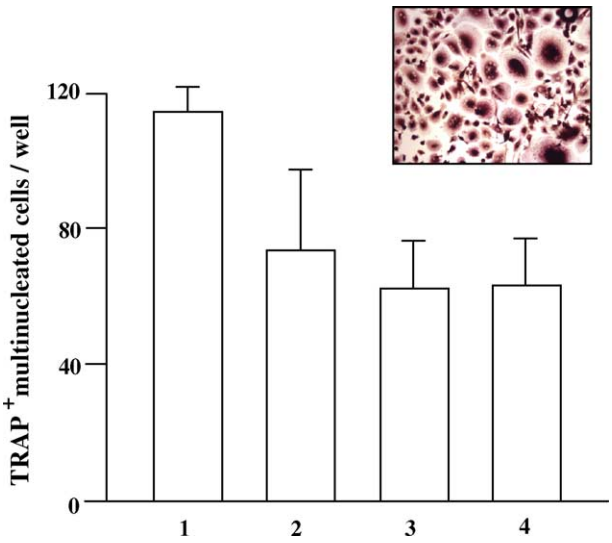

Fig. 1. Primary human osteoclasts cultures. Primary osteoclasts cultures, prepared as described in Section 2, were treated for 14 days with 25 ng/mL M-CSF plus 30 ng/mL RANKL and PTH 10^{-7} M and subsequently stained for TRAP (insert). Multinucleated (>3 nuclei), TRAP-positive cells obtained in four independent experiments are shown. Cells were counted in three separated wells (data are average ± SEM). The TRAP + cells represent 5–10% of total number of cells.

with monoclonal antibody GSR1, recognizing the macrophage-associated antigen CD14, not expressed by osteoclasts (data not shown) [44].

3.2. *In vitro effects of the HIV-1 NF-kappaB decoys on interactions of NF-kappaB transcription factors to ^{32}P-labeled NF-kappaB binding sites*

Double stranded decoy molecules mimicking the NF-kappaB binding sites (sense strand 5′-CGC TGG GGA CTT TCC ACG G-3′) present in the long terminal repeat (LTR) of the human immunodeficiency type 1 virus (HIV-1) are known to be able of suppressing the interactions between NF-kappaB transcription factors and target NF-kappaB binding sites, exhibiting a differential efficiency in relation to the NF-kappaB site [39]. These HIV-1 NF-kappaB decoys inhibit with high efficiency interactions of NF-kappaB transcription factors with nonsymmetric binding sites (as those present within the HIV-1 LTR and in the promoter sequences of several eukaryotic genes) [39,45–50], exhibiting lower inhibitory activities on symmetric NF-kappaB binding sites (as those present in Ig-k enhancers) [39]. This behaviour is also obtained using as model system NF-kappaB binding sites from the promoter of two genes expressed by osteoclasts, such as MITF [45] and TNF-α [46] (Fig. 2). The homology of HIV-1, MITF and TNF-α NF-kappaB sites is shown in Fig. 2A. Fig. 2B and C shows the effects of the NF-kappaB decoy of binding of NF-kappaB p50 to ^{32}P-labeled DNA/DNA carrying MITF (Fig. 2B) and TNF-α (Fig. 2C) NF-kappaB binding sites. As it is clearly evident, suppression of interactions between NF-kappaB factor and ^{32}P-labeled MITF NF-kappaB DNA is obtained using 3–6 ng per reaction of HIV-1 NF-kappaB decoy (Fig. 2B). By sharp contrast, some interaction between NF-kappaB factor and ^{32}P-labeled TNF-α NF-kappaB DNA is still evident using 400 ng per reaction of HIV-1 NF-kappaB decoy. In conclusion, the data shown in Fig. 2 demonstrate that the activity of the HIV-1 NF-kappaB decoy affects mostly interaction between proteins belonging to NF-kappaB superfamily and nonsymmetric NF-kappaB binding sites. This was confirmed using oligonucleotides mimicking the NF-kappaB binding sites present in the human HLA-DRA [50], IL-2 [48] and interferon-β [48] genes.

Since p50 acts in total cellular extracts as an heterodimer [47], crude nuclear extracts, including those from human OCs were employed to confirm the results obtained with purified NF-kappaB p50. We employed to this aim the osteoclast-like cell line, GCT23, obtained from a human giant cell bone tumour previously characterized for its osteoclast phenotype [33]. This was done for two reasons: the first is related to the difficulty in obtaining sufficient amounts of nuclear extract from human primary OCs. The other reason is that, in any case, the proportion of primary OCs was found to be low (usually not exceeding 5–10% of the total number of cells produced by the two-phase cell culture), thus rendering difficult the interpretation of the EMSA assay. On the contrary, the percent of GCT23 cells exhibiting an osteoclast phenotype was found to be 95–98%. The results obtained shown in Fig. 2 show that nuclear factors from GCT23 cells exhibit binding activity to NF-kappaB binding sites (Fig. 2D). Fig. 2E and F confirms the data presented in Fig. 2B and C, showing that HIV-1 NF-kappaB decoys inhibit the binding of ^{32}P-labeled MITF and TNF-α double stranded target oligonucleotides to nuclear extracts from GCT23 cells. Also in this case, the inhibitory activity of HIV-1 NF-kappaB was found to be lower than that of TNF-α NF-kappaB (Fig. 2F and C). Control experiments demonstrate that HIV-1 NF-kappaB decoys do not inhibit the binding of nuclear factors to ^{32}P-labeled NF-IL-2A, GATA-1 and STAT-1 mers (data not shown).

The finding that OCs nuclear factors exhibit NF-kappaB binding activity is important, since it justifies investigations aimed at determining in this cellular system biological effects of decoy molecules against NF-kappaB-related proteins.

3.3. *Effects of the HIV-1 NF-kappaB decoy on primary human osteoclasts*

Since the transcriptional factor NF-kappaB has been reported to be important for the expression of several osteoclast-specific genes, we tried to alter osteoclast development by inhibiting NF-kappaB action by the decoy approach. The effects of the double-stranded decoy ODN targeting NF-kappaB was analysed on apoptosis of isolated multinucleated cells in terms of apoptosis. We treated primary OCs with the NF-kappaB HIV-1 decoy molecule

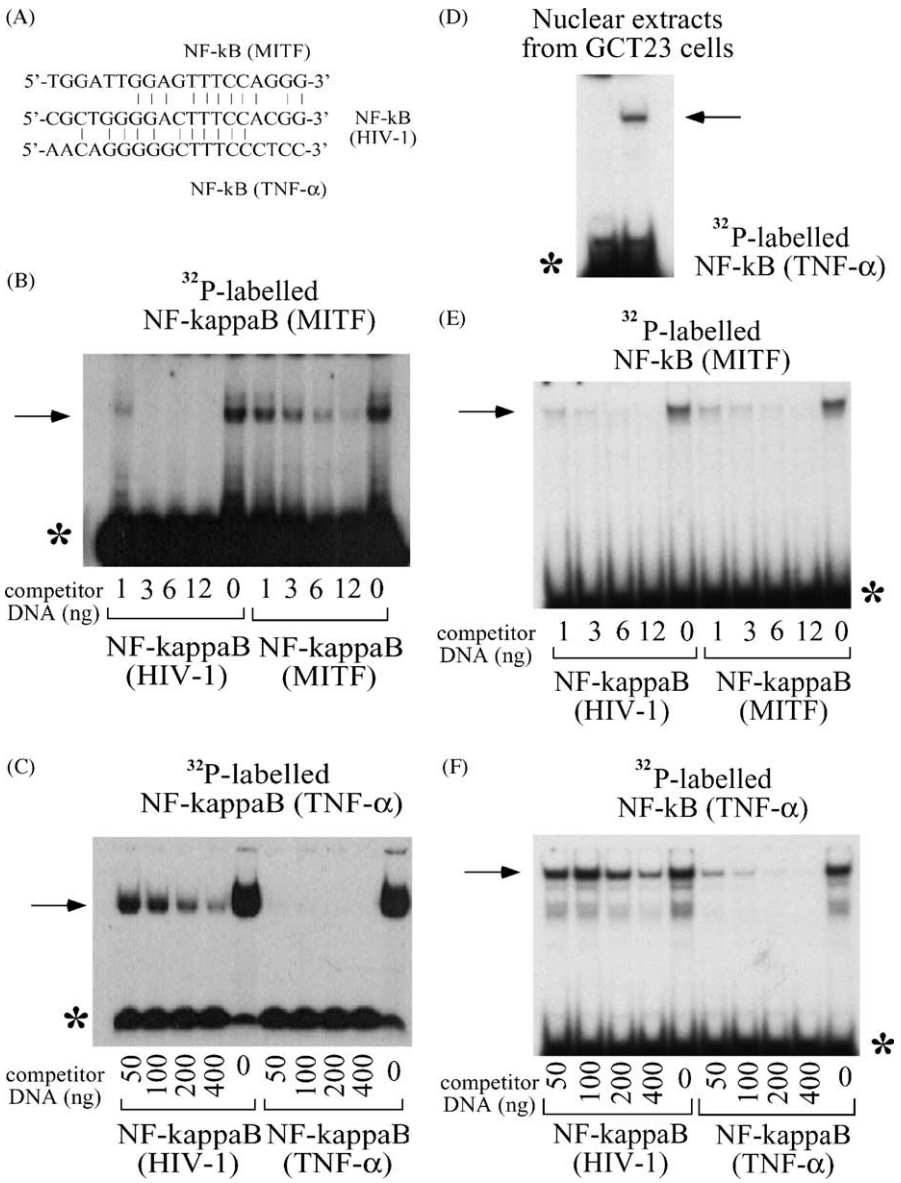

Fig. 2. (A) Sequence of the employed ODN carrying NF-kappaB binding sites. (B and C) The effects of the HIV-1 NF-kappaB decoy on interactions between purified NF-kappaB p50 transcription factors with ^{32}P-labeled DNA/DNA molecules carrying nonsymmetric MITF (B) and symmetric TNF-α (C) NF-kappaB binding sites. (D–F) Effects of HIV-1 NF-kappaB decoy on DNA/protein interactions when crude nuclear extracts are used. (D) Nuclear extracts from the human GCT23 osteoclast-like cell line were demonstrated to interact with the ^{32}P-labeled DNA/DNA molecules carrying TNF-α NF-kappaB binding sites. The effects of HIV-1 LTR, MITF and TNF-α cold competitors on nuclear factors binding to ^{32}P-labeled DNA/DNA molecules carrying MITF (E) and TNF-α (F) NF-kappaB binding sites are also shown. In all the competition experiments, NF-kappaB p50 (B and C) or GCT23 nuclear extracts (D–F) were pre-incubated for 30 min with the indicated amounts of HIV-1, MITF and TNF-α NF-kappaB DNA/DNA molecules and then target ^{32}P-labeled DNA/DNA molecules were added as indicated and the binding reactions carried out for further 30 min. After binding, the complexes were separated by electrophoresis. Protein/DNA complexes are shown by arrows. Asterisks (∗) indicate free ^{32}P-labeled DNA/DNA molecules.

complexed with PC:DOTAP cationic liposomes using DNA and lipid dosages previously determined in other cells types [37] to retain functional activity without affecting cell viability. In any case, since data from literature indicate that a same lipid formulation can display a differential toxicity on different cultured cells, a first set of experiments was addressed to investigate whether the employed liposomal formulations display cytotoxicity in osteoclasts (Fig. 3). The adherent cells were treated with different amounts of cationic liposomes in combination with unrelated (scrambled) ODN, and viable cells were quantified by a colorimetric assay with thiazolyl blue (MTT assay) [42]. The percentage of surviving cells after treatment is reported in Fig. 3. Notably, treatment with 1 μg/μL of DNA/liposome complex failed to yield any cytotoxic effect on the total cell population and on osteoclasts (see Fig. 3B and C, respectively), indicating that the conditions employed for the decoy approach did not decrease cell viability.

The same experimental conditions were employed using the HIV-1 NF-kappaB decoy. Mature osteoclasts and

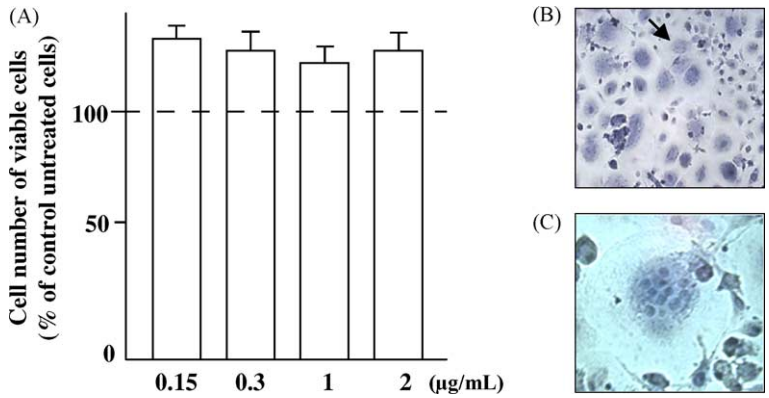

Fig. 3. Effect on cell survival of PC:DOTAP cationic liposome complexed with scrambled oligonucleotide at different concentrations (0.15, 0.3, 1 and 2 μg/mL). (A) The data obtained by counting the viable cells (up to 3 days of cell culture) with a colorimetric assay based on MTT (see Section 2) are reported. Results are expressed as number of viable cells and are the mean ± SEM of three independent experiments ($P < 0.05$). For analysis of cell morphology, phase-contrast images of total cell population and single osteoclast, after liposome/DNA treatment (2 μg/mL), are shown in panel B (original magnification 20×) and panel C (original magnification 80×), respectively.

TRAP-positive precursors were treated with 1 μg/μL of the decoy molecules for 48 hr and then subjected to apoptosis analysis. Morphological analysis of NF-kappaB decoy-treated osteoclasts demonstrated cell retraction in comparison with unrelated ODN control-treated osteoclasts (see Fig. 4) indicative of apoptosis. Several nuclei showed morphological changes consistent with nuclear damage. Therefore, after treatment with the decoy against NF-kappaB on day 14 (when osteoclasts differentiation was complete), a deep change in cell survival of osteoclasts was observed.

To confirm apoptosis, TUNEL staining of fragmented DNA was performed. As shown in Fig. 4 NF-kappaB decoy caused a programmed cell death at a very early stage with more than 13-fold increase in the percentage of apoptotic osteoclasts (Table 1).

We next examined whether ODNs modulate induced apoptosis when added at different times with different doses. Fig. 5 shows the time-course and the dose dependence of the effect of NF-kappaB decoy on the stimulation of the apoptosis. There was not a significative difference in the number of osteoclasts with TUNEL-positive nuclei at different time of decoy treatment, but the increased dose of ODN up to 2 μg/μL caused a significant increase in the apoptotic cells.

In order to provide additional data related to apoptotic pathways involved, the decoy-treated cells were immunostained with antibody directed against Caspase 3 [36]. As shown in Fig. 6, multinucleated osteoclasts were strongly positive for Caspase 3 after the decoy treatment.

Table 1
Apoptosis analysis

Treatment	Mean% ± SEM of osteoclasts with TUNEL-positive nuclei
Control	6.6 ± 1.75
PC:DOTAP	5.16 ± 2.35
PC:DOTAP + scrambled decoy	6.5 ± 3.12
PC:DOTAP + NF-kappaB decoy	79.3 ± 3.84*

* $P < 0.001$.

Therefore, NF-kappaB decoys activate, in these non-proliferating terminally differentiating cells, the early pathway of apoptosis. In order to determine whether the

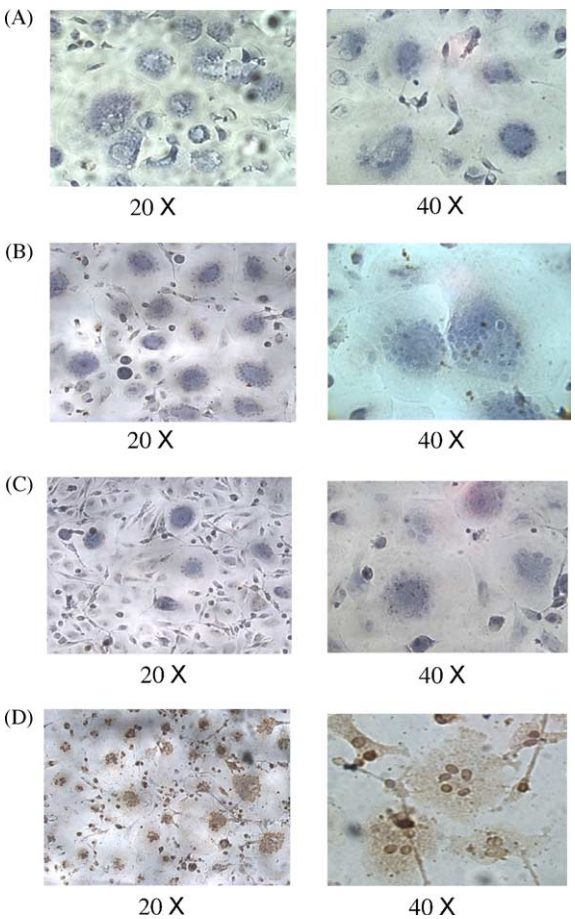

Fig. 4. Detection of apoptosis by TUNEL assay in human osteoclasts cells. Cells, cultured as described in legend to Fig. 3, were transiently transfected with liposomes alone (B), 1 μg of scrambled oligonucleotide complexed with cationic liposome (C), 1 μg of NF-kappaB decoy oligonucleotide complexed with cationic liposome (D) or remained untreated (A). Cells were then photographed at the 40× magnification.

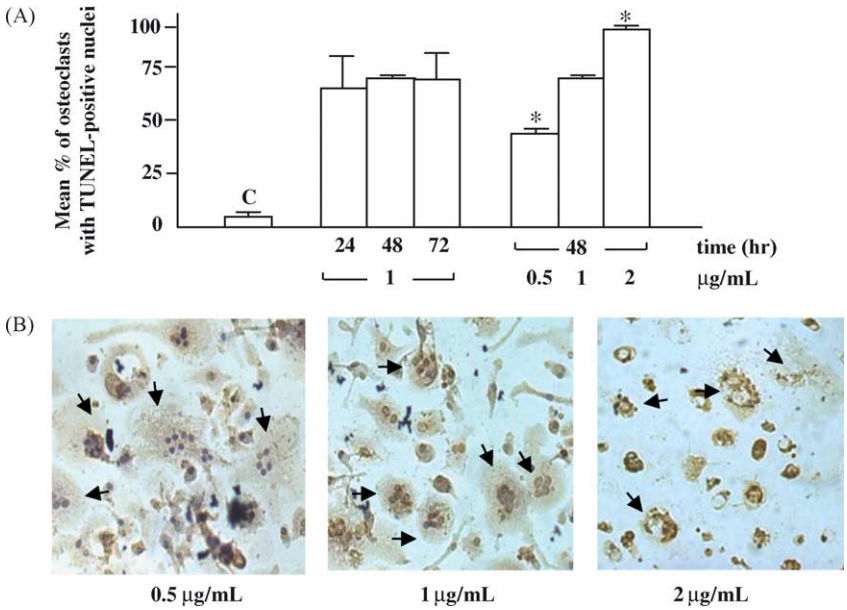

Fig. 5. Time and dose-dependence of the stimulation of apoptosis. Cells were transiently transfected for 24, 48 and 72 hr with 1 μg of NF-kappaB decoy oligonucleotide complexed with cationic liposome and with 0.5, 1 and 2 μg of NF-kappaB decoy oligonucleotide for 48 hr. The mean% ± SEM of osteoclasts with TUNEL-positive nuclei is reported in the graph of the panel A ($^{*}P < 0.001$ with respect to treatment with NF-kappaB decoy at 1 μg/μL for 48 hr). The presence of apoptotic osteoclasts after treatment with different doses of NF-kappaB decoy oligonucleotide for 48 hr is shown in the panel B (20× magnification).

apoptotic pathway induced by HIV-1 NF-kappaB decoy is associated with inhibition of NF-kappaB activity, two experiments were performed. In the first, the efficiency of NF-kappaB decoys was evaluated on the expression of IL-6, a typical target of NF-kappaB [35]; in the second, accumulation of NF-kappaB-related proteins was determined. The results obtained demonstrate that treatment with NF-kappaB decoy do not affect NF-kappaB content, causing on the contrary a strong decrease of the expression of IL-6.

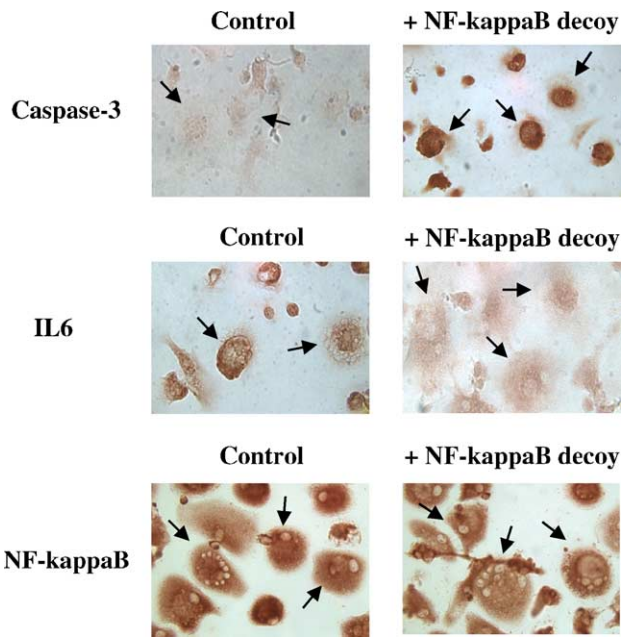

Fig. 6. Differential expression of Caspase 3 (upper panel), IL-6 (middle panel) and NF-kappaB (lower panel) in human osteoclasts analysed by immunocytochemistry with specific antibodies after incubation with liposomes alone (control) or 1 μg of NF-kappaB decoy oligonucleotide complexed with cationic liposome (+NF-kappaB decoy) for 48 hr.

These results give informations on the mechanism of action of the NF-kappaB decoy ODNs on human OCs. First, the evidence that no inhibition of accumulation of NF-kappaB proteins occurs during treatment (Fig. 6, lower panel) allow us to exclude direct effects of NF-kappaB decoys on NF-kappaB mRNA accumulation and translation. Second, the strong inhibition of IL-6 expression (Fig. 6, middle panel) suggest that NF-kappaB dependent biological functions are impaired, possibly by inhibition of the molecular interactions between NF-kappaB and target DNA sequences (Fig. 2).

In conclusion, these results have both theoretical and practical implications. From the theoretical point of view, they suggest that NF-kappaB is an important transcription factor in the regulation of viability of precursors and mature OCs. From the practical point of view, they firmly demonstrate that NF-kappaB decoy administration increased the prevalence of osteoclasts apoptosis.

3.4. Perspectives

The analysis of the activity of OCs after specific pharmacological treatments has been limited by difficulties in isolating these cells from the human skeleton. Therefore, OCs formed from cultures of peripheral blood mononuclear precursors represent an useful experimental model

system for gene expression analysis and pharmacological modulation of the phenotype. The technique, herein described, of OC isolation in combination with the decoy approach, is expected to allow the precise determination of the role of cellular and specific transcription factors on osteoclast formation and activity. In addition, at least in theory, it should also possible to set up experimental conditions that may be transferred, in a second step, in the study of specific bone disorders and in clinical orthopedics and orthodontics.

In this respect, it should be underlined that bone resorption and remodelling is an highly complex physiological process requiring the function of osteoclasts. Production and differentiation of these cells are regulated by multiple local factors and different hormones. It is well known that the appearance of osteoclasts at specific sites in alveolar bone is essential not only to bone turnover, but also to orthodontic tooth movement [51,52]. In fact, orthodontic tooth movement depends on alveolar bone remodelling. This highly active process induces site-specific bone formation and resorption by cytokines released from periodontal ligament (PDL) cells [53]. There are several evidences that osteoclast precursors are recruited through blood vessels in the periodontium which leads to formation of functional osteoclasts. A complete understanding of the biological processes accomplishing osteoclasts formation and recruitment may have an important impact also in clinical orthodontics. By using the decoy approach here described, we could be able to monitor and alter osteoclast life spans, controlling the efficiency of tooth movement through increase of portions of bone promoting osteoclast apoptosis by decoy against NF-kappaB. Even if further studies addressing (a) the identification of candidate genes regulated by NF-kappaB important for osteoclast survival and (b) the mechanism by which NF-kappaB inhibition stimulates osteoclast apoptosis (including the detailed analysis of the effects of NF-kappaB decoys on all the pro-apoptotic and apoptosis-associated genes) are necessary, we suggest that the induction of apoptosis after NF-kappaB decoy treatment obtained in primary osteoclasts from peripheral blood, could be also obtained in other tissue environments and used in the control of bone formation. Accordingly, induction of apoptosis in osteclasts could be a strategy for the treatment of a large variety of osteopenic diseases, such as osteoporosis (including that associated to hereditary diseases such as thalassemia), arthritis, Paget syndrome, tumour-associated osteolytic metastases [54,55].

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