Local *in vivo* administration of a decoy oligonucleotide targeting NF-κB induces apoptosis of osteoclasts after application of orthodontic forces to rat teeth

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Abstract. In this study, we report the *in vivo* effects of a decoy oligonucleotide targeting the nuclear factor κB (NF-κB) on osteoclasts during forced orthodontic tooth movement in rats. Wistar rats were subjected to orthodontic forces, in the absence or presence of treatment with a decoy molecule mimicking a nonsymmetric NF-κB binding site (5'-CGC TGG GGA CTT TCC ACG G-3'). TUNEL staining of fragmented DNA revealed that treatment with NF-κB decoy but not with scramble double-stranded oligodeoxynucleotides (ODN) induced a high level of osteoclast apoptosis *in vivo*. Immunohystochemical analysis for death receptor Fas revealed strong positivity only in samples treated with NF-κB decoys, demonstrating that osteoclasts are sensitive to death induction via Fas signaling. Induction of apoptosis in osteoclasts could be a strategy for treatment of excessive osteoclast activity in pathologic conditions such as osteoporosis, peri-articular osteolysis, inflammatory arthritis, Paget's syndrome and tumour-associated osteolytic metastases.

Introduction

Double-stranded oligodeoxynucleotides (ODN) containing the consensus binding sequence of a transcription factor is a promising approach to treat several diseases by modulating transcriptional regulation (1). This transcription factor decoy (TFD) strategy results in the attenuation of the authentic cis-trans interaction, leading to decreased binding levels of transcription factors to endogenous cis-elements. The technique has been proven effective *in vitro* and *in vivo*, suggesting its use in therapy (1-3). Great attention has been recently focused on the applications of TFD strategy against the nuclear factor κB (NF-κB) (3-5).

NF-κB belongs to a family of inducible transcription factors which modulate the expression of several genes involved in differentiation, embryonic development, apoptosis, tumorigenesis, metastasis, inflammatory and proinflammatory processes (6,7).

With regard to bone, NF-κB is absolutely essential for osteoclastogenesis, as it induces the activation of genes critical for osteoclast differentiation and activity (8,9). Osteoclasts (OCs) are multinucleated cells derived from hematopoietic stem cells through a multistep process and are the sole bone resorbing cells, through different mechanisms which are not fully characterized (10).

Signaling events involved in the regulation of OC differentiation and function include several molecules and processes (11). A growing body of studies report that RANKL (also called TNF-related activation-induced cytokine, TRANCE) binds to its receptor RANK (also called TRANCE receptor) expressed in OCs, resulting in the activation of several signaling cascades. RANK is also able to bind to the soluble decoy receptor osteoprotegerin (OPG), which, reducing RANKL availability, opposes RANK signaling. In addition to NF-κB, the activated signaling pathways include extracellular signal-regulated kinase (ERK), c-Jun terminal kinase (JNK), and p38 mitogen-activated protein kinase through recruitment of the TNF receptor-associated factors (TRAFs). OC environment also shows specific sensitivity to most hormones and cytokines that influence bone resorption through modulation of the balance of RANKL and OPG (11).

OCs are essential for skeletal development and remodeling throughout the lifespan of animal and man. Accordingly, molecular characterization of osteoclast differentiation is of potential clinical importance (12). Overactivity of OCs is described in several human bone diseases, such as Paget's disease of bone, expansile skeletal hyperphosphatasia and familiar expansile osteolysis (13,14). The clinical phenotype of these diseases is uncontrolled bone remodeling, with the presence of many, often enlarged, OCs. Even if the majority of the genes involved in these diseases are yet to be identified,

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evidence suggest that a critical role is played by genes linked to the NF-κB signalling pathway (15).

Dental problems in diseases of OC-activation affecting the maxillo-mandibular bone have also been reported (16). For example, early tooth loss in severe conditions has been reported as a diagnostic feature. In addition, the increased number and activity of OCs play a critical role in the regulation of alveolar bone resorption during orthodontic tooth movement (17,18).

Under the experimental conditions established in our laboratory and as previously reported, NF-κB activity was markedly reduced by using a specific decoy strategy against NF-kB in human primary OCs from peripheral blood (19). A functional consequence of this treatment was a very significant OC apoptosis associated with a decrease in the expression of interleukin-6 (IL-6), a typical target of NF-κB. In order to clarify the effect of this decoy strategy on the apoptosis of OCs *in vivo*, we designed the experiments here reported, aimed at regulating alveolar bone resorption during orthodontic tooth movement in rats subjected to orthodontic forces.

Materials and methods

Animal treatment. Ten adult male Wistar rats with an average body weight of 400 g were used. Two animals were used as control and subjected only to orthodontic forces, and eight animals were subjected to orthodontic forces in combination with decoy treatment. The rats were kept in stainless-steel cages supplied with a self-wash system, air conditioning and lighting in agreement with the Italian guidelines on the housing of laboratory animals. The maxillary molars were moved mesially by means of Sentalloy (GAC International, Inc., Bohemia, NY) closed-coil springs delivering a constant force of 25 g according to King *et al* (20). A schematic diagram of the orthodontic device applied to rat molars for the induction of mesial traction force is illustrated in Fig. 1A. Sentalloy closed-coil springs were first attached to the molars (Fig. 1B and C), holes were made on the incisors with a diamond burr (0.07 mm) (Fig. 1D) and ligature ties were made (Fig. 1E and F). The positioning of the appliance was performed under general anesthesia induced by an intra-

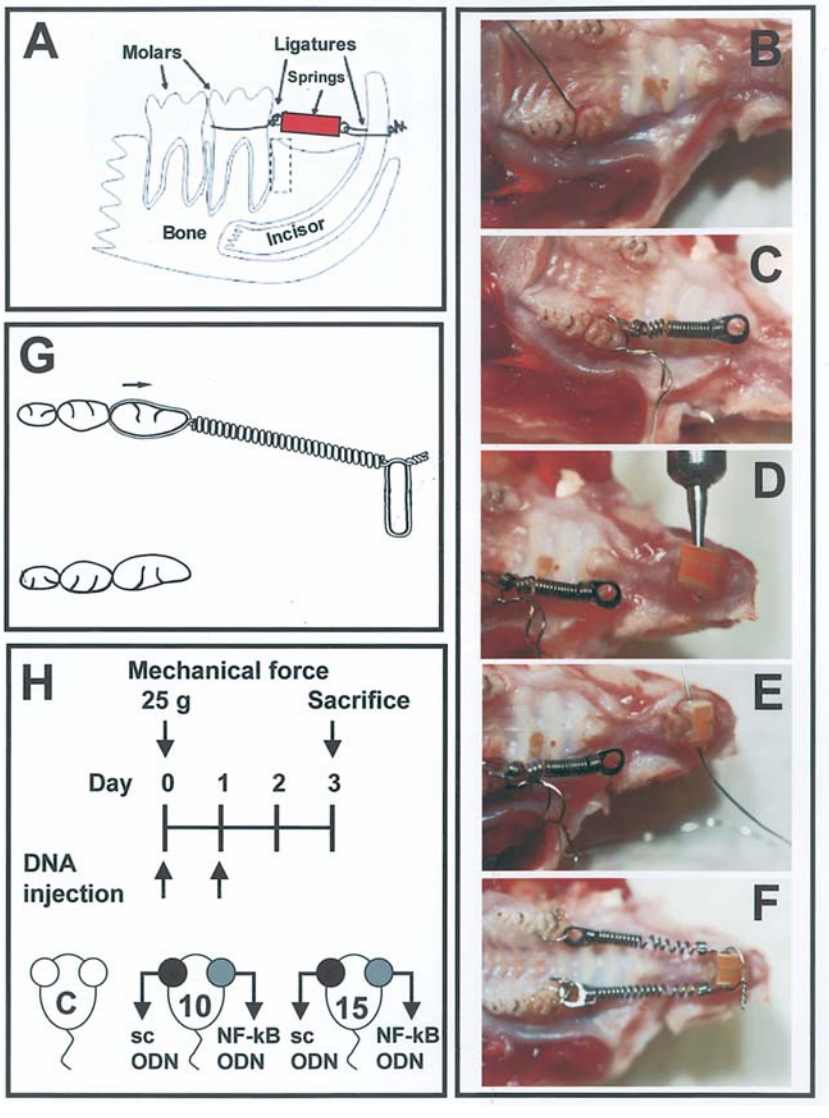

Figure 1. Schematic diagram of the orthodontic device applied to rat molars for the induction of mesial traction force (A). The dashed rectangle indicates hard tissue collected for molecular analysis. The orthodontic device *in situ* applied to rat molars for the induction of mesial traction force (B-F). Sentalloy closed-coil spring attached to the molar (C), a hole is made on the incisors with a diamond burr (0.07 mm) (D) and ligature ties are made (E,F). The scheme of the orthodontic device is depicted in (G). Experimental strategy is depicted in (H).

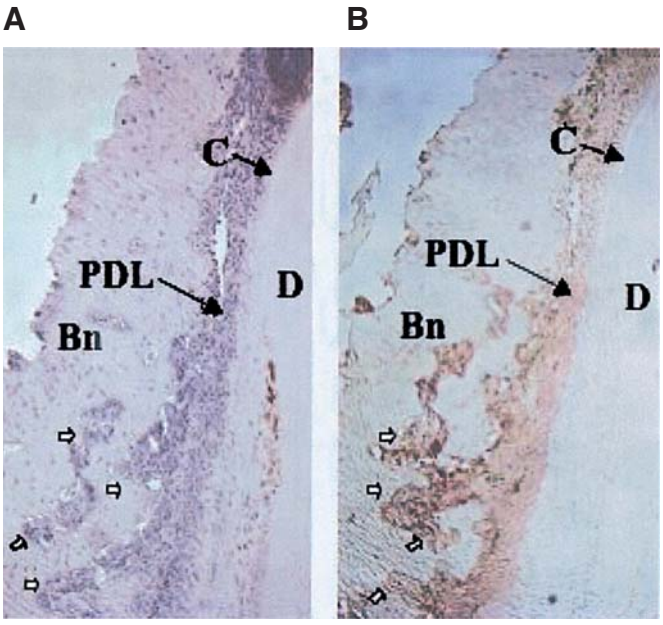

Figure 2. Paraffin sections of mesial tooth surfaces. (A) Haematoxylin staining of normal tooth structure. Periodontal ligament (PDL) was readily recognized, dentin (D), cementum (C), and bone (Bn). (original magnification, x10). (B) The TRAP analysis of the same sections was reported. The presence of mature osteoclasts is indicated by white arrows.

muscular injection (a mixture of 87 mg/kg body weight of ketamine and 13 mg/kg of xylazine) and maintained for 4 days in combination or not with the decoy treatment.

Decoy oligonucleotides. As decoy molecule against the transcription factor NF-κB, ODN mimicking the non-symmetric NF-κB binding site were used (5'-CGC TGG GGA CTT TCC ACG G-3'). NF-κB decoy molecule (10-15 μg) or the scramble oligonucleotide (5'-CAC AAA GTG TAA CAG TCT-3') was complexed with lipofectAMINE 2000 transfection reagent (Invitrogen Corp., Carlsbad, CA) and injected at the test and control sites respectively.

Biological assays. Each excised molar was fixed with 4% paraformaldehyde and decalcified in 10% ethylenediaminetetraacetic acid (EDTA) solution for 7 weeks at room temperature. The bone tissues were then embedded in paraffin for the preparation of 4-μm sagittal sections of the mesiobuccal root of the upper first molar. The presence of multinucleated OCs was detected by analyzing their positivity to the TRAP enzyme (Tartrate-resistant acid phosphatase) (Acid phosphatase kit n. 386, Sigma Aldrich, St. Louis, MO, USA). The level of apoptosis was analyzed by the DeadEnd colorimetric apoptosis detection system (Promega, Madison, WI, USA) according to the manufacturer's instructions. The experiments were performed in fixed samples, yielding information on processes captured at the time when the fixation of tissues had occurred.

Results

In order to determine whether NF-κB decoy molecules are able to induce *in vivo* apoptosis of osteoclasts, Wistar rats were subjected to orthodontic forces, in the absence or in the presence of the decoy treatment. The maxillary molars were moved mesially by means of Sentalloy closed-coil springs delivering a constant force of 25 g according to King *et al* (20) (Fig. 1A). The orthodontic forces were applied as follows: a) Sentalloy closed-coil springs were first attached to the molars, b) holes were made on the incisors with a diamond burr and c) ligature ties were made (Fig. 1B-F). Where the decoy treatment was applied, a split-mouth design was used, considering the palatal, the mesial and the buccal sites of the left upper molars as test and the right controlateral as internal controls (Fig. 1H). NF-κB decoy molecule (10-15 μg) or the scramble oligonucleotide were complexed with lipofectAMINE 2000 transfection reagent and injected at the test and control sites, respectively. The treatment was repeated two times (every 24 h) and, after 48 h from the last injection, the rats were sacrificed. Each excised molar was fixed as described in the Materials and methods section for 7 weeks and the bone tissue was then embedded in paraffin for the preparation of 4-μm sagittal sections of the mesiobuccal root of the upper first molar. In order to analyze the morphology of the tooth sections, haematoxylin staining was performed (Fig. 2A).

The presence of multinucleated OCs was detected by analyzing their positivity to the TRAP enzyme. Examination of paraffin sections show resorption lacunae associated with giant cells, in particular on the mesial root surfaces of teeth subjected to orthodontic forces that caused a high recruitment of osteoclasts (Fig. 2B).

The TUNEL staining of fragmented DNA revealed that treatment with NF-κB decoy, but not with scramble ODN, induces a very high level of OC apoptosis (Fig. 3A).

In agreement with these data, immunohistochemical analysis for death receptor Fas (a member of the tumor necrosis factor receptor family, TNFR) (21) revealed a significant positivity only in the sample treated with NF-κB decoy (Fig. 3B) but not with scramble ODN. This demonstrates that osteoclast cells are sensitive to death induction via Fas signaling and confirms that Fas is implicated in osteoclast apoptosis (21).

Discussion

The data obtained in this study demonstrate that *in vivo* osteoclasts obtained after application of orthodontic forces to rat teeth are induced to apoptosis after treatment with a decoy oligonucleotide targeting NF-κB transcription factors. These results extend to an *in vivo* experimental system our *in vitro* observations on osteoclasts isolated from peripheral blood (3,19).

The results allow us to propose that the inhibition of NF-κB activity, by the *in vivo* transfer of NF-κB decoy, results in the suppression of the transcription of key genes controlling OC survival. This clearly supports the validity of a NF-κB decoy strategy by the *in vivo* OC transfection of decoy ODN containing the NF-κB cis-element. Accordingly, it is likely, on the basis of the data reported here, that the previously set up *in vitro* experimental conditions may be transferred to the study of specific bone disorders and to clinical orthodontics (19).

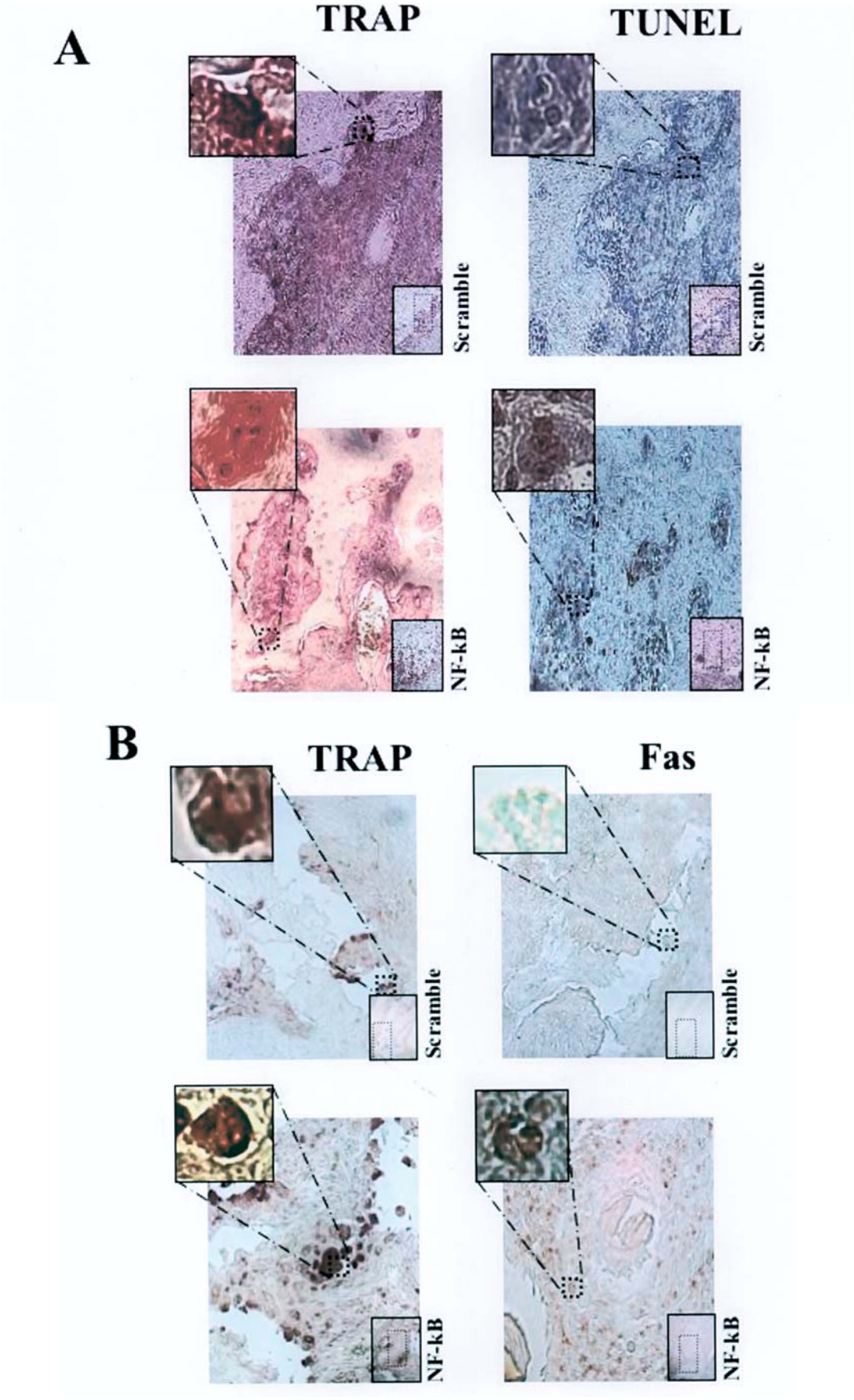

Figure 3. Induction of apoptosis following treatment with the NF-κB decoy. (A) The same sections were subjected to TRAP and TUNEL assay. Brown staining indicates the presence of apoptotic nuclei. Red staining corresponds to TRAP-positive cytoplasm. After TUNEL assay the sections have been haematoxylin counterstained. (B) Immunohistochemical reaction for Fas of the corresponding NF-κB and scramble ODN-treated teeth. The presence of brown staining indicates the positivity to Fas. On the left panel, the TRAP analysis of the same section is shown. Scramble, scramble ODN-treated tooth; NF-κB, NF-κB ODN-treated tooth.

In this respect, by using the decoy approach described here, we are able to monitor and alter osteoclast lifespans, controlling the efficiency of tooth movement through the increase of portions of bone promoting osteoclast apoptosis.

Accordingly, the induction of apoptosis in osteoclasts may be a strategy for the treatment of excessive osteoclast activity in pathologic conditions such as osteoporosis (including that associated to hereditary diseases such as thalassemia), peri-articular osteolysis, inflammatory arthritis, Paget's syndrome and tumour-associated osteolytic metastases (22-24).

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