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Expression of TRAIL and its receptors DR5 and DcR2 in orthodontic tooth movement

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Summary. Background. TRAIL is a transmembrane protein that induces apoptosis in various tissues including alveolar bone. Its in vitro expression can be activated by several methods, such as RANKL administration and cell scraping. Expression of TRAIL and its receptors DR5 and DcR2 was examined in osteoclast-like cells to analyze their effects on cell lifespan and to explore their role in orthodontic tooth movement.

Materials and Methods. Osteoclast-like cells were differentiated from a mouse hematopoietic cell line by stimulation with RANKL for 24 h (T1), 72 h (T2) or 5 days (T3); some cultures were then scraped. Immunostaining for TRAIL, DR5 and DcR2 was evaluated by immunocytochemistry and Western blot analysis in control and treated cells.

Results. Significantly greater TRAIL expression was found in treated osteoclast-like cells at T1 and T3 both on immunocytochemistry and Western blotting. TRAIL expression peaked at T1 and T3 in correspondence with DcR2 and DR5 maxima, respectively.

Conclusions. These data may contribute to a better understanding of the mechanisms regulating tooth movement and to improve the accuracy of orthodontic treatments.

Key words: TRAIL, RANKL, Tooth movement, Scraping.

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Introduction

Apoptosis, or genetically programmed cell death, is characterized by shrinkage, cytoplasmic membrane blebbing, chromatin condensation and DNA fragmentation. This homeostatic mechanism acts through two main pathways: the extrinsic (or death receptor-mediated) and the intrinsic (or mitochondrial) pathway (Bhardwaj andand Aggarwal, 2003; Chaudhari et al., 2006). Some death receptors, like Fas, tumor necrosis factor α (TNF-α) and TNF-α-related apoptosis inducing ligand (TRAIL) have been described in detail (Bhardwaj andand Aggarwal, 2003). In particular, the levels of TNF-α have been seen to increase significantly during orthodontic tooth movement (OTM) (Lowney et al., 1995).

TRAIL is a type II transmembrane protein that induces apoptosis by binding to DR4 and DR5, its death domain-containing receptors. DR5 recruits Fas-associated death domain (FADD) and caspase-8, leading to formation of a death-inducing signaling complex and to caspase-8 activation (Baetu andand Hiscott, 2002; Falschlehner et al., 2007; Chen et al., 2009). Once activated, caspase-8 can in turn activate the executioner caspase-3, which fulfils its role by destroying the cell's cytoskeletal and reparative proteins, a process that culminates in DNA fragmentation (Chaudhari et al., 2006; Colucci et al., 2007). Furthermore, TRAIL interacts with at least three "decoy" receptors, without the death effector domains, DcR1, DcR2 and osteoprotegerin (Bhardwaj andand Aggarwal; 2003, Colucci et al., 2007; Robinson et al., 2007; Chen et al., 2009), whose expression protects TRAIL-sensitive cells from TRAIL-induced apoptosis (Bhardwaj andand Aggarwal, 2003).

TRAIL-induced effects have been described in various normal tissues, and cells like tymocytes, neurons, hepatocytes and osteoclasts may be sensitive to regulation by TRAIL. TRAIL expression can be induced during OTM, which is characterized by osteoclast-mediated bone resorption. Another molecule involved in osteoclastogenesis is ligand of receptor activator of nuclear factor κ (RANKL), a peptide that shares homologies with members of the TNF family (Tyrovola et al., 2010).

Although several investigators have analyzed the activity of RANKL and TRAIL, there are few studies of their role in bone resorption during OTM.

One study found that RANKL enables osteoclast precursors to differentiate into mature osteoclasts in the presence of macrophage colony stimulating factor (M-CSF), and that TRAIL may play a role in osteoclast apoptosis (Roux et al., 2005); according to another investigation TRAIL is probably a redundant negative regulator of physiological osteoclastogenesis (Zauli et al., 2004). The levels of RANKL during OTM have been seen to rise after application of mechanical stimulation (Nishijima et al., 2006; Tyrovola et al., 2008, 2010; Xie et al., 2008; Brooks et al., 2009; Yamaguchi, 2009; Baloul et al., 2011); however, little research has been done on the effects of TRAIL on osteoclast activity during OTM, even though their lifespan could be the keystone to bone remodeling.

OTM involves local remodeling of alveolar bone (Ren et al., 2005; Baloul et al., 2011) in response to the application of a mechanical force and involves three stages: an initial tipping phase (cell activation), a lag phase with osteoclast recruitment and initiation of bone resorption, and a post-lag phase, when tooth movement actually occurs (Reitan, 1951, 1967). Application of a mechanical force results in bone resorption by osteoclasts on the compression side of the teeth, and in bone formation by osteoblasts on the opposite (tension) side. In fact, during OTM tooth movement within the periodontal space causes stretching and compression of collagen fibers and changes in cellular activity (Krishnan and Davidovitch, 2006; Masella and Meister, 2006). The latter events can occur only if osteoclasts are able to destroy the areas of hyalinized tissue on the compression side via a combination of inflammatory cytokines and blood flow interruption (Brooks et al., 2009). Resorption on the compression side continues as long as there are active osteoclasts in the alveolar bone. The mechanism by which these cells are cleared from tissues during OTM remains unclear; some researchers have hypothesized that it may be cell death (Noxon et al., 2001). Several studies have reported that osteoclast lifespan play an important role in OTM.

While osteoclast precursors from the periodontal ligament (PDL) are responsible for removal of this tissue, on the tension side mechanical stress leads to differentiation of pre-osteoblasts into mature osteoblasts (Yamaguchi, 2009).

Osteoblastogenesis and bone formation are regulated by hormones, growth factors, cytokines, mechanical loading, diet, aging and other unidentified factors. In particular, osteoblasts synthesize and secrete RANKL and M-CSF to stimulate osteoclastogenesis (Manolagas, 1995; Boyle et al., 2003; Eghbali-Fatourechi et al., 2003).

The purpose of this investigation was: i) to evaluate the expression of TRAIL, DR5 (the death receptor) and DcR2 (the decoy receptor) in osteoclast-like cells after induction of osteoclast activity by RANKL administration; ii) to assess whether scraping can provide a mechanical insult capable of simulating OTM, suggested by Krishnan and Davidovitch (2006) as a tooth movement model; and iii) to document any osteoclast death.

Materials and methods

Osteoclast cultures

Osteoclast-like cells were differentiated from RAW 264.7 cells (Cell Culture Centre, Brescia, Italy), a mouse hematopoietic cell line, by stimulation with recombinant RANKL (Sigma Chemical Company St. Louis, MO, USA) as described previously (Krits et al., 2002). Briefly, RAW 264.7 cells were plated at a density of 20,000 cells/cm^2 in 6-well plates or on coverslips in 24-well plates and cultured in Dulbecco's modified medium plus 10% bovine serum albumin (BSA; Sigma). Cells were incubated at 37°C in humidified 95% air/5% CO_2 mixture and stimulated by exposure to 70 ng/ml GST-RANKL for 24 h (T1), 72 h (T2) or 5 days (T3). GST-RANKL expression and purification have been described in detail (Krits et al., 2002). After 3 days the culture media, RANKL and the vehicle were replenished. Differentiation of RAW 264.7 cell into osteoclast-like cells was established by direct examination under a light microscope. RANKL-stimulated cultures were used for experiments if they displayed well-spread giant cells forming a near-monolayer. Vehicle-treated cultures contained mononuclear cells. After 5 days, cells were either prepared for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and the MTT assay or fixed for immunostaining.

Cell scraping

Another set of RAW 264.7 cells were grown for 24 h (T1) 72 h (T2) or 5 days (T3) in the presence of 70 ng/ml GST-RANKL and then scraped with a plastic scraper. Cells were either immediately harvested in SDS-PAGE sample buffer, or allowed to settle and harvested after 1, 3, 6 or 12 h. Samples were boiled, spun at 200,000 x g for 30 min to remove nucleic acids and kept at -70°C until they were subjected to SDS-PAGE and blotting.

Immunocytochemistry

Immunostaining for TRAIL, DR5 and DcR2 was performed on control RAW 264.7 cells, on RANKL-treated T1, T2 and T3 cultures, and on RANKL-treated T1, T2 and T3 cultures scraped after 24 h. Briefly, cells were fixed with 4% phosphate-buffered para-formaldehyde for 20 min at room temperature (r.t.) and quenching was performed with a solution of 2% hydrogen peroxide (H_2O_2) and 10% methanol in phosphate-buffered saline (PBS) for 1 min. Cells were permeabilized in fresh 0.3% Triton X-100 solution in PBS for 5 min. They were then treated with 5% BSA in PBS for 1 h at r.t.; BSA is a blocking agent used to prevent non-specific antibody binding. Cells were subsequently incubated overnight with primary rabbit polyclonal anti-TRAIL (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), anti-DR5 or anti-DcR2 (Novus Biologicals, Littleton, CO, USA) antibody at 1:200 working dilutions. A fluorescein isothiocyanate (FITC)-labeled anti-rabbit antibody (1:100, Santa Cruz Biotechnology) was used as the secondary antibody. Coverslips containing the cells were washed, mounted in PBS/glycerol (50:50), and placed on glass microscope slides. They were examined with a Nikon Eclipse TE200 fluorescence microscope and photographed with a digital camera (Canon, Japan). Positive cells were counted over the entire coverslips.

Evaluation of immunostaining

TRAIL, DR5 and DcR2 staining was classified as either negative or positive. Positive staining was defined as presence of fluorescence on the edge of the black background within the cytoplasm or in the immediate lacunar/pericellular space. Staining intensity and the proportion of immunopositive cells were also determined by fluorescence microscopy. Intensity of staining (IS) was graded on a five-point scale as: 0 = no detectable staining, 1 = weak staining, 2 = moderate staining, 3 = strong staining, 4 = very strong staining. The proportion of cells immunopositive for TRAIL, DR5 or DcR2 (extent score = ES) was evaluated independently by three investigators (two anatomical morphologists and a histologist) and scored as a percentage of the final number of 100 cells into five categories: 0 = <5%; + = 5–30%; ++ = 31–50%; +++= 51-75%, and ++++ = >75%. Counting was performed at X200 magnification.

Positive and negative controls

Positive and negative tests of the specificity of the primary antibodies at the protein level included exposure of basal cell carcinoma tissue to an immunoperoxidase process (positive control). Immunolabeling for TRAIL, DR5 and DcR2 was found in both membrane and cytoplasm. As a negative control, RAW 264.7 cells were treated with normal rabbit serum instead of the specific antibody.

Protein extraction

RANKL-treated RAW 264.7 cells (T1, T2 and T3 cultures) were homogenized in a Polytron homogenizer using a lysis buffer containing 150 mM NaCl, 50 mM Tris–HCl (pH 7.5), 5 mM ethylenediaminetetraacetic acid (EDTA), 1 mM Na_3VO_4, 30 mM Na pyro-phosphate, 50 mM NaF, 1 mM acid phenyl-methyl-sulfonyl-fluoride, 5 μg/ml aprotinin, 2 μg/ml leupeptin, 1 μg/ml pepstatin, 10% glycerol, and 0.2% Triton X-100. The homogenates were then spun at 14,000 rpm for 10 min at 4°C. The protein concentration of the supernatant was determined by the Bradford method.

Western blot analysis

Equal amounts of protein were subjected to SDS–PAGE on 10% gels, transferred onto Hybond ECL nitrocellulose membranes (Amersham Pharmacia Biotech, Amersham, UK) for 1 h, and analyzed by immunoblotting with a primary polyclonal anti-TRAIL antibody (BD Transductions Laboratories, Franklin Lakes, NJ, USA; 1:1000), a secondary peroxidase-conjugated anti-rabbit antibody (Amersham Pharmacia Biotech; 1:10,000), or a polyclonal rabbit anti-DR5 or anti-DcR2 antibody (both from Santa Cruz Biotechnology). Detection was performed with a chemiluminescence assay (ECL; Amersham Italia, Milano, Italy).

All experiments were performed at least three times; signal intensity was analyzed using a digital imaging analysis system (1D Image Analysis Software; Kodak Scientific Imaging, New Haven, CT, USA). α-tubulin

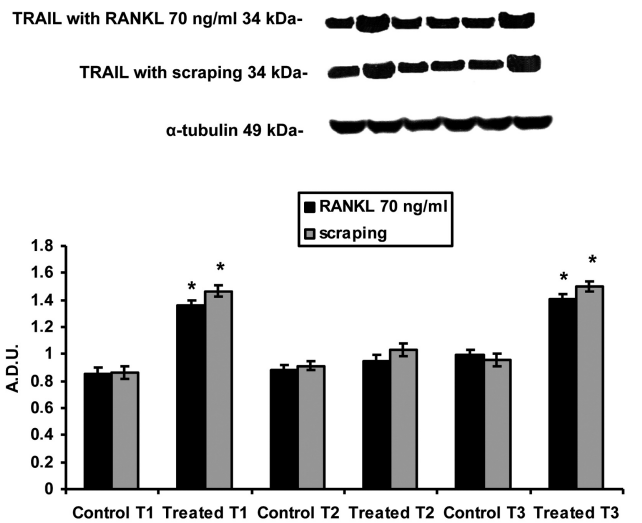

Fig. 1. TRAIL expression in osteoclast-like cells treated with RANKL 70 ng/ml or RANKL 70 ng/ml and scraping as determined by Western blot analysis at different times (T1, T2, T3). Data are from three independent experiments and show TRAIL relative expression (mean ± SEM) calculated as arbitrary densitometric units (ADU).

(Santa Cruz Biotechnology) was used as an internal control to validate the amount of protein loaded onto the gels.

Bands were measured densitometrically and relative density was calculated based on the density of the α-tubulin bands in each sample. Values were expressed as arbitrary densitometric units (ADU) corresponding to signal intensity.

Statistical analysis

All variables were normally distributed. Comparisons between two means were tested with Student's *t* test and comparisons among means with ANOVA. P-values <0.01 were considered as statistically significant.. All data were analyzed with the SPSS program (SPSS® release 16.0, Chicago, IL, USA).

Cohen's kappa was applied to measure inter-observer agreement and averaged over all three to evaluate overall agreement using the grading: 0-0.2 (slight), 0.21-0.40 (fair), 0.41-0.60 (moderate), 0.61-0.80 (substantial), and 0.81-1.0 (almost perfect).

Results

Trail

T1 and T3 osteoclast-like cells treated with RANKL and scraping showed significantly increased TRAIL expression compared with control cells both on immunocytochemical analysis and on Western blotting (Fig. 1). Scraped cells displayed a higher level of TRAIL expression (IS: 4; ES: ++++) than those merely subjected to RANKL administration (IS: 3; ES: ++++) (Fig. 2A,B). Immunostaining was both in the cytoplasm and in the membrane. Few or no immunostained cells were detected in control samples (Fig. 2C).

DR5

A significantly increased expression of the death receptor DR5 compared with control cells was

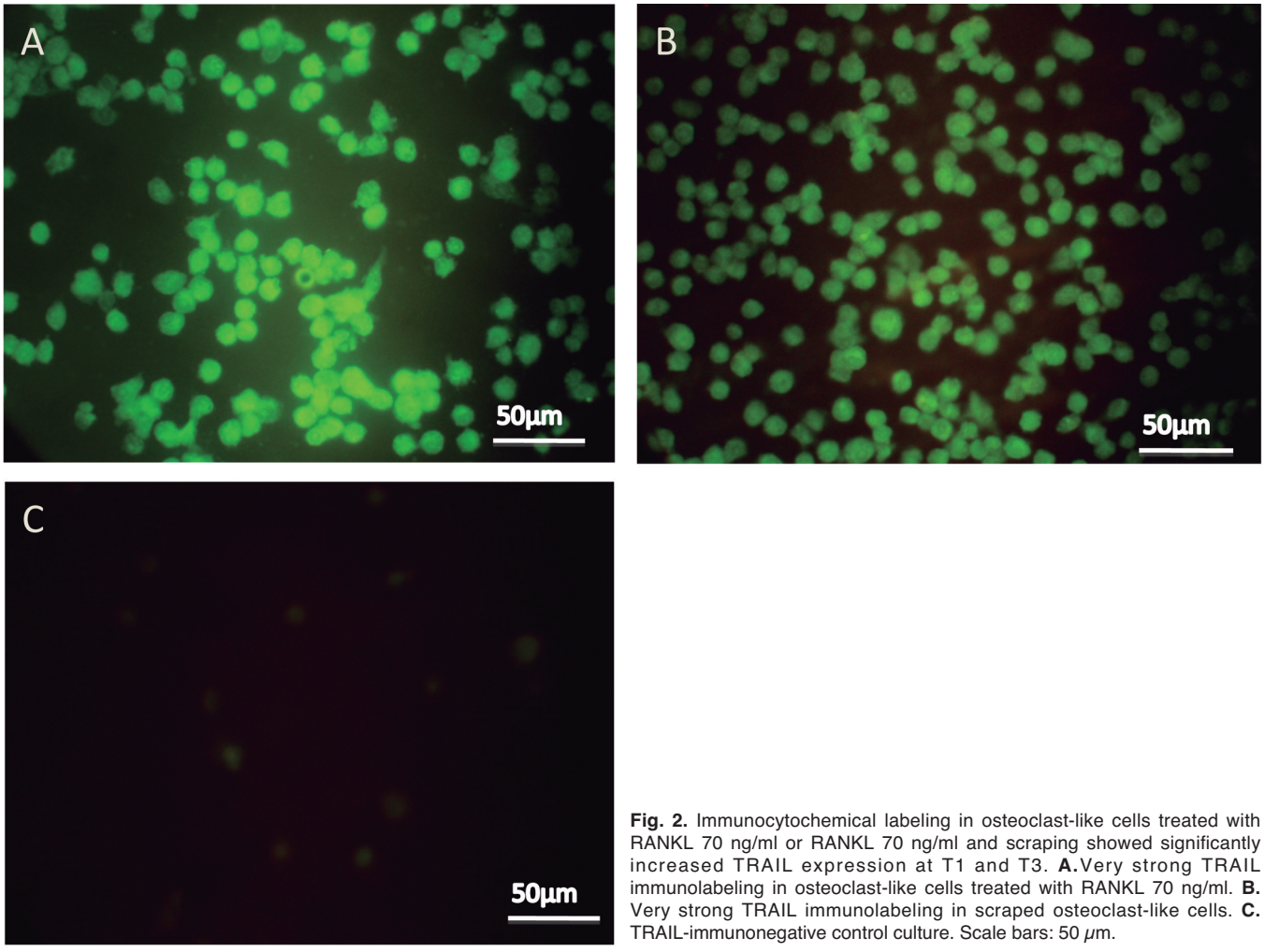

Fig. 2. Immunocytochemical labeling in osteoclast-like cells treated with RANKL 70 ng/ml or RANKL 70 ng/ml and scraping showed significantly increased TRAIL expression at T1 and T3. **A.** Very strong TRAIL immunolabeling in osteoclast-like cells treated with RANKL 70 ng/ml. **B.** Very strong TRAIL immunolabeling in scraped osteoclast-like cells. **C.** TRAIL-immunonegative control culture. Scale bars: 50 µm.

demonstrated at T3 both by immunocytochemistry and by Western blotting (Fig. 3). Again, scraped cells showed a greater expression of DR5 (IS: 4; ES: ++++) (Fig. 4A) than those merely exposed to RANKL (IS: 3; ES: +++) (Fig. 4B). No significant differences were seen in treated cells at T1 and T2 compared with control cultures. Few or no immunostained cells were detected in control cultures (Fig. 4C).

DcR2

Osteoclast-like cells treated with RANKL and scraping exhibited a significant increase of the decoy receptor at T1, both on immunocytochemistry (Fig. 5A,B) and on Western blotting (Fig. 6) compared with controls. In particular, scraped cells showed a higher DcR2 expression (IS: 4; ES: ++++) compared with those exposed to RANKL alone (IS: 3; ES: ++++). Immunostaining was both in the cytoplasm and in the membrane. Few or no immunostained cells were observed in control cultures (Fig. 5C).

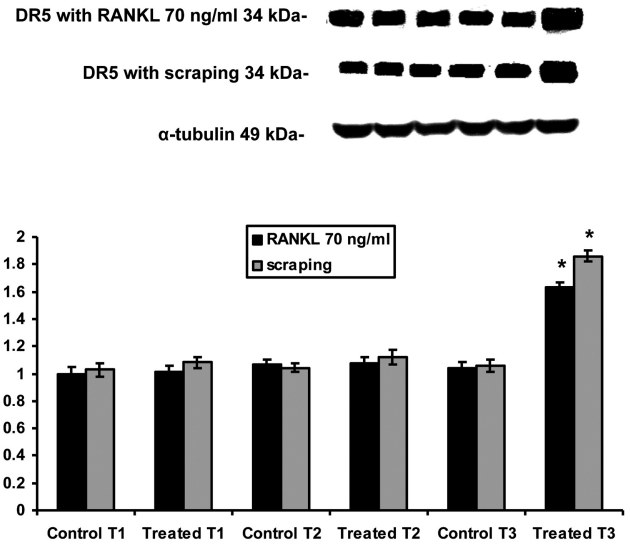

Fig. 3. DR5 expression in osteoclast-like cells treated with RANKL 70 ng/ml or RANKL 70 ng/ml and scraping as determined by Western blot analysis at T1, T2, and T3). Data are from three independent experiments and show the relative expression (mean ± SEM) of DR5 calculated as arbitrary densitometric units (ADU).

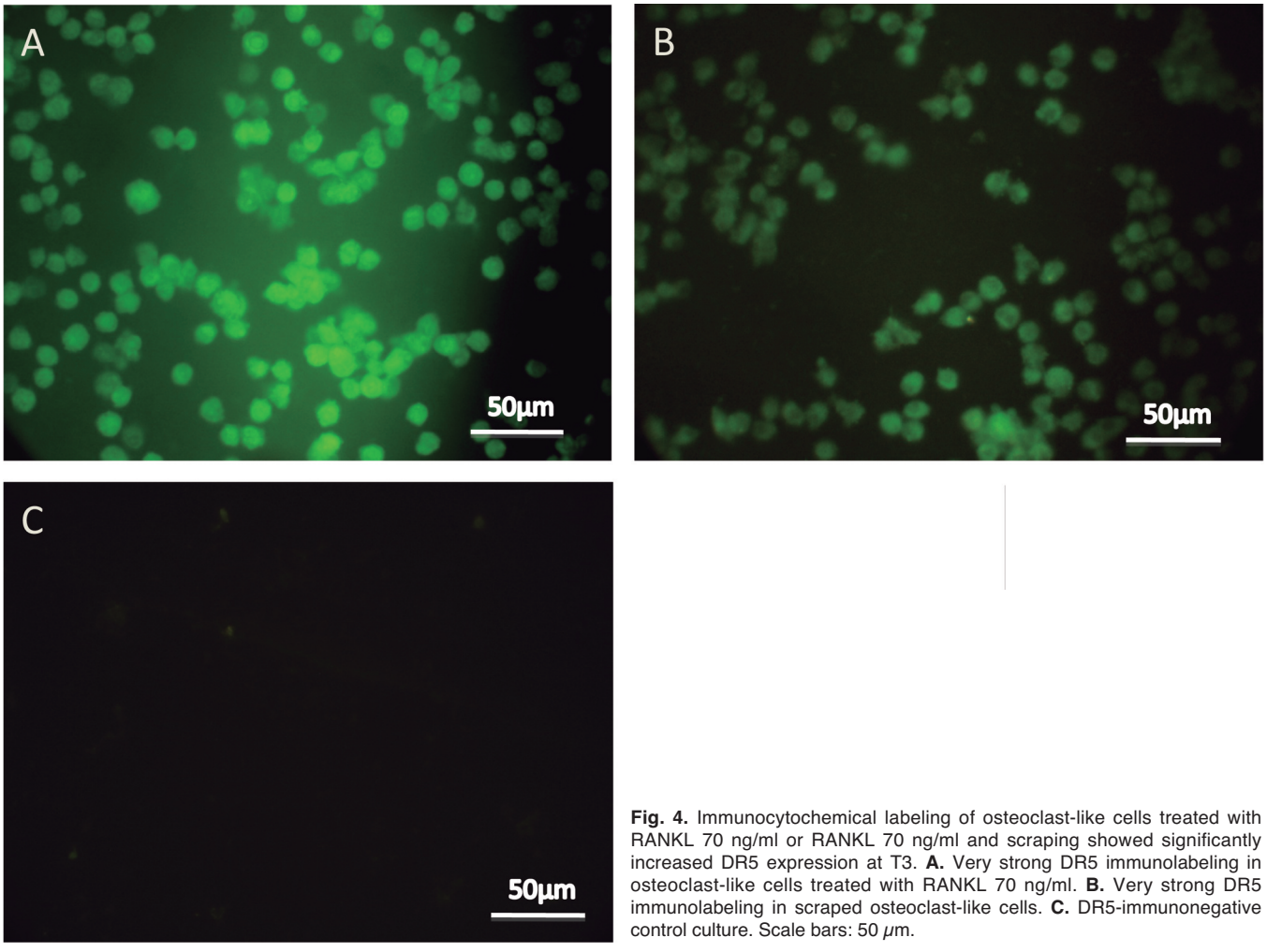

Fig. 4. Immunocytochemical labeling of osteoclast-like cells treated with RANKL 70 ng/ml or RANKL 70 ng/ml and scraping showed significantly increased DR5 expression at T3. **A.** Very strong DR5 immunolabeling in osteoclast-like cells treated with RANKL 70 ng/ml. **B.** Very strong DR5 immunolabeling in scraped osteoclast-like cells. **C.** DR5-immunonegative control culture. Scale bars: 50 μm.

Discussion

This study evaluates the in vitro expression of TRAIL and its receptors DR5 and DcR2 in osteoclast-like cells treated with RANKL, exposed to RANKL and scraping, or grown as control cultures.

The immunocytochemical and Western blotting findings documented an increased expression of TRAIL in both sets of treated cells compared with the control cultures. The increase was significant at T1 and T3, and at both time points TRAIL expression was higher in the cells also subjected to scraping. Interestingly, analysis of the expression of TRAIL and its receptors disclosed that the TRAIL peak at T1 corresponded to the maximum expression of the decoy receptor DcR2, whereas the TRAIL peak at T3 corresponded to the peak expression of the death receptor DR5.

These findings may reflect a different influence of TRAIL on osteoclasts in relation to the time elapsed from the application of mechanical stress (scraping, simulating OTM). At the beginning of force application, i.e. T1, TRAIL might induce cell differentiation and development through its anti-apoptosis receptor DcR2; once this impulse is spent, expression of this decoy receptor would revert to its normal level. At this point (T3) expression of the death receptor DR5 would increase, leading in turn to apoptotic cell death and promoting cell turnover. In synthesis TRAIL would exert modulation and differentiation functions at T1 and induce cell apoptosis at T3. A peak of osteoclast apoptosis, observed between 5 and 7 days from stimulus application (Noxon, 2001), seems to confirm our data. This mechanism would be important in regulating the balance between resorption and formation characterizing bone tissue and plays an essential role in OTM.

In fact, it is reasonable to speculate that OTM would continue as long as an active osteoclast population were present in the alveolar bone, and that the limit to this process would therefore be osteoclast death.

Tooth movement by orthodontic force application is characterized by remodeling in dental and paradental tissues, including pulp, PDL, alveolar bone, and gingiva (Nishijima et al., 2006). Their exposure to varying degrees of magnitude, frequency, and duration of mechanical loading induces extensive gross and microscopic changes. Several molecules can evoke

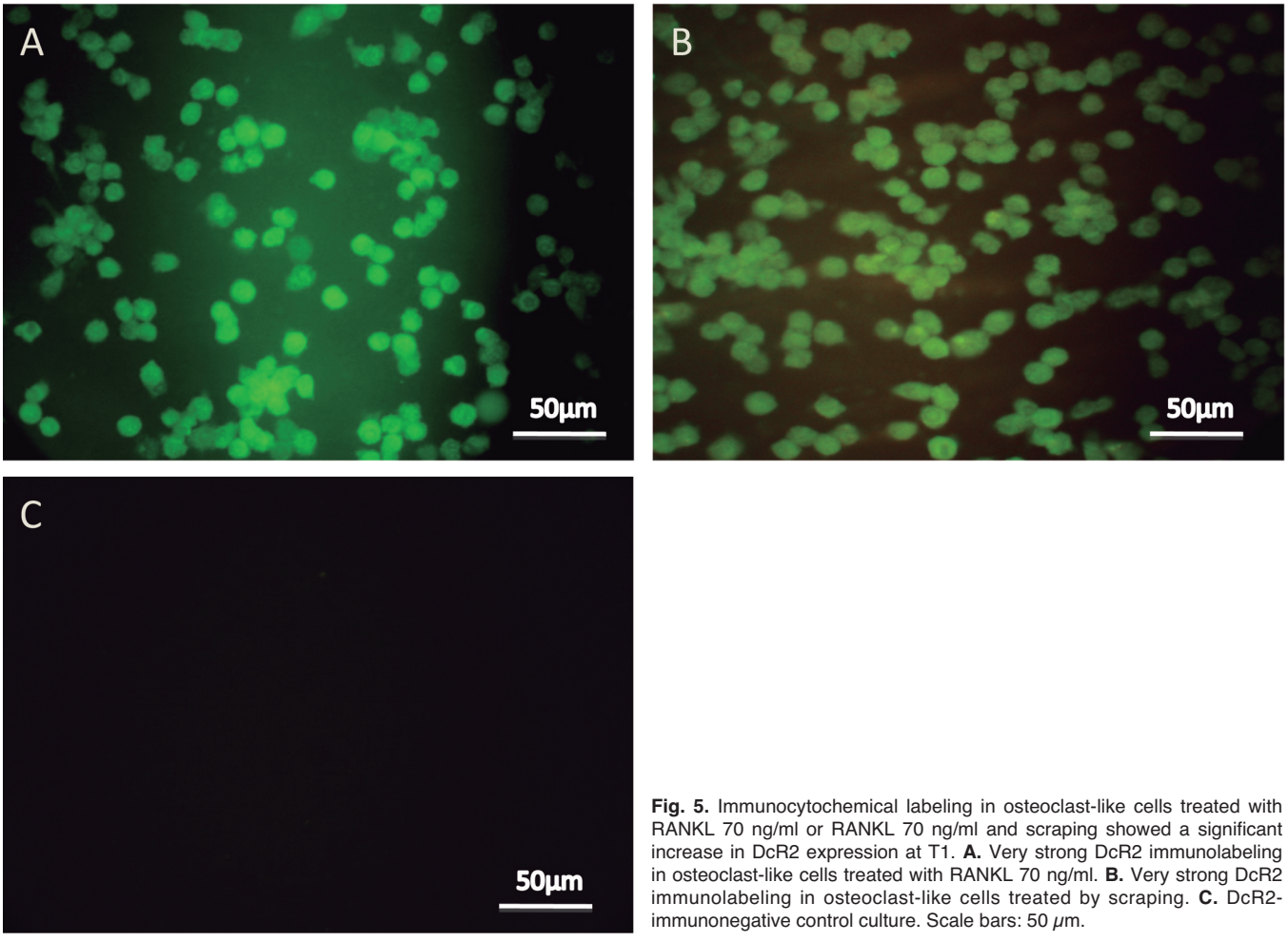

Fig. 5. Immunocytochemical labeling in osteoclast-like cells treated with RANKL 70 ng/ml or RANKL 70 ng/ml and scraping showed a significant increase in DcR2 expression at T1. **A.** Very strong DcR2 immunolabeling in osteoclast-like cells treated with RANKL 70 ng/ml. **B.** Very strong DcR2 immunolabeling in osteoclast-like cells treated by scraping. **C.** DcR2-immunonegative control culture. Scale bars: 50 μm.

responses by various cell types in and around the teeth, providing a favorable microenvironment for tissue deposition or resorption (Ren et al., 2005; Krishnan and Davidovitch, 2006; Boyce and Xing, 2007; Baloul et al., 2011).

OTM causes stretching and compression of collagen fibers and changes in cell activity in the periodontal space. As mentioned above, tissue disruption on the compression side is achieved by circulating macrophages and bone marrow osteoclasts (Krishnan and Davidovitch, 2006; Masella and Meister; 2006, Brooks et al., 2009). RANKL promotes maturation and fusion of pre-osteoclasts into activated osteoclasts and stimulates their bone resorption activity (Nishijima et al., 2006). Furthermore, compression during OTM causes an increase in RANKL secretion (Tyrovola et al., 2010).

RANKL (or ODF, TRANCE, or OPGL) has recently been identified as a member of the membrane-associated TNF ligand family and is an important regulatory molecule in osteoclastogenesis (Xing et al., 2005; Yamaguchi, 2009; Nakano et al., 2010). It has also been detected in osteoblasts and PDL cells during experimental tooth movement (Ogasawara et al., 2004). The important role of the TNF family in osteoclast biology is further supported by the finding that several other TNF family members including TNF-α, FasL and TRAIL also participate in osteoclast differentiation, function, survival and/or apoptosis (Feng, 2005).

TRAIL has a potent cytotoxic activity and induces apoptosis in susceptible cells (Zauli et al., 2004; Chaudhari et al., 2006; Falschlehner et al., 2007; Yen et al., 2008; Chen et al., 2009). Moreover, mounting experimental evidence indicates that it plays a regulatory role in various tissues such as neurons, hepatocytes and osteoclasts (Colucci et al., 2007). In this study we examined the expression of TRAIL and its receptors in osteoclast-like cells treated with RANKL and subsequently by scraping.

A literature search on the role of RANKL in osteoclast differentiation highlighted the work by Krishnan and Davidovitch (2006) on cytoskeleton-extracellular matrix (ECM) interactions during OTM. According to these researchers cells are motors for tissue modeling and remodeling, and most cell types are sensitive to mechanical loads. The responses of dental and paradental cells to mechanical loads involve an interplay between intra- and extracellular structural elements, and among biochemical messengers. Applied mechanical forces are transduced from the strained ECM to the cytoskeleton, inducing cytoskeletal reorganization, secretion of stored cytokines, ribosomal activation, and gene transcription. The ECM molecules involved in this process include collagen, proteoglycans, laminin, and fibronectin (Krishnan and Davidovitch, 2006).

The role of mechanotransducers in transforming mechanical force into biochemical signals has been studied extensively in recent years (Krishnan and Davidovitch, 2006; Masella and Meister, 2006; Xie et al., 2008; Baloul et al., 2011). The mechanisms underlying cellular reactions to mechanical forces are critical in orthodontics.

The mechanical stimulus applied in our work, i.e. osteoclast scraping, proved to be able to induce the biological reactions that lead first to cell differentiation and later to apoptosis. The effects induced by this technique on our cultures demonstrate the scope for a fine control of OTM by stopping the active phase of bone remodeling via apoptosis induction. Osteoclast scraping, which triggered the typical reaction cascade occurring in tooth movement, is therefore not merely a model for OTM, but also a means for precision control of tooth movement. We documented how osteoclast activation and programmed death can be induced: the former event may be likened to the beginning of orthodontic treatment by application of orthodontic appliances, whereas osteoclast death would determine the end of active treatment. Understanding the biological events underlying OTM is crucial in clinical practice. Ideally, treatment could be accurately programmed by administration of apoptosis-inducing substances to stop OTM.

Studies of the osteoclast life cycle may provide interesting suggestions for the assessment of the timing for orthodontic treatment. A thorough knowledge of cell behavior during OTM could aid clinicians in planning appliance activation and reactivation. Improving our understanding of these biological processes, including cell recruitment and clearance, and how they correlate to common orthodontic manipulations, could improve OTM efficiency. Biochemical regulation would also be a useful way to control osteoclast functional lifespan.

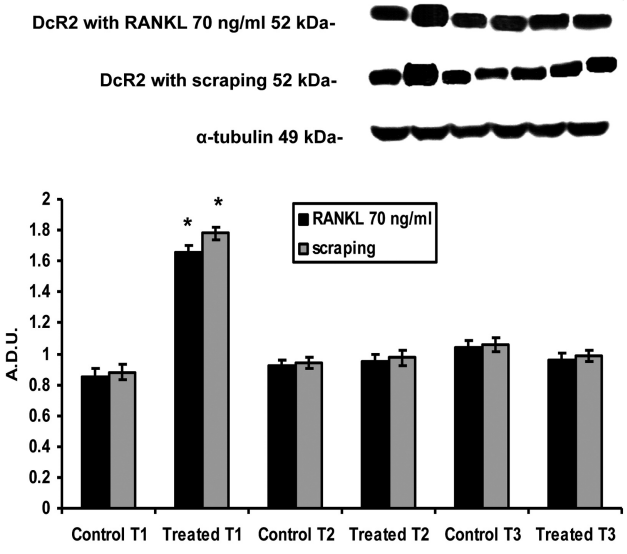

Fig. 6. DcR2 expression in osteoclast-like cells treated with RANKL 70 ng/ml or RANKL 70 ng/ml and scraping as determined by Western blot analysis atT1, T2, and T3. Data are from three independent experiments and show the relative expression (mean ± SEM) of DcR2 calculated as arbitrary densitometric units (ADU).

Conclusions

TRAIL expression by osteoclast-like cells was greater in cells treated both with RANKL and with scraping than in control cells..

Expression of TRAIL receptor DcR2 peaked at T1 and decreased at T3; whereas DR5 expression peaked at T3.

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