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The presence of TNF-α and TNFR1 in aseptic root resorption. A preliminary study

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Background: It is hypothesised that osteoprotegerin (OPG), as an osteoclast antagonist, may offer molecular control over the process of orthodontic root resorption. Previous work investigating OPG in a rat periodontal ligament (PDL) ankylosis model found no inhibitory effect on osteoclasts and odontoclasts when given at a recommended dosage of 2.5 mg/kg. It was considered that traumatically-induced PDL inflammation produces mediators and cytokines with the ability to stimulate clast cell differentiation and counter the effects of OPG.

Aims: The present study investigated the presence of Tumour Necrosis Factor Alpha (TNF-α) and its receptor Tumour Necrosis Factor Receptor 1 (TNFR1) in a PDL sterile inflammatory model.

Methods: Dry ice was applied for 15 minutes to the upper right first molar crown of eighteen, 8-week-old, male Sprague-Dawley rats of which 9 were injected with OPG at a dose of 2.5 mg/kg of body weight at the time of freezing. After 7 days, the rats were sacrificed and each maxilla processed for immunohistochemical identification of TNF-α and TNFR1.

Results: Results showed the presence of root resorption in varying amounts and locations in both experimental and control rats. Reparative processes appeared greater in the OPG-treated rats, often with the presence of an ankylotic union. Immunolabelling showed the presence of TNF-α and TNFR1 in the sterile inflammation located mainly in the interradicular PDL area. More definitive labelling appeared in OPG-treated rats.

Conclusion: The results indicated that TNF-α, and its receptor TNFR1, by their presence, may modify OPG effectiveness by offering an alternative pathway for osteoclast formation, which counters the anti-resorptive effects of OPG.

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Introduction

The complex interaction between osteoblasts and osteoclasts is important in orthodontic tooth movement as well as in root resorption but much remains unclear. Osteoclasts are recruited to specific sites to resorb bone which subsequently undergoes regeneration by osteoblastic deposition and calcification of bone matrix. Resorption and bone formation are therefore linked in a coupled process, the uncoupling of which, leads to the reduced bone mass seen in osteoporosis or, in some rare cases, the accumulation of bone leading to osteopetrosis. In addition, osteoclast-derived bone remodelling seen in orthodontic tooth movement often has the adverse consequence of sterile inflammatory external root resorption.[1]

The identification and investigation of the osteoprotegerin/RANK/RANK ligand system[2] has been considered a major breakthrough in the understanding of bone biology. It has been generally accepted that osteoclastic bone resorption precedes osteoblastic bone formation in the normal bone remodelling cycle.[3] Osteoclast differentiation initiated through direct cell-to-cell contact between osteoblasts and osteoclast precursors has been demonstrated by Udagawa[4] and Takahashi et al.[5] Furthermore, it has been revealed that osteoblasts express initiating factors that are recognised by receptors on osteoclast precursors which, upon interaction, initiate osteoclast differentiation.[6] The initiating factors have been identified as the RANK ligand produced by the osteoblast and its RANK receptor on the osteoclast

precursor. Additionally, OPG has been identified as a decoy for RANK,[2,7,8] which competitively binds to RANKL, thereby inhibiting osteoclastogenesis and subsequent bone resorption.

The inhibitory effects of OPG during external inflammatory root resorption have been previously investigated.[9] Results indicated that a recommended OPG dosage of 2.5 mg/kg did not inhibit root resorption and the conclusion suggested the possible involvement of other inflammatory cytokines. Tumour necrosis factor alpha (TNF-α) has previously been shown to be involved in the RANK/RANKL induction of osteoclasts[10] and to directly induce osteoclast differentiation.[11] TNF-α-mediated osteoclast differentiation was not inhibited by OPG.[11] Furthermore, two cell surface receptors (TNFR) for TNF-α have been identified and, of importance, is TNFR1.[12] TNFR1 is essential and known to mediate most of the biological properties of TNF-α via a cascade of cellular kinases.[13] Marrow cultures expressing only TNFR1 have demonstrated more osteoclasts in comparison to wild type cultures,[14] indicating that TNF-α possibly enhances osteoclastogenesis via its receptor. It is hypothesised that the direct induction of osteoclast recruitment by TNF-α is due to the enhanced RANK expression and sensitization of precursor cells to RANKL.[11] Given the likely interplay between the TNF/TNFR and the OPG/RANK/RANKL systems, the present study aimed to investigate the involvement of TNF-α and TNFR1 in the presence and absence of OPG, in a model of induced sterile inflammation and necrosis.

Materials and methods

Experimental animals

Eighteen 8-week-old, male Sprague-Dawley rats were used and treated under the ethical regulations for animal experiments as approved by the Ethics Committee of The University of Adelaide (M-4-04). Each animal weighed between 250-300 g at the start of the experiment and increased in weight normally during the experimental timeline.

Experimental protocol

Each rat was anaesthetised with a combination of Hypnorm (fentanyl citrate 0.315 mg/ml and fluanisone 10 mg/ml) and Hypnovel (midazolam hydrochloride 5 mg/ml) in a 1:1 ratio with sterile water and administered at a dosage of 2.7 ml/kg of body weight. The anaesthetised rats were placed on a specially constructed rack which stretched the mouth open through the use of metal rings looped around the upper and lower incisors. With cheek and tongue retraction, the upper right first molar was carefully frozen for a period of fifteen minutes by the application of customized pellets of dry ice (CO_2 at -81° C). Following the application of cold, the tissues were allowed to thaw slowly under anaesthesia and the animals recovered under a heat lamp to prevent hypothermia. The upper left first molar was left unfrozen and served as a control. In nine randomly selected rats, osteoprotegerin, as a single dose at a concentration of 2.5 mg/kg, was subcutaneously administered into the hind leg of each animal immediately after freezing.

Immunohistochemistry

Seven days after the thermal insult, all animals were sacrificed under anaesthesia via the intracardiac perfusion of 30 ml of 4 per cent paraformaldehyde fixative. Each maxilla was dissected out and immersed in the same fixative for 24 hours, rinsed in phosphate-buffered saline (0.4M pH 7.4) for 24 hours and then decalcified using 4 per cent EDTA in phosphate buffer. Subsequently the tissues were placed in 70 per cent alcohol before processing through graded alcohols and paraffin wax impregnation in a Shandon Citadel 2000 automatic processor. Coronal plane wax sections of 5 μm were cut and mounted in sequential order on aminopropyltriethoxysilane (APT) coated slides.

Every third slide was labelled using the avidin-biotin (ABC) peroxidase immunolabelling technique for TNF-α (polyclonal antibody to mouse TNF-α, HyCult biotechnology, Uden, The Netherlands) and counterstained with haematoxylin. Each subsequent slide was labelled for TNFR1 (rabbit polyclonal to TNF Receptor 1, Abcam, Cambridge, United Kingdom) and counterstained with haematoxylin. A positive reaction was identified as dark brown staining and validated by comparison with a positive control of adenocarcinoma tissue. Negative control sections were provided by the deletion of the primary antibody. Light microscopy was used to examine the areas of interest which were the interradicular region and all

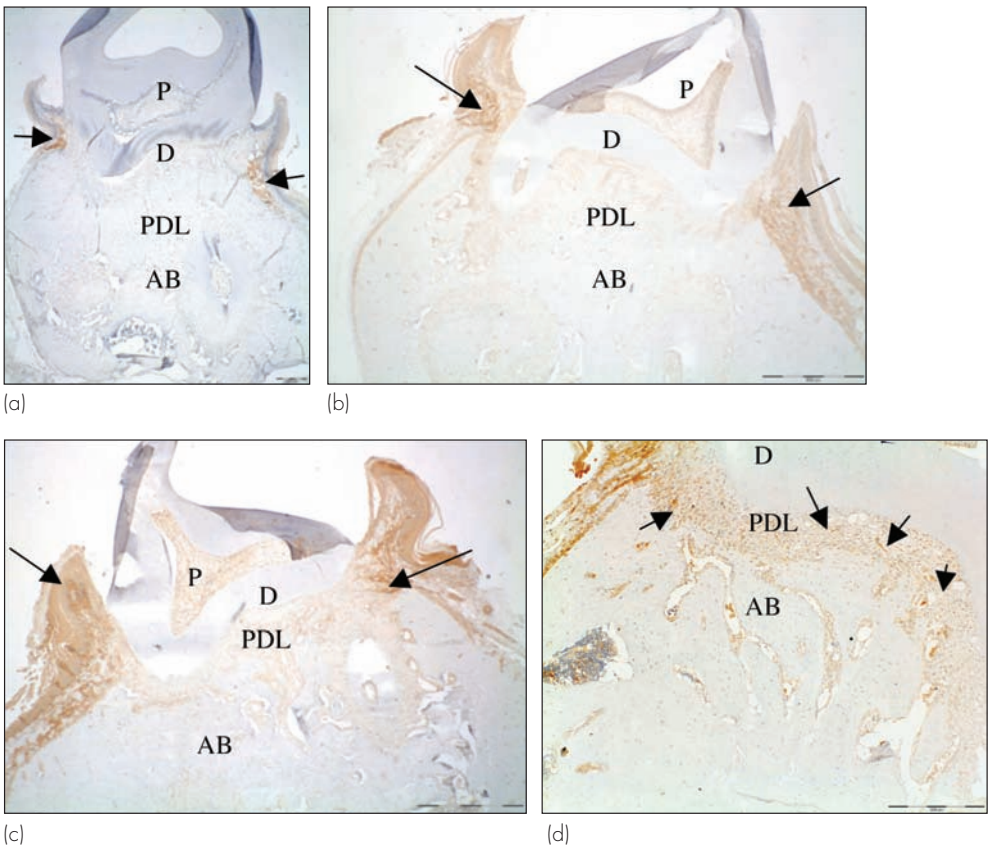

Figure 1. (a) TNF-α staining of non-frozen tooth in OPG administered rat (Scale bar equals 200μm). Arrows show positive staining in the gingival tissues. (b) TNF-α staining of non-frozen tooth in non-OPG-administered rat (Scale bar equals 500μm). Arrows show positive staining in the gingival tissues. (c) TNF-α staining 7 days after freezing in non-OPG administered rat (Scale bar equals 500μm). Arrows show positive staining in gingival tissues. (d) TNF-α staining 7 days after freezing in OPG administered rat (Scale bar equals 200μm). Arrows show staining in the periodontal ligament. Arrows show positive staining. D = Dentine, PDL = periodontal ligament, AB = alveolar bone P = Pulp

root surfaces. In order to achieve this, the tooth was divided into thirds comprising buccal root, palatal root and interradicular area. Resorption lacunae were visually counted and evaluated in every third section.

Results

TNF-α

A summary of the results is presented in Table I. No positive staining was seen in the furcation region of the PDL of non-frozen teeth in OPG-administered and non-OPG administered rats. Positive staining was seen in the gingival tissues and in the marrow spaces of the alveolar bone (Figure 1A, Figure 1B). However, diffuse staining occurred in the PDL in non-OPG administered rats in the frozen and non-frozen teeth (Figure 1B and Figure 1C). Therefore, no distinct pattern in the staining was seen in the PDL of non-OPG administered rats.

OPG-administered rats showed positive staining in necrotic pulp tissue in the experimentally frozen teeth as well as in the periodontal interradicular area (Figure 1D). The positive staining was seen in the PDL, in the gingival tissues, in nerve tissue and in the marrow spaces of the alveolar bone.

Multinucleate giant cells in resorption lacunae were observed along the palatal roots of the frozen teeth in OPG-administered and non-OPG administered rats, but these areas did not show positive label for TNF-α (Figure 2).

In several specimens and independent of OPG administration, material with an osseous-like appearance and surrounded by positive TNF-α staining was seen in the interradicular area (Figure 3). This osseous-like material extended from the alveolar crest to the interradicular cemental surface in the furcation area. The amount of the osseous-like material varied from isolated islands to complete obliteration of the

Table I. Summary of results found in the present study. Numbers in brackets indicate the number of animals from the total used.

	Non-OPG		OPG	
	Non-frozen	Frozen	Non-frozen	Frozen
TNF-α	-	-	-	+ 6/9
TNFR1	+ (9/9)	+ (9/9)	+ (9/9)	++ (9/9)
Osseous-like material	No	Yes (2/9)	No	Yes (5/9)
Resorption lacunae	No	Yes	No	Yes

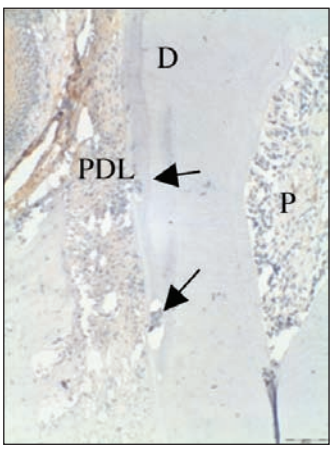

Figure 2. Resorption lacunae on the palatal root 7 days after freezing in OPG administered rat. Arrows show no positive staining for TNF-α in the resorption lacunae (Scale bar equals 50μm). D = Dentine, PDL = periodontal ligament, P = Pulp

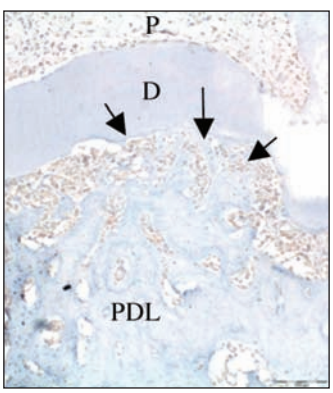

Figure 3. Frozen tooth in OPG administered rat. Arrows show positive staining for TNF-α surrounding the osseous-like material (Scale bar equals 100μm). P = Pulp, D = Dentine, PDL = periodontal ligament, AB = alveolar bone

PDL which, therefore, indicated the high likelihood of ankylosis. Positive TNF-α label was seen within the PDL in the cytoplasm of the ovoid-shaped fibroblast-like cells and within the extracellular matrix.

TNFR1

A summary of results is presented in Table I. Positive staining was seen in control and experimental teeth in OPG-administered and non-OPG administered rats. In non-frozen teeth of the non-OPG administered rats, positive staining for TNFR1 was seen within the PDL surrounding fibroblasts and randomly within pulp tissue. Strong positive staining was seen in ovoid-shaped cells lining the alveolar crest. These cells were uniform in shape and size and followed the contour of the alveolar ridge (Figure 4A). This was a consistent finding in all non-frozen teeth in OPG and non-OPG administered rats (Figure 4C) and in experimentally-frozen teeth of the non-OPG administered rats (Figure 4B).

In experimentally-frozen teeth of OPG-administered rats, positive staining for TNFR1 was seen within the PDL, in the dental pulp and gingival tissue. Within the furcation region, staining for TNFR1 was strong but diffuse. The positive labelling was not only localised within the cells of the PDL but was also seen in the extracellular matrix (Figure 4D).

OPG-administered rats showed resorption lacunae containing multinucleate giant cells which stained positively for TNFR1. These lacunae were seen randomly dispersed on the palatal roots of frozen experimental teeth (Figure 5).

Ovoid-shaped lining cells showed a strong positive staining for TNFR1 (Figure 4D). The ovoid cells were seen in areas containing the osseous-like cells in a line of demarcation between the PDL and the alveolar bone. In OPG-administered rats, the ovoid lining cells were seen surrounding the region of osseous-like material in the furcation region (Figure 6). Diffuse, strong, positive labelling for TNFR1 was also seen in areas of PDL in the furcation region not containing the osseous-like material.

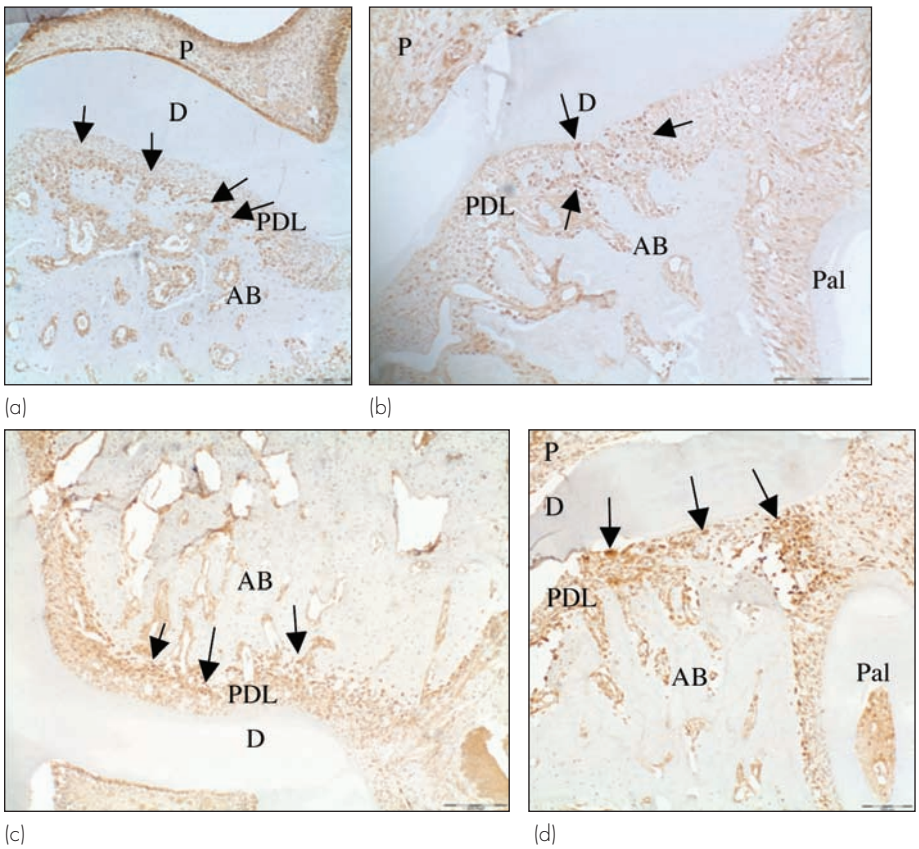

Figure 4. (a) TNFR staining in non-frozen tooth in non-OPG administered rat; (b) Frozen tooth in a non-OPG administered rat; (c) Non-frozen tooth in OPG administered rat; (d) Frozen tooth in OPG-administered rat. Arrows show positive staining for TNFR (Scale bar equals 100μm). P = pulp, D = Dentine, PDL = periodontal ligament, AB = alveolar bone, Pal = Palatal root

Discussion

A frozen tooth model has been used to produce aseptic inflammation, necrosis and subsequent root resorption in an attempt to mimic orthodontic root resorption.[9] Previous studies using periodontal cryotherapy by Wesselink et al.[16] and Tal et al.[17] reported resorption of the alveolar bone and cementum. In the present study, it was histologically evident that cryotherapy caused necrotic destruction of the underlying pulpal and periodontal tissues. Osteoclastic cell activity was evident on the bone and cementum surfaces in areas affected by the freezing process.

The present study qualitatively assessed the presence of TNF-α and TNFR1 immunostaining in the resorption model. Because OPG is a member of the TNF superfamily, it was considered that cross-reactivity could occur with the TNF-α polyclonal antibody. This was addressed in an earlier pilot study which used the only TNF-α monoclonal antibody available at that time. Breast adenocarcinoma tissue, known to react positively with TNF-α, was used with a variety of antigen retrieval techniques. No positive labelling was seen in any of the sections with any of the retrieval protocols. The TNF-α monoclonal antibody was, therefore, found to be unreliable and was discarded in favour of the TNF-α polyclonal antibody. It is deemed unlikely that cross-reactivity between the TNF-α polyclonal antibody and OPG existed, as not every OPG-administered specimen revealed positive labelling for TNF-α. However, this needs re-verification with a reliable TNF-α monoclonal antibody. Earlier studies have shown that TNF-α might be associated with osteoclast

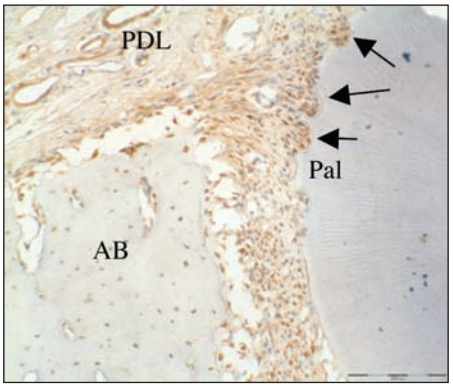

Figure 5. Resorption lacunae on the palatal root of an OPG administered frozen tooth. Arrows show positive staining for TNFR1 (Scale bar equals 100μm). PDL = periodontal ligament, AB = alveolar bone, Pal = Palatal root.

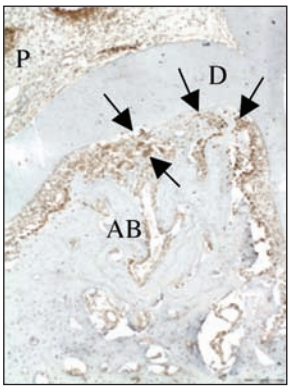

Figure 6. Frozen tooth in an OPG administered rat. Arrows show an area of osseous-like material with TNFR1 positive cells lining this region. Diffuse positive staining seen in the remaining PDL in the bifurcation region (Scale bar equals 100μm). P = pulp, D = Dentine, AB = alveolar bone

differentiation[14,18] and bone remodelling.[19] Mechanical loading with an orthodontic appliance demonstrated that expression of TNF-α was detected 12 hours after loading but three days later had returned to base levels.[20] It was concluded that the local enhancement of TNF-α preceded an increase in tartrate-resistant acid phosphatase (TRAP) activity and the number of TRAP-positive cells. In the present study, OPG, as an osteoclast inhibitor, did not prevent resorption lacunae from forming nor prevent their occupation by multinucleate giant cells. It is possible that these cells did not stain positively for TNF-α, despite forming via the TNF-α pathway, if the expression of TNF-α had reverted to its basal level three days after the thermal insult as reported by Andrade et al.[20]

Nevertheless, positive staining for TNFR1 was seen in resorption lacunae. As TNFR1 is the major receptor for TNF-α, the positive staining results for TNFR1 support the possibility that TNF-α can induce osteoclastic differentiation in the presence of OPG. Azuma et al.[18] showed TNF-α strongly induced differentiation of macrophage colony stimulating factor (M-CSF)-dependent bone marrow macrophage cells into mature osteoclasts via the TNFR1 receptor in the presence of OPG. The TNFR1 results indicated a generalised receptor presence throughout the PDL in control and experimental rats but the addition of OPG qualitatively appeared to increase the intensity of staining. A recent study has shown that cultures from TNFR1-null mice generate significantly fewer osteoclasts compared to their wild-type counterparts.[21] It was concluded that TNFR1 played a critical role in direct and indirect induction of osteoclastogenesis and that TNFR1 expression by osteoclast precursors is required for osteoclastogenesis. The presence of OPG may result in the increased expression of TNFR1, thereby allowing osteoclast differentiation to occur following the thermal insult. This provides a possible reason why stronger staining for TNFR1 in OPG-administered rats and the presence of resorption lacunae containing multinucleate giant cells was noted.

Because bone degradation is usually balanced by bone formation, the presence of TNFR1 in bone, particularly in osteoblasts, indicated that TNF-α may be involved with the osseous-like material within the PDL. After the cold thermal insult, the PDL healed, not only by repopulation of cells possibly derived from the gingival and PDL mesenchymal phenotypes, but also from the marrow spaces in alveolar bone. Transient ankylosis has been previously noted by Blomlof et al. [22] during reparative cementum formation after instrumentation of root surfaces in monkeys. It is possible that the osseous-like cells observed in the present study could be a result of periodontal wound healing and, therefore, may be transient in nature. Transient ankylosis has been noted for up to five weeks, resulting in a mineralized tissue layer apposed to, but not attached to, the cementum surface. A lamellar appearance resembling alveolar bone has been described.[23] This formation may be due to the osteoblastic lining cells and the osteoblast lineage of undifferentiated mesenchymal cells in the PDL apical to the damaged area.[23] As was clear in the control samples, a layer of ovoid cells lined the alveolar bone in the interradicular area and from the

location and the shape of these cells, their identity was likely osteoblastic. It is possible that, stimulated by the resorptive activity, the osteoblasts formed islands of osseous-like tissue in appositional contact with the intact cemental surface and thereby initiated the ankylotic process. The identification and mechanism of this ankylosis requires further study.

The presence of an increased amount of osseous-like material in the PDL in OPG-administered rats suggests that the osteoblast-osteoclast interaction may have an impact on the expression of the PDL osteoblastic potential.[24,25] A coupling factor between bone resorption and bone formation has been suggested with osteoclasts potentially providing a stimulatory source for osteoblast formation.[26] It is further possible that OPG causes an increase in the factor (or factors) that are released by osteoclasts to cause osteoblast formation which, in turn, results in a decreased net bone loss and, therefore, a bone mass increase. In addition, that these factors could belong to the TNF superfamily and their effects be mediated via TNFR1, is an intriguing supposition that requires further investigation.

Conclusion

The current study indicates that OPG, given at a dosage of 2.5 mg/kg, does not inhibit osteoclast formation in a freezing-induced aseptic inflammatory model. Known to stimulate osteoclast formation, TNF-α was qualitatively found to a greater extent in OPG-administered rats and matched by an increase in the presence of TNFR1. Osteoclast formation may therefore occur through TNFR1 in the presence of OPG. The presence of osseous-like material also suggests that OPG and TNF-α may contribute to an increase in osteoblastic expression and a net bone increase in the PDL. Further studies assessing the interrelationship between OPG, TNFR1 and the osseous-like material are now required to clarify the role of TNFR1, in both osteoclastic and osteoblastic activity and the phenomenon of ankylosis in aseptic inflammation-induced root resorption.

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