Localization of RANK, RANKL and osteoprotegerin during healing of surgically created periodontal defects in sheep


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Background and Objective: Modeling of periodontal bone regeneration in a large animal enables better examination of the spatial and temporal regulation of osteogenesis and the remodeling of the healing defect. RANK, RANKL and osteoprotegerin (OPG) are known to be important regulators of bone healing. The aim of this study was to create periodontal defects surgically in a large animal model and to examine bone regeneration and the expression of RANK, RANKL and OPG proteins in the defect site during bone regeneration.

Material and Methods: Periodontal defects were made in the furcation of the second mandibular premolar of sheep. Wound healing was examined 6 h, and 1, 4 and 6 wk after surgery and in control tissue. The teeth and defect region were decalcified and paraffin embedded. Immunohistochemistry for RANK, RANKL and OPG was conducted. Osteoclasts were identified using TRAP staining.

Results: The defects were examined at different time points after surgery and by 6 wk the defect region had fully regenerated with new bone, albeit less dense than that in the unwounded controls. RANK-positive osteoclasts were present at the edge of the wound from week 1 and were found within the defect at week 6, corresponding to osteoclast activation and bone remodeling. RANKL staining increased from week 1 compared with unwounded tissue, and peaked at 4 and 6 wk, as the osteoblast numbers increased. At the same time, OPG immunostaining was high in controls and at week 6, suggesting that it may act to block RANKL and control the bone remodeling within the defect.

Conclusion: Distinctive temporal and spatial expression patterns for RANK, RANKL and OPG proteins were observed during healing of surgically created periodontal wounds in a sheep model. The research identifies possible therapeutic approaches to periodontal bone repair via modulation of these members of the tumor necrosis factor family.

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Bone remodeling is a continuous process and is tightly controlled. It involves osteoclastic bone resorption in balance with osteoblastic bone synthesis (1). Control of this balance is crucial for skeletal growth and development (2,3) as well as for regulation of calcium homeostasis (4). Various systemic and local factors, including hormones, cytokines, growth factors and transcription factors, contribute to the control of bone remodeling.

Three members of the tumor necrosis factor (TNF) family – RANK, its ligand (RANKL) and its inhibitor osteoprotegerin (OPG) – appear to play pivotal roles in bone remodeling (5). Therefore, understanding their expression in different tissues and under varying clinical situations is highly relevant to health professionals who treat conditions involving osseous healing (1,6).

RANK is expressed on osteoclast precursors and osteoclasts (7,8) and induces intracellular signals leading to osteoclastogenesis upon ligand binding with RANKL. RANKL, also known as TNF-related activation-induced cytokine, osteoclast differentiation factor, OPG ligand and stromal osteoclast-forming activity, is localized on the cell membrane of osteoblasts and bone marrow stromal cells and is known to enhance the differentiation, activation and survival of osteoclasts via cell-cell interaction with osteoblast/stromal cells (8,9). RANKL is also found in a soluble form which actively binds to RANK on osteoclasts (10). OPG is an extracellular protein and a member of the TNF family, and is known to be produced by osteoblasts and bone marrow stromal cells (7,11,12).

The essential role of RANK–RANKL in osteoclast development was confirmed by a series of experiments targeting their regulation. In-vivo treatment of mice with RANKL activates osteoclasts, promotes bone loss and causes severe hypercalcemia (7). In contrast, severe osteopenosis and suppression of tooth eruption were seen in RANKL knockout mice because osteoclastogenesis was blocked (13). Mice deficient in OPG exhibit severe osteoporosis, with loss of trabecular bone and dramatic decreases in bone strength (14). Conversely, systemic administration of OPG to normal mice led to significantly increased bone mineral density and bone volume, and decreased osteoclast numbers (11,12).

Periodontitis is an inflammatory oral disease initiated by bacterial infections, resulting in the destruction of the periodontium, including the alveolar bone that supports the teeth. The periodontium consists of epithelium, fibrous connective tissue, tooth cementum and bone, in an intimate and functional relationship. Successful resolution of periodontitis requires regeneration of these tissues, and bone healing is a crucial part of periodontal regenerative therapy. However, molecular control and the expression of RANK, RANKL and OPG proteins in the periodontium during wound healing are poorly understood. A better understanding of the expression of these proteins during periodontal wound healing may lead to new insights in pharmacological and/or tailored gene therapy that may eventually traditional nonsurgical and surgical treatments for periodontal diseases.

Sheep have been identified as an appropriate animal model for dental research as they have premolar and molar teeth with a periodontium similar to the human dentition. At the University of Otago, initial work established that naturally occurring periodontitis in sheep could be used as a model for human periodontitis (15,16). A surgical model was then developed, using the bifurcation region of the second mandibular premolar, for testing periodontal regenerative procedures (17–20). Anatomically, this bifurcation region in sheep premolars has similar proportions to that of a small human mandibular molar. The metabolic rate of sheep is also similar to that of humans (21), and there are similarities in size, weight and general physiology (22,23).

The aim of this study was to localize the expression of RANK, RANKL and OPG proteins in relation to bone healing, using a surgically created periodontal bifurcation model in sheep.

Material and methods

Animals and surgical procedures

Ten cross-bred, 2-year-old female sheep were used. All procedures were undertaken with prior approval from the University of Otago Animal Ethics Committee (AEC 50-80). Two sheep were randomly selected as unwounded controls and were killed at time 0. In the test group, Class II furcation defects were surgically created on the buccal (lateral) surface of the mandibular second premolars (Fig. 1) of eight sheep, as previously described (17–20).

The sheep were anesthetized using intravenous injection of 20 mg/kg thiopental via the cephalic vein, then intubated through endotracheal tubes and connected to the anesthetic machine. Anesthesia was maintained by halothane (1–2%) and nitrous oxide/oxygen (1:2). With the sheep anesthetized, the oral cavity was aseptically prepared for surgery. A sulcular incision was made from the mid-buccal aspect of the third mandibular premolar to the mesial aspect of the first mandibular premolar, and then a relieving incision was made out into the diastema region that lies between the anterior teeth and the premolars. A full-thickness mucoperiosteal flap was then raised (Fig. 2A). The buccal surface of the crown of the second premolar tooth was thoroughly cleaned using a dental curette. Buccal bone on the second premolar was removed to identify the entrance and the fornix of the furcation. An access portal was created by making sharp vertical nicks on the mesial and distal surfaces of the tooth using a chisel and a mallet. Then, a horizontal nick was made to join the two earlier nicks (Fig. 2B). A thin buccal plate of bone was removed, exposing the fornix of the furcation.

A large round dental bur (number 5) in a contra-angle dental drill motor (Beaver-V; Osaka Electric Co. Ltd., Los Angeles, CA, USA) was used at 30 × 10³ r.p.m. to expose the furcation and root surfaces further. Copious irrigation with 0.9% sterile saline (Baxter Healthcare Pty Ltd., Auckland, New Zealand) was administered.
throughout the drilling process. A triangular defect was extended 5 mm vertically and horizontally, as measured from the fornice of the furcation. Following bone removal, a dental curette was used to remove cementum and residual periodontal ligament from the exposed root surface. The base of the defect was identified by notching the adjacent root surface with a small round number 3 dental bur. The mucosal flap was then repositioned and closed with 3-0 Vicryl sutures (Johnson & Johnson Medical Pty Ltd., Auckland, New Zealand).

Immediately following surgery, 2 mL of long-acting local anesthetic (Bupivacaine HCl, 5 mg/mL; Astra Zeneca, Auckland, New Zealand) was administered to each surgical site to minimize postoperative discomfort. Postoperatively the sheep were transferred to a recovery area where they were closely monitored by veterinary staff for 3 d. During this 3-d recovery period, the animals received daily treatment of 4 mg/kg bodyweight (1ml/12.5 kg) of the anti-inflammatory drug carprofen via intramuscular injection (Pfizer Animal Health, Auckland, New Zealand), 5 mL of the antibiotic streptopen (250 mg of dihydrostreptomycin sulfate and 200 000 IU procaine penicillin G per mL of suspension; Glaxo Animal Health, Auckland, New Zealand) and had their mouth syringed with antiseptic mouthwash (20 mL of 0.2% chlorhexidine gluconate in aqueous solution). The sheep were then returned to pasture at the Animal Breeding Station for the designated healing period.

Tissue collection and morphological analysis
Sheep were killed by formalin perfusion via the carotid arteries under anesthetic overdose. Two sheep were killed at each time point: 0 h (i.e. no-surgery control), 6 h postsurgery (surgical baseline) and then 1, 4 and 6 wk postsurgery. A block of alveolar bone measuring approximately 1 cm3 with the second premolar tooth in situ was harvested bilaterally from each sheep using a hand saw and immediately fixed in 10% neutral buffered formalin (LabServ, Biolab Ltd, Auckland, New Zealand) for 48 h at 4°C with agitation. The specimens were decalcified with 10% ethylenediaminetetraacetic acid disodium salt (cat. no. 108418; Merck, Brunswick, Germany), pH 7.4, for 6–11 wk. Specimens were gently agitated at 4°C and the ethylenediaminetetraacetic acid disodium salt was changed twice weekly. The end point for decalcification was determined when no visible precipitate formed after an ammonium oxalate test. When decalcified, the tissue was washed overnight in phosphate-buffered saline (PBS), dehydrated, cleared and embedded in paraffin.

The specimens were sectioned in a mesio-distal plane along the long axis of the tooth, from the buccal surface to the mid-portion of the blocks. Serial sections were cut at a thickness of 4 μm and mounted on slides coated with 3-aminopropyltriethoxysilane (Lab Scientific Inc., Highlands, NJ, USA). Step serial sections were either stained with hematoxylin and eosin or assigned to immunohistochemical protocols for RANK, RANKL and OPG, as described below. Osteoclast activity was identified through histochemical demonstration of TRAP.

Immunohistochemistry
Paraffin sections were dewaxed, rehydrated and rinsed thoroughly with...
distilled water. The sections for anti-RANK and anti-OPG immunostaining were pretreated with 0.1% trypsin (Sigma-Aldrich Co., St. Louis, MO, USA) in 0.1% CaCl₂ (pH 7.8) for 10 min at room temperature. No pretreatment agents were used for the anti-RANKL IgG1. Sections were placed in a humidified chamber and incubated for 30 min in PBS containing 20% rabbit serum (Sigma Chemical Co.) and with 1% bovine serum albumin. The sections were then incubated overnight at 4°C with one of the following antibodies: 10 µg/mL of mouse anti-human RANKL (Clone EL19F; cat. no. sc-74260; Santa Cruz Biotechnology Inc., Dallas, TX, USA); 4 µg/mL of mouse anti-human RANK (Clone 9A725; cat. no. sc-52951; Santa Cruz Biotechnology, Inc.) or 2.5 µg/mL of mouse anti-human OPG/TNFRSF11B (Clone 98A1071; cat. no. ab79064; Sapphire BioScience Pty Ltd., Waterloo, NSW, Australia). The antibodies were diluted in PBS containing 1% bovine serum albumin and 5% rabbit serum. Following incubation, the sections were rinsed with PBS for 45 min, and all sections were treated with a biotinylated rabbit anti-mouse IgG-H&L-F(ab)₂ secondary antibody (cat. no. ab5671; Sapphire Bioscience Pty Ltd.) at 1 µg/mL and incubated for 1 h at room temperature. Then, the sections were washed in three changes of PBS for 20 min. Endogenous peroxidases were quenched with 0.3% H₂O₂ (Merck) in methanol for 10 min, followed by washing with PBS for 10 min. The sections were labeled with avidin–biotin–horseradish peroxidase (Vectorstain Elite ABC, Vector Laboratories, Burlingame, CA, USA), followed by 3,3′ diaminobenzidine (Sigma Chemical Co.) for up to 3 min. The sections were counterstained with hematoxylin for 5 s and cover-slipped using DePeX (Merck).

**TRAP staining**

Sections were dewaxed using xylene and rehydrated using a graded series of ethanol. TRAP staining was carried out using an Acid Phosphatase Leukocyte kit (cat. no. 387A; Sigma-Aldrich Co.) and was prepared according to the manufacturer’s instructions. Each section was incubated with 500 µL of TRAP solution for 4 h at 37°C and protected from light. Then, the slides were rinsed thoroughly in warm water for 2 min, counterstained with hematoxylin for 5 s and cover-slipped using Aquatex aqueous mounting agent (Merck).

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**Fig. 3.** Photomicrographs showing premolars stained with hematoxylin and eosin and scanned at low power to reveal the relationship between furcation (F)/defect (De)/furcation wound area (FWA) (dotted lines) and the surrounding structures. (A) The unwounded furcation (F) at baseline. (B) The defect (De) created within the furcation, shown 6 h postsurgery as evidence of the extent of the wound formation. (C) The furcation wound area (FWA) at 4 wk postsurgery. (D) The FWA was filled with bone by 6 wk. The root region (R) was evident in all images, and in A, B and D pulp and dentine were evident within the root, whereas the sectioning of C revealed only dentine. AB, alveolar bone. (Scale bar = 500 µm.)
Photography and scoring of immunohistochemical staining

Images were captured and digitized at a standardized magnification using a JVC TK1281 color video camera (SciTech Pty Ltd, Preston, Victoria, Australia) fitted to a Vanox-T binocular microscope (Olympus New Zealand PTY LTD, Auckland, New Zealand). Sections produced at each time point were examined after staining for RANKL, RANK and OPG. If no positive cells were observed within the furcation wound area or the immediate surrounding bone, they were scored as not detected. The presence of osteoclasts within the furcation wound was scored as follows: +, 1–2 positive osteoclasts per section; ++, 3–10 positive osteoclasts per section; and ++++, more than 10 positive osteoclasts per section. When considering RANKL in osteoblasts and OPG, the scoring equated to small, moderate and large numbers of positive cells.

Results

Histological evidence of bone formation

For comparison, the unwounded bifurcation region (0 h, control) is shown in Fig. 3A. Examples of healing wounds from the test animals that were killed after 6 h (surgical baseline), and after 4 and 6 wk, are shown in Fig. 3B, 3C and 3D, respectively. As expected, the unwounded control demonstrates intact alveolar bone, periodontal ligament, cementum and dentine (Figs 3A and 4A). At 6 h, the furcation wound was clearly delineated, with no signs of new-bone formation evident (Fig. 3B). The furcation wound was partially filled with a mixture of new bone and organized fibrous connective tissue at 4 wk (Figs 3C and 5G). By 6 wk, the furcation wound was filled with new bone; however, this appeared to be less dense than that of the unwounded control specimens (Fig. 3D).

Immunohistochemistry controls

Negative controls with IgG at a matched concentration were run simultaneously with all antibodies and showed no background staining. The antibodies used in this research were all specific monoclonals and were tested, before use, on paraffin-embedded mouse embryos and remodeled sheep bone to confirm cross-species reactivity. RANKL was present in association with osteoblasts and osteoclasts in the developing mouse mandible and maxilla, RANK was associated with osteoclasts and OPG was associated with the connective tissue in the developing mandible. The lack of background endorsed these findings. Immunostaining of the sheep tissue was consistent with staining of the antigens of interest. The unwounded furcation area (Fig. 4) stained with anti-OPG, and no RANK and RANKL were detected, as would be expected in stable bone. RANK was detected only in association with osteoclasts in the furcation. RANKL was detected primarily in osteoblasts; however, osteoclasts within the areas of remodeling also staining positive for RANKL and OPG, and this is discussed later.

Immunohistochemistry for RANKL, RANK and OPG

Table 1 gives an overview of the immunohistochemical staining for osteoblasts, osteoclasts and tissue over the study time periods examined. Figure 4A shows the unwounded control (0 h) at a higher magnification. Only very limited numbers of osteoblasts and osteoclasts were observed, with no RANKL immunostaining; and osteoclasts stained only very occasionally, and weakly, for RANK (Fig. 4B, 4C; Table 1). Strong OPG staining was seen localized to osteoclasts, osteocytes and the connective tissue matrix within the furcation (Fig. 4D).

In the test specimens, RANKL, RANK and OPG proteins were all expressed during the healing process. After 1 wk, RANKL staining was present on some osteocytes at the
periphery of the alveolar bone, but not on the mature osteocytes (Fig. 5A). Osteoblasts were relatively scarce at 1 wk; however, where present, they did express RANKL. Osteoclasts were evident by week 1 on the bone adjacent to the wound and these stained for RANKL as did osteoclasts (Fig. 6A). Osteoclasts were observed and these stained for RANK within the furcation wound area (Fig. 6B). Strong staining of OPG protein was evident 6 wk after wounding (Fig. 6C).

**Histochemical identification of osteoclasts: TRAP staining**

TRAP staining was carried out to verify the presence of osteoclasts. Osteoclasts stained positive for TRAP at all time points. Consecutive serial sections showed TRAP-positive osteoclasts (Fig. 7A), which were also positively labeled with the RANK antibody (Fig. 7B). This labeling confirmed that the cells expressing RANK were indeed osteoclasts.

**Discussion**

The present study characterized the temporal expression pattern of RANK, RANKL and OPG proteins during healing in a sheep periodontal wound model. To our knowledge this is the first study to demonstrate the expression of RANK, RANKL and OPG proteins in periodontal wound healing and the first to do so in the sheep model.

The expression of these proteins changed after wounding. RANK was expressed only by osteoclasts. From week 1, osteoclasts staining positive for RANK were associated with the remodeling of bone at the edge of the furcation wound area; by week 6, such osteoclasts were also associated with the new bone within the furcation wound area. RANKL immunostaining was present around some peripheral immature osteocytes and most of the osteoclasts throughout the healing periods. The RANKL and OPG immunostaining of osteoclasts was unexpected; however, the detection of RANKL bound to receptor would be consistent with this finding. Strong immunostaining of osteoclasts

At 4 wk, strong RANKL staining was observed on osteoblasts and immature osteocytes at the periphery of the wound where new bone was evident (Fig. 5B). Osteoclasts all stained with RANKL immunohistochemistry. Osteoclasts did not stain for RANK within the furcation wound area where bone was being produced at week 4, but were positive on remodeling bone at the edge of the original wound (Fig. 5D, 5E). OPG stained moderately at 4 wk (Fig. 5G).
Table 1. Immunohistochemical staining of cells within the furcation defect

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<tr>
<th>Study time point</th>
<th>RANKL Osteoblasts</th>
<th>RANKL Osteoclasts</th>
<th>RANK Osteoclasts</th>
<th>OPG Tissue</th>
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<tr>
<td>Control</td>
<td>ND</td>
<td>ND</td>
<td>+</td>
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<tr>
<td>6 h postsurgery</td>
<td>ND</td>
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<td>1 wk postsurgery</td>
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<td>6 wk postsurgery</td>
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Scoring was as follows for RANKL in osteoblasts and osteoprotegerin (OPG) in tissue: +, a small number of positive cells; ++, a moderate number of positive cells; and ++++, a large number of positive cells. For osteoclasts within the furcation wound, RANK/ RANKL positivity was scored as follows: +, 1–2 positive osteoclasts per section; ++, 3–10 positive osteoclasts per section; and ++++, more than 10 positive osteoclasts per section. ND, no positive staining detected.

for RANKL and RANK, and weak to medium immunostaining for OPG, has been found by others during bone remodeling and is thus consistent with our findings (24,25). Also consistent are the high serum levels of RANKL and OPG protein and high tissue production during remodeling, indicating that these ligands could act in an endocrine and paracrine manner and contribute to the high levels of immunostaining of osteoclasts with RANKL and OPG associated with areas where bone is remodeling (26). RANKL immunohistochemical staining was also associated with cuboidal osteoblasts and was stronger later in the wound-healing process when more cuboidal osteoblasts were evident. OPG was seen around osteoblasts, osteoclasts, osteocytes and in the connective tissue matrix, and was highest in the connective tissue matrix of the unwounded bone and at 6 wk postwounding.

In the present study, RANK expression at 1 wk is consistent with an increase in osteoclast activation and bone remodeling at the edge of the FWA at this time. This strong immunostaining for RANK protein, compared with the unwounded control tissue, could suggest an increase in the number of RANK receptors on the osteoclast precursors as a result of RANKL binding (7,13) and would be consistent with active resorption of the alveolar bone on the periphery of the wound. RANKL was not present in unwounded tissue but was induced in the early stages of healing to be present by week 1 of healing.

Spatial changes in RANKL-positive osteoblasts and osteoclasts were observed from 1 to 6 wk. As the healing progressed, the localization of osteoblasts and osteoclasts with RANKL-positive immunostaining changed, as did the intensity of their staining. RANKL-positive cells were confined to the furcation wound at 1 wk postsurgery, but were found both within and on the edge of the wound area at 4 and 6 wk postsurgery, suggesting remodeling as well as new-bone formation.

The interaction between RANKL and RANK is competitively inhibited by OPG, which specifically binds to RANKL and inhibits its activity (11,27,28) by preventing binding to RANK (9,28). In the unwounded control it would be expected that this interaction would minimize the activation of osteoclasts. The reduced OPG seen in the present study at 1 wk postsurgery is consistent with an increase in bone remodeling during the early stages of bone healing. By 4 and 6 wk, although high levels of RANKL expression were observed, correspondingly high levels of OPG were found and may indicate a control of osteoclast activation. The results of the present study therefore suggest that the balance between RANKL and OPG may be important, not only in maintaining bone homeostasis, but also in controlling healing of an osseous wound. Therefore, our observations have confirmed a previous study on the role of OPG in osteoclastogenesis (11) and expand the role of OPG to periodontal wound healing in sheep.

This study is the first to demonstrate these proteins in a periodontal wound model. Their regulation during healing suggests that research into the potential therapeutic use of OPG proteins may be worthwhile in periodontal wound healing. Early addition of exogenous OPG within the wound may reduce osteoclastic resorption and accelerate osteoblast activity. Other animal models have shown that OPG therapy is effective at blocking
osteoclast activation. OPG administration in rodents prevented ovariectomy-induced bone loss (11), reduced periodontitis-induced bone destruction (29) and suppressed bone erosion in arthritis (30,31). OPG therapy also improved bone formation in tendon-to-bone wounds in rabbits (32) and has been extended to humans for treatment of humoral hypercalcaemia of malignancy (33) and osteoporosis (34). The present study suggests that investigation of OPG therapy using this model of surgically created periodontal defects would be worthwhile.

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References


