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DOI: 10.2478/aoj-2014-0011

A qualitative investigation of RANKL, RANK and OPG in a rat model of transient ankylosis

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Introduction: Previous studies have found ankylosis occurs as a part of the inflammatory process of aseptic root resorption initiated in a rat model.[1] The physiologic mechanisms behind the development of dentoalveolar ankylosis and healing response are still unclear. While receptor activator of nuclear factor-κβ ligand (RANKL), receptor activator of nuclear factor-κβ (RANK) and osteoprotegerin (OPG) have gained momentum in the understanding of resorption, no study to date has investigated their role in dentoalveolar ankylosis.

Aims: The aims of this study were to investigate if, and when, ankylosis occurred in the rat PDL, whether the resolution of ankylosis occurred with time and, finally, to observe the expression of RANKL, RANK and OPG during the ankylotic process.

Materials and methods: Dry ice was applied for 20 minutes to the upper right first molar crown of 15 eight-week-old, male Sprague-Dawley rats. An additional three rats served as untreated external controls. Groups of three rats were sacrificed after the thermal insult on day 0, 4, 7, 14 and 28 respectively. Each maxilla was dissected out and processed for histological examination and RANKL, OPG and RANK immunohistochemistry.

Results: By the use of light microscopy and H&E staining, no ankylosis was detected in the external control group and the experimental groups at days 0 and 4. On day 7, disruption within the periodontal ligament was observed in the interradicular region and the initial signs of ankylosis were seen in the form of finger-like projections extending from the alveolar bone towards the cementum. Fourteen days after the thermal insult, all animals exhibited extensive ankylosis that spanned the entire interradicular periodontal space. At 28 days, the development of ankylosis appeared to have ceased and repair was observed, together with an intact periodontal ligament in all but one rat. Positive staining results were obtained with RANKL, RANK and OPG antibodies. The expressions of RANKL, RANK and OPG were similar in the external control group, 0-, 4-, and 28-day experimental groups. In the 7- and 14-day experimental groups, RANKL, RANK and OPG were expressed in the blood vessels within the ankylotic regions.

Conclusions: During the development of ankylosis and its resolution, it was concluded from their simultaneous presence that there is a complex interaction between RANKL, RANK and OPG that requires further investigation.

(Aust Orthod J 2014; 30: 143–151)

Received for publication: May 2014

Accepted: August 2014

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Introduction

Bone remodelling is a life-long process in which bone is removed from the skeleton by osteoclasts and new bone is added by osteoblasts. In a homeostatic state, bone resorption and formation are balanced so that old bone is continuously replaced by new bone tissue.[2,3] An understanding of the molecular mechanisms which regulate osteoclast formation and activation has gained momentum since the discovery of the RANKL/RANK signalling system. It has been accepted that osteoclastic bone resorption precedes osteoblastic bone formation in a normal bone remodelling cycle.[4] Osteoclast differentiation is initiated through direct cell-to-cell contact between osteoblasts and osteoclast precursors. The initiating factors have been identified as the RANK ligand produced by the osteoblast and its RANK receptor found on the osteoclast cell precursor.[5] Osteoprotegerin (OPG) is a protein produced by osteoblasts, stromal cells and other formative cells in the PDL,[6,7] which has been identified as a decoy receptor for RANK.[8-10] OPG competitively binds to

RANKL, thereby inhibiting osteoclastogenesis and subsequent bone resorption. In recent times, the dominant RANKL/RANK/OPG pathway in the regulation of bone resorption has been demonstrated in animal models, in which these molecules have been genetically removed or over-expressed. In mouse studies, the negation of RANKL or RANK resulted in severe osteopetrosis and defective tooth eruption due to a lack of osteoclasts.[11-14] In contrast, mice with negated OPG displayed osteoporosis resulting from increased numbers and activity of osteoclasts.[15,16] However, the over-expression of OPG results in increased cortical and cancellous bone mass associated with reduced osteoclast numbers.[17]

Orthodontic tooth movement relies on the interaction between osteoblasts and osteoclasts and on the preservation and homeostasis of the periodontal ligament (PDL) space. An important property of the PDL is its ability to maintain its width over time. According to Nanci and Bosshardt,[18] cells within the PDL during development and regeneration secrete molecules which play a role in regulating the extent of mineralisation and preventing the fusion of cementum with surrounding bone, i.e. ankylosis. When ankylosis occurs, all orthodontic tooth movement ceases until such time as the ankylotic union is removed.

The aetiology of ankylosis is still unclear. Biederman[19] suggested that a discontinuity in the PDL must be present, which is followed by bony deposition; or there must be a direct local ossification of the PDL itself. However, recent work has indicated that calcifications may develop within the hyalinised areas of the PDL during orthodontic tooth movement.[20] Resorption in combination with ankylosis has been observed after dental and periodontal ligament trauma associated with tooth reimplantation,[21,22] transplantation[23] and the luxation of teeth.[24]

Currently, there are few reports which have examined the role of the RANKL/RANK/OPG system in an ankylotic model. Hence, the present study aims to investigate the expression of RANKL/RANK/OPG in experimentally-induced ankylosis.

Materials and methods

Experimental animals

Eighteen eight-week-old, male Sprague-Dawley rats were randomly divided into five experimental groups of three animals plus one control group and treated under ethical regulations for animal experiments as approved by the Ethics Committee of The University of Adelaide (M-04-2004, M-023-2007).

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 twenty minutes by the application of customised 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.

All animals were sacrificed under anaesthesia via intracardiac perfusion of 30 ml of 4% paraformaldehyde fixative. The external control group of three animals was sacrificed on the same day immediately following the onset of deep anaesthesia and without the application of the dry ice. The five experimental groups of three animals were sacrificed at 0, 4, 7, 14 and 28 days respectively after the thermal insult.

Each maxilla was dissected out and immersed in the same fixative for 24 hours, rinsed in a phosphate-buffered saline (0.4M pH 7.4) for 24 hours and then decalcified using 4% EDTA in phosphate buffer. Subsequently, the tissues were placed in 70% 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. The teeth were cut to provide serial sections through the furcation region and arranged so that consecutive sections were placed on the same slide. Serial slide staining in the sequence of haematoxylin and eosin, RANKL antibody, OPG antibody and RANKL antibody was performed. In this way, 200 sections were taken from the furcation region of each first molar tooth.

Immunohistochemical staining for RANKL, RANK and OPG

Using the streptavidin-biotin complex (ABC) method, prepared slides were processed for immunohistochemical (IHC) staining. No antigen retrieval procedure was required for RANKL. Heat-antigen retrieval with tris-EDTA (pH 9) at 65°C was required for both RANK and OPG. Optimal dilutions were derived for RANKL, RANK and OPG antibodies at 1:50, 1:100 and 1:100 respectively. Rat knee joints were used as positive controls for RANKL and OPG while human tonsillar tissue was used for RANK. Primary antibodies were omitted in the negative controls.

Results

Haematoxylin and eosin

No animals in the 0- and 4-day groups showed ankylosis (Figure 1a). Both experimental and control teeth were histologically similar to those of the external control group. Under histological examination, ankylosis was present within the interradicular periodontal space in all experimental teeth in the 7- and 14-day observation groups and in one rat in the 28-day observation group (Figure 1b, c). At day 28, no ankylosis was observed in the other two experimental rats of the group (Figure 1d). No ankylosis was present in any of the internal control teeth of all groups. The interradicular regions of the internal control teeth of all groups were histologically similar to the external control group, i.e. no ankylosis was present and a vital PDL was maintained at all times.

The experimental molars of animals in the 7-day group showed disruption in the form of periodontal ligament interradicular hyalinisation. The initial signs of ankylosis were observed as fine, finger-like projections extending through the PDL from the alveolar bone to the cementum, with ankylotic union seen mainly within the furcation area (Figure 1b). Connective tissue cells and blood vessels were interspersed amongst the ankylotic projections. Tooth and bone resorption was rarely seen in the interradicular region.

Fourteen days after tooth freezing, all animals exhibited ankylosis. This was extensive and occupied almost the entire interradicular region (Figure 1c). The ankylosis consisted of solid bone-like material with fine trabeculae, extending from the alveolar bone to the root surface. Blood vessels and connective tissue cells were observed but the PDL was almost entirely replaced by bone-like deposition. Resorption could be seen affecting the root surface adjacent to the ankylotic area. Changes were observed within the pulp chamber which exhibited considerable dentine formation (Figure 1c).

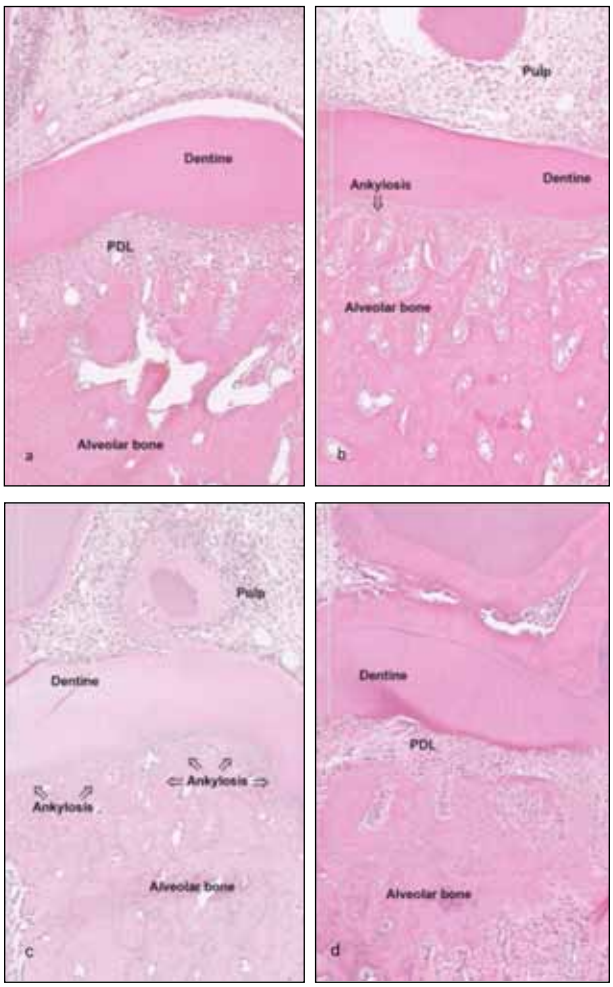

Figure 1. (a) H&E staining of an experimental tooth in the experimental 0-day observation (Magnification 10x). (b) H&E staining of experimental tooth at 7 days. Initial signs of ankylosis are indicated by the arrow (⇒) (Magnification 10x). (c) H&E staining of experimental tooth at 14 days. Widespread ankylosis is indicated by arrows (⇒) (Magnification 10x). (d) H&E staining of experimental tooth at 28 days. No sign of ankylosis was noted but there is formation of secondary dentine within the pulp chamber (Magnification 10x).

Twenty-eight days after the thermal insult, the development of ankylosis appeared to have ceased in all but one animal. The repair of the ankylotic areas was observed with the appearance of an intact and seemingly normal periodontal ligament that extended throughout the entire interradicular region. However,

the pulpal changes persisted and were similar to the changes seen in the 14-day group, except that there was an additional increase in dentine formation (Figure 1d).

Immunohistochemical staining

The control teeth, at all time observations, showed the same expression of RANKL, OPG and RANK as the experimental teeth at external control and 0-day observation.

RANKL

The positive expression of RANKL was similar in the experimental teeth at the external control, 0-day observation and 4-day observation periods. RANKL was expressed in a few isolated cells within the PDL in the furcation area. RANKL staining was mainly observed in the vicinity of blood vessels within the alveolar bone near the PDL and cells lining the alveolar bone but was not detected in cells lining the cementum (Figure 2a). At 7 days, positive labelling in the experimental teeth was associated more frequently with cells around blood vessels (Figure 2b). At 14 days, positive labelling in the experimental teeth was associated with cells around blood vessels as well as osteocyte-like cells surrounded by newly formed osteoid (Figure 2c). At 28 days, the positive labelling was similar to the external control, 0-day, and 4-day groups with RANKL expressed in isolated cells within the PDL in the furcation area and in the vicinity of blood vessels within the alveolar bone adjacent to the PDL (Figure 2d).

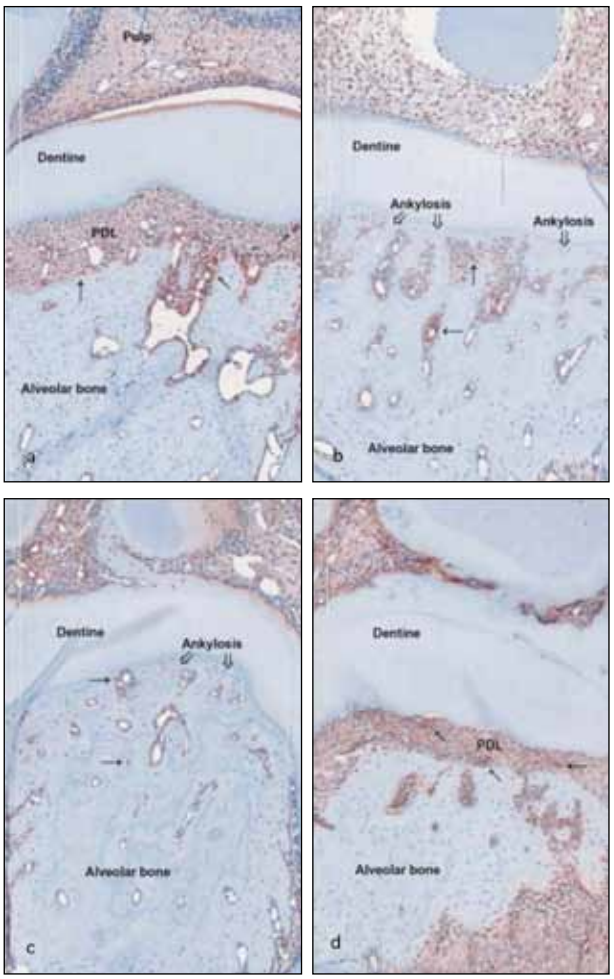

Figure 2. (a) RANKL staining of an experimental tooth in the experimental 0-day observation group as indicated by arrows (→) (Magnification 10x). (b) RANKL staining of an experimental tooth in the experimental 7-day observation group, as indicated by arrows (→). Areas of ankylosis indicated by arrows (⇒) (Magnification 10x). (c) RANKL staining of an experimental tooth in the experimental 14-day observation group, as indicated by arrows (→). Areas of ankylosis indicated by arrows (⇒) (Magnification 10x). (d) RANKL staining of an experimental tooth in the experimental 28-day observation group, as indicated by arrows (→) (Magnification 10x).

OPG

A positive expression of OPG was noted in experimental and control teeth at external control, 0-day and 4-day observation periods. Positive label was expressed in a few isolated cells within the PDL, but was mainly observed in the vicinity of blood vessels within the alveolar bone near the PDL and cells lining the alveolar bone. Occasionally, OPG label was located around blood vessels lying close to the tooth surface (Figure 3a). At the 7-day observation period, labelling was similar to the previous time points, with positive staining associated more frequently with cells around blood vessels (Figure 3b). At 14 days, in addition to the previously noted labelling, positive labels were associated more frequently with cells around blood vessels as well as osteocyte-like cells surrounded by newly formed osteoid (Figure 3c). At 28 days, positive labelling was similar to the 0- and 4-day observation. Positive labelling was noted in cells within the PDL, but appeared to be more concentrated along the surface of the root and alveolar bone (Figure 3d).

RANK

A positive expression of RANK was noted in experimental and control teeth at external control, 0-

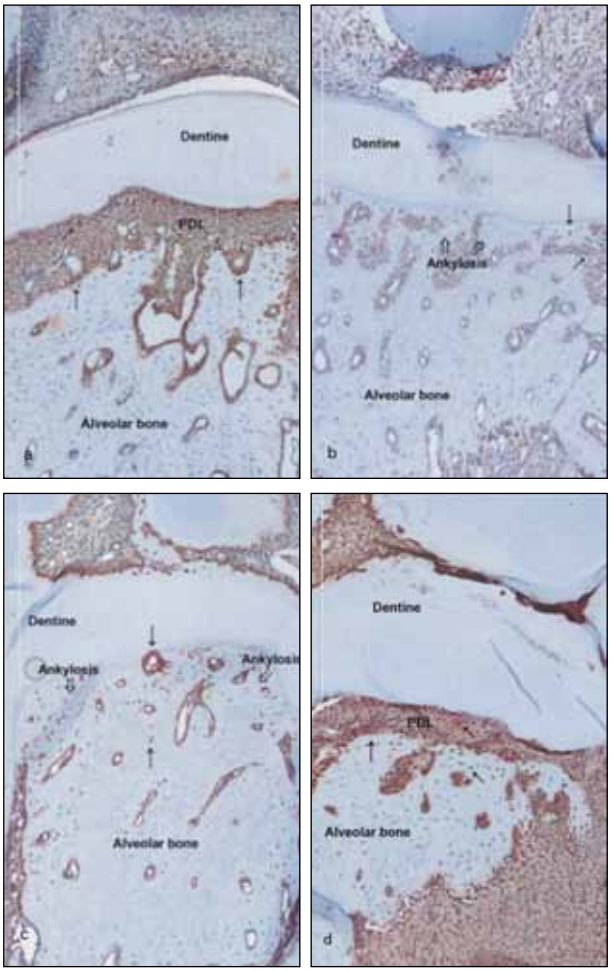

Figure 3. (a) OPG staining of an experimental tooth in the experimental 0-day observation group as indicated by arrows (→) (Magnification 10x). (b) OPG staining of an experimental tooth in the experimental 7-day observation group, as indicated by arrows (→). Areas of ankylosis indicated by arrows (⇒) (Magnification 10x).
(c) OPG staining of an experimental tooth in the experimental 14-day observation group, as indicated by arrows (→). Areas of ankylosis indicated by arrows (⇒) (Magnification 10x). (d) OPG staining of an experimental tooth in the experimental 28-day observation group, as indicated by arrows (→) (Magnification 10x).

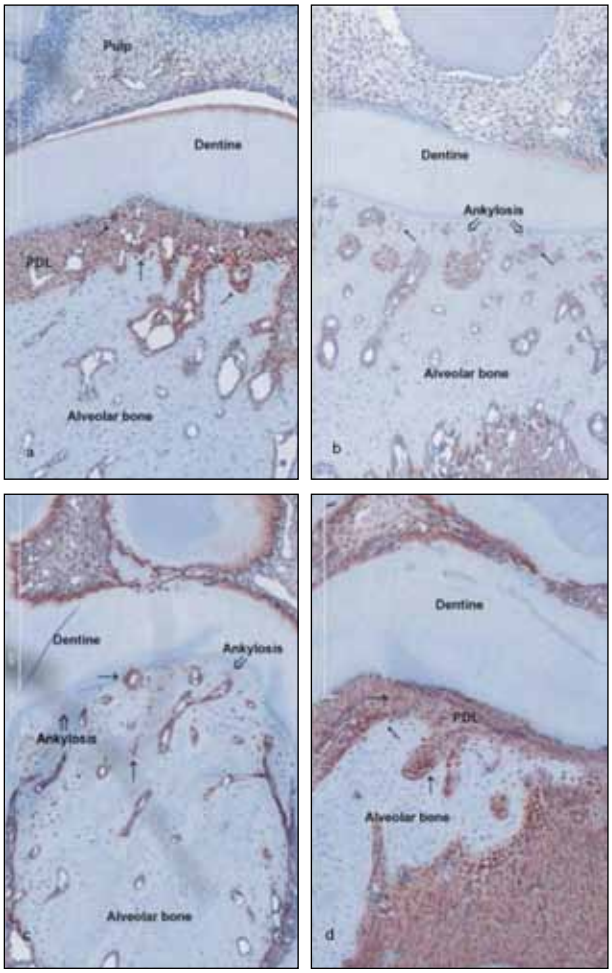

Figure 4. (a) RANK staining of an experimental tooth in the experimental 0-day observation group, as indicated by arrows (→) (Magnification 10x). (b) RANK staining of an experimental tooth in the experimental 7-day observation group, as indicated by arrows (→). Areas of ankylosis indicated by arrows (⇒) (Magnification 10x).
(c) RANK staining of an experimental tooth in the experimental 14-day observation group, as indicated by arrows (→). Areas of ankylosis indicated by arrows (⇒) (Magnification 10x). (d) RANK staining of an experimental tooth in the experimental 28-day observation group, as indicated by arrows (→). There was no sign of ankylosis (Magnification 10x).

and 4- day time points. The distribution of RANK label was similar to that of RANKL expression. RANK was expressed in a few isolated cells within the PDL, but was mainly observed in the vicinity of blood vessels within the alveolar bone near the PDL and cells lining the alveolar bone (Figure 4a). At 7 days, in addition to the previously noted distribution, positive labels were associated more definitively in cells around blood vessels (Figure 4b). At 14 days, the experimental teeth showed positive labels associated with cells around blood vessels as well as osteocyte-like cells surrounded by newly formed osteoid (Figure 4c). At 28 days, positive label distribution was similar to the 0- and 4-day groups and was seen in cells within the PDL but appeared to be more concentrated along the surfaces of the root and alveolar bone (Figure 4d).

Discussion

Using rats in experiments has several advantages, including similarity of the rat PDL with that of the human, their low cost and minimal genetic variability.[25,26] However, data derived from rat studies must be interpreted with caution before conclusions for human application are drawn.[27]

The thermal insult procedure used in the present study was adapted from an established protocol.[28] The thermal insult was standardised over each observation time group in an attempt to produce consistent results. All of the animals underwent the same thermal insult over the same time period and with the same pressure directed onto the occlusal surface of the rat molar. The freezing of the molar crowns likely transmitted the thermal insult through the tooth, resulting in the necrosis of bone lining cells and adjacent periodontal tissue. The observed ankylotic union may be attributed to a reparative response by unaffected cells and blood vessels in adjacent tissues and alveolar bone.[29] Ankylosis was a consistent observation in all of the experimental animals in the 7- and 14-day observation groups.

Andreasen,[29] in his time-related study of periodontal healing and resorption activity, described two types of ankylosis. The first developed in old resorption lacunae in which resorption of cementum and dentine preceded alveolar bony union. The second occurred when the periodontal ligament was replaced by bone without prior resorption of cementum. The alveolar bone and cemental surface appeared to be in close contact, either fused together or possibly separated by a thin amorphous layer. The ankylosis encountered in the present study was of the appositional rather than the replacement type. Histologically, there was no sign of preceding resorption in the ankylotic areas, which is in agreement with previous findings.[28] Observations at days 4 and 7 after the thermal insult showed that the enthusiastic healing response by the periodontal ligament and surrounding interradicular alveolar bone resulted in overgrowth of hard tissue and subsequent ankylotic union.

The close proximity of blood vessels observed in the interradicular anklyotic regions of the 7- and 14-day experimental groups was consistent with the pattern of woven bone formation adjacent to newly formed blood vessels during the healing of tooth extraction sockets.[30]

At day 28 following the thermal insult, ankylosis was not evident in two of the three experimental molars of that group. It appeared that ankylosis had been resolved in the animals between day 14 and day 28 to the extent that a vital PDL was re-established over the entire interradicular region. Although vascular pericytes have been suggested as playing a role in promoting osteogenic cells in the development of ankylosis,[31] they have also been associated with areas of resorption in which osteoclastic precursors may migrate out of blood vessels and differentiate into actively resorbing osteoclasts. The physiologic mechanisms behind the maintenance of the periodontal space and the prevention of dentoalveolar ankylosis are still largely unknown. The reduction in ankylosis between days 14 and 28 may be attributed to upregulated resorptive activity. In all animals which developed ankylosis, a vital PDL was evident adjacent to the ankylotic union, even at the peak of ankylosis observed at day 14. A vital PDL has previously been established as essential in the resolution of ankylosis.[32,33] This could possibly be due to the PDL acting as a source of clast-like cells, which may also be derived from new blood vessels and the repopulation of marrow cells. It is likely that upregulated resorption may serve as a reparative mechanism of the PDL by removing regions of reactive hard tissue.

In the present study, positive staining results were obtained with the RANKL, RANK and OPG antibodies at an optimal dilution of 1:50, 1:100 and 1:100, respectively. The antibodies produced positive and reliable staining in all sections. RANKL, RANK and OPG expression were similar in all right and left molars of the external control tissues and the control left side molars in all the experimental groups. In the interradicular region of the experimental teeth, the expression of RANKL, RANK and OPG were similar in the external control group, 0-day, 4-day and 28-day experimental groups. RANKL, RANK and OPG expressions were mostly associated with blood vessels in the PDL, lining cells and apparently random cells within the PDL. When ankylosis was observed in the 7-day and 14-day groups, RANKL, RANK and OPG were expressed in the region of blood vessels within the ankylotic area.

The discovery of RANKL, RANK and OPG has greatly enhanced the understanding of bone biology. RANKL is a member of the TNF superfamily, is expressed by osteoblasts and their precursors and is necessary for osteoclastogenesis.[34] RANKL activates its receptor, RANK, which is expressed on osteoclasts and their precursors, to promote osteoclast differentiation and activation. The effects of RANKL are blocked by the secretory glycoprotein OPG, which acts as a decoy receptor for RANKL.[17,35] The regulation of RANKL and OPG is dependent on many cytokines and hormones, which affect osteoclast function, and

the RANKL/OPG ratio is critical in establishing the balance between bone formation and bone resorption.[17] To date, no study has comprehensively investigated the immunohistochemical expression of RANKL, RANK and OPG in a model of transient ankylosis. Throughout the entire observation period of this study, from the development of ankylosis (day 7 and peak at day 14) to its absence (at day 28), RANKL, RANK and OPG were positively identified in the serial sections of the interradicular region.

In the present study, the expression of RANK was found in the region of osteoclast-like cells, blood vessels in the PDL and on the lining cells of the interradicular alveolar bone. During osteoclastogenesis, osteoclast precursors are usually recruited from the blood vessels that interact with cells expressing RANKL in the PDL to form pre-osteoclasts, which subsequently migrate to PDL hard tissue surfaces to form active osteoclasts.[36] RANK immuno-reactivity, observed in the vicinity of PDL blood vessels, was likely to be associated with osteoclast precursors.

Kawamoto et al.[37] demonstrated immuno-localisation of RANKL in the rat periodontium. RANKL was detected in spindle-shaped mesenchymal cells around blood vessels near the alveolar bone surface and on elongated cell processes near blood vessels. RANKL immuno-reactivity around blood vessels within the PDL appeared to be more intense on the tooth side than on the alveolar bone side, which was also demonstrated in the present study. RANKL, present on cells within the PDL, may interact with osteoclast precursors recruited from the blood vessels. The precursors differentiate into pre-osteoclasts, which migrate toward the bone surface to form active osteoclasts. This suggests that RANKL, together with RANK and OPG, plays a crucial role in determining osteoclast differentiation and function. Further research to better understand the role of RANKL in the establishment and resolution of ankylosis is required.

Wada et al.[38] have suggested that OPG produced by fibroblasts in the PDL prevents the late differentiation of osteoclasts. In the present study, the presence of OPG may be associated with PDL fibroblasts, which in turn may play a role in regulating the PDL response to the cold thermal insult. Previous studies have shown that OPG is expressed by vascular endothelial cells.[39] It was therefore suggested that OPG participates in the regulation of vascular injury, inflammation and homeostasis. The expression of OPG by vascular endothelial cells may explain the immuno-localisation of OPG in the vicinity of the blood vessels. Studies of disease models often use the RANKL/OPG ratio as an index in determining bone resorption.[40] When the expression of RANKL is upregulated and OPG is downregulated, the RANKL/OPG ratio increases in favour of osteoclastogenesis and vice versa.[41] It is likely that the RANKL/OPG ratio played an important role in the establishment of ankylotic union between days 4 and 14, when the RANKL/OPG ratio would be expected to be reduced below normal. However, between days 14 and 28, the RANKL/OPG ratio likely increased, promoting osteoclastogensis and resorptive activity in order to resolve the ankylotic union. It is possible that, although all three proteins were present at the same time, each may exert its significance by dominating and affecting bone metabolism. It may therefore be inferred from the simultaneous expression of these proteins that there is a complex interaction of RANKL, RANK and OPG. Cytokines such as IL-1, TNF-α and TGF-β have also been demonstrated to interact with bone cells and have the ability to stimulate osteoclastic activity independently of the RANKL/OPG ratio.[1,42,43] While the RANKL/RANK/OPG triad appears to be the main regulator of resorption/apposition coupling, there may be other pathways through which osteoclastic differentiation may occur. Further research is required to explore the mechanisms of these secondary pathways.

Conclusions

From the findings of the present study, it was concluded:

1. The transient or permanent state of ankylosis is dependent on the initial extent of the PDL injury and the regenerative potential of the PDL as shown by the histological findings.
2. The formation and resolution of ankylosis appears time dependent, as it was extensive in the experimental teeth at day 14 and had resolved in the majority of the experimental teeth at day 28.
3. The PDL has the ability to regenerate and resolve ankylosis, as revealed by the histological findings in the interradicular PDL of experimental teeth, which were similar to the control teeth at day 28.
4. An unknown/unidentified factor/factors are involved in re-establishing a vital PDL in areas of ankylosis.

5. RANKL, RANK and OPG were all expressed simultaneously in both experiment and control teeth at all observation time periods.
6. The exact interaction between RANKL, RANK and OPG remains incompletely understood in transient ankylosis.
7. Further investigations are required to define the complex interrelationship between RANKL, RANK and OPG within the PDL.

The aims of this study have been met and open a new door for further investigation into the complex relationship of RANKL, RANK and OPG. The possibility that ankylosis can be transient and the PDL can regenerate is an exciting area for further investigation.

Acknowledgments

The authors are grateful for the financial support provided by the Australian Society of Orthodontists Foundation for Research and Education.

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