PRESENCE OF TUMOUR NECROSIS FACTOR-α AND TUMOUR NECROSIS FACTOR RECEPTOR 1 IN ASEPTIC ROOT RESORPTION

Submitted in partial fulfilment of Doctor of Clinical Dentistry (Orthodontics)

By

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Table of Contents

Table of Contents 2
Figures and Tables 5
Section 1 5
Section 2 5
Abstract 6
Declaration 8
Acknowledgements 9
SECTION 1 10
Introduction 10
Literature Review 11
The Periodontium 11
Periodontal fibres 12
Collagen 13
Oxytalan, Elaunin and Elastin Fibres 14
Cells of the periodontal ligament 16
The fibroblast 16
The osteoblast 17
The cementoblast 20
Epithelial cells 21
Hard tissues 22
Cementum 22
Alveolar bone 23
Blood vessels 24
Osteoclasts 24
The origin of osteoclasts 24
Osteoclast morphology 26
Mechanisms of resorption 27
Fate of osteoclasts 32
Overview of osteoclasts and controlling factors 32
Osteoprotegerin (OPG) 33
Action 34
Structure of OPG 35
Regulation of OPG 35
RANKL 36
RANK 38
Nomenclature 38
Tumour Necrosis Factor-α 39
Structure 41
Receptors 41
Effect in Bone 43
Effect on RANK/RANKL/OPG 44
Effect on osteoclasts 45
Immunohistochemistry 47
The Avidin-Biotin Technique 48
Immunolabelling consideration factors 51
Conclusions 52
References: 53
Statement of Purpose 78
Hypothesis 78

Aims 78
SECTION 2 79
Article 1 79
Presence of TNF-α and TNFR1 in aseptic root resorption 80
ABSTRACT 82
INTRODUCTION 83
MATERIALS AND METHODS 85
Experimental Animals 85
Experimental Protocol 85
Immunohistochemistry 86
Observations 87
TNF-α 87
TNFRI 88
DISCUSSION 90
Images 95
Acknowledgements 105
Reference 106
Article 2 110
Immunohistochemical Investigation of Tumour Necrosis Factor Alpha 111
Abstract 113
INTRODUCTION 114
MATERIALS AND METHODS 116
Materials 116
Methods 116
Immunohistochemistry 117
2006 Study 118
2009 Study 118
RESULTS 120
2006 Study. 120
2009 Study 121
DISCUSSION AND CONCLUSIONS 122
REFERENCES 125
APPENDIX 1 127
Materials 127
Polyclonal antibody to mouse TNF-α 127
Polyclonal antibody to rabbit TNFR1 127
Datasheet for Osteoprotegerin FcOPG 129
ANTICOAGULANT - HEPARIN 131
Solutions 132
Phosphate buffered saline (PBS) pH 7.4 132
Fixative 132
4% Paraformaldehyde/0.1 M phosphate buffer pH 7.4 132
Decalcifying Agent 133
4% EDTA in phosphate buffer 133
Avidin-Biotin Peroxidase Technique For Paraffin Sections 133
Methanol/Hydrogen Peroxide Blocking Solution 133
Citrate (C) 133
Normal Horse Serum (NHS) 134
Streptavidin Horseradish Peroxidase-Conjugated antibody (SPC) 134
Peroxidase Substrate Solution (DAB) 134

APPENDIX 2 136
Tissue dehydration and paraffin embedding 136
Slide coating procedures for Immunohistochemistry 137
Mayer Lillie Haematoxylin and Eosin Staining Method 138
Haematoxylin 139
Eosin 139
Immunohistochemical Staining Method 140
2006 Study - TNF-α Staining 140
2009 Study – TNF-α and TNFR1 Staining 142

Figures and Tables

Section 1

Figures

1. Diagrammatic representation of OPG/RANK/RANKL interaction…….. pg 36
2. Diagrammatic representation of Avidin-Biotin Technique..,…….pg 49
3. Diagrammatic representation of Avidin-Biotin-Peroxidase Complex..... pg 50

Section 2

Article 1

Table

1. Summary of results found in present study…….pg 90

Figures

1. TNF-α staining of control tooth in OPG administered rat……pg 95
2. TNF-α staining of control tooth in non-OPG-administered rat……pg 96
3. TNF-α staining in experimental tooth in non-OPG administered rat……...pg 97
4. TNF-α staining in experimental tooth in OPG administered rat……...pg 98
5. Resorption lacunae on the palatal root in an experimental tooth in OPG administered rat…….pg 99
6. TNF-α staining in experimental tooth in OPG administered rat around osseous-like material…….pg 100
7. TNFR1 staining in control tooth in non-OPG administered rat……...pg 101
8. TNFR1 staining in experimental tooth in OPG administered rat….....pg 102
9. TNFR1 staining in resorption lacunae in OPG administered rat……....pg 103
10. TNFR1 staining in experimental tooth in non-OPG administered rat…….pg104
11. TNFR1 staining in experimental tooth in OPG administered rat……....pg 105

Article 2

Figures

1. TNF-α staining in experimental tooth in OPG administered rat in 2006…..pg 120
2. TNF-α staining in experimental tooth in OPG administered rat in 2009….. pg 121
3. TNF-α staining in experimental tooth in OPG administered rat after antibody retrieval in 2009…….pg 121

Abstract

The osteoclast antagonist osteoprotegerin (OPG) can hypothetically offer molecular control over the process of orthodontic root resorption. Unpublished work investigating OPG in a rat model found no inhibitory effect on osteoclasts and odontoclasts when given at a dosage of 2.5mg/kg. It was hypothesised that traumatically induced periodontal ligament (PDL) inflammation produced mediators and cytokines with the ability to stimulate clast cell differentiation and antagonize the effects of OPG. A pilot study conducted in 2006 found Tumour Necrosis Factor Alpha (TNF-α) to be present to a greater extent in OPG-treated rats. It was concluded that TNF-α may moderate OPG effectiveness. The present study investigated the presence of TNF-α and its receptor Tumour Necrosis Factor Receptor 1 (TNFR1) in a PDL sterile inflammatory model. Dry ice was applied for 15 minutes to the upper right first molar crown of 18, eight-week-old male Sprague-Dawley rats. These were evenly divided into experimental and control groups. The experimental group was injected with OPG at a dose of 2.5 mg/kg of body weight. After seven days, the rats were sacrificed and their maxilla processed for immunohistochemical identification of TNF-α and TNFR1. Results showed root resorption to be present in varying amounts and locations. 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 of the periodontium, mainly in the interradicular area. There appeared to be more noticeable labelling in OPG-treated rats. The results indicated that TNF-α and its receptor TNFR1 were present and may modify OPG effectiveness by offering

an alternative pathway for osteoclastc formation, thereby challenging the potential anti-resorptive effects of OPG. Results from immunohistochemical reactions are strongly influenced by technical and interpretative problems and, in some instances, may result in false positive or negative outcomes. Differences in results were obtained between the pilot study and the current study conducted three years apart using the same animal material and immunohistochemical protocol. The pilot study conducted in 2006 investigating the presence of Tumour Necrosis Factor alpha (TNF-α) found positive staining in an induced sterile inflammation animal model. The current study using the same animal material in 2009 found a remarkable difference in results. In 2006, a multispecies detection kit was used and no antigen retrieval was required. Results showed a strong, generalised, positive staining for TNF-α within the periodontal ligament. At the end of this study, the unused tissue was packaged and stored for a period of three years. In 2009, the same tissue and antibody were used in a parallel immunohistochemical investigation but no positive result was found. The original protocol was reviewed and the antibody concentration and antigen retrieval was optimised with a new staining protocol being developed. Results showed diffuse positive staining in six of the nine specimens. It was concluded that other processing and storage factors were involved in the loss of antigenicity during the time period between studies.

Declaration

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Dr. Linda Gayle Curl

Dated

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SECTION 1

Introduction

External root resorption is an adverse and unwelcome consequence of orthodontic treatment. The predominant resorbing cells are osteoclasts which are multinucleated cells derived from the pluripotential haemopoietic stem cell. Osteoclast differentiation occurs through the osteoblast secretion of RANKL, binding to the RANK receptor expressed on osteoclasts. Osteoprotegerin (OPG) is a member of the tumour necrosis factor receptor superfamily and serves as a decoy receptor inhibiting the osteoclast-osteoblast interaction and therefore preventing osteoclast formation[1]. Previous investigations in a rat model found OPG did not inhibit osteoclast formation at the manufacturer's recommended dosage of 2.5mg/kg[2]. It was therefore hypothesised that other factors were involved. Tumour necrosis factor alpha (TNF-α) has been shown to be among the many proinflammatory cytokines that stimulate osteoclastogenesis[3]. TNF-α works through two cell surface receptors, Tumour necrosis factor receptor 1 (TNFR1) and Tumour necrosis factor receptor 2 (TNFR2). TNFR1 is known to mediate most of the biological properties of TNF-α and marrow cultures expressing only TNFR1 have more osteoclasts in comparison to wild type cultures. Cultures containing only TNFR2 contained fewer osteoclasts[4]. It would be expected that resorptive conditions would favour the dominance of TNF-α and TNFR1. This study therefore aims to investigate the relationship of TNF-α and TNFR1 in both OPG and non-OPG administered rats.

Literature Review

The Periodontium

The periodontium is a unique structure formed by highly vascular tooth supporting connective tissue. This is composed of the root cementum, periodontal ligament, alveolar bone and gingiva[5]. The PDL develops in conjunction with root formation and has an origin from the dental follicle which is derived from neural crest cells. With a width ranging from 0.15 to 0.38 millimetres, the PDL is a very small structure but is of great functional importance. The PDL is involved in tooth support, proprioception and regulation of alveolar bone volume[6]. This structure is located between the cementum covering the root surface and the alveolar bone. The PDL merges with the dental pulp at the root apex and the gingival connective tissue at the cervical region above the alveolar crest.

The main volume of the PDL is comprised of a collagenous fibre network which inserts into both the cementum and the alveolar bone. The supportive fibres run obliquely from the alveolar bone and attach apically into the tooth. This supportive mechanism is believed to protect the surrounding tissues from damage, especially those within the periapical region[7]. The collagen fibre bundles are well defined and are embedded in ground substance, which consists largely of proteoglycans, glycoproteins, and glycolipids. Variants of the elastic fibre, elaunin, and oxytalan also occur within the PDL[8].

Glycoproteins, proteoglycans, hyaluronate, water, mineral, growth factors and cells comprise the non-fibrous components of the PDL. The cellular constituents are osteoblasts and osteoclasts, fibroblasts, epithelial cell rests of Malassez, macrophages, undifferentiated mesenchymal cells and cementoblasts. These cells form, maintain and repair both the ligament and the adjacent alveolar bone and cementum. The rapid matrix remodelling of the PDL and its ability to adapt to alterations in mechanical loading forms the basis of orthodontics[8].

Periodontal fibres

During embryonic tooth development, the dental follicle gives rise to cementum, the periodontal ligament and alveolar bone, while the dental papilla gives rise to odontoblasts and dental pulp[5]. Hoffman[9] studied the development potential of the dental follicle via transplantation of the developing tooth bud into a subcutaneous site. Hoffman demonstrated that the cementum and PDL originated from the dental follicle as the transplanted tissue formed all elements of the periodontium. Conclusions from this study were not drawn about the development of the alveolar bone. Ten Cate[10] investigated the origin of the progenitor cells for cementum, alveolar bone and PDL fibroblasts through the use of transplanted tooth buds previously labelled with tritated thymidine. Results showed a strong labelling of cementoblasts and PDL fibroblasts but weak labelling of osteoblasts. Ten Cate [10, 11] concluded that as only the dental follicle proper was transplanted along with

the tooth bud, it must be the origin of the progenitor cells for cementum, alveolar bone and PDL fibroblasts.

Collagen

The collagen component of the PDL is comprised of a mixture of type I and III fibres as described by Ten Cate[8]. There is no consensus of the precise arrangement of these fibre bundles as there have previously been technical problems associated with histological preparations[12, 13].

Ten Cate[8] noted that as the tooth develops, and during the onset of root development, the fibre groups were formed from changes occurring within the dental follicle. At the beginning of root development, fibres appear loosely structured. This changed with the eruptive position of the tooth which caused the fibres to become densely packed[14]. During tooth eruption, as the PDL matures, the fringe fibres merge across the width of the ligament to form the principal fibre bundles[5]. The fibre bundles form distinct groups with each bundle resembling a spliced rope. This formation allows continuous remodelling of the PDL without compromise to the overall structure and function of the fibres[8]. Investigations by Cho and Garant[5] found the fibre bundles which originate at the surface of the newly formed root dentine to be in a close relation to highly polarised fibroblasts. Ten Cate[8] named these fibre groups the alveolar crest group, the horizontal group, the oblique group, the apical group and the interradicular group. The view that collagen exists as gingival, crestal, horizontal, oblique and apical fibre groups was accepted

even though it was unclear whether they extended right across the PDL space[14].

The orientation of PDL fibres is important for the functioning of the PDL. Melcher and Bowen[12] showed PDL fibres to be embedded within the cementum and the alveolar bone. This structure facilitates the supportive and cushioning function of the collagen. Histological evidence from Cohn[15] suggested that principal collagenous fibres pass through the alveolar process as Sharpey fibres. This was supported by Ten Cate[8] who also suggested the principal collagen fibre bundles to be embedded in the cementum as Sharpey's fibres. Sharpey's fibres are either entirely mineralised (when embedded in primary acellular cementum) or partially mineralised (when embedded in cellular cementum)[5]. This led to the concept of continuity between the fibre systems of adjacent teeth.

The PDL is also comprised of type IV collagen which is found in the basement membrane of neurovascular bundles and epithelial rests of the PDL[16]. This type of collagen does not form fibrils, but it has been hypothesised to have a structural role in maintaining the integrity of the PDL by anchoring the elastic system to the vasculature[16].

Oxytalan, Elaunin and Elastin Fibres

Fullmer[17] first demonstrated the presence of immature elastic fibres, oxytalan and elaunin in the PDL. These have been reported to form a three-dimensional network within the PDL[18, 19]. It is thought that this network of

immature elastic fibres links the tooth cementum to peripheral periodontal blood vessels. Oxytalan fibres are considered to be numerous and dense in the cervical region of the ligament[8]. Chavrier *et al.*[20] showed that oxytalan fibres are the likely precursors of elastin fibres. Sims[21] hypothesised the function of the oxytalan fibre meshwork to be part of a proprioceptor system for the regulation of PDL vascular flow but this has yet to be fully demonstrated. Due to the anatomical correlation between the distribution, direction and branching patterns of oxytalan fibres and blood vessels Chantawiboonchai *et al.*[22] have suggested a specialized physiological role within the PDL. Ten Cate[8] suggested that oxytalan fibres regulate vascular flow in relation to tooth function because of their elastic properties which allow expansion in response to tensional variations.

Currently, there is limited understanding of the function and distribution of elaunin fibres. Rosenbloom[23] reported that elastin fibres were found in the PDL of certain species and tooth type. Ten Cate[8] described elaunin fibres as another form of elastic tissue consisting of microfibril bundles of collagen embedded within a small quantity of elastin. This view supported Ross[24] who described mature elastin fibres as being comprised of microfilaments, some of which are embedded in an elastin core. Ten Cate[8] speculated that rapid turnover within the PDL prevented the maturation of its elastic component.

Cells of the periodontal ligament

The PDL contains many different cell populations and is considered a highly cellular connective tissue. Progenitor cell populations proliferate and migrate in the developing PDL as shown by Palmer[25]. These cells may synthesize bone, cementum and extracellular matrix within the PDL. McCulloch[6] identified several cell populations within the healthy PDL. These included fibroblasts, endothelial cells, epithelial cell rests of Malassez, sensory cells (such as Ruffini-type end organ receptors), osteogenic and osteoclastic cells and cementoblasts. Berkovitz[26] suggested that these cellular components may be subdivided into synthetic, destructive, or epithelial depending on their characteristics. Synthetic cells are comprised of fibroblasts, osteoblasts and cementoblasts[27, 28]. Destructive cells are comprised of osteoclasts and odontoclasts and are the prime cells involved in bone and tooth resorption[29, 30].

The fibroblast

Fibroblasts are located between the collagenous fibres of the PDL[24]. Ten Cate[8] reported heterogeneous populations of fibroblasts with different functional characteristics. Freeman and Ten Cate[31], and Ten Cate[11], demonstrated that PDL fibroblasts near the cementum are derived from the ectomesenchymal cells of the dental papilla, while fibroblasts near the alveolar bone are derived from perivascular mesenchyme. Fibroblasts are seen histologically as flattened irregular discoid in shape with a single distinct prominent nucleus[26, 32, 33]. This appears to change with age as Sasaki and

Garant[34] noted in the aged PDL of rats, multinucleated fibroblasts, which they hypothesised to be derived from the fusion of mononuclear cells or through faulty cell division.

The PDL fibroblast is characterised by the ability to achieve a high turnover of the extracellular matrix with a main emphasis on collagen turnover. As such, the cell is comprised of extensive cytoplasm which contains an abundance of organelles which are associated with protein synthesis and secretion[35]. Ligament fibroblasts are aligned along the general direction of the fibre bundles and have extensive processes that wrap around the fibre bundles[8]. These processes appear to be interconnected with gap and adherence-type gap junction[36]. Ten Cate[8] hypothesised that the well-developed cytoskeleton and prominent actin network of the fibroblast indicates the functional demands placed on the cell that requires an ability to change shape and migrate throughout the PDL

The osteoblast

Osteoblasts are derived from mesenchymal cells located on the periosteal surfaces and within bone marrow stroma. These cells act together with osteoclasts to remodel and maintain bone. Differentiation of the osteoblast occurs through a complex series of differentiation steps which is influenced by a number of transcription factors, growth factors and hormones. Core binding factor a1 (Cbfa1) also known as osteoblast-specific transcription factor-2

(Osf2) is the main transcription factor involved in the differentiation of osteoblasts[37].

The primary function of the osteoblast is the formation, calcification and induction of new bone[38]. This is achieved by the secretion of a complex mixture of bone matrix proteins and organic matrix, predominantly containing collagen. These cells are located in a single cellular layer attached to the periosteal or endosteal surface where new bone is being formed[39]. In the periodontium, osteoblasts are situated on the surface of alveolar bone[26] as a layer of cuboidal cells aligned together between Sharpey fibres[40]. Osteoblasts are located at a distance from mineralised bone matrix and are separated via a layer of thick unmineralised matrix known as the osteoid seam[38].

Periodontal osteoblasts are similar in overall appearance to osteoblasts found elsewhere in the body. The active osteoblast is a cuboidal cell with a strong basophilic cytoplasm due to the large amounts of rough endoplasmic reticulum[26], mitrochondria and vesicles which is associated with active secretion of matrix[38]. The osteoblast secretes bone matrix proteins such as proteoglycans, gylcoproteins and glutamic acid upon the network of type I collagen fibres[38]. Mariotti[41] detailed the important role of osteoblasts in the production of bone matrix components, such as collagenous and non-collagenous proteins in the form of osteocalcin, osteonectin, osteopontin, matrix gla protein, plus numerous other phospho-, sialo-, and glyco-proteins.

Mackie[38] specified the glycoproteins produced by osteoblasts as including osteonectin/SPARC, tenascin-C, fibronectin and members of the thrombospondin family. It was speculated that these glycoproteins are likely to be involved primarily in the regulation of bone cell adhesion, migration, proliferation and/or differentiation in bone development and remodelling[38].

Mackie[38] suggested that osteoblasts are indirectly responsible for the mineralization of the inorganic component of the osteoid, by hydroxyapatite crystals. It has been hypothesised that the osteoblast-derived phosphate-containing proteins of the bone matrix such as bone sialoprotein are involved in the initiation of hydroxyapatite crystal formation[38]. Bellows *et al.*[42] indicated the possible role for alkaline phosphatase in the mineralization process through an observation that, when alkaline phosphatase activity is blocked, the mineralization of bone matrix is prevented.

Osteoblasts form a coordinated communication system throughout bone via cell-to-cell interactions. These involve the secretion of paracrine factors, short-range signalling molecules and direct cell-cell adhesion, either via gap junctions or desmosomes[43]. Stains and Civitelli[44] noted that the gap junctions present between osteoblasts are composed of two juxtaposed hemichannels present on each surface of adjacent cells. Gap junctions form a transcellular channel that permits rapid movement of ions, metabolites and secondary messengers between adjoining cells. Gap junctions are also formed with osteocytes that are incorporated in the mineralized matrix.

Kubota *et al.*[45] found osteoblasts to be involved in the regulation of osteoclast differentiation. Osteoblasts express two major regulatory factors, OPG and RANKL, which causes osteoclast differentiation inhibition or promotion respectively. Buckwalter *et al.*[46] theorised a role for osteoblasts in calcium homeostasis indirectly through their regulation of osteoclast differentiation and therefore activity.

The cementoblast

Cementum is the avascular mineralized tissue covering the root surface and forms the interface between root dentine and the PDL. In a study by Cho[47] it was demonstrated that cells from the dental follicle proper invaded the intercellular spaces of the root sheath. These cells were identified as precementoblasts on the basis of their enlarged cell bodies containing numerous profiles of rough endoplasmic reticulum, lysosomes and Golgi complex. These findings were supported by Diekwisch[48] who showed that early cementogenesis arose from follicular mesenchymal cells which penetrate Hertwig's epithelial root sheath. However, Brossard and Selvig[49] disagreed with these findings and stated that virtually nothing was known about cementoblast origin, differentiation and cell dynamics during normal development, repair and regeneration. Even though there are morphologic similarities between cementoblasts and osteoblasts[50] the response of these cells to type 1 collagen, osteocalcin (OC) and bone sialoprotein (BSP) genes shows a clear distinction between the two cell phenotypes[51]. Lindskog *et*

al.[52] showed that cementoblasts express parathyroid hormone receptors but, unlike osteoblasts and bone lining cells, they do not retract in response to parathyroid hormone to expose the root surface to preosteoclasts.

Epithelial cells

Hertwig's epithelial root sheath originates from the inner and outer enamel epithelial cells of the tooth germ. Embryologic development consists of a double layer of epithelial cells that extend apically and are continuous with the rim of the enamel organ[5]. Ten Cate[8], suggested from the results of his study, that the remnants of Hertwig's epithelial root sheath remain as aggregations of one or two epithelial cells within the PDL. Thomas and Kollar[53] supported this finding and further suggested Hertwig's epithelial root sheath to be involved in the differentiation of the root odontoblasts. Spouge[54] contended that the epithelial cells serve a function that, as yet, remains undetermined. As non-functional cells usually disappear through apoptosis, the persistence of epithelial cell rests lends itself to the suggestion that these cells undergo activity and may serve an as yet undetermined function. During orthodontic loading, Reitan[55] observed that, on the "compression" side, hyalinization occurred with no regeneration of the epithelial cell rests, even though there was subsequent regeneration of the connective tissue elements of the PDL. In a later study of orthodontic root resorption, Brice *et al.*[56] reported epithelial cell rests in a repairing area. These authors went on to suggest epithelial cell rests were involved in mediating repair by cementogenesis. In an ankylosis model, Loe and Waerhaug[57] observed that

ankylosis and root resorption did not occur when a PDL containing epithelial cell rests was retained. They further suggested that epithelial cell rests may be the factor limiting resorption and therefore play a role in the maintenance of the periodontal space. This suggestion was further supported by Lindskog *et al.*[58].

Hard tissues

Cementum

Cementum lines the root dentine and provides attachment for the PDL fibres. During tooth formation, cementogenesis and periodontal fibre attachment occurs at the same time. This caused Bosshardt and Schroeder[59] to report that a cellular distinction between the two processes was difficult. Schroeder[60] classified cementum as cellular or acellular based on the presence or absence of cementocytes. These categories were further divided intro intrinsic and extrinsic fibre cementum depending on the presence of collagen. McCullough *et al.*[61] and Lang *et al.*[62] presented additional findings suggesting that cellular cementum might be derived from cells that are extrinsic to the PDL. Lindskog and Blomlöf[63] provided evidence suggesting that osteoblasts, as well as cementoblasts, might contribute to cementum formation, particularly in reference to reparative cementum.

Cementum, bone and dentine are similar in both organic and inorganic content[64]. Srivicharnkul[65] estimated the mineral content of cementum to be

65 per cent, whereas the mineral content of dentine was estimated at 70 per cent on a wet-weight basis. Selvig and Selvig[66] found bone to be comprised of 70 per cent inorganic salts, whereas cellular cementum contained 46 per cent. Butler *et al.*[67] reported type I collagen to predominate in bone, while, in cementum, type I collagen and a small percentage of type III collagen is present.

Alveolar bone

As the development of the alveolar process is dictated by tooth formation and eruption, Schroeder[60] concluded the alveolar process to be a tooth-dependent bony structure. Alveolar bone is a specialized mineralized connective tissue consisting by weight of 33% organic matrix; 28% type 1 collagen, and 5% noncollagenous proteins, which include osteonectin, osteocalcin, bone morphogenetic proteins, bone proteoglycans and bone sialoprotein[68]. The organic matrix is permeated by hydroxyapatite (HA) ($Ca_{10}(PO_4)_6(OH)_2$) which comprises the remaining 67% of bone[68]. The organic matrix of bone is comprised of collagen, predominantly type I, noncollagenous proteins and lipids[69]. Schroeder[60] described alveolar bone as 0.1 to 0.4 mm thick and consisting of Haversian systems, lamellated and bundle bone. Four separate cell types are responsible for the formation, resorption and maintenance of the osteoarchitecture; the osteoprogenitor cell, the osteoblast, the osteocyte and the osteoclast.

Blood vessels

The PDL is a highly vascularised connective tissue. Berkovitz[27] indicated that blood vessels comprise a substantial proportion of the PDL in comparison to other connective tissue systems. Ten Cate[8] indicated that the vascularity of the PDL reflects its high cellular and extracellular turnover. Using light microscopy Johnson and Pylypas[70] showed a change in the vascular distribution throughout the ligament with a higher concentration at the apical region. Weekes and Sims[71] and Lee *et al.*[72, 73] revealed a varying distribution with changes along the length and across the width of the PDL. The entry points of the vessels also vary. A majority of vessels enter from the apical aspect but Kindlova[74] showed a collateral circulation through the alveolar bone and gingiva. This collateral circulation provides an added nutritive and hydrostatic support mechanism for the tooth.

Osteoclasts

The origin of osteoclasts

Osteoclasts are multinucleated cells derived from the pluripotential haemopoietic stem cells, and are the main cell involved in bone resorption[75-77]. Two discrete types of osteoclasts have been identified[78], the mononuclear and the multinuclear, which arise from a monocyte/macrophage precursor in haemopoietic tissue. Rivollier[79] showed the fusion of the mononuclear precursors into multinucleated osteoclasts, occurred near the bone surface. Meunier *et al.*[80] indicated that osteoclasts are uncommonly seen in bone but are located either on endosteal surfaces, within Haversian systems or on the periosteal surfaces. Walker[81] demonstrated, through transplantation of bone

marrow in osteopetrotic mice, that osteoclasts form in and are derived from bone marrow tissue. Coccia *et al.*[82] utilized this finding in bone marrow transplantation for the treatment of juvenile osteopetrosis. Kahn and Simmons[83] also used transplantation studies and showed that osteoclasts are host derived and are present in marrow, spleen and circulating peripheral blood.

The osteoclast differentiation pathway is common to that of the macrophage and dendritic cells. A haematopoietic stem cell that is common to lymphocytes, red blood cells, platelets, granulocytes and mononuclear phagocytes, progresses through the colony-forming unit for granulocytes and macrophages to the preosteoclast and multinucleated cell until the mature active resorbing osteoclast is formed[84]. Therefore, a promyeloid precursor can differentiate into either an osteoclast, a macrophage or a dendritic cell, depending on exposure to NF-κB ligand (RANKL), also called tumour necrosis factor-related activation-induced cytokine (TRANCE), osteoprotegerin ligand (OPGL) or osteoclast differentiation factor (ODF)[85-88].

The most recent major breakthrough was the discovery that RANKL is critical for osteoclastogenesis, leading to an understanding of the steps involved to form a mature osteoclast. Receptor activator of NF-κB ligand as a tumour necrosis factor family cytokine, induces the differentiation of osteoclasts in the presence of macrophage-colony stimulating factor (M-CSF) *in vitro*[89].

Osteoclast morphology

Osteoclasts are large multinucleated cells found in resorption bays or Howship's lacunae, on the surface of bone[40, 75]. As osteoclasts possess motility, they have been observed to move between resorption sites on the surface of bone[90-92]. In routine staining with toluidine blue, results show less organelles associated with protein production for secretion[91]. Ribosomes are present in the cytoplasm, either singly or in clusters, suggesting some protein synthesis for intracellular use[91]. Osteoclasts have an abundance of mitochondria which contain electron-dense granules. These granules contain calcium suggesting that the mitochondria function as a calcium storage depot[93]. The most characteristic appearance of the osteoclast is the "foamy" or vacuolated appearance of the cytoplasm due to the extensive lysosomal system[91].

The ruffled border, first named by Scott and Pease[94], is a unique feature of the osteoclast and is considered by Väänänen[95] to be the resorbing organ. Using high power light microscopy, infoldings of the basement membrane appear as a complex series of finger-like projections within close contact with the hard tissue surface. Väänänen[95] contended that the ruffled area was formed by the fusion of intracellular acidic vesicles with the region of plasma membrane facing the bone. The ruffled border appears inside the sealing zone, and has several characteristics of late endosomal membrane as shown by Akamine[96] through the use of immunocytochemical studies.

Mechanisms of resorption

Steps in a resorption cycle[97]

1. Formation of multinucleated cell from mononuclear precursors and recruitment to resorption site.
2. Attachment of osteoclast to mineralised matrix including organisation of actin to ring-like structure and formation of sealing zone.
3. Polarization of plasma membrane and formation of two specific membrane domains, ruffled border and functional secretory domain.
4. Bone resorption including extra-cellular dissolution of apatite crystals and fragmentation of collagen fibrils and other proteins in the resorption lacuna.
5. Removal of resorption products from the resorption lacuna to the functional secretory domain by transcytosis and their secretion into the circulation.
6. Depolarisation of the cell, detachment and reorganisation of the cytoskeleton and relocation to a new resorption site or apoptosis.

Many factors have been hypothesised to control both the site of resorption and osteoclast recruitment but the major determinants are still unknown. Complement, macrophage inflammatory protein-1α, human osteogenic protein-1 in combination with 1,25,dihydroxyvitamin D3 have all been suggested[98-100] as possible control mechanisms. Suda *et al.*[101] suggested that osteoclast recruitment is more likely to be related to local factors which Zaidi *et al.*[102] established are governed by hormonal, cytokine, growth and

colony-stimulating factor interaction. Importantly, tumour necrosis factor receptor types 1 (TNFR1) and 2 (TNFR2) are found to differentially regulate osteoclastogenesis[4], while interleukin-1 (IL-1) and tumour necrosis factor (TNF), by stimulating the inflammatory process, lead to osteoclast recruitment and bone resorption[103].

Osteoclast formation from mononuclear progenitors occurs at the designated site of resorption. Differentiation is followed by the attachment of the osteoclast to the bony surface[104-106]. Integrin receptors bind to the Arg-Gly-Asp (RGD) sequence found in a variety of extracellular matrix proteins and act as an anchor for the osteoclast to attach while resorption occurs[92]. Nesbit *et al.*[107] identified three main integrins, $\alpha_v\beta_3$ also called the vitronectin receptor, $\alpha_2\beta_1$ also called the collagen receptor and $\alpha_v\beta_1$. Nakamura *et al.*[108] found osteoclasts expressed a high proportion of $\alpha_v\beta_3$ integrin receptors which bind to many extracellular proteins including osteopontin and bone sialoprotein. Using RGD containing peptides and blocking antibodies to $\alpha_v\beta_3$, Nakamura *et al.*[108] showed the blockage of this integrin inhibited bone resorption and revealed the importance of $\alpha_v\beta_3$ integrin in osteoclast regulation.

The osteoid layer interferes with osteoclastic attachment to bone and therefore the process of resorption[109]. Chambers *et al.*[109] showed the osteoblast-derived collagenase (MMP-1) facilitated osteoclastic attachment by degrading surface osteoid allowing cell contact with the underlying resorption-stimulating bone mineral. Chambers *et al.*[110] and Chambers and Fuller[111]

concluded that the non-mineralised collagen layer needed to be removed from the bone surface before an osteoclast could attach and cause bone degradation. Teitelbaum *et al.*[112] identified the importance of close contact between the osteoclast and bone for the creation of an isolated area containing the acidic extracellular resorptive microenvironment. During the resorption process, osteoclasts are tightly sealed to the bone surface, and hence the resorption area is isolated from the extracellular environment[40].

Osteoclasts form a specialized sealing area which is comprised of a prominent peripheral ring derived from filaments containing F-actin[113]. Marchisio *et al.*[114] described numerous punctuate structures of F-actin filaments and termed these podosomes. Kanehisa *et al.*[115] and Lakkakorpi and Väänänen[104] observed an increase in the number of podosomes arranged in a peripheral ring when the cell is resorbing. It was concluded that the cytoskeleton changes configuration to form an actin-ring upon cellular attachment. Marchisio *et al.*[116] showed podosomes mediated the attachment of osteoclasts to extra-cellular matrix via the $\alpha_v\beta_3$ integrin as they gathered to form a circular structure in the plasma membrane facing bone matrix. Once densely packed, they disappear at the same time as F-actin forms in a thick ring-like structure near the plasma membrane facing bone. Väänänen[97] identified actin-associated proteins, vinculin and talin, arranged around the actin ring.

During the initiation of bone resorption, osteoclasts become polarized. This creates three distinct membrane domains; a ruffled border, a sealing zone and a functional secretory domain. Lakkakorpi *et al.*[117] and Lakkakorpi and Väänänen[104] showed the organization of osteoclast cytoplasm as the cells become highly polarized through changes in plasma membrane morphology[118]. The sealing area of bone attachment separates the ruffled border and the basolateral membrane into distinct basal membrane specializations. The ruffled border is an extended interconnection between the cell and the extracellular environment, which allows for an increased exchange of metabolites. It is enclosed within the sealing zone. In an activated osteoclast, acidic intracellular vesicles fuse to the plasma membrane of the ruffled border within the actin ring[119]. Upon vesicular fusion acid is released into the resorption space to begin the dissolution of apatite crystals[120]. Osteoclasts express specialized proteins including a vacuolar-type H^+-ATPase that drives acid secretion[78, 121, 122]. Using immunocytochemistry, Baron *et al.*[123] characterized the biosynthetic pathway delivering lysosomal enzymes to the resorption lacuna.

To maintain ion equilibrium and pH balance, the secretion of acid is counteracted by the movement of chloride anions via specific membrane chloride channels[124]. Continuous acid production is facilitated by carbonic anhydrase II-facilitated CO_2 hydration accompanied by bicarbonate/chloride exchange. Ravensloot *et al.*[125] found that intracellular pH recovery was assisted by a Na^+-H^+ exchange process.

Salo *et al.*[126] suggested the functional secretory domain is an area of the ruffled border responsible for the uptake of bone degradation products from the resorption lacuna[126-129]. The degraded organic and inorganic products are transported through the osteoclast and released into the extracellular environment via the specialised and polarized basal membrane[126]. Studies by Nesbitt and Horton[130] and Mostov and Werb[131] confirmed the transcytosis of proteins liberated from the mineralized matrix to the basolateral membrane for extracellular release.

Tartrate-resistant acid phosphatase (TRAP) is an enzyme of unknown biological function. This enzyme is localized in transcytotic vesicles transporting bone matrix degradation products from the resorption lacuna to a functional secretory domain in the basolateral membrane in active resorbing osteoclasts[132]. TRAP is capable of generating reactive oxygen species (ROS), which in turn are capable of destroying organic bone matrix components, suggesting that they may be targeted to further destroy initial matrix degradation products in the transcytotic vesicles[132, 133]. Results showed that when recombinant TRAP was incubated with type I collagen in the presence of H_2O_2, collagen and other proteins were cleaved into small peptide fragments. This suggested that TRAP may have a role in the degradation of extra cellular proteins during their transcytosis[127].

Fate of osteoclasts

Once an osteoclast has fulfilled its resorption task it may undergo fission back to its mononuclear cell, or it may undergo apoptosis. Solari *et al.*[134] demonstrated with fluorescent markers injected into mature multinucleated osteoclast cells that mononuclear cells may bud from the giant parent cell. These newly budded mononuclear cells were then able at a later stage to coalesce to re-form multinucleated cells.

A study by Kameda *et al.*[135] showed the morphological nuclear changes, DNA fragmentation, and biochemical changes during osteoclast death are similar to those found in apoptosis in other cell systems. These two different fates of the osteoclast show that our understanding of the ultimate fate of osteoclasts is, at best, confused[134].

Overview of osteoclasts and controlling factors

Osteoclasts are multinucleated giant cells that form from fusion of mononuclear precursors and have the capacity to resorb bone. Several groups independently cloned the long-sought osteoclast differentiation factor in 1997-1998[136-139] which is now widely called RANKL. RANKL expressed on the cell surface of osteoblasts/stromal cells binds to its receptor RANK which is expressed on the surface of haematopoietic osteoclast precursor cells. This interaction between RANKL and RANK induces a signalling and gene expression cascade which leads to the differentiation and maturation of osteoclasts. Osteoprotegerin (OPG) can bind to RANKL and act as a decoy

receptor thereby blocking the interaction between osteoblasts and osteoclast precursors resulting in an inhibition of osteoclast formation.

RANKL, when in the presence of M-CSF, is sufficient to cause osteoclastogenesis without the need for other cells, co-cultures, cytokines, hormones or growth factors[84]. RANKL also has the capacity to activate osteoclasts, stimulate inactive osteoclasts to bone resorption[140-142] and to inhibit osteoclast apoptosis[141].

Osteoprotegerin (OPG)

OPG is a member of the tumour necrosis factor receptor (TNFR) superfamily[139, 143]. In 1997 two independent laboratories discovered OPG through cDNA sequencing projects and protein purification[143, 144]. Tsuda *et al.*[144] termed the protein purification discovery "osteoclastogenesis inhibitory factor" (OCIF) because it inhibited osteoclast formation. OCIF was shown to be identical to OPG through cDNA sequencing[144]. OPG has also been previously known as TR1[145], and FDC receptor-1 (FDCR-1) [146].

Overexpression of OPG was found to decrease the number of trabecular osteoclasts and increase the mineralized trabecular bone[143]. Simonet *et al.*[143] concluded that the overexpression of OPG increased bone mass through the inhibition of the terminal stages of osteoclast differentiation. Simonet *et al.*[143] have also demonstrated an inhibition of the development of

tartaric acid-resistant acid phosphatase positive osteoclasts and TRAP activity in osteoclasts following the administration of OPG-Fc fusion protein. Bucay *et al.*[147] and Mizuno *et al.*[148] produced OPG-deficient mice which showed an increase in mortality associated with a higher incidence of vertebral and enchondral bone fractures. Terminal severe osteoporosis was shown by Hofbauer[149] in OPG-knockout mice. This resulted in decreased bone mineral density with thin cortical bone in the femur, pelvis and destruction of the femoral growth plates. Bucay *et al.*[147] found an increase in bone turnover with histological results showing OPG-deficient mice had numerous osteoclasts and osteoblasts with lamellar bone being replaced with woven bone. Aubin and Bonnelye[150] showed adult OPG-deficient mice to display calcification of the aorta and renal arteries suggesting, that while OPG is produced by osteoblastic cells, it is not restricted to bone tissue and is expressed in a wide variety of cell and tissue types, including the aorta and other large arteries.

Action

OPG inhibits *in vitro* osteoclastogenesis by directly binding to its receptor on osteoclasts/stromal cells interrupting the signalling from osteoblasts/stromal cells to osteoclast progenitors[139]. The concentration of M-CSF appears crucial for osteoblast-mediated osteoclast formation as a high concentration of M-CSF suppresses osteoclast formation. Yasuda *et al.*[1] surmised that M-CSF produced by osteoblasts/stromal cells is essential for the proliferation and differentiation of osteoclast progenitors. *In vitro* it has been shown that

the addition of OPG to pre-existing osteoclasts, causes a disruption to the formation of the F-actin ring and, therefore, diminishes the resorption capacity of these cells[151].

Structure of OPG

Analyses of the structure of OPG have shown a hydrophobic profile, a lack of transmembrane and cytoplasmic domains and it is concluded that OPG is a secreted protein[1, 143, 145, 152]. Hofbauer[153] reported that OPG is synthesized as a monomer and assembled as a homodimer within the cell, and is secreted as a disulphide-linked homodimer into the extracellular compartment. The OPG homodimer displayed a higher heparin-binding ability and a higher calcium-lowering potency in normal rats[153]. Analysis of the structure-function relationship of the OPG protein revealed that the integrity of all four cysteine-rich motifs at the N-terminus is necessary and sufficient for the inhibition of osteoclastogenesis[153].

Regulation of OPG

Hofbauer[154] showed OPG inhibited osteoclastogenesis induced by 1α,2,5$(OH)_2$vitaminD_3[144, 152] , prostaglandin (PG)E_2[152], parathyroid hormone (PTH)[144, 152], interleukin -1[152] and interleukin-11[144, 152] .

RANKL

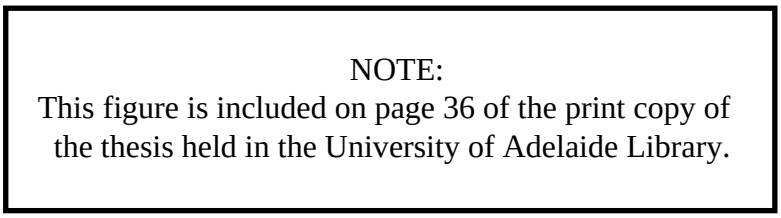

Figure 1. Diagrammatic representation of osteoblast/osteoclast/OPG interaction[155].

Suda *et al.*[86] isolated an OPG-binding molecule expressed by a $1,25(OH)_2D_3$-treated stromal cell line known to support osteoclastogenesis. This molecule was termed “osteoclast differentiation factor” (ODF) because of its ability to cause osteoclastogenesis in cell and bone organ cultures. Expression of ODF by osteoclast/stromal cells was also found to be stimulated by all hormones known to cause osteoclast formation in previously used co-culture systems. Lacey *et al.*[140] found the same OPG-binding molecule by using a myelomonocytic cell cDNA library, this was termed OPG-binding ligand (OPGL). Results showed that OPGL-knockout mice experienced severe osteopetrosis with a complete lack of osteoclast or resorptive activity. There was total occlusion of the bone marrow spaces within endosteal bone and a

defect in tooth eruption. OPGL-knockout mice were shown to have the normal osteoclast precursors but no osteoclasts. This finding indicated that OPGL was an essential requirement for osteoclast formation[140]. Conclusions from Lacey *et al.*[140] suggested that OPGL stimulated both osteoclast differentiation and activity.

Wong *et al.*[156] identified TRANCE as a gene induced by stimulation of the T cell receptor. TRANCE is known to be involved in the JNK (c-Jun N-terminal kinase) kinase-apoptosis pathway in T lymphocytes and dentritic cells[157].

It was observed that when recombinant soluble TRANCE was added *in vitro* to a culture of osteoclasts, there was a resultant increased pseudopodial motility and associated cell spreading. This motility and spreading of osteoclasts was localized in broad focal pseudopods, and was associated with rhythmic contraction of the osteoclast cell body[158]. A study by Fuller *et al.*[141] found TRANCE induced osteoclast formation and resorption is inhibited by OPG. Myers *et al.*[159] discovered RANKL, a molecule identical to TRANCE during a search for proteins that bind to RANK. RANK is a TNFR family member that is highly expressed in muscle and thymus and implicated in the differentiation of monocytes and dendritic cells.

Studies have found many different factors that up-regulate OPGL (RANKL) mRNA steady state levels in marrow stromal and osteoblastic cells. These at present are 1α,25-(OH)2D3, IL-11, PTH, and PGE_2[139, 153, 160]. Pro-inflammatory cytokines IL-1β and TNF-α have recently been demonstrated to stimulate OPGL mRNA steady-state levels in primary marrow stromal

cells[154]. Tsukii *et al.*[161] found that osteoclast-like cells which expressed tartrate-resistant acid phosphatase and calcitonin receptors and were capable of forming pits on dentine slices were suppressed by $1\alpha,25(OH)_2D_3$, PTH, PGE_2 or IL-1[161]. This indicated that hormones and cytokines may regulate bone resorption indirectly by increasing the production of RANKL.

RANK

RANK was originally described by Anderson *et al.*[136] as a receptor on T cells and dendritic cells. RANK was found to be a polypeptide belonging to the type I transmembrane protein class. RANKL bound specifically and with high affinity to RANK located on osteoclastic lineage cells. It was found that activation of RANK by RANKL was necessary and sufficient for osteoclastogenesis to occur[162, 163]. Hsu *et al.*[162] found RANK to be the same receptor as osteoclast differentiation and activation receptor (ODAR). Anderson *et al.*[136] were the first to report that RANK overexpression is sufficient to activate NF-κB. Myers *et al.*[159] also found RANK, expressed on mature rat and human osteoclasts when treated with RANKL, resulted in activation of NF-κB and Ca^{2+} signalling in these cells.

Nomenclature

The sequencing of OPGL and ODF revealed that the molecules were identical to other members of the TNF ligand family called TRANCE (TNF-related activation-induced cytokine) or RANKL (receptor activator of NF-κB ligand). ODF/SOFA/OPGL/RANKL/TRANCE are all the same molecule and are now universally referred to as RANKL. OPG/OCIF/TR1/FDCR-1 are the same

molecule and are now referred to as OPG. RANK/ODAR are the same molecule and will be referred to as RANK as decided by the American Society for Bone Mineral in 2000[164].

Tumour Necrosis Factor-α

Tumour necrosis factor (TNF) is a major mediator of apoptosis as well as inflammation and immunity, and it has been implicated in the pathogenesis of a wide spectrum of human diseases, including sepsis, diabetes, cancer, osteoporosis, multiple sclerosis, rheumatoid arthritis, and inflammatory bowel diseases. From previous studies it is known that RANKL in the presence of M-CSF is required for osteoclastogensis to occur *in vitro*. It has also been shown that genetic manipulation of this system results in expression of the extremes of skeletal phenotype (osteopetrosis and osteoporosis) *in vivo*. Hofbauer *et al.*[165] suggested that there is a strong control mechanism over this system and, therefore, other hormones and cytokines closely regulate osteoclastogenesis and bone resorption by modulating the expression of RANKL and OPG by osteoblastic cells. It has been revealed that pro-inflammatory cytokines are important mediators in the regulation of osteoclastic alveolar bone resorption as well as odontoclastic root resorption. Yeung *et al.*[3] showed IL-1β, IL-6, IL-11, IL-17 and TNF-α to be pro-inflammatory cytokines that stimulate osteoclastogenesis by increasing the expression of RANKL and decreasing the expression of OPG. Nakashima *et al.*[166] showed the inhibitory cytokines to be IL-13, interferon-γ, and

transforming growth factor-β, which suppress the expression of RANKL and enhance the expression of OPG.

Interleukin-1 (IL-1) and tumour necrosis factor alpha (TNF-α) have been shown to play a role in orthodontically induced tooth movement and in the pathogenesis of several inflammatory periodontal diseases[167, 168]. In studies undertaken by Saito *et al.*[169] and Sandy *et al.*[170], during orthodontic tooth movement, cytokines were detected in both the periodontal tissues and the crevicular fluids. Lossdorfer *et al.*[167] hypothesised that under physiological conditions, TNF-α and IL-1α are present in the rat periodontium as they are secreted by inflammatory cells, gingival fibroblasts and PDL cells, and may therefore contribute to the remodelling process in physiological tooth drifting[171]. Reviews by Manolagas[172] and Pacifici[173] considered that, as in the bone microenvironment, osteoclast differentiation is regulated by a coordinated synthesis and action of cytokines produced by bone marrow stromal cells and osteoblasts.

TNF is divided into two structurally and functionally related proteins, TNF-α or cachetin[174, 175] and TNF-β or lymphotoxin[176]. TNF-α is produced mainly by monocytes and/or macrophages, whereas TNF-β is a product of lymphoid cells. TNF-α and TNF-β bind to the same cell surface receptors[177] and are very similar in their range of activities. TNF-α is a multifunctional cytokine[178] with actions ranging from proliferation, differentiation and maintenance of phenotype to apoptosis in various cell types[179]. TNF action upon a cell leads

to the activation of multiple signal transduction pathways, kinases and transcription factors. The multifunctional ability of this cytokine is due to the TNF receptor which is found on almost all cell types[178, 180]. To date there are 12 identified TNF receptors of which most are type 1 transmembrane proteins with a characteristic cysteine-rich pseudorepeat in the extracellular region[181].

Structure

TNF-α has a precursor sequence, proTNF-α, a type II transmembrane protein, that serves to anchor the TNF-α polypeptide in the plasma membrane[182]. A small section of this peptide is released through proteolytic cleavage by the metalloproteinase-disintegrin TNF-α convertase (TACE) which is a member of the disintegrin and metalloprotease (ADAM) family[183, 184]. Kriegler *et al.*[185] hypothesised that the transmembrane form of TNF-α exposed on the outside of the cell as well as acting as a precursor of released TNF-α, can also bind to a receptor on an adjacent cell and engage in intercellular communication.

Receptors

Schall *et al.*[186], through the use of cDNA cloning, and Dembic *et al.*[187], through protein purification, identified two cell surface TNF receptors (TNFR), 55kDa (TNFR1) and 75kDa (TNFR2). The potency of these two receptors is still under debate. Thoma *et al.*[188] concluded, through the use of receptor-specific blocking antibodies, that a 75kDa TNFR is unable to initiate responses to TNF-α, and that the 55kDa TNFR is essential for TNF receptor function in several cell lines. Results from Yongchaitrakul *et al.*[171] showed

that TNFR1 was the major receptor for TNF-α. TNFR1 is known to mediate most of the biological properties of TNF-α, such as the activation of NF-κB and programmed cell death through its death domain[189]. Chen and Goeddel[190] indicated in their review that TNFR1 could activate a cascade of kinases which ultimately result in the activation of RANKL and JNK pathway.

In contrast, Reinhard *et al.*[191] showed TNFR2 interacts directly with TRAF-2 which activates both the NF-κB and c-Jun amino-terminal kinase (JNK) pathways. Under pathological conditions, Abu-Amer *et al.*[4] have shown that TNFR1 promotes osteoclastogenesis whereas TNFR2 acts as an inhibitor.

A trimeric form of TNF binds one of the two cell surface receptors, either TNFR1 or TNFR2, to initiate a signal cascade that causes inflammatory gene activation, and selective gene repression. Once TNF binds to its receptor on the cell surface, the receptor then associates with intramembrane cytosolic proteins that bind to downstream signals[192]. The first association is with TNF receptor-associated death domain (TRADD), a cytosolic protein that then branches the signalling down two different pathways. The first pathway activates TNF receptor associated factors (TRAFs) 1 and 2, which ultimately cause the activation of nuclear factor kappa B (NF-κB) transcription factor[192]. This molecule, signalling through TNFR1 promotes osteoclast differentiation in vitro and in vivo[193].

Abu-Amer *et al.*[4] found that in marrow cultures expressing only TNFR1 had more osteoclasts in comparison to wild type cultures, whereas those cultures having only TNFR2 contained fewer osteoclasts. These results are consistent with the conclusion that TNF enhances basal osteoclastogenesis via TNFR1 and suppresses it by TNFR2[4].

Effect in Bone

Mundy *et al.*[194] described multiple effects of TNF-α on bone cells. TNF-α inhibits DNA and collagen synthesis and osteocalcin gene expression in osteoblasts, but it stimulates the synthesis of proteolytic enzymes such as plasminogen activators and matrix metalloproteinases, and cytokines such as interleukin (IL)-8, IL-6 and M-CSF[195-198].

Kitaura *et al.*[193] found that upon exposure of high-dose TNF-α there was an induced increase in osteoclastogenesis. The direct induction of osteoclast recruitment by TNF-α is due to the enhanced RANK expression and sensitization of precursor cells to RANKL.

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Boyce[199] showed that early marrow progenitors embark on a path toward pre-osteoclasts under the influence of M-CSF, while TNF, IL-1 and RANKL promote a downstream progression toward the functional osteoclast phenotype. These soluble factors signal via the receptors, for MCSF (c-fms), TNFR1, ILIR, and RANK, respectively. Once the receptors are occupied, the

transcription factors c-fos and Nf-κB enter the nucleus to regulate gene transcription. In this way, the expression of genes coding for the mature osteoclast phenotype is activated, including tartrate-resistant acid phosphatase, carbonic anhydrase II and the receptors for calcitonin and vitronectin[192].

Nanes[192] showed that bone cultures treated with TNF had an increase in calcium release and suppression of matrix protein production, suggesting stimulation of osteoclasts causing bone resorption and inhibition of formation. The role of TNF as a stimulator of osteoclastogenesis has been confirmed by numerous investigations[172, 173, 200].

Effect on RANK/RANKL/OPG

Thomson *et al.*[201] and Konig *et al.*[202] showed TNF-α to be a potent bone-resorbing factor which stimulates osteoclastic bone resorption *in vitro* and *in vivo* by activating NF-κB through an intracellular mechanism that overlaps that of RANKL. Hofbauer *et al.*[154] established that IL-1β and TNF-α increased RANKL mRNA and OPG mRNA steady-state levels. The results of this study confirmed the hypothesis of Zou *et al.*[203], that TNF-α mediates RANKL stimulation of osteoclast differentiation through an autocrine mechanism. Osborn *et al.*[204], during an investigation into the human immunodeficiency virus showed that TNF and IL-1 were potent activators of NF-κB. Le *et al.*[205] demonstrated that genes regulated by TNF were similarly affected by other

cytokines, mainly by IL-1, but less commonly by interferons and growth factors.

Geboes *et al.*[206] found that systemic TNF-α mediated an increase in peripheral osteoclast precursors by up-regulating the expression of c-fms, the receptor for M-CSF. M-CSF is an essential factor in survival of osteoclast precursor cells. In the arthritis model Goboes *et al.*[206] found that the development of arthritis coincided with increased systemic production of TNF-α, expansion of osteoclast formation in the periphery, and an increase in the RANKL:OPG ratio in the joint. RANKL and TNF-α were present in the synovium and it was hypothesised that they may act synergistically on osteoclastogenesis. *In vitro,* Geboes *et al.*[206] found a strong synergistic effect of TNF-α and RANKL on osteoclastogenesis. These results were in accordance with the findings of Fuller *et al.*[207]. Results from this study concluded that TNF-α and RANKL act synergistically during osteoclastogenesis[206].

Effect on osteoclasts

As well as affecting bone resorption through interaction via the RANKL system, TNF-α can stimulate proliferation and differentiation of osteoclast precursors directly[154] or indirectly via osteoblasts[208]. According to Boyce[209], most factors known to stimulate osteoclast formation, such as TNF-α, IL-1, IL-6, PTH, 1α,25-(OH)2D3, and prostaglandins, bind to receptors on stromal cells/osteoblastic cells rather than binding to receptors on osteoclast

progenitors to induce the release of osteoclast-stimulating factors. In a study by Azuma *et al.*[178], results showed TNF-α strongly induced differentiation of M-CSF dependent bone marrow macrophage cells into mature osteoclasts through the TNRI (p55) receptor. These results demonstrated the importance of M-CSF in TNF-α induced osteoclastogenesis. The presence of TRAP positive cells was lower in TNF-α induced cells when compared to cells induced by RANKL. When OPG was added there was no effect on the TNF-α induced cells demonstrating that TNF-α osteoclastogenesis acts directly on osteoclast precursors via a pathway other than RANK/RANKL. The results from Kobayshi *et al.*[210] confirmed the importance of M-CSF on TNF-α induced osteoclasts. No TRAP positive cells were formed when M-CSF was not added to the bone marrow cell culture. Kobayshi also established that TNF-α stimulated TRAP positive cells through TNFR. When blocking antibodies against TNFR1 and TNFR2 were added to the cells, there was a strong inhibition of TRAP positive cells demonstrating that TNF-α worked independently from the OPG/RANK/RANKL system and directly on the cell via cell surface receptors.

Fuller *et al.*[207] also investigated TNF-α involvement and relationship with RANKL in osteoclast formation. Results showed that TNF-α was not affected by a blockade of RANKL via OPG suggesting direct activity of TNF-α on the osteoclast. Kudo *et al.*[200] in a similar experiment had similar results and confirmed Fuller's conclusions of direct TNF-α activity. Fuller *et al.*[207] showed that TNF-α caused activation of pre-existing osteoclasts and osteoclast cell spreading similar to RANKL-induced osteoclasts. Fuller *et*

al.[207] demonstrated that a lower concentration of TNF-α was needed for the activation of pre-existing osteoclasts than for the induction of osteoclastogenesis.

These results are different to those obtained during experimental arthritis in which Kong *et al.*[85] showed that OPG prevented bone erosion. Li *et al.*[211] indicated that TNF-α failed to induce a significant osteoclastogenic response in RANK-deficient mice. Lam *et al.*[212] suggested that TNF-α induced osteoclastogenesis required the precursors to be primed by RANKL. Ragab *et al.*[213] demonstrated TNF-α, lL-1β and lL-6 act in a synergistic fashion to produce a nett increase in RANKL activity. Fuller *et al.*[207] concluded that it was likely that the main mechanism by which TNF-α induced osteoclast differentiation was through synergy with RANKL because this might occur at concentrations below that which induces pro-inflammatory changes in tissues.

Immunohistochemistry

Immunohistochemistry is a research and diagnostic tool that allows the location and visualization of antigens in tissue through the combination of antigen-antibody interaction and microscopy. The majority of antigens are macromolecules that contain short sequences known as epitopes which provide a binding site for an antibody raised to that particular antigen[214].

An indirect method using unlabelled primary antibodies directed against the tissue antigen was developed to improve immunolabelling sensitivity[215] A second labelled antibody, directed against the primary antibody created an

increased magnification of the antigen-antibody interaction when viewed under the microscope[216]. The magnification is a result of several secondary antibodies being able to react with different antigenic sites on the primary antibody.

Avrameas[217] and Nakane[218] proposed the use of horseradish peroxidase in the direct and indirect immunolabelling methods. The linked peroxidase enzyme could be detected using a variety of different histochemical techniques of which the diaminobenzidine (DAB) reaction is the most widely used. The visualization reaction forms a solid brown stable deposit at the antigenic site, which may be seen under light microscopy. This labelling method allowed tissues to be dehydrated and permanently prepared for later archiving[216]. Work by Sternberger[219] and Nadj *et al.*[220] developed the peroxidase-anti-peroxidase complex (PAP) which increased the amount of enzyme available for the DAB reaction thereby amplifying the response of the interaction.

The Avidin-Biotin Technique

The avidin and biotin complex (ABC) was developed by Hsu *et al.*[215, 221, 222] and relies on the high binding affinity between biotin and avidin. Biotin is a low molecular weight vitamin that can be covalently linked to a primary antibody to produce a biotinylated complex. When this is added to a section, it localizes to the sites of an antigen. Avidin is a glycoprotein commonly found in egg white and has four binding sites for biotin. When avidin, bound to

horseradish peroxidase (HRP), is added to the biotinylated antibody, a tight irreversible bond is formed thereby locating the peroxidase complex at the site of the antibody-antigen reaction (Figure 2)[215, 221, 223].

NOTE:
This figure is included on page 49 of the print copy of the thesis held in the University of Adelaide Library.

Figure 2

Avidin-Biotin Technique

Biotin (represented by the square, is covalently lined to the primary antibody [**λ**] which binds to the tissue antigens (represented by triangles).
Avidin (represented by **+**) has four binding sites for biotin and can be chemically conjugated to horseradish peroxidase (represented by octagonal). The addition of peroxidase-conjugated avidin as a secondary reagent will allow tight binding of the complex to the biotinylated primary antibody, localizing to the site of the antibody-antigen reaction[215].

Figure 3

Avidin-Biotin-Peroxidase Complex (ABC) Technique

This technique employs a primary antibody with specificity against the antigen under study, a secondary biotinylated antibody, and a tertiary, ABC complex. The latter comprises a lattice-like three dimensional formation of avidin-biotin-peroxidase molecules which serves to localise several molecules of horseradish peroxidase at the site of antibody-antigen reaction in the tissue section[215].

The ABC technique has been further refined by the use of streptavidin which is a protein derived from the bacterium *Streptomyces avidinii* and has four identical subunits, each with a biotin-binding site (Figure 3). As this protein

has a neutral charge, it produces less background staining in comparison with avidin[215, 221].

Immunolabelling consideration factors

A major factor when considering histological preparations is the preservation of cells and tissues in a stable reproducible state[224]. The process of fixation preserves tissues by inhibiting lysosomal enzymes and preventing putrefactive changes within the tissue[224]. It has been recognized that immunoreactive antigens are progressively lost during the fixation process as fixatives denature tissue proteins by coagulation, by forming additive compounds or by a combination of both of these processes[225]. This causes conformational changes in the structure of the protein and inactivation of enzymes. The resulting complexes may structurally differ from the unfixed proteins. It is therefore important when performing an immunohistochemical study to use a fixative that will provide adequate fixation without major structural modifications. Many chemical fixatives are commonly used in pathology and can be classified into three categories: aldehydes, alcohols, and heavy metal fixatives. The most widely used is the aldehyde fixative, formaldehyde[226]. According to Glauert[227], paraformaldehyde is a preferred fixative compared with formaldehyde as it produces fewer cross-links and is less likely to mask tissue antigens in comparison with other aldehydes.

Conclusions

It can be seen from the review of the literature the factors involved in inflammatory induced root resorption are many and varied. Extensive investigation has been carried out on some of the involved areas, but the whole picture is still incomplete. The emerging involvement of cytokines such as TNF-α, on the OPG/RANK/RANKL interaction may lead to a better understanding and possible control over osteoclast differentiation. The recent identification of TNFR1 as the primary receptor involved in osteoclast formation allows a more precise area for further investigation. This study aims to investigate the interrelationship between TNF-α, TNFR1 and OPG in sterile inflammatory root resorption in an animal model

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Statement of Purpose

Hypothesis

It is hypothesized that the cell surface receptor of TNF-α, TNFR1 is present in the periodontal ligament of sterile inflammatory root resorption in an animal model. It is hypothesized that there will be a larger presence of TNFR1 in OPG-administered-rats.

Therefore, it is intended that the proposed research evaluate the above hypothesis which would then have a significant flow on benefit for the medical and dental researchers who have not found consistency with OPG's inhibitory effects

Aims

1. To investigate the presence of TNF-α, and TNFR1 in sterile inflammatory root resorption in both the presence and absence of OPG.
2. To explore the differences in TNF-α results found between the pilot study in 2006 and the present study in 2009

SECTION 2

Article 1

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Presence of TNF-α and TNFR1 in aseptic root resorption

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Presence of TNF-α and TNFR1 in aseptic root resorption

ABSTRACT

The osteoclast antagonist osteoprotegerin (OPG) can hypothetically offer molecular control over the process of orthodontic root resorption. Unpublished work investigating OPG in a rat model found no inhibitory effect on osteoclasts and odontoclasts when given at a dosage of 2.5mg/kg. It was hypothesised that traumatically induced periodontal ligament (PDL) inflammation produced mediators and cytokines with the ability to stimulate clast cell differentiation and antagonize the effects of OPG. 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. Dry ice was applied for 15 minutes to the upper right first molar crown of 18, eight week old male Sprague-Dawley rats. These were evenly divided into experimental and control groups. The experimental group was injected with OPG at a dose of 2.5 mg/kg of body weight. After seven days, the rats were sacrificed and their maxilla processed for immunohistochemical identification of TNF-α and TNFR1. Results showed root resorption to be present in varying amounts and locations. 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 of the periodontium mainly in the interradicular area. There appeared to be more noticeable labelling in OPG-treated rats. The results indicated that TNF-α and its receptor TNFR1 were present and may modify OPG effectiveness by offering an alternative pathway for osteoclastc formation, thereby challenging the potential anti-resorptive effects of OPG.

INTRODUCTION

Orthodontic tooth movement achieved by osteoclast-induced bone remodelling often has the adverse consequence of sterile inflammatory root resorption (Brezniak and Wasserstein, 2002). The interaction between osteoblasts and osteoclasts is important in both tooth movement and root resorption. However, a complete understanding of the biological interactions involved in these processes is still unclear. The identification and investigation of the OPG/RANK/RANKL system (Simonet et al., 1997) was considered a 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 (Phan et al., 2004). Osteoclasts are recruited to a specific resorptive site where they remove bone which subsequently undergoes repair by osteoblastic deposition of bone matrix. Resorption and bone formation are thus a coupled process. Uncoupling of this process leads to the reduced bone mass seen in osteoporosis or, in some rare cases the accumulation of bone leading to osteopetrosis. Osteoclast differentiation initiated through direct cell-to-cell contact between osteoblasts and osteoclast precursors has been demonstrated by Udagawa (2002) and Takahashi et al. (1999). It has been further shown that osteoblasts express surface domains that are recognized by receptors on osteoclast precursors which, upon interaction, initiate osteoclast differentiation (Takayanagi, 2005). These receptors have been identified as the RANK ligand (RANKL) on the osteoblast and its RANK receptor on the osteoclast precursor. OPG was identified as a decoy receptor for RANK (Simonet et al., 1997; Tsuda et al., 1997; Yun et al., 1998) which

competitively binds to the RANKL receptor thereby inhibiting osteoclastogenesis and subsequent bone resorption. The inhibitory effects of OPG during sterile inflammatory root resorption have been previously investigated (Dreyer, 2002). Results indicated that the manufacturer's recommended dosage of 2.5mg/kg OPG did not inhibit sterile inflammatory root resorption and the conclusions suggested the possible involvement of other inflammatory cytokines. TNF-α has previously been shown to have an involvement in the RANK/RANKL induction of osteoclasts (Yeung, 2004) and to directly induce osteoclast differentiation (Fuller et al., 2002). When studied with OPG, TNF-α-mediated osteoclast differentiation was not shown to be inhibited by OPG (Fuller et al., 2002). Furthermore, two cell surface receptors for TNF-α have been identified; 55kDa (TNFR1) and 75kDa (TNFR2) (Abu-Amer et al., 2000). TNFR1 is essential for TNFR receptor function in a variety of cell lines as shown by Thoma (Thoma et al., 1990). TNFR1 is known to mediate most of the biological properties of TNF-α, such as the activation of NF-κB. TNFR1 can activate a cascade of kinases which results in the activation of RANKL and the C-Jun NH_2-terminal kinase (JNK) pathway as indicated by Chen and Goeddel (2002). In marrow cultures expressing only TNFR1 it was found there were more osteoclasts in comparison to wild type cultures (Abu-Amer et al., 2000). These results are consistent with the conclusion that TNF-α enhances osteoclastogenesis via TNFR1. Also, upon exposure of high-dose TNF-α an induced increase in osteoclastogenesis has been found (Kitaura et al., 2004). The direct induction of osteoclast recruitment by TNF-α is due to the enhanced RANK expression and sensitization of precursor cells to RANKL (Fuller et al., 2002). The present

study aims to investigate the presence of TNF-α and TNFR1 in sterile inflammatory root resorption in both the presence and absence of OPG.

MATERIALS AND METHODS

Experimental Animals

Eighteen, eight-week-old, male Sprague-Dawley rats were used. All animals were treated under ethical regulations for animal experiments as approved by the Ethics Committee of The University of Adelaide (M-4-04). Each animal weighed between 250-300g at the start of the experiment and increased 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 at a dosage of 2.7ml/kg of body weight. The rats were placed on a specially constructed rack which stretched the mouth open through the use of looping metal rings around both the upper and lower incisors. The upper right first molar was frozen for a period of fifteen minutes by application of customized pellets of dry ice (CO_2 at $-81^{o}C$). Following the application of ice, the tissues thawed 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 was administered as

a single dose at a concentration of 2.5mg/kg subcutaneously into the hind leg of each animal immediately after freezing.

Immunohistochemistry

Seven days post-thermal insult, all animals were sacrificed via cardiac perfusion with 30ml of 4% paraformaldehyde fixative into the left ventricle. On completion of perfusion, the maxilla was dissected out and immersed in 4% paraformaldehyde for 24 hours, rinsed in phosphate-buffered saline (0.4M pH 7.4) for 24 hours and then decalcified using 4% EDTA in phosphate buffer. Radiographic examination was used to determine full decalcification which took between four and six weeks. The tissues were placed in 70% alcohol before automatic processing with a Shandon Citadel 2000 automatic processor. Dehydration was achieved through graded alcohols before paraffin wax impregnation. Coronal sections of 5 μm were cut and mounted on aminopropyltriethoxysilane (APT) coated slides in sequential order of three sections per slide.

Extensive methodologies with different antigen retrieval protocols were employed to optimise the antibody concentration and staining result. Every third slide was stained 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. Every subsequent slide was stained for TNFR1 (rabbit polyclonal to TNF Receptor 1, Abcam, Cambridge, United Kingdom) and counterstained with haematoxylin. Positive staining was identified as dark

brown staining and validated by comparison with a positive control of adenocarcinoma tissue. Negative control was achieved though deletion of the primary antibody in the experimental tissue.

Observations

TNF-α

A summary of results are presented in Table 1. No positive staining was seen in the furcation region of the periodontal ligament of control 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 (Fig 1, Fig 2). However, diffuse staining occurred in the periodontal ligament in non-OPG administered rats and this was the same for both the experimental and control teeth and therefore considered background staining (Fig 2 and Fig 3). Therefore, no positive staining was seen in the periodontal ligament of non-OPG administered rats.

OPG administered rats showed positive staining in the necrotic pulp tissue in the experimental frozen teeth. In six of the OPG administered specimens, positive staining was seen in the interradicular area in experimental frozen teeth. The positive staining can be seen surrounding the cells of the periodontal ligament, in the gingival tissues, in nerve tissue and in the marrow spaces of the alveolar bone (Fig 4).

Resorption lacunae were seen along the palatal roots of the frozen teeth in both OPG administered and non-OPG administered teeth. These were identified as containing multinucleated cells in lacunae. These areas did not

show positive label for TNF-α (Fig 5). Resorption lacunae were found on the palatal root in a greater number in comparison to the buccal root. The tooth arbitrarily was divided into thirds, buccal root, palatal root and interradicular area. Resorption lacunae were evaluated according to their distribution.

In some specimens, osseous-like material was seen in the interradicular area surrounded by positive TNF-α staining (Fig 6). These regions were seen in five of the OPG administered rats and two of the non-OPG-administered rats. This osseous-like material extended from the alveolar crest to the cemental surface in the bifurcation area. The amount of the osseous-like material varied from isolated islands to complete obliteration of the PDL. Positive TNF-α label was seen within the cytoplasm of the surrounding ovoid-shaped cells and within the extracellular fluid.

TNFRI

A summary of results are presented in table 1. Positive staining was seen in both control and experimental teeth in both OPG-administered and non-OPG administered rats. In control teeth of non-OPG administered rats, positive staining for TNFRI was seen within the periodontal ligament surrounding the fibroblasts and randomly within the pulp tissue. Strong positive staining was seen in ovoid-shaped cells which appeared to be lining the alveolar crest. These cells were uniform in shape, size and followed the contour of the alveolar ridge (Fig 7). This was a consistent finding in all control teeth in both OPG and non-OPG administered rats and in experimental frozen teeth in non-OPG administered rats.

In experimental frozen teeth of OPG administered rats, positive staining for TNFRI was seen within the periodontal ligament, in the pulp tissue and gingival tissue. Within the bifurcation region, staining for TNFRI was strong and diffuse. The positive staining was not only localised within the cells of the PDL but was also seen in the extracellular space (Fig 8).

Resorption lacunae were seen in OPG administered rats. These contained multinucleated cells which stained positive for TNFRI. These lacunae were seen randomly on the palatal roots in frozen experimental teeth (Fig 9).

In the specimens containing the osseous-like material, the ovoid-shaped lining cells showed a strong positive staining for TNFRI (Fig 10). In the non-OPG administered rats, the ovoid cells could be seen lining the regions of osseous-like material showing a line of demarcation between the PDL and the alveolar bone. In OPG administered rats, the ovoid lining cells could be seen surrounding the osseous-like material in the bifurcation region (Fig 11). The diffuse, strong positive staining for TNFRI could also be seen in regions not containing the osseous-like material in the bifurcation region.

Table 1, Summary of results found in the present study

	Non-OPG		OPG	
	Control	**Experimental**	**Control**	**Experimental**
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

DISCUSSION

The frozen tooth model was used to produce aseptic necrosis and subsequent root resorption in an attempt to mimic orthodontic root resorption. Previous studies using cryotherapy by Wesselink *et al.* (1986) and Tal *et al.* (1991) showed resorption of the periodontal hard tissues. The frozen tooth model was chosen for its simplicity and effectiveness. In this study, it was histologically evident that cryotherapy caused the necrotic destruction of the underlying pulpal and periodontal tissues. Osteoclastic cell activity was evident on the hard tissue surface in areas radially affected by the freezing process.

This study visually assessed the results of TNF-α and TNFRI staining. OPG is a member of the TNF superfamily and the possibility exists for cross-reactivity between the TNF-α polyclonal antibody and OPG. This was addressed in a pilot study by using TNF-α monoclonal antibody (of which only one was present on the market at the time) but it was found not to stain in the known positive control tissue of breast adenocarcinoma. The TNF-α monoclonal antibody was, therefore, found to be unreliable and was discarded in favour of

the TNF-α polyclonal antibody. It is unlikely that cross-reactivity between the TNF-α polyclonal antibody and OPG existed, as not every specimen which had been administered OPG showed a positive staining for TNF-α. This does need to be verified with a reliable TNF-α monoclonal antibody. In a recent publication (Capparelli et al., 2003) investigating OPG in a rat model, results showed a rapid and sustained reduction in osteoclast numbers with a peak serum concentration for OPG occurring within 12 hours of treatment. These serum levels remained relatively high at days five and 10 but reduced up to day 30.

Previous studies showed that TNF-α might be associated with osteoclast differentiation (Abu-Amer et al., 2000; Azuma et al., 2000) and bone remodelling (Horowitz et al., 2001). When mechanical loading with an orthodontic appliance was used, it was demonstrated that expression of TNF-α was detected 12 hours after loading but 3 days later had returned to basal levels (Andrade et al., 2007). It was concluded that the local upregulation of TNF-α preceded the enhancement in TRAP activity and the number of TRAP-positive cells. In the present study OPG, which is known to block the interaction of RANK and RANKL and therefore inhibit osteoclastic formation, did not inhibit resorption lacunae from forming. It was histologically seen that these resorption lacunae contained multinucleated cells. Counterstaining with TRAP would show if these cells were TRAP-positive and, therefore, osteoclasts/odontoclasts. It is possible that these resorption lacunae did not stain positive for TNF-α, despite osteoclasts forming via the TNF-α pathway, because the expression of TNF-α may have reduced back to its basal level after 3 days as seen by Andrade *et al*. (2007). Nevertheless, positive staining

for TNFRI was seen in the resorption lacunae. TNFRI is the major receptor for TNF-α and can activate a cascade of kinases which ultimately result in the activation of RANKL and the JNK pathway (Chen and Goeddel, 2002; Fuller et al., 2002). Our results showing positive staining for TNFRI in the resorption lacunae containing multinucleated cells, support the possibility that TNF-α can cause osteoclastic differentiation in the presence of OPG; this has been shown previously by Azuma *et al*. (2000). The present study originally aimed to undertake staining with TNFRII which is known to inhibit osteoclast formation(Abu-Amer et al., 2000), but no suitable TNFRII receptor antibody was available at the time. The TNFRI results indicated a generalised presence throughout the PDL in both control and experimental rats but the addition of OPG appeared to increase the intensity of staining. Results in the published literature show TNFRI to be the major receptor for TNF-α and it is known to mediate most of the biological properties of TNF-α (Yongchaitrakul et al., 2006). Therefore, our results showing generalised staining are consistent with these known facts. A recent study has shown that cultures from TNFRI-null mice generate significantly fewer osteoclasts compared to their wild-type counterparts (Abu-Amer et al., 2004). It was then concluded that TNFRI plays a critical role in direct and indirect induction of osteoclastogenesis and that TNFRI expression by osteoclast precursors is required for optimal osteoclastogenesis. The presence of OPG may result in the increased expression of TNFRI, thereby allowing the osteoclastic differentiation to occur from the thermal insult. This could be why a stronger staining for TNFRI in OPG administered rats and the presence of resorption lacunae was noted.

Bone degradation is balanced by bone formation and the presence of TNFRI in bone, including osteoblasts, indicated that TNF-α may be involved with the presence of osseous-like material in the frozen PDL. After insult, the PDL heals in the same way as other types of connective tissue. Repopulation of cells has been thought to arise from the gingival and PDL mesenchymal phenotypes but cells from the marrow spaces in alveolar bone are also thought to be involved. Transient ankylosis has been previously noted by Blomlof *et al*. (1994) during reparative cementum formation on an instrumented root surface in monkeys. It is possible that the osseous-like material seen in the present study could be a result of periodontal wound healing and, therefore, may be transient in nature. Transient ankylosis has been observed for up to five weeks leaving a mineralized tissue layer apposed to, but not attached to, the dentine surface while showing a lamellar appearance resembling alveolar bone. 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 (Lang et al., 1995). As can be seen in the control samples, there is a layer of cells lining the alveolar bone in the interradicular area. Due to the location and the shape of these cells, it is likely that these cells are osteoblasts. Stimulated by resorptive activity, the osteoblasts form islands of osseous-like tissue which initiates the ankylotic process. In the present study, it can be seen that the alveolar bone is in appositional contact with the intact cemental surface. It is possible that the hard tissues are either fused together or in very close approximation, possibly separated by a thin amorphous layer. The presence of an increase in 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 (Pinkerton et al., 2008; Wescott et al., 2007). A coupling factor between bone resorption and bone formation has been suggested with osteoclasts potentially being a source of stimulus for osteoblast formation (Martin, 2004). It may be 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 nett bone loss and, therefore, a bone mass increase. It may be that these factors belong to the TNF superfamily and could work through the TNFRI mechanism.

In conclusion, our study suggests that OPG given at a dosage of 2.5mg/kg does not inhibit osteoclast formation in a freezing induced aseptic necrosis model. This suggests that either TNF-α or other factors that cause osteoclastogenesis are involved. TNF-α, which is known to cause osteoclastic formation, was found to a greater extent in OPG administered rats. This is consistant with an increase in the presence of TNFRI which suggests osteoclast formation may occur through TNFRI in the presence of OPG. The presence of osseous-like material also suggests that OPG and TNF-α may participate in an increase in osteoblastic expression and a nett bone increase in the PDL. Further studies looking into the interrelationship between OPG, TNFRI and the osseous-like material are now required for better understanding of the role of TNFRI in both osteoclastic and osteoblastic activity and the phenomenon of ankylosis in aseptic necrosis-induced root resorption.

Images

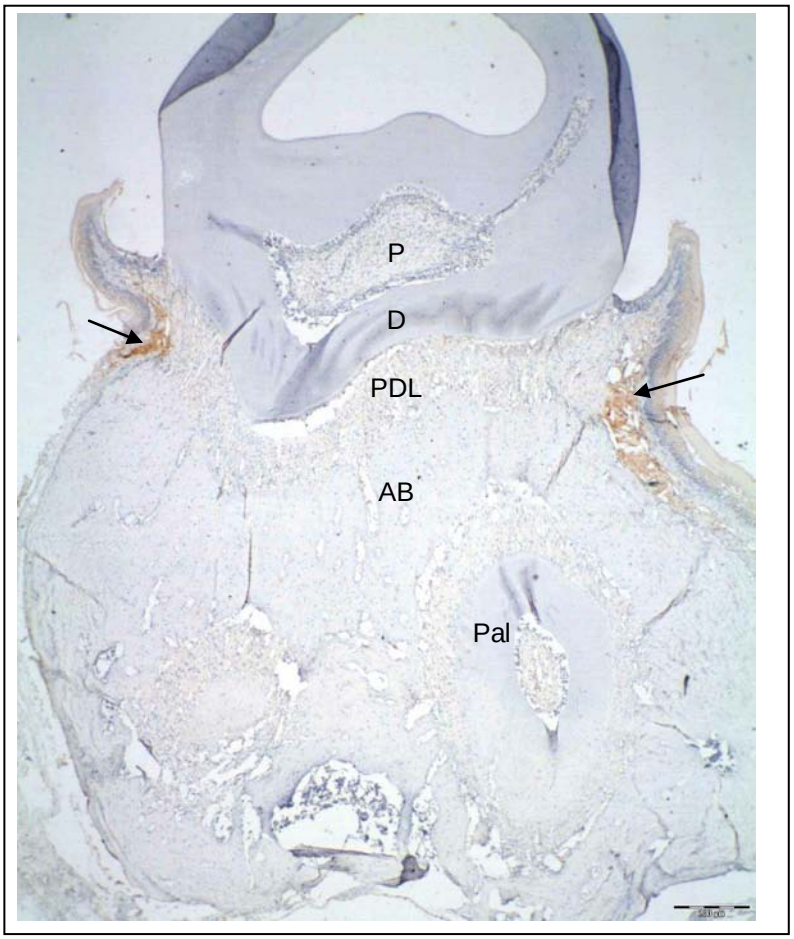

Fig 1. TNF-α staining of control tooth in OPG administered rat (Scale bar equals 200µm) Arrows show positive staining in the gingival tissues. P = pulp, D = Dentine, PDL = periodontal ligament, AB = alveolar bone, Pal = Palatal root

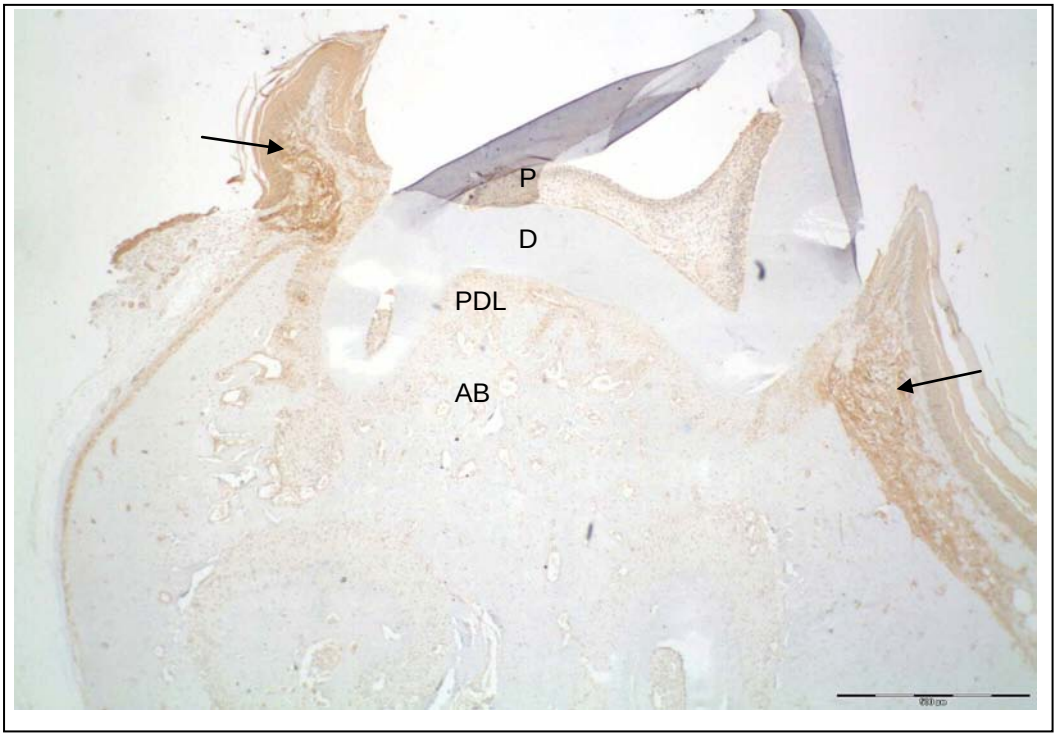

Fig 2. TNF-α staining of control tooth in non-OPG-administered rat (Scale bar equals 500μm) Arrows show positive staining in gingival tissues. P = pulp, D = Dentine, PDL = periodontal ligament, AB = alveolar bone

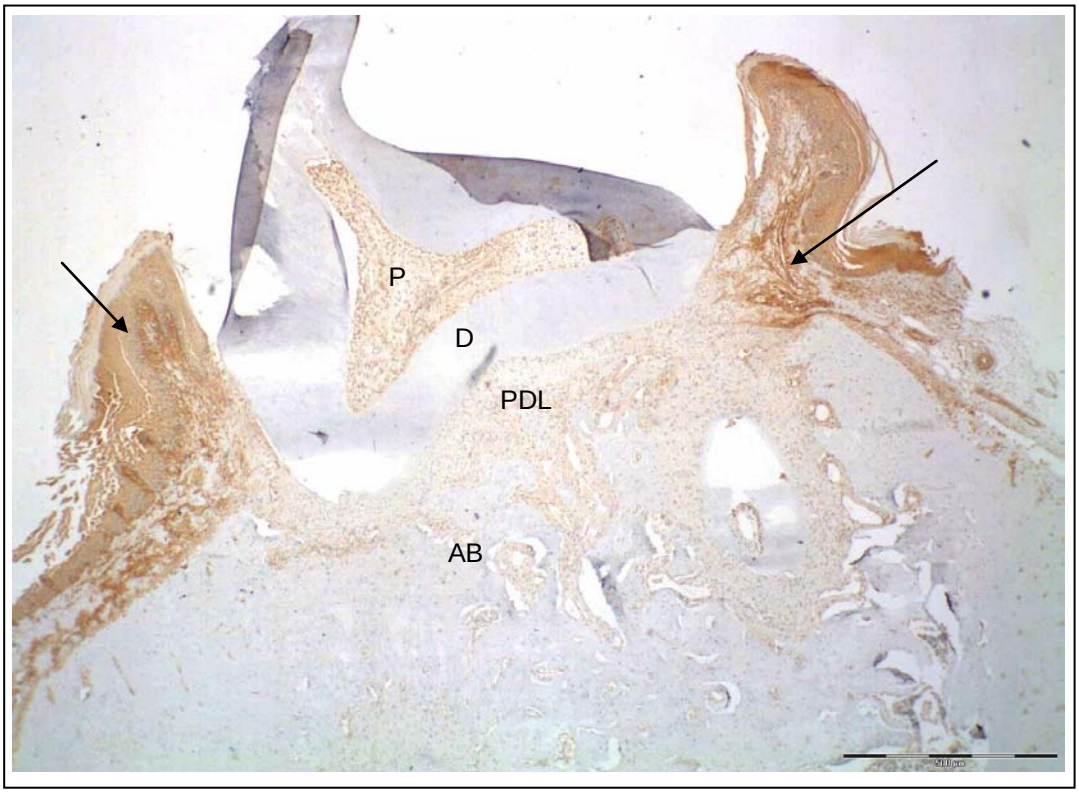

Fig 3. TNF-α staining in experimental tooth in non-OPG administered rat (Scale bar equals 500μm). Arrows show positive staining in gingival tissues. P = pulp, D = Dentine, PDL = periodontal ligament, AB = alveolar bone

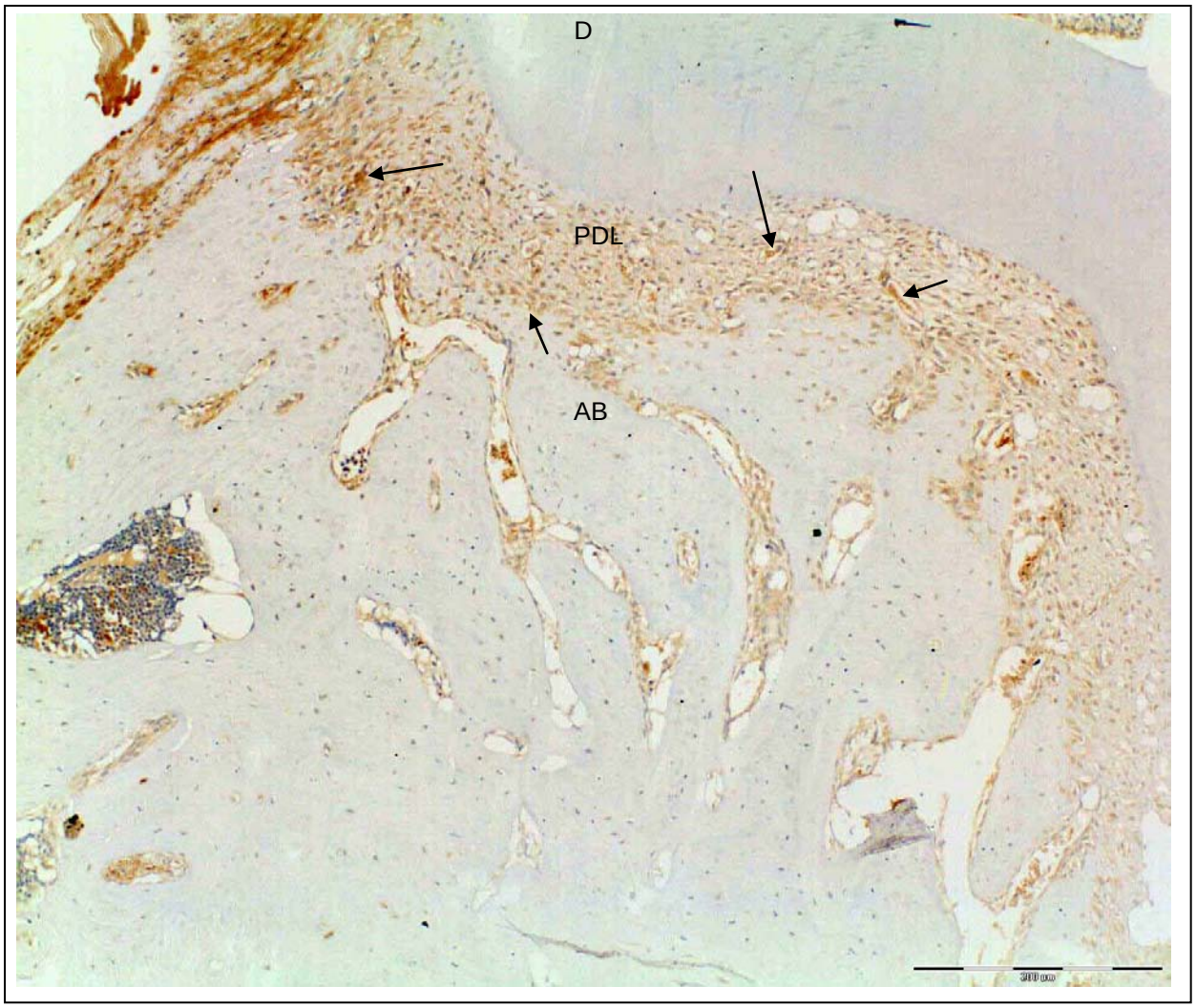

Fig 4. TNF-α staining in experimental tooth in OPG administered rat (Scale bar equals 200μm). Arrows show positive staining in the periodontal ligament. D = Dentine, PDL = periodontal ligament, AB = alveolar bone

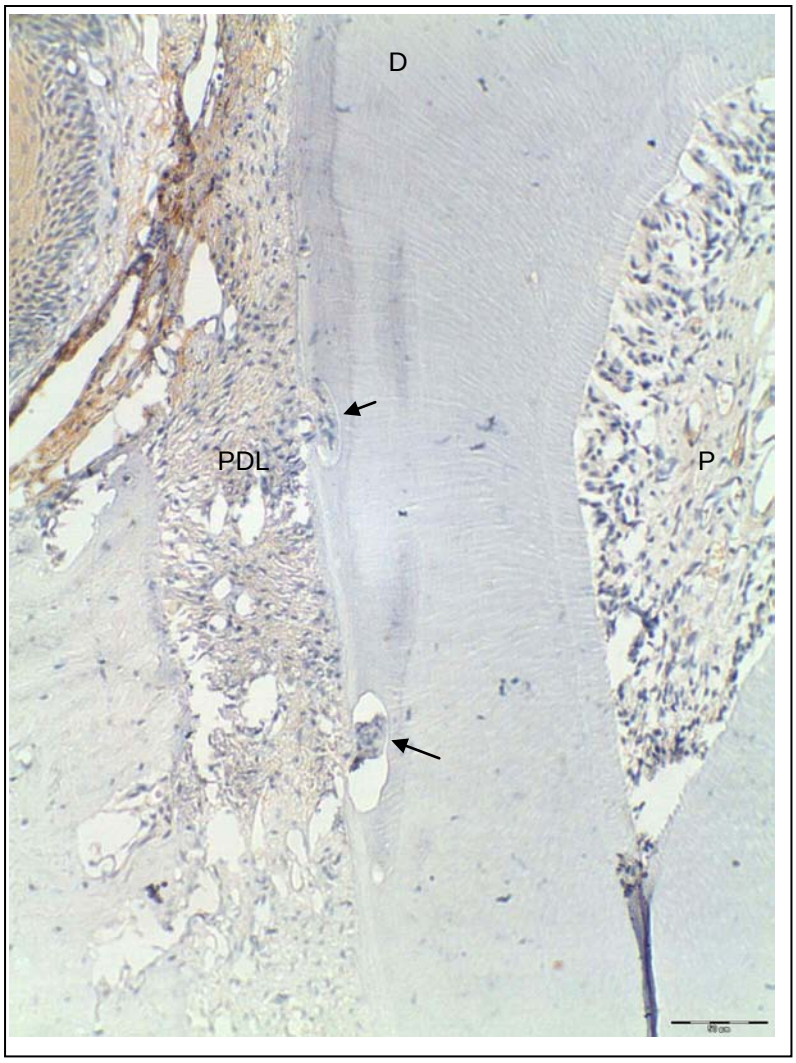

Fig 5. Resorption lacunae on the palatal root of an experimental tooth in OPG administered rat arrows show no positive staining for TNF-α in the resorption lacunae (Scale bar equals 50µm). D = Dentine, PDL = periodontal ligament, P = pulp.

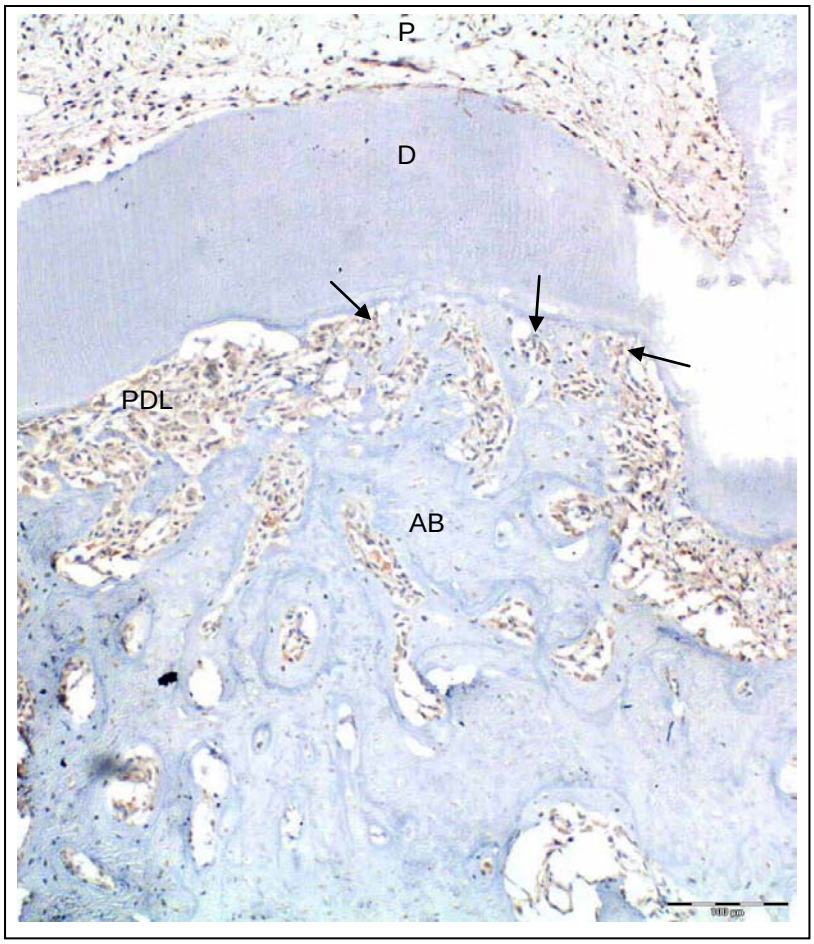

Fig 6. Experimental tooth in OPG administered rats. Arrows show positive staining for TNF-α surrounding the osseous-like material (Scale bar equals 100µm) P = pulp, D = Dentine, PDL = periodontal ligament, AB = alveolar bone

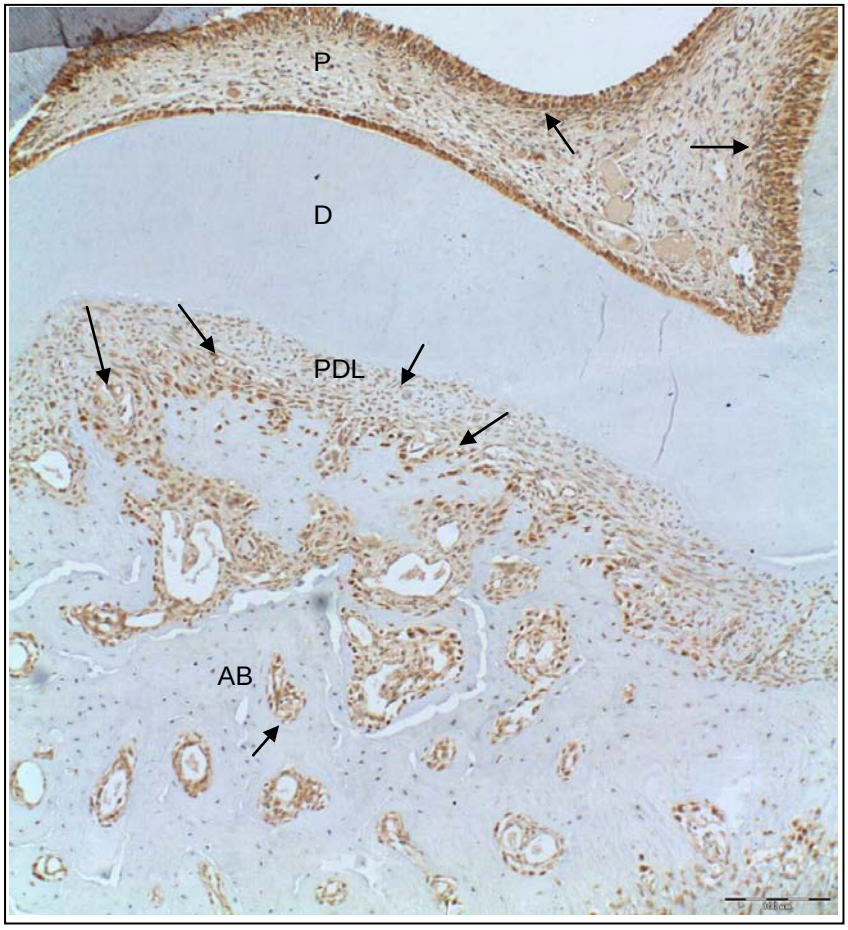

Fig 7. Control tooth in non-OPG administered rat. Arrows show positive staining for TNFRI in the PDL, pulp and cells lining the alveolar bone (Scale bar equals 100µm). P = pulp, D = Dentine, PDL = periodontal ligament, AB = alveolar bone

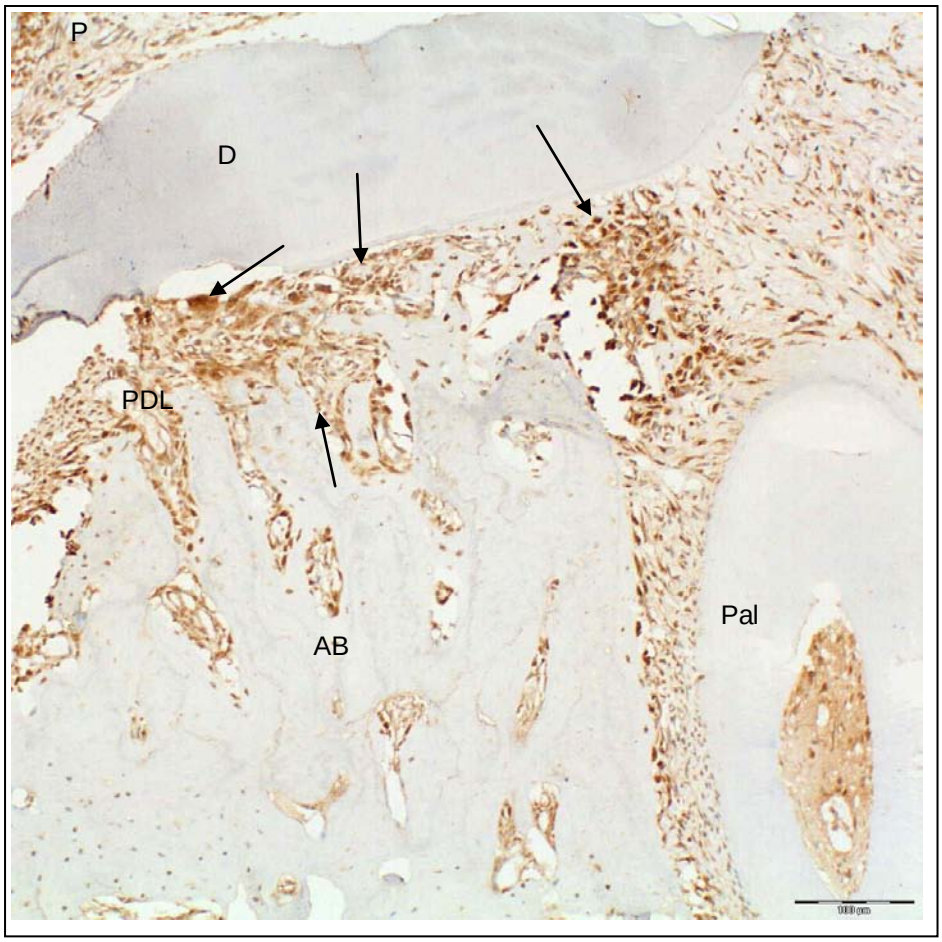

Fig 8. Experimental frozen tooth in OPG-administered rat. Arrows show strong diffuse positive staining for TNFRI within the bifurcation region, (Scale bar equals 100µm). P = pulp, D = Dentine, PDL = periodontal ligament, AB = alveolar bone, Pal = Palatal root

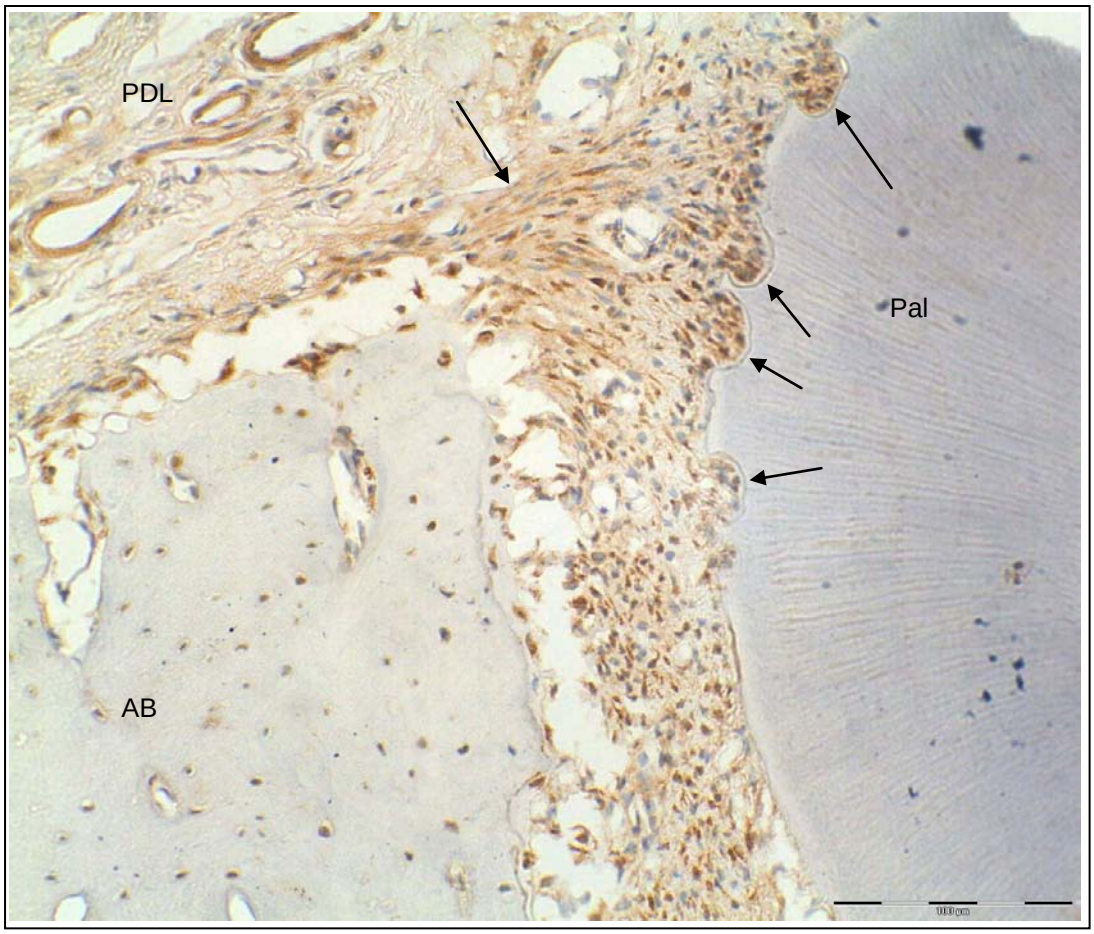

Fig 9. Resorption lacunae on the palatal root of an OPG administered experimental frozen tooth. Arrows show positive staining for TNFRI. (Scale bar equals 100µm). PDL = periodontal ligament, AB = alveolar bone, Pal = Palatal root

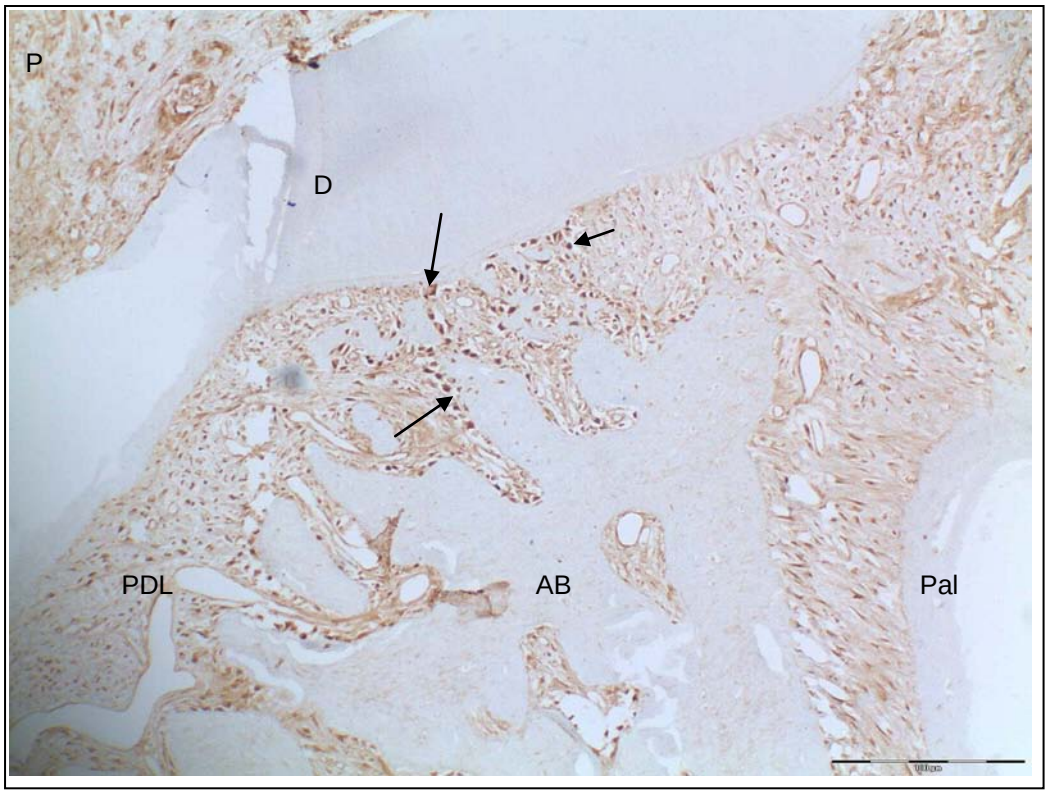

Fig 10. Experimental frozen tooth in a non-OPG administered rat. Arrows show an area of osseous-like material with TNFRI positive cells lining the surface (Scale bar equals 100µm). P = pulp, D = Dentine, PDL = periodontal ligament, AB = alveolar bone, Pal = Palatal root

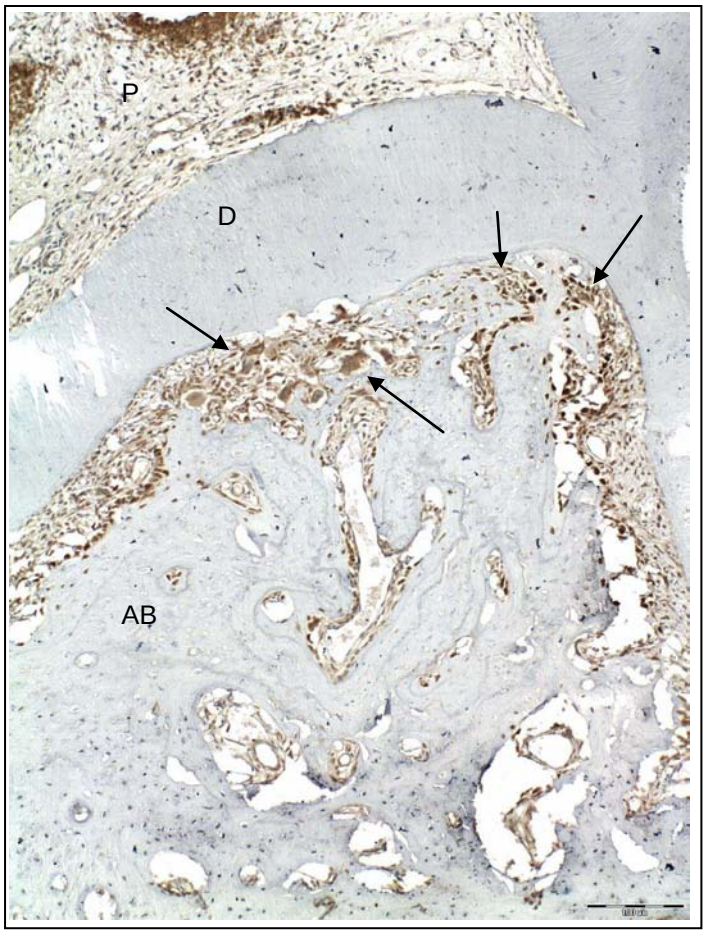

Fig 11. Experimental frozen tooth in an OPG administered rat. Arrows show an area of osseous-like material with TNFRI positive cells lining this region. Diffuse positive staining seen in the remaining PDL in the bifurcation region (Scale bar equals 100µm). P = pulp, D = Dentine, AB = alveolar bone,

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Article 2

This article has been written for submission to the Australian Orthodontic Journal

Immunohistochemical Investigation of Tumour Necrosis Factor Alpha

(Australian Orthodontic Journal)

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Immunohistochemical Investigation of Tumour Necrosis Factor Alpha

Abstract

Outcomes of an immunohistochemical reaction are often used for research and medical diagnostic purposes. Results are strongly influenced by technical and interpretative problems and, in some instances, may result in false positive or negative outcomes. This report reviews the difference in results obtained between two studies conducted three years apart using the same animal material and immunohistochemical protocol. An initial investigation conducted in 2006 into the presence of Tumour Necrosis Factor alpha (TNF-α) found positive staining in an induced sterile inflammation animal model. Later research using the same animal material in 2009 found a remarkable difference in results. The material was generated by a 15 minute application of dry ice to the upper right first molar crown of 18, eight week old male Sprague-Dawley rats which were equally divided into experimental and control groups. The experimental group was injected with OPG at a dose of 2.5 mg/kg of body weight immediately after the thermal insult. After seven days, the rats were sacrificed and their maxilla processed for immunohistochemical identification of TNF-α. In 2006, a multispecies detection kit was used and no antigen retrieval was required. Results showed a strong, generalised, positive staining for TNF-α within the periodontal ligament. At the end of this study, the unused tissue was packaged and stored for a period of three years. In 2009, the same tissue and antibody were used in a parallel immunohistochemical investigation but no positive result was found. The original protocol was reviewed and the antibody concentration and antigen retrieval was optimised with a new staining protocol being developed. Results showed diffuse positive staining in six of the 18 specimens. These

results differed markedly from the original study. It was concluded that processing and storage factors were involved in the loss of antigenicity during the time period between studies.

INTRODUCTION

Immunohistochemistry (IHC) is a research and diagnostic tool that allows the location and visualization of tissue antigens through an immunological reaction revealed by light microscopy. IHC staining has now become an essential part of histocytopathology diagnosis and analysis and the result of the IHC reaction should be interpreted properly. False results may be related to technical and interpretative difficulties and can sometimes result in an incorrect finding. Many factors involved in the IHC analysis may influence the final outcome with a single mistake in any step causing a final false negative result. IHC works through unlabelled primary antibodies being directed against the tissue antigen. The majority of antigens are macromolecules that contain short sequences known as haptens which provide a binding site for an antibody raised to that particular antigen[1]. A second labelled antibody directed against the primary antibody, amplifies the antigen-antibody interaction which, when labelled with a chromogen, is viewed under the microscope[2]. The primary antibody is able to be viewed microscopically as a result of several chromogen-labelled secondary antibodies binding to different antigenic sites on the primary antibody. Nakane[3] proposed the use of horseradish peroxidase as a replacement for fluorochromes in the direct and indirect immunolabelling protocols. The linked peroxidase enzyme was detected via a variety of histochemical techniques of which the

diaminobenzidine (DAB) reaction is the most widely used. The visualization reaction forms a solid brown stable deposit at the antigenic site, which may be seen under light microscopy. The avidin and biotin complex (ABC) was developed by Hsu[4] and relies on the high binding affinity between biotin and avidin. As a low molecular weight vitamin, biotin can be covalently linked to a primary antibody to produce a biotinylated complex[4] to identify and localize the sites of an antigen. Avidin is a glycoprotein commonly found in egg white and has four binding sites for biotin[4]. When avidin, bound to horseradish peroxidase (HRP), is added to the biotinylated antibody, a tight irreversible bond is formed thereby locating the peroxidase complex at the site of the antibody antigen reaction[4]. The ABC technique has been further refined by the use of streptavidin which has four identical molecular subunits, each with a biotin-binding site[5]. As this protein has a neutral charge it produces less background staining in comparison with avidin[5]. There are many variables within the IHC staining protocol where difficulties may arise which affect the final outcome. These areas include tissue processing, especially tissue fixation and factors which may result in loss of tissue antigenicity such as inappropriate storage which can result in oxidisation of the tissue and changes in the level of antigen expression[6, 7]. In previous unpublished work[8] TNF-α was located in rat periodontium through IHC analysis. Later work[9] using the same animal material found a difference in the both the location and intensity of TNF-α staining. This report investigates the difference in IHC results using the same animal experimental tissue and antibody but with different IHC methodology.

MATERIALS AND METHODS

Materials

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

Methods

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 at a dosage of 2.5ml/kg of body weight. The rats were placed on a specially constructed rack which stretched the mouth open through the use of looping metal rings around both the upper and lower incisors. The upper right first molar was frozen for a period of fifteen minutes by application of customized pellets of dry ice (CO_2 at -81^{o}C). Following the application of ice, the tissues thawed slowly under anaesthesia and the animals recovered under a heat lamp. The upper left first molar was left unfrozen and served as a control. Osteoprotegerin was administered immediately after the thermal insult as a single dose at a concentration of 2.5mg/kg subcutaneously into the hind leg of nine randomly selected animals.

Immunohistochemistry

Seven days post thermal insult, the animals were anaesthetised and sacrificed via cardiac perfusion with 30ml of 4% paraformaldehyde fixative into the left ventricle. On completion of perfusion, the maxilla was dissected out and immersed in 4% paraformaldehyde for 24 hours, rinsed in phosphate-buffered saline (PBS 0.4M pH 7.4) for 24 hours and then decalcified using 4% ethylenediaminetetra-acetic acid (EDTA) in phosphate buffer. Radiographic examination was used to determine full decalcification which took between four and six weeks. The tissues were placed in 70% alcohol before automatic processing with a Shandon Citadel 2000 automatic processor. Dehydration was achieved through graded alcohols before paraffin wax impregnation. Coronal sections of 5 μm were cut and mounted on aminopropyltriethoxysilane (APT) coated slides in sequential order with three sections per slide.

Every third slide was stained 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. Every tenth slide was stained with Mayer's haematoxylin and eosin (H&E) for orientation.

Positive staining was confrmed as dark brown staining compared with a positive control of adenocarcinoma tissue. Negative control was achieved through the absence of the primary antibody in the experimental tissue.

2006 Study

In this study a multispecies kit was used and the procedures followed the ABC technique (Signet, Dedham, Massachusetts, USA). Sections were dewaxed using xylene and alcohol solutions. The slides were placed in a 30% solution of H_20_2 in methanol in order to block endogenous peroxidase activity. Treated sections were washed in PBS (pH 7.4) prior to incubation in normal horse serum for five minutes. Excess serum was then shaken off and the slides were incubated with the primary antibody of TNF-α (polyclonal antibody to mouse TNF-α, HyCult biotechnology, Uden, The Netherlands) in a concentration of 1:20 (diluted with phosphate buffered saline) overnight in a humidity chamber. Successive incubations in biotinylated linking antibody from the kit and streptavidin peroxidase occurred prior to the application of DAB as a peroxidase substrate. The slides were washed after the DAB stage and lightly counterstained with haematoxylin. The slides were then mounted with aquatex (Merck, Darmstadt, Germany). Positive staining was seen throughout the periodontal ligament of OPG administered rats.

After this study, the remaining unstained slides were placed in a slide box and stored in a dark cupboard at room temperature. The remaining block sections were placed in the refrigerator and stored at 4^{o} C

2009 Study

The slides and remaining block sections were retrieved from storage and staining was attempted using the same protocol as 2006. No positive results were found. The TNF-α primary antibody was then optimised for both concentration and antigen retrieval. This was done at different concentrations

from 1:50 – 1:600 using no retrieval, EDTA and citrate buffer (10mM sodium citrate pH 6) in a water bath. It was found that a concentration of 1:50 TNF-α primary antibody with citrate buffer retrieval for two hours in an 80^{o}C water bath gave the best positive results in the control specimen of adenocarcinoma tissue. Consequently, experimental slides were dewaxed and taken to absolute alcohol. Endogenous peroxidase was blocked with 0.5% H_2O_2 in methanol at room temperature for 30 minutes. Slides were then rinsed in phosphate buffered saline pH 7 (PBS). Antigen retrieval was achieved by placement of slides into citrate buffer heated to 80^{o} C in a water bath and incubated for 2 hours. This was then removed and allowed to cool to below 50^{o}C before being rinsed twice in PBS. The slides were then incubated in 3% normal horse serum (NHS) for 30 minutes, drained and then incubated with the primary TNF-α (polyclonal antibody to mouse TNF-α, HyCult biotechnology, Uden, The Netherlands) at a 1:50 concentration overnight in a humidity chamber. Subsequently, slides were rinsed in PBS and incubated with the secondary antibody 1:250 for 30 minutes, rinsed in PBS and incubated with streptavidin peroxidase 1:500 for 15 minutes. DAB staining occurred over an optimised time of 7minutes. The slides were then rinsed in PBS and lightly counterstained with Mayer's haematoxylin, dehydrated and clear mounted. Positive results were seen in six of the OPG administered rats within the interradicular area of experimental frozen teeth.

RESULTS

2006 Study

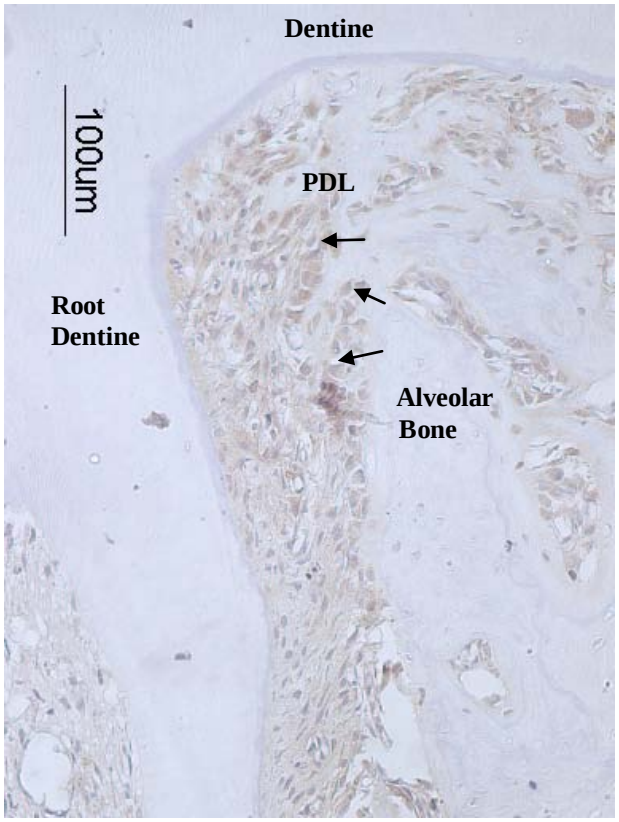

Figure 1
Arrows show positive DAB staining for TNF-α in ovoid cells of the periodontal ligament in a experimental tooth in an OPG administered rat molar.

TNF-α was distributed throughout the periodontal ligament of the upper right first molar indicating an inflammatory response. The labelling was seen surrounding ovoid cells within the periodontal ligament. This positive staining was seen in the cervical third of the section and at both the buccal and palatal root apex. The staining seen was strong and generalised (Fig 1).

2009 Study

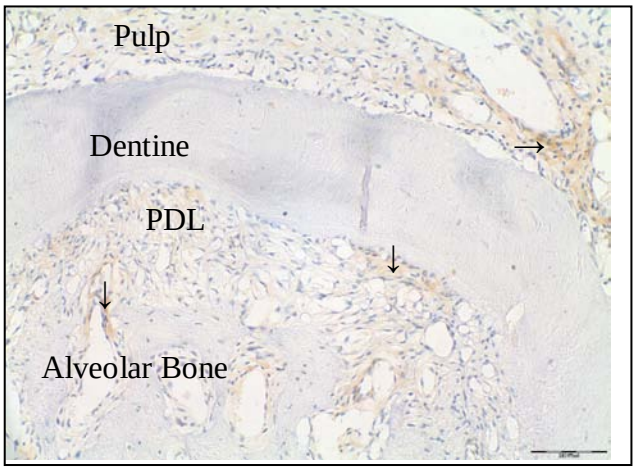

Figure 2
Arrows show background DAB staining for TNF-α in the periodontal ligament in an experimental tooth in a OPG administered rat molar before antigen retrieval (Scale bar equals 200µm).

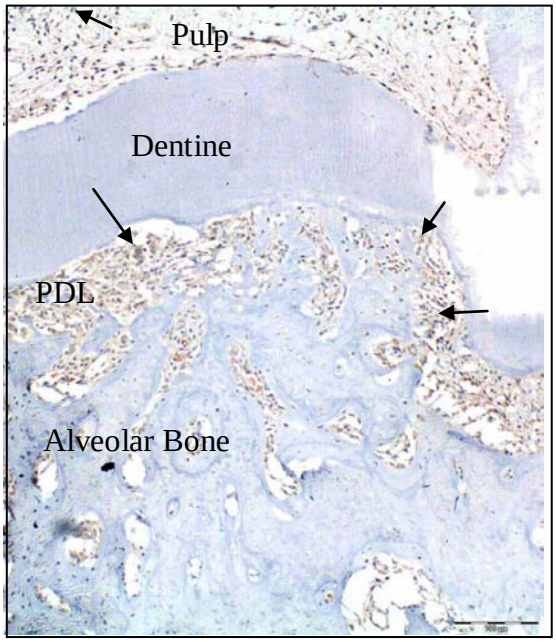

Figure 3
Arrows show positive DAB staining for TNF-α in the periodontal ligament in an experimental tooth in an OPG administered rat molar after antigen retrieval (Scale bar equals 100µm).

Before the use of antigen retrieval no positive staining was seen within the periodontal ligament in the experimental teeth of OPG administered rats (Fig 2), only background staining was noted. After the application of antigen retrieval positive DAB staining was seen in the frozen pulp tissue (Fig 3). Only six of the 18 specimens showed positive staining in the interradicular area, with the remaining 12 specimens not showing any positive staining for TNF-α.

The staining was seen surrounding the cells of the periodontal ligament, within the gingival tissues, in nerve tissues and marrow spaces of the alveolar bone. The staining seen was diffuse, pale and with some background staining present.

DISCUSSION AND CONCLUSIONS

The results showed a change had occurred between the material used in the initial study in 2006 and the subsequent study in 2009. As the tissue and antibody used were the same in both studies the results showed remarkable differences. The study in 2009 showed indifferent results with either no positive staining or less TNF-α staining and more background staining. The original protocol failed in the second study which required new antibody optimisation and use of antigen retrieval. Factors which may have influenced the results require consideration. The preservation of cells and tissues in a stable state is a fundamental requirement. It has been recognised that immunoreactive antigens are progressively lost during the fixation process as fixatives denature tissue proteins by coagulation, forming additive compounds or by a combination of both of these processes[10]. This causes conformational changes in the structure altering tertiary and quaternary organisation of proteins which may hamper the link to the antibody[7]. Paraformaldehyde produces fewer cross-links and is less likely to mask tissue antigens in comparison to other aldehydes. The use of formaldehyde-fixed paraffin-embedded tissue for immunohistochemical evaluation provides an alternative to fresh or frozen tissues and allows the material to be archived for retrospective or further studies. Formalin-fixation is a time-dependent process

in which an increase in time results in a continuation of aldehyde groups binding to proteins to a point of equilibrium[11]. Many studies have shown that prolonged fixation results in a decreased antigenicity[10, 12], and there is possibly an optimal timeframe for fixation to occur. If it takes approximately 25 hours for formalin to penetrate a $1cm^3$ sphere of tissue[13], it is possible that lack of adequate fixation occurred in the material used, although this was presumably addressed by the use of cardiac perfusion of paraformaldehyde during the sacrifice of the rats. A recent study by Webster et al.[13] showed that after inadequate fixation, tissue deep within the specimen degrades, thereby altering the protein structure with possible loss of the antigens being investigated. Webster et al.[13] found that with most antibodies, moderate variations in fixation times up to 7 weeks should not significantly affect the immunohistochemistry result. However, the effects of prolonged formalin-fixation are antibody-dependent. It could, therefore, be possible that the tissue being used in the 2009 study was inadequately fixed as it was deeper into the specimen. The unfixed tissue located deep in the specimen would ultimately be fixed with ethanol as a dehydrating agent during paraffin embedding. Ethanol is a coagulative fixative which removes water molecules and results in destabilising hydrophobic bonding of the proteins, altering their tertiary structure with the ultimate result of protein denaturation[7]. It is, therefore, possible the antigen retrievals employed may have been inappropriate and or the immunoreactivity decreased due to protein breakdown over the three year storage period.

The aged material used may have resulted in loss of the antigen. It has been shown that the age of sections is a crucial factor governing the success of

IHC staining for many different antibodies[14]. Bussolati et al.[7] suggests that antigen loss is a result of slow protraction of the cross-linking reactions of formaldehyde, with a progressive transformation of aminomethylol groups to a more stable methylene bridge. These authors further suggest this can be reversed with antigen retrieval procedures. Blind et al.[6] hypothesised that antigen loss can occur through chemical (pretreatment in H_2O_2 bath), thermal (storage under dry heat) and photo oxidation (exposure to ultraviolet irradiation). Jacobs et al.[15] reported inappropriate storage of tissue sections could lead to a false-negative result. Jacobs et al.[15] found that storing sections in a refrigerator at 4^0C and the use of paraffinisation was the most effective approach to preventing antigen degradation. Blind et al[6] concluded that prolonged storage of tissue sections could hamper IHC results. In general, it was found that loss of antigenicity could be caused by photo-oxidation induced by UVA irradiation and drying as a result of UVA light through glass windows and from electric light sources. Blind et al.[6] recommended appropriate storage of tissues should include protection against light and heat with the addition of possible cooling.

It is, therefore, likely that the storage of the tissues may have been inadequate with the resultant loss of antigens through photo-oxidation. This may have led to the false negative results as seen. In conclusion, it can be seen that for optimal results in IHC tissue preparation, proper fixation and storage is of vital importance.

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APPENDIX 1

Materials

Polyclonal antibody to mouse TNF-α

(HyCult biotechnology, Uden, The Netherlands)

Form: Liquid, 1ml (100 µg/ml) 0.2 µm filtered antibody solution in PBS, containing 0.02% sodium azide and 0.1% bovine serum.

Species: Rabbit IgG

Use: For immunohistology, flow cytometry and Western blotting dilutions to be used depending of detection system applied. It is recommended that users test the reagent and determine their own optimal dilutions. The typical starting working dilution is 1:10.

Application: The antibody can be used for inhibition of biological activity. Furthermore the antibody is useful for immunohistology on both frozen and paraffin sections, flow cytometry, immuno assay and Western blotting.

Storage: Product should be stored at 4^{o}C.

Polyclonal antibody to rabbit TNFR1

(abcam, Cambridge science park, Cambridge UK)

Form: Liquid, 1.00mg/ml. Borate buffered saline pH 8.2

Reactivity: Human, Mouse, Rat, Rabbit, Xenopus laevis, Cow, Dog, Drosophila melanogaster, Hamster, Monkey, Pig, and Sheep

Use: For immunohistology, flow cytometry and Western blotting dilutions to be used depending of detection system applied. The typical starting working dilution is 1:1000.

Cellular

Localization: Type 1 membrane protein and secreted

Storage: Product should be stored at 4^{o}C (1-2 weeks).

Datasheet for Osteoprotegerin FcOPG

Amgen, California, USA

Batch number: 35000F8

Concentration: 10 mg/ml

Presentation: 1 ml vials

Storage: -70°C

Administration: Subcutaneously

OPG presented in 1 ml vials at a concentration of 10 mg/ml. This was diluted to vials containing 1 mg/ml. Guidelines for OPG dosage is based on weight; therefore, each rat was weighed before OPG administration.

For OPG concentration = 2.5mg/kg

Also known as group 2.5/experimental

Rats weight = 250g = 0.25 kg

Amount of OPG required per rat

= concentration x weight

= 2.5 mg x .25 kg

= 0.625 mg

Therefore the amount of solution required

= prepared dilution x OPG required

= 1 mg/ml x 0.625 mg

= 0.625 ml of solution

Therefore, for a concentration of 2.5 mg/kg 0.625 ml of solution was administered subcutaneously.

Total amount of OPG = 0.625 x 9 = 5.625 ml

Note this does not include material lost during transfer (i.e. left in vials, needles etc), or possibility of losing animals that have been treated and expired before harvesting.

ANTICOAGULANT - HEPARIN

Heparin Injection B.P. (containing no antiseptic) was supplied in 1 ml plastic ampoules (David Bull Laboratories, Mulgrave, Australia).

Contained 1000 units (IU) per 1 ml.

Dosage: 0.02 ml of heparin sodium per 100 g of body weight

Route: Intravenous injection via femoral vein

Shelf life: Discard unused heparin after vial seal is broken

Storage: Below 25°C

Solutions

Phosphate buffered saline (PBS) pH 7.4

Preparation of 30x concentrate

1052g NaCl

165.6g $NaH_2PO_4.H_2O$

Dissolve $NaH_2PO_4.H_2O$ in 1000ml distilled water while heating and stirring

Add 2800ml distilled water and dissolve NaCl into solution

Cool to room temperature

Adjust pH of solution to 6.23-6.24 with 5N NaOH (requires approx 190ml)

(5N NaOH: 40g NaOH in 200ml distilled water)

Preparation of working solution

Dilute concentrate 1/30 with distilled water (e.g. 1666ml 30 x concentrate in 48334ml distilled water)

To mix the working solution, fill a 1 litre cylinder and then pour back into the drum. Do this twice…

Fixative

4% Paraformaldehyde/0.1 M phosphate buffer pH 7.4

Preparation

160g Paraformaldehyde

H_20 1600mls heated

NaOH dropwise to clarify

800mls 0.4M Sodium Phosphate buffer pH 7.4

Add distilled H_2O till 4000mls.

Decalcifying Agent

4% EDTA in phosphate buffer

Reagents:

Phosphate buffer	- Parts A and B
EDTA	- 80 gm

Procedure:
To 280 ml of part A and 720 ml of part B add 1 L of distilled water and EDTA to give a pH of 7

For **10% EDTA** in phosphate buffer, 200 g of EDTA was added to the 2 L of phosphate solution.

Avidin-Biotin Peroxidase Technique For Paraffin Sections

Methanol/Hydrogen Peroxide Blocking Solution

Hydrogen peroxide 100vol 30%w/v

8.3ml hydrogen peroxide in 500ml methanol

Citrate (C)

10mM sodium citrate pH 6

Distilled water……. 5000ml

5N NaOH……25ml

Citric acid……10.5g

Normal Horse Serum (NHS)

Add 1 ml aliquot of horse serum to 29ml PBS

Streptavidin Horseradish Peroxidase-Conjugated antibody (SPC)

Spreptavidin horseradish peroxidise-conjugate antibodies (SPC) are provided lyophilised

Concentrate is made by dissolving the lyophilised powder in distilled water: 1mg/0.5ml, i.e. 5mg vial: add 2.5ml distilled water

The concentrate is aliquotted in 0.5ml lots and stored in the freezer (<-10^{o}C)

Working solution: 1µl SPC concentrate/ml NHS

Peroxidase Substrate Solution (DAB)

Stock Solution

(3, 3'-diamino benzidine, Sigma Cat # D-5637)

Prepare stock solution of DAB 75mg/ml (i.e. 25g/333.3 ml distilled water)

Aliquot 300µm into microcentrifuge tubes and store at -20^{o}C

It is recommended that DAB be used in a fume hood or on a laminar flow bench.

Used DAB solution should be neutralised by adding equal quantities 0.2M potassium permanganate and 2.0M Sulphuric acid.

Allow mixture to stand overnight. It is now non-mutagenic. Following day decolourise the mixture with Ascorbic acid. Add powder until colour disappears. Discard solution down drain

Working Solution

Part A: 0.2N HCl

1.7ml conc HCl……8.5ml cmHCl

100ml distilled water……500ml distilled water

Part B: 0.2M Tris

6g Tris (Sigma Cat # T-1378)……30gm TRIS

250ml distilled water……1250ml distilled water

13ml A + 13ml B + 24ml distilled water

Adjust pH to 7-65-7.70

Add 50µl hydrogen peroxide and 1 aliquot DAB stock solution

Use immediately

APPENDIX 2

Tissue dehydration and paraffin embedding

Shandon Citadel 2000 automatic processor (Shandon Industries, Pittsburgh, Pennsylvania)

The following automatic procedure was used for the impregnation of tissues with paraffin wax prior to embedding:

1.	70% alcohol	1 h
2.	70% alcohol	1 h
3.	80% alcohol	3 h
4.	90% alcohol	3 h
5.	100% alcohol	4 h
6.	100% alcohol	4 h
7.	100% alcohol	4 h
8.	70% histoclear/alcohol	4 h
9.	100% histoclear	5 h
10.	100% histoclear	5 h
11.	Hot vacuum	1 h

Tissues were then embedded in paraffin using a Reichert Jung Histostat.

Slide coating procedures for Immunohistochemistry

Method

1. Load slides into racks and wash with detergent and rinse thoroughly in tap water. Alternatively load racks using cotton gloves and proceed to step 3
2. Dry slides thoroughly
3. Rinse in absolute ethanol
4. Dip in silane solution for a few seconds
5. Rinse in absolute ethanol
6. Rinse in deionised water
7. Dry thoroughly and store in boxes

Silane (Working) Solution:

10ml 3-aminopropyl-triethoxy-silane (Sigma Cat # A-3648) in 500ml absolute ethanol

This solution should be used for about 300 slides (10 racks) and then discarded

Mayer Lillie Haematoxylin and Eosin Staining Method

1.	Remove wax	
	Xylol	2 x 2 min
	Absolute alcohol	2 x 2 min
2.	Dip in water to check for complete wax removal	
3.	Stain in haematoxylin	5 min
4.	Wash in running water	1 min
5.	Differentiate in 0.5% HCl	one dip
6.	Wash in running water	10 min
7.	Blue in dilute alkali (NH_4OH in alcohol)	2 dips
8.	Counterstain in eosin	30 s
9.	Differentiate in 70% alcohol	3 dips
10.	Dehydrate and clear	
	Absolute alcohol	2 x 2 min
	Xylol	2 x 2 min
11.	Mount and coverslip using DePex	

Haematoxylin

Formula:

Haematoxylin	5 g
Ammonium alum	50 g
Glycerol	300 ml
Distilled water	700 ml
Sodium iodate	1 g
Glacial acetic acid	20 ml

Eosin

Formula:

Eosin	10 g
Potassium dichromate	5 g
Saturated picric acid	100 ml
Absolute alcohol	100 ml
Distilled water	800 ml

Immunohistochemical Staining Method

2006 Study - TNF-α Staining

- Deparaffinization and rehydration
    - Xylene – 2min
    - Xylene – 2min
    - Alcohol (100%) – 2min
    - Alcohol (100%) – 2min
    - Distilled water –check deparaffinization
- Dry around specimen with Tissue and mark around specimen with PAP pen
- Rinsing in Phosphate Buffered Saline (PBS) pH 7.4 – 10min
- Blocking of endogenous peroxidase in 140ml methanol + 60ml H_2O_2 (30% H_2O_2) – 10 min
- Rinsing in PBS – 5min
- Change PBS solution and rinse again – 5min
- Preincubation with BSA (5% Normal Horse Serum) – 5min
- Tap off excess serum (do not rinse)
- Incubation with TNF-alpha antibody diluted 1:20 in humid chamber overnight at room temperature
- Tap off excess serum
- Incubation with secondary antibody (from the kit) humid chamber at room temperature – 45min
- Tap off excess serum

- Incubation with streptavidin-biotin complex, humid chamber at room temperature – 20min
- Tap off excess serum
- DAB chromagen – 5min
- Rinse in distilled water
- Counterstain with Mayer's haematoxylin 5 sec (filter haematoxylin solution twice before use)
- Rinse in tap water
- Rinse in distilled water
- Pat dry with filter paper
- Mount with Aquatex medium (water soluble)

2009 Study – TNF-α and TNFR1 Staining

1. Mount sections on silane-coated slides and dry over air heater until wax has melted.
2. Dewax and take sections to absolute alcohol
    a. Xylene……..2min
    b. Xylene…….2min
    c. Absolute alcohol……2min
    d. Absolute alcohol……2min
    e. Rinse in PBS
3. Block enogenous peroxidase with 0.5% H_2O_2 in methanol at room temperature……30min
4. Rinse in PBS……..2 x 3min
5. Antigen retrieval
    a. Place 250ml of citrate into a plastic pot and place into the water bath, heat to 80^0C
    b. Place slides into plastic racks and place into the pot and keep in water bath……120min
    c. Remove pots and allow cooling below 50^0C. This takes approximately 30min
    d. Discard citrate solution
6. Rinse in PBS buffer……2 x 3min
7. Circle section with PAP pen and incubate in 3% NHS…….30min

8. Drain NHS and incubate with primary antibody at room temperature overnight (concentration TNF-α 1:50), (concentraction TNFR1 1: 2000)
9. Rinse in PBS buffer……..2 x 3min
10. Incubate with secondary antibody (conentraction1 in 250)……30min
11. Rinse in PBS buffer……2 x 3min
12. Incubate with streptavidin peroxidase (concentration 1 in 500)…..15min
13. Rinse in PBS buffer……..2 x 3min
14. Apply peroxidase substrate solution (DAB)……..7min
15. Rinse in PBS buffer……2 x 3 min
16. Lightly counterstain with Mayer's haematoxylin
    a. Haematoxylin……..1min
    b. Wash in running water……..5min
    c. Differentiate in 0.5% HCl……..1 dip
    d. Wash in running water……10min
17. Dehydrate
    a. Absolute alcohol……..2min
    b. Absolute alcohol……2min
    c. Xylene……2min
    d. Xylene……2min
18. Wipe around section to dry
19. Mount in DePex solution