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DOI: 10.2478/aoj-2000-0009

Original Article

The immunohistochemical response of the rat periodontal ligament endothelium to an inflammatory stimulus

Andrew Toms
MSc, BDS, FDSRCS, LDSRCS, MOrthRCS, DOrthRCS
Postgraduate research student

Bren Gannon
BSc, PhD
Associate Professor

Colin Carati
BSc(Hons), PhD
Senior Lecturer
Microcirculation Laboratory

School of Medicine
Flinders University of South Australia
South Australia
Australia

Recently, inflammation has been recognised as an important co-requisite to orthodontic tooth movement. When such a reaction is initiated, the process of up-regulation of certain adhesion molecules may occur, resulting in the extravasation of leukocytes. This may stimulate progenitor/precursor pathways and signals that regulate the biological responses resulting in tooth movement. We propose that up-regulation of leukocyte adhesion molecules occurs in response to orthodontic forces, resulting in circulating monocyte attraction, extravasation and differentiation into osteoclasts, which are responsible for bone resorption that results in orthodontic tooth movement. To investigate this hypothesis, it is necessary to determine whether periodontal ligament (PDL) endothelium responds to inflammatory stimuli as other organs do. We studied the normal distribution of endothelial adhesion molecule ICAM-1 within PDL vessels, and then the following exposure to an inflammatory endotoxin. The rat PDL blood vessels expressed ICAM-1 in response to the inflammatory stimulus, similar to other organs, suggesting that the inflammatory responses are similar. Whether and where in the PDL microvascular bed orthodontic forces cause up-regulation of ICAM-1 needs to be established.

Aust Orthod J 2000; 16: 61–68

Key Words: adhesion molecules, inflammation, periodontal ligament, tooth movement

Received for publication: September 1999
Accepted: April 2000

Introduction

The periodontal ligament (PDL) is a thin, dense connective tissue that occupies the periodontal space between the root of the tooth and the alveolus. Blood vessels take up 11 per cent of the volume of the ligament,[1] and this hydrostatic cushion, together with the periodontal fibres, helps to resist displacing forces. The PDL is also involved in the mechanisms whereby a tooth reaches and maintains its functional position, which include tooth eruption, tooth support and drift.

Orthodontic tooth movement is a complex process involving the application of a mechanical force that causes a remodelling of the PDL and dentoalveolar tissues.[2–5] This process, although well studied, is still not completely understood. Recently, it has been recognised that inflammation is an important co-requisite to orthodontic tooth movement.[6] Cellular and vascular changes, as well as inflammatory mediators, growth factors and neuropeptides, have been identified in the supporting dentoalveolar tissues around teeth subjected to orthodontic forces.[7–10] This has led to the assumption that interactions between cells producing these substances, such as leukocytes, nerves, immune and paracrine cells, may orchestrate the tooth movement process once an orthodontic force has been applied.

Inflammation is a response of vascular tissue, blood leukocytes and extracellular tissues to an injury of a biological, physical or chemical nature. There is an initial vasoconstriction, which is followed by vasodilatation, an increase in vascular permeability, vascular stasis, oedema, leukocyte attraction and their subsequent vascular margination, adherence to endothelial cells and diapedesis.[11]

The importance of inflammation in the biology of tooth movement is that it would appear to be aseptic in nature. Intravital microscopy has shown that continuous compression, imitating initial orthodontic forces, causes increased vascular permeability[12,13] and leukocyte migration from the microvasculature into the extracellular spaces of the PDL.[14] The electron microscope has shown these cells to be monocytes and polymorphs, which suggests that cells from the monocyte line differentiate into macrophages and osteoclast precursor cells. There is also evidence to support the concept that osteoclasts arise by fusion of mononuclear phagocytes derived from blood monocytes.[15–17] Whilst the presence of mononuclear cell lines has been observed in the PDL and dentoalveolar tissues undergoing orthodontic tooth movement, change in the number and distribution of lymphocytes and granulocytes has not been reported.[18,19] Absence of such cells lends weight to the hypothesis that experimental orthodontic tooth movement involves an aseptic inflammatory response.

Further evidence of the role of inflammation in tooth movement has been shown in experiments where orthodontic forces induced a neurogenic component of inflammation[6] through calcitonin gene-related peptide (CGRP), causing vaso-dilatory effects,[20] proliferation of endothelial cells[21] and up-regulation of endothelial adhesion molecules.[22]

The mechanism of leukocyte emigration from the microvasculature into the tissues involves a variety of molecules expressed on both the circulating blood leukocyte and endothelial cell surface. Increasing evidence suggests that leukocyte interactions with the vascular endothelium play a crucial role in the process of inflammation.[23–25] Initially, leukocytes are thought to roll along the endothelial cell via interactions between selectin molecules and carbohydrate ligands. This is followed by firm adhesion via the leukocyte adhesion molecule, LFA-1 (also named CD11a/CD18) and its ligand, intercellular adhesion molecule type 1, ICAM-1 (also named CD54) on endothelial cells.[11] It is the up-regulation of these adhesion molecules that is responsible for the endothelial response of inflammation. The leukocytes are attracted to the endothelium as a result of pro-inflammatory chemo-attractants (which include cytokines) being released at sites of inflammation that subsequently cause up-regulation of the adhesion molecules. Once attached to the endothelium, adhesion and aggregation of leukocytes occur prior to their transendothelial migration or diapedesis into the extracellular matrix.[23]

Leukocytes circulate within blood vessels and do not interact with the endothelial surface of the vasculature under normal physiological conditions, although it has been shown that a small number of ICAM-1-positive endothelial cells do occur in normal rat gastric mucosa.[25]

Due to the inherent physiological movement that takes place continuously between the tooth and its bony socket wall, the endothelial cells of the PDL microvasculature may well have a sub-inflammatory level of up-regulation of ICAM-1, similar to that seen in the gastric mucosa of the rat.[25] However, when an orthodontic force is applied to a tooth, up-regulation of leukocyte adhesion molecules may follow, which results in the extravasation of leukocytes. This may stimulate progenitor/precursor pathways and signals that lead to osteoclastic bone resorption and orthodontic tooth movement. The different homing and re-circulation behaviours of leukocytes depend upon the expression of specific adhesion receptors by leukocytes and endothelial cells. The expression of these receptors is finely regulated according to cell type, functional state and anatomical location, building up a complex network of interactions that simultaneously involve several of these receptors working as signallers or controllers for leukocyte homing and migration.

The first aim of this study was to develop an immunohistochemical staining technique for the endothelial adhesion molecule ICAM-1 within the vessels of the PDL of rat molars, and to identify the normal distribution of the adhesion cell molecule. The second aim was to investigate whether an inflammatory stimulus, induced by an inflammatory endotoxin, would alter the expression of this molecule in the microvasculature of the PDL. Panes *et al.*[26] compared the up-regulation of ICAM-1 on endotoxin-challenged endothelial cells in different tissues of the rat. Their positive findings for up-regulation of ICAM-1 in response to this endotoxin, formed the basis for this investigation. The up-regulation of ICAM-1 on the endothelial cells of PDL vessels in response to an inflammatory endotoxin would demonstrate that their response is similar to that demonstrated by other tissues in response to inflammatory stimuli.

Materials and method

Sprague-Dawley male rats, eight weeks old, were used in this study. All animals were housed in polycarbonate cages; a standard pellet diet and tap water were given *ad libitum*. Anaesthesia was obtained by intraperitoneal injection of pheno-

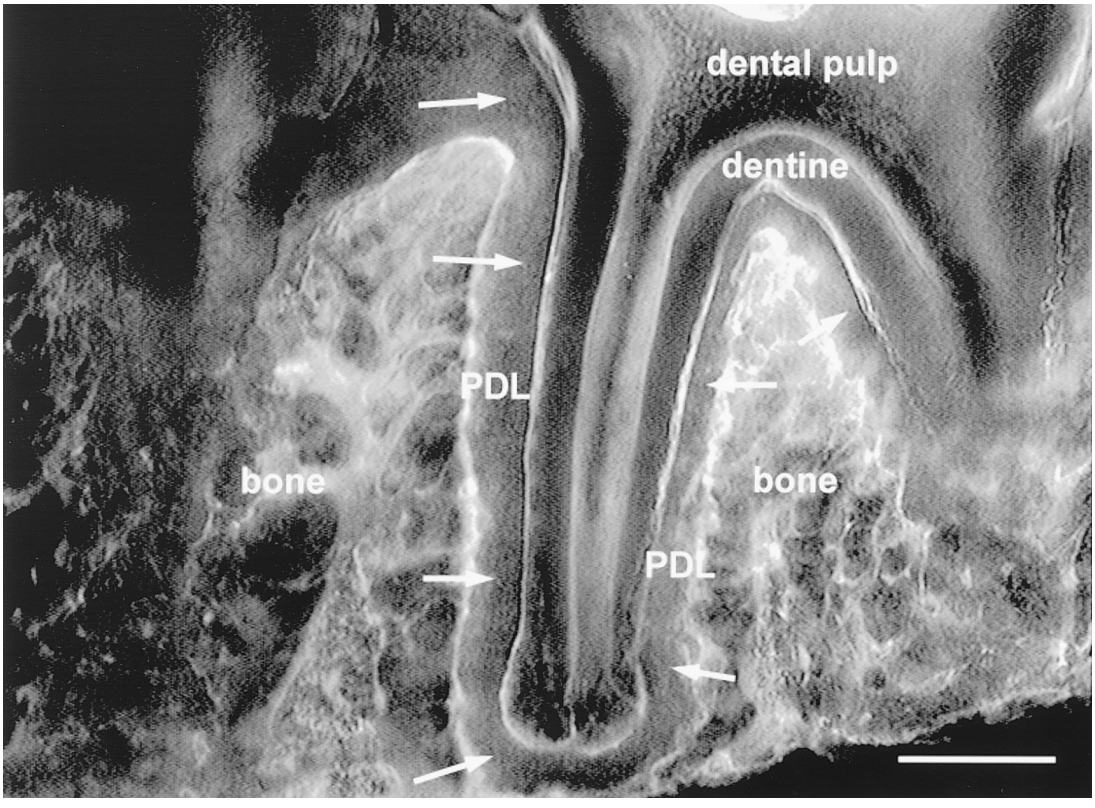

Figure 1. Mandibular section in a control animal showing minimal labelling for ICAM-1 in the PDL (arrowed), suggesting a low immunoreactivity under normal conditions. Bar = 200µm.

barbitone sodium 60 mg/kg* at a dose of 1mL/kg body weight. The animals were anaesthetised and placed on a heating pad throughout the experiment.

The endotoxin lipopolysaccharide from *Salmonella abortus aqui*† was injected at a dose of 3 mg/kg.[26] The animals were monitored and sacrificed by an overdose of anaesthetic after eight hours.

The mandibles were dissected out of the experimental (*n*=6) and control (*n*=6) animals and placed in Zamboni's fixative at 4°C. The mandibles were sectioned with a phosphate buffered saline (PBS) lubricated diamond wheel giving approximately 180–240µm thick sections, showing the molar roots, the PDL, dental pulp, gingival tissue and bone.

The mandible sections were blocked with 10 per cent normal donkey serum for two hours. This pre-incubation step helps to prevent or lessen any non-specific binding and background fluorescence. Once the donkey serum had been applied, the slides were placed in a humid chamber to prevent drying out of the sections. After this pre-incubation, the 1° antibody, mouse/anti-rat ICAM-1 was applied and left on the tissue sections in the humid chamber for four days. The primary antibody is made up to the required dilution with 10 per cent normal donkey serum in PBS with 0.01 per cent sodium azide, to reduce non-specific binding of antibodies to the tissue. Once the four days had elapsed, the sections were washed in antibody diluent in 5 per cent normal donkey serum (4 x 30 min), and turned every 15 minutes, before applying the 2° antibody and incubating in the humid box for three days. After a final wash in PBS, the sections were mounted under sealed cover-slips in 20 per cent buffered glycerol (pH 8.6).

The primary antibody (ICAM-1) dilution used for the mandibular sections was 1:800. The secondary antibody dilution for CY3 was 1:100.

The following antisera were used in this study: mouse monoclonal antibodies against rat CD54;‡ donkey anti-mouse secondary antibodies labelled with the immunofluorescent marker CY3 (yellow excitation, 488 nm); and normal donkey serum§. All chemical solutions were made from research grade reagents from BDH,|| unless otherwise stated.

The slides were viewed with an Olympus BX50 fluorescence microscope, and the images captured using the National Institute for Health (USA) Image Capture System V1.6, running on a Macintosh 7600 computer and manipulated in Adobe Photoshop V5.0.

* Boehringer Ingelheim, Artarmon, NSW Australia.

† Sigma, St Louis, Mo. USA.

‡ ICAM-1; Pharmingen, San Diego, Ca. USA.

§ Jackson Immuno Research, Westgrove, Pa. USA.

|| Poole, Dorset UK.

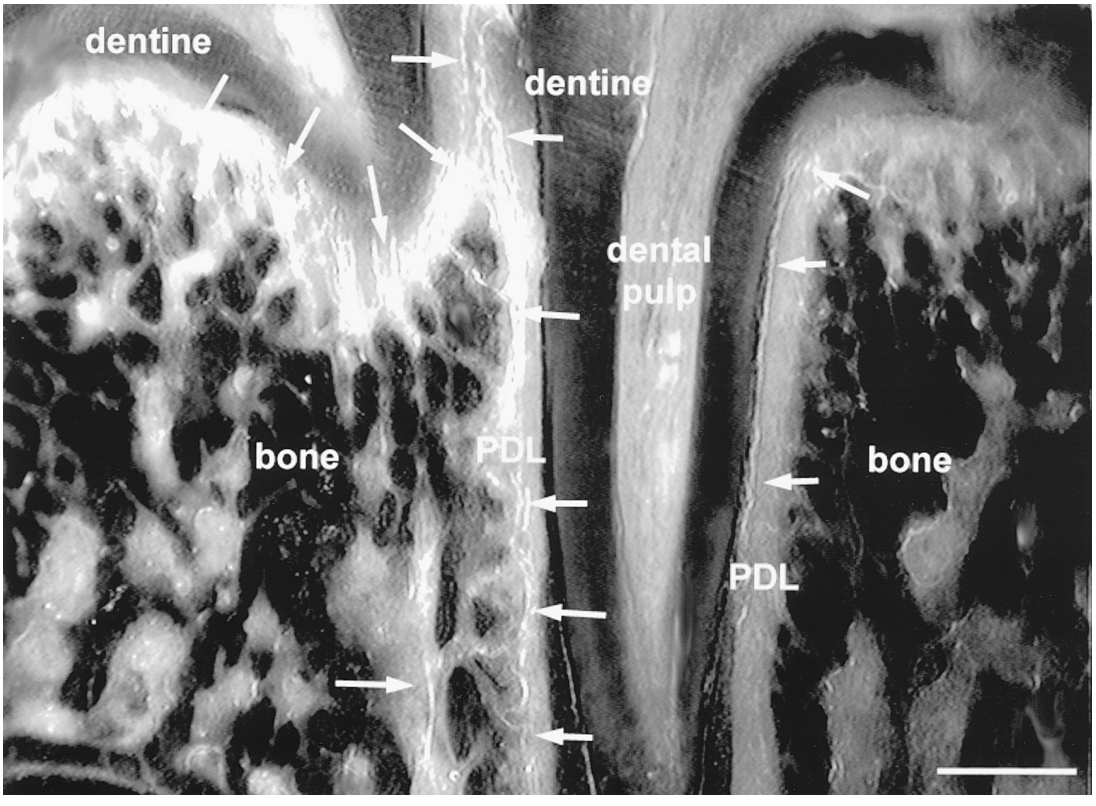

Figure 2. Mandibular section showing ICAM-1 labelled vessels (arrowed) in response to the inflammatory stimulus. The labelled vessels, both arteriolar and venular, can be seen in the PDL, gingival crest, pulp tissue and alveolar bone. Bar = 200μm.

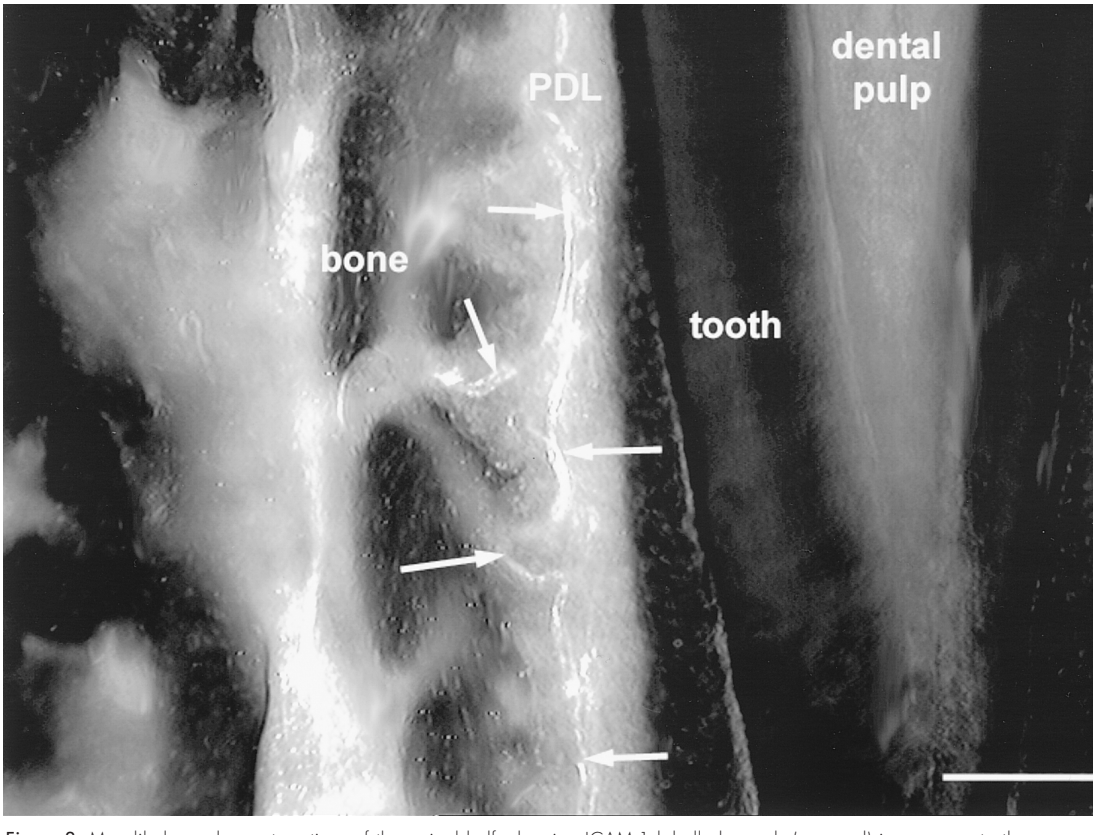

Figure 3. Mandibular molar root section, of the apical half, showing ICAM-1 labelled vessels (arrowed) in response to the inflammatory stimulus in both the PDL and alveolar bone. Bar = 100μm.

Results

When compared with the controls, the animals that received the inflammatory endotoxin showed a significant increase in the number of ICAM-1 labelled blood vessels, both arteriolar and venular, within the dento-alveolar structures (Figures 1 and 2). These changes were consistent across all the experimental mandibular molar sections. There appeared to be no evidence of ICAM-1 antibody cross-reactivity with other cell types in the tissue sections.

Due to the thickness of the sections (180–240 µm), it was not always possible to see continuous blood vessels in one focal plane, but by changing the focal planes, vessels could be followed through the tissue sections.

There appeared to be minimal immunoreactivity of the dento-alveolar microvasculature under basal conditions, although a background immunofluorescence of red blood cells was seen in some control sections within blood vessels in the PDL, dental pulp and alveolar bone.

The increased microvascular ICAM-1 labelling seen in the sections of the endotoxin-treated animals (Figures 2, 3, 4 and 5) was evenly distributed in the PDL, gingival tissue and bone. The vessels in the dental pulp were not as consistently labelled as the other tissues, which may be a result of their more friable structure.

It has been shown for the first time that the blood vessels in the rat PDL stain for ICAM-1 in response to inflammatory stimuli in a similar manner to other organs, such as the gut.[25] The images confirm the previously reported distribution of PDL blood vessels, which branch and anastomose forming vascular complexes around molar roots, and being denser towards the apices where plexuses are often seen· (Figure 5).

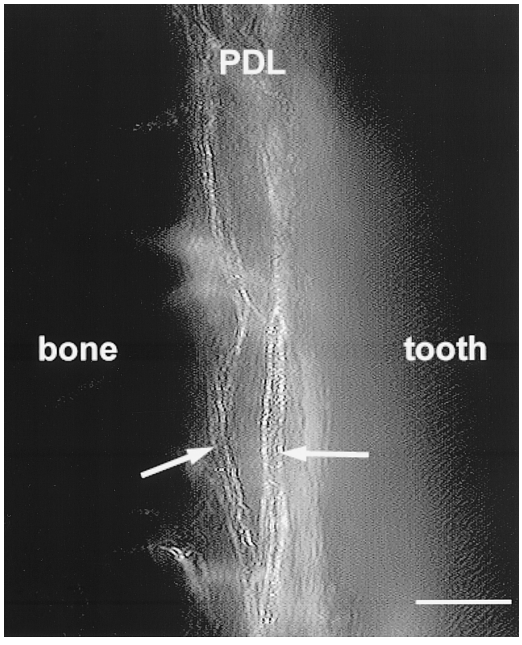

Figure 4. Mesial aspect, at the mid third level, of a mandibular molar. The ICAM-1 labelling of the endothelium of the PDL vessels is clearly seen in this endotoxin treated sample. Bar = 50µm.

Discussion

The cells of the PDL are considered to play an important role in maintaining homeostasis of the periodontium during tooth movement.[27] The body's responses to orthodontic forces are regulated by the nervous, endocrine and immune systems, the PDL returning to a stable state after a period of transition. The aim of this study was to identify the normal distribution of the adhesion cell molecule ICAM-1 in control animals, and to show for the first time, up-regulation of PDL microvascular endothelial cells for ICAM-1 in an inflammatory situation induced by treatment with an endotoxin.

Orthodontic tooth movement is accomplished by applying mechanical forces to teeth, producing physical and biochemical reactions in the PDL that result in the remodelling of alveolar bone.[28] Resorption of bone occurs in areas of pressure whilst bone formation occurs in areas of tension.[4] The effect that orthodontic tooth movement has on the PDL has been examined at the microscopic[29–32] and ultrastructural level.[1,33–38] Recently, the emphasis has focused on the understanding of both the physiology of bone and the microvasculature, and their respective and combined roles in orthodontic tooth movement.[2,6,7] It is now recognised that the biological process of tooth movement involves a series of responses that are inflammatory in nature,[6–17] and that the vascular endothelial cells and leukocytes play a pivotal role. ICAM-1 was shown to be present in the PDL blood vessels in this study, which is consistent with reports of "biological stress" causing general inflammation and leukocyte extravasation.[26,39] Activation of the tooth moving process begins when the external mechanical signal is transferred into physical movement of tissue fluids inside and out of the PDL, in association with adhesion molecule up-regulation and distortion of cells, extracellular matrix, alveolar bone and nerve endings. This leads

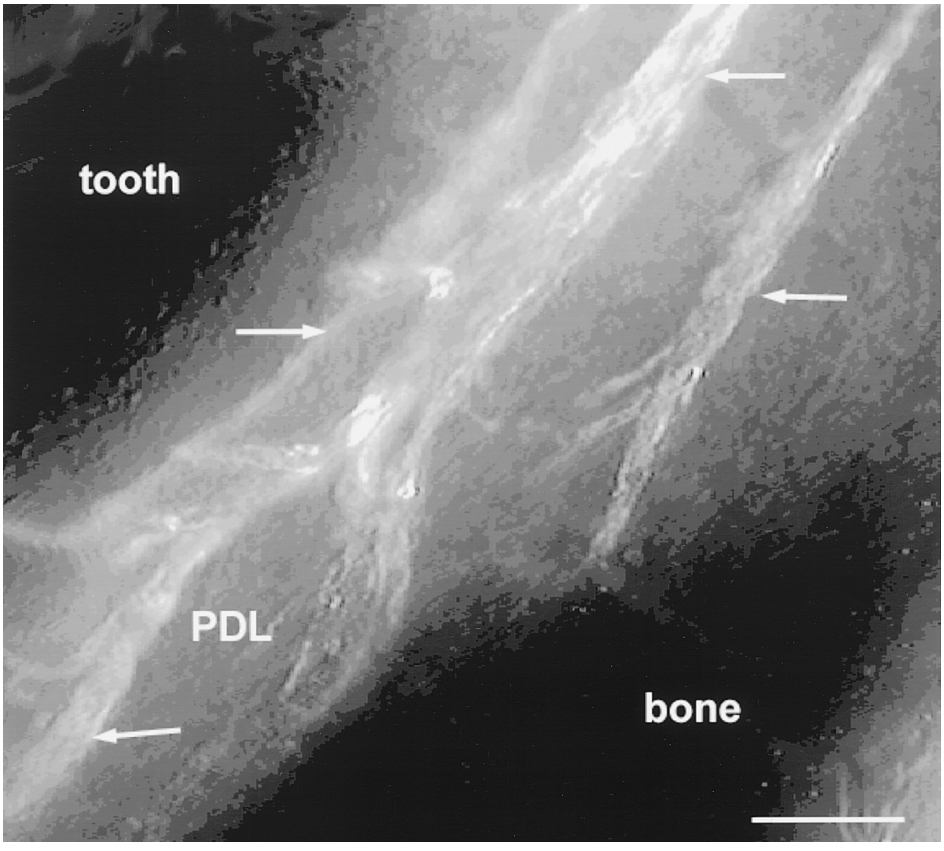

Figure 5. Apical region of a mandibular molar showing an increased density of ICAM-1 labelled PDL vessels. The increased number of blood vessels in this region suggest the existence of an apical vascular plexus. Bar = 30μm.

to an alteration of cellular shape and ion channel permeability, neuropeptide release from nerve endings, capillary vasodilation and migration of leukocytes into the extravascular areas, together with generation of streaming potentials and piezoelectric effects.[40] Cell activation leads to local production of cytokines or growth factors thought to mediate local regulatory mechanisms of bone remodelling. Story[41] reported an inflammatory process in the stressed PDL, even when light forces were applied. This inflammatory process was confirmed in an ultrastructural investigation using extrusive orthodontic forces, where an early sign of inflammation, in the form of diapedesis, was found at the endothelial junctions of postcapillary sized venules in the rat PDL.[42] It has been shown that the endothelial cells of blood vessels subjected to mechanical strain show an up-regulation of ICAM-1, and an increased adhesion of leukocytes to the strained cells.[43] The interaction of leukocytes with endothelium has been shown to produce chemokines, which may serve in cell activation, as well as extravasation and recruitment of additional leukocytes during the inflammatory response.[44]

The different homing and re-circulation behaviours of leukocytes, such as neutrophils, monocytes and lymphocytes, depend upon the expression of specific adhesion receptors by leukocytes and endothelial cells. The expression of these receptors is finely regulated according to cell type, functional state and anatomical location, building up a complex network of interactions that simultaneously involve several of these receptors working as signallers or controllers for leukocyte homing and migration. Endothelial adhesion molecules allow leukocytes, which are normally free in the blood stream, to attach to vessel walls prior to migrating through them, and are considered to play crucial roles in many aspects of inflammation and tumour metastasis. Many receptors are involved and the differential control of their expression on particular cell sub-populations makes

the process both versatile and complex. Butcher[45] suggested that the various combinations of these adhesion molecules give rise to the selectivity seen in the leukocyte/endothelial cell interactions.

Clearly, inflammation is an important co-requisite in the process of orthodontic tooth movement. The role of the inflammatory adhesion molecule ICAM-1 in the recruitment of leukocytes in the PDL and their subsequent role in the process of alveolar bone resorption and deposition is intriguing. It could be that the up-regulation of adhesion molecules on both blood leukocytes and endothelial cells is the first step in the process of tooth movement.

It is of interest that all vessels within the PDL seemed to be up-regulated by this level and duration of endotoxin treatment. It now remains to be seen whether orthodontic forces cause up-regulation of ICAM-1, and where in the PDL microvascular bed this occurs. The use of confocal microscopy in future experiments should allow microvessels to be viewed as 3D reconstructions, which will overcome the difficulties of viewing microvessels in relatively thick tissue sections using a standard fluorescent microscope.

Acknowledgments

The authors would like to thank the Australian Society of Orthodontists' Foundation for Research and Education for its financial support of this PhD research project; and Kate Davidson and the Lung Physiology Laboratory at the School of Medicine, Flinders University of South Australia.

Address for correspondence and reprints:

Dr A. P. Toms
Department of Anatomy
School of Medicine
Flinders University of South Australia
PO Box 2100
South Australia 5001
Australia
Email: andrew.toms@flinders.edu.au

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