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Blood vessel response to pan-endothelium (RECA-1) antibody in normal and tooth loaded rat periodontal ligament

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SUMMARY Immunolabelling of the normal rat molar periodontal ligament (PDL) with RECA-1 antibody, an endothelial cell surface marker, demonstrated the endothelium in the different categories of blood vessels. The intensity of immunolabelling was similar for venous capillaries (VC), post-capillary-sized venules (PCV), and collecting venules (CV). Arterial capillaries (AC) and terminal arterioles (TA) showed a different response, both having a high intensity of endothelium and smooth muscle cell labelling, whether they were located in the PDL or alveolar bone. An experimental, continuous loading of ≈100 g was applied unilaterally to the mandibular molars for 10 minutes. In the PDL apical compression zone this load resulted in a loss of RECA-1 immunolabelling of the VC, PCV, and CV. Adjacent to the alveolar crest, where shear and tension loads were judged to have occurred, there was enhanced immunoreactivity of VC, PCV, and CV. In the loaded PDL, the AC and TA, irrespective of their location in the ligament or bone, showed strong immunofluorescence of their endothelium and the enveloping smooth muscle layer. Vessel and PDL immunofluorescence were analysed with standardized grey scale densitometry, and the data subjected to ANOVA. Comparison between individual vessel means showed significant differences ($P < 0.05$). Control teeth showed no immunostaining difference between the coronal and apical region vessels, whereas in the loaded teeth the overall cervical vessel endothelium had a significantly higher value than the apical vessel endothelium ($P < 0.001$). These findings demonstrate that the endothelium of this microvascular bed can undergo significant immunoreactivity changes when exposed to short-term, continuous, tooth loading.

Introduction

Endothelial cells constitute the lining of the blood vessel system in the PDL. In other microvascular beds, these cells promote physiological homeostasis by providing an active interface between the circulating blood and the extravascular tissues. This interface controls blood vessel function through the generation of vasoactive mediators, which determine dilation or constriction (Haynes, 1995). The endothelial cells are physiologically very active and release a cascade of compounds that regulate vascular tone (Suzuki and Kashiwagi, 1991; Campbell *et al*., 1996) and respond to shear stress exerted by the blood flow (Ayajiki *et al*., 1996). However, the function of the PDL blood vessel endothelium when subjected to mechanical stress has received only limited histological assessment (Reitan, 1951; Rygh *et al*., 1986) and electron microscopic investigation (Lew *et al*., 1989; Clark *et al*., 1991; Tang *et al*., 1993).

Orthodontic tooth movement results in an inflammatory response with vascular dilatation and increased permeability (Rygh *et al*., 1986; Davidovitch *et al*., 1992; Rygh and Brudvik, 1995). In this context, some consideration has been given to the effect of mechanical stress on PDL microvascular permeability using light

microscopy (Iida *et al*., 1992) and electron microscopy approaches (Cooper *et al*., 1990; Tang and Sims, 1992). Substantial attention has also been directed to the immunohistochemistry of inflammatory mediators released by the cell population of the PDL when affected by orthodontic tooth movement (Davidovitch *et al.,* 1992; Davidovitch 1995; Vandevska-Radunovic *et al.,* 1997). Nevertheless, the immunohistochemistry of the blood vessel wall in the unstressed and stressed PDL has remained virtually unexplored.

Current concepts of the biomechanics of tooth movement tend to emphasize the application of physical moment principles (Shroff *et al*., 1997) and PDL response to varying test loads relative to tooth size (Melsen, 1986; Rygh *et al*., 1986; Brudvik and Rygh, 1994). In reality, the definition of optimal loads required for the mechanical signal to initiate biomolecular orthodontic tooth movement should be related to a knowledge of physiological indicators; for example, functional gradients in vessels where endothelium-dependent shear stress varies from 10 to 100 dynes/cm^2 in the microcirculation. It is this response gradient, and a variety of other response gradients in cell and vessel function, that preserve the cell–cell and cell–matrix interactions (Skalak and Price, 1996).

In the endothelial cell there is a vast array of regulatory molecules (Hwa *et al.,* 1996; Campbell *et al*., 1996; Webb, 1997) including vasoconstrictor agents, such as endothelin, angiotensin II, and endothelium derived relaxing factors, in particular nitric oxide (Dinerman *et al.,* 1993). However, the signal transduction pathways of receptors mediating the release of these endothelial agents in the PDL have not been fully determined.

Pan-endothelium (RECA-1) is a monoclonal antibody developed as a cell-specific probe to rat endothelial cell antigens for studies of the role of endothelial cells in various physiological and pathological processes (Duijvestijn *et al.*, 1992). Those investigators noted that polyclonal antibodies commonly used for identifying endothelial cells in human and murine species were not specific, and did not identify all rat vascular endothelium. Particular reference was made to the von Willebrand factor that failed to reveal all rat vascular endothelium. Duijvestijn *et al.* (1992) tested the reactivity of two RECA IgG1 isotypes against four other monoclonal antibodies previously described as reactive with rat endothelium. Comparisons of endothelium immunoreactivity across a variety of rat tissues showed that all monoclonal antibodies had different fine specificities and only RECA-1 showed reactivity with all endothelium in the tested tissues. The authors emphasized that, although heterogeneity exists among vascular endothelial cells, RECA-1 expression demonstrated that all vascular endothelial cells shared unique cell-specific molecules.

The present study was conducted to test the hypotheses that RECA-1 antibody would identify the endothelium of blood vessels in normal rat molar PDL and alveolar bone, and that RECA-1 immunoreactivity may be modulated when the PDL was subjected to stress.

Materials and methods

The protocol for this investigation was approved by the Animal Ethics Committee of the University of Adelaide. Eight male Sprague–Dawley rats, aged 90 days, with a mean weight of 350 ± 50 g were used for the study. The rats were anaesthetized with a mixture of veterinary Hypnorm (Fentanyl citrate 0.315 mg/ml and fluanisone 10 mg/ml—Batch No. 95A30(1)429; Jansson-Cilag Ltd, Sanderton, High Wycombe, UK), Hypnovel (Midazolam 5 mg/ml—Batch No. B248 MFDO6 1996; Roche, Basel, Switzerland), and distilled water in a volume ratio of 1:1:1. A dose of 0.9 ml/300 g of body weight was administered by intra-peritoneal injection.

Following the onset of anaesthesia, a small rubber pad, 8 × 3 × 1.5 mm glued to a plastic cheek retractor, was placed between the molar teeth on one side. A two-piece head frame (Figure 1) was constructed from 1.9-mm diameter stainless steel wire sliding into stainless steel tubing with an internal diameter of 2.0 mm. Orthodontic latex elastics 5/16 inch × 2 ounces (TP Orthodontics, La Porte, IN, USA), held by soldered spurs, activated the frame and applied a load against the mandibular border for 10 minutes to exert an intrusive molar action via

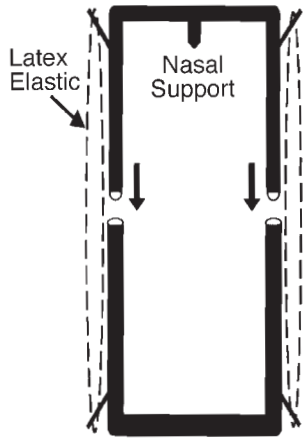

Figure 1 Telescoping head frame constructed from stainless steel wire and 2.0-mm ID tubing. The frame, activated with latex orthodontic elastics, exerted a closing pressure against the lower border of the mandible, and to the rubber pad between the maxillary and mandibular molars.

the intermolar pad. Elastic loading, was subsequently measured with a Correx tension gauge (Haag-Streit AG, Lieberfeld, Bern, Switzerland) at the frame intrusion position. Elastic action per side ranged from 80 to 120 g with a mean value of 100 g. The rubber pads were randomly allocated between left and right molar sides with the contralateral unloaded molars providing the controls. The rats were guillotined and the heads placed in Zamboni's fixative at 4°C for 24 hours with the loaded frame *in situ*. The mandibles were surgically removed, hemi-sectioned and sliced sagittally through the long axis of the first molars with a 0.03-mm diamond disc (van Moppes, Amsterdam, The Netherlands) attached to an Isomet low speed saw (Buehler, Lake Bluff, IL, USA) rotating at 500 rpm and continuously flooded with phosphate buffered saline (PBS) at 4°C. Whole tissue sections were ≈150 μm thick and provided blocks of molar teeth with intact PDL for direct immunohistochemical staining. At least two sections were obtained from each side of the jaws, thus providing 16 matched experimental and control sections for evaluation.

Working dilutions for antibody applications were predetermined by cross-check tests. The primary antibody (mouse × rat RECA-1 pan-endothelium MCA970, 20–50 μg/ml; Serotec Ltd, Oxford, UK) required a working dilution of 1:8. Comparable tests for the secondary CY3 conjugated antibody (donkey × mouse 715-165-151, 1.4 mg/ml; Jackson Laboratories, West Grove, PA, USA) determined a dilution of 1:100.

Molar sections were processed for immunostaining in sets by dehydration through an ethanol series to 100 per cent and permeablized in 4 per cent DMSO (dimethylsulphoxide) in PBS. After being washed with PBS at pH 7.4 for 1 hour, they were immersed in 10 per cent donkey serum in PBS for 2 hours as a pre-incubation step to reduce background staining. This procedure was followed by incubation at a room temperature of 20°C in a humid chamber for 72 hours with the primary antibody (Serotec) at a 1:8 titre in 10 per cent donkey serum. The sections were then washed with PBS and re-incubated for 48 hours in secondary antibody (Jackson) conjugated with CY3 diluted 1:100 in double strength PBS antibody dilulent. After a final rinse in PBS, the preparations were mounted in Mowiol-glycerol + 2.5 per cent DABCO. Negative control sections were processed simultaneously, omitting primary and/or secondary antibodies (Vandevska-Radunovic *et al.,* 1994). Because RECA-1 is a derived antibody from lymph glands (Duijvestijn *et al*., 1992), as a control system it was not possible to eliminate the antibody by pre-incubation absorption with the original antigen, which is not identified or available.

The tissues were scanned with a BioRad MRC-1000 confocal unit (BioRad, Hemel Hempstead, Herts, UK) using a krypton/argon laser with a 568/10 nm yellow excitation filter and 560 DRLP dichroic splitter coupled with a 605-DF-32 emission filter, linked to a Nikon Diaphot 300 inverted microscope in fluorescence mode using a Nikon x20 lens NA 0.4. The images were captured as digital files with a computer-linked CoMOS (BioRad) image analysis software program in serial sections with stacked layers of varying thickness to create 3-D reconstructions of antibody distribution along the vessel walls. The optical section thickness was maintained at 1 μm and the separation at 2 μm, while the image stack number varied from 1 to

49. For all optical sectioning the noise to signal ratio was kept constant (Guilak, 1994).

Statistical analysis

Since immunoreactivity enhancement proved to be a consistent loading effect, the fourth animal killed from the third experimental group of four rats was designated for vessel statistical analysis. From each blood vessel a randomly selected region was sampled to provide data for the quantitative analysis of RECA-1 antibody expression as grey scale intensity-coded readings. Vessel and ligament immunofluorescence was compared between control and experimental sides by standardized densitometry analysis for statistical evaluation. Representative blood vessels were matched according to type and regions and sampled together with their surrounding PDL. The areas of immunofluorescence were intensity coded in grey-scale using the CoMOS program set to 256 levels of grey with 0 being black and 256 being white.

Randomly sited samples of 10 densitometry readings were taken at equivalent intervals along the length of the wall from each of four PCV and one AC in the coronal region, and two apical region CV and AC vessels. For the collection of the PCV and AC data a sampling box of 20 × 13 pixels was used on a picture area of 145 × 97 μm. The pixel size was 0.415 μm. As a comparison, recordings of 10 randomly selected, adjacent PDL background regions were made with a box size of 60 × 40 pixels (Figure 2). Where large, apical CV were present in a cross-section, the readings were made at 10 evenly distributed distances around the endothelial wall using a box size of 20 × 13 pixels on a picture area of 384 × 256 μm. The box size for these adjacent PDL backgrounds was 60 × 40 pixels. Confocal images were viewed on a computer screen at a ×5.3 zoom for small vessels, the PCV, AC, and TA, and a ×2.0 zoom for the large CV. All the samples and readings were taken by an independent observer.

An analysis of variance (ANOVA) was used (Genstat 5, Release 3, Reference Manual, 1993) to test the means for each vessel type using the PDL background as a covariate. A preliminary analysis showed that there were no significant correlations between the wall and background measurements for any of the vessels. For this reason, no correction was necessary for the background measurements. The 10 values for the wall and for the background that were taken for each vessel were first averaged, and the average measurements were subjected to ANOVA. Independence of the observations was assured because only vessel averages were considered. The residuals were examined and were found to be at least approximately normally distributed. Use of a log transformation did not significantly improve the normality of the residuals. The raw data were, therefore, analysed.

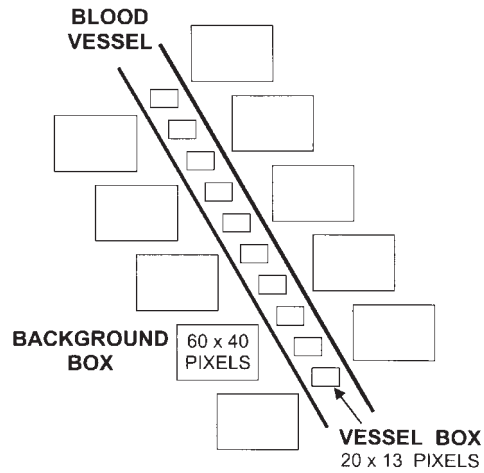

Figure 2 The vessel analysis system. Random samples of 10 densitometry readings were taken from each vessel at equivalent intervals along the length of the wall. A sampling box of 20 × 13 pixels was used on a picture area of 145 × 97 μm. For comparison, 10 random, adjacent PDL background regions were recorded with a box size of 60 × 40 pixels. For large apical CV in cross-section, 10 evenly distributed readings were made around the endothelial wall. The vessel and PDL box sizes were constant and the picture area was 384 × 256 μm.

Results

RECA-1 consistently labelled the endothelial cell linings of the PDL microvascular bed, the vessels in the alveolar bone and pulp, and also stained the smooth muscle of AC and TA. Morphologically, the vessel types, which comprised

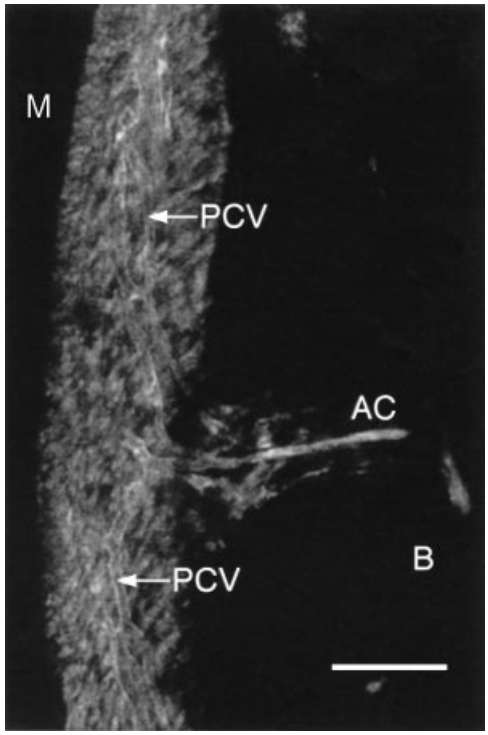

Figure 3 An AC in the alveolar bone forming VC and PCV branches that fed the PDL venule system. Strong immunofluorescence was present in the terminal arteriole endothelium and its smooth muscle. The weak level of RECA-1 expression in the capillaries and venules suggested a low immunoreactivity under basal conditions. Image number 20 from a stack of 24 slices totalling 48 μm. Control section. First molar: mesial side of the distal root opposite the mid root region. AC, arterial capillary; PCV, post-capillary-sized venule; B, alveolar bone; M, molar. Bar = 100 μm.

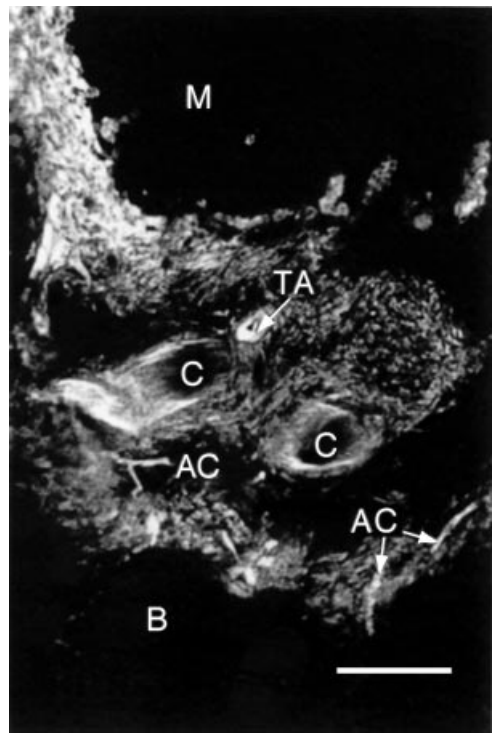

Figure 4 The apical region of control PDL demonstrating the immunoreactivity in large CV, the AC and a TA. Where arterial segments were viewed in cross-section, immunofluorescence extension into the abluminal smooth muscle layer was evident. Extended focus image of 36 slices totalling 70 μm. Control section. Distal root of the first molar. AC, arterial capillary; TA, terminal arteriole; C, collecting venule; B, alveolar bone; M, molar. Bar = 100 μm.

AC, TA, VC, PCV, and CV, could be readily identified in normal and loaded PDL tissue. There was no evidence that RECA-1 antibody cross-reacted with other cell types in the PDL, such as macrophages, fibroblasts, or osteocytes. Despite the compression load transmitted to the apical PDL tissues, the lumina of the CV appeared to remain patent.

In control sections, the PCV were present throughout the ligament. All AC and TA extending from the alveolar bone into the middle and apical ligament regions were precisely demarcated by their more intense fluorescence compared with that of the VC, PCV, and CV (Figures 3 and 4). At the alveolar bone boundary, where the AC entered the PDL, they formed a branching network of VC and PCV, which showed a marked reduction in fluorescence. Within the apical third of the ligament, the CV were positively identified by their large diameters which exceeded 30 μm. The immunoreactivity of their endothelium was weak and presented a somewhat striated appearance. Apical region TA and AC showed an intense RECA-1 immunofluorescence of their surrounding smooth muscle as well as the endothelium, although these two structures could not be separately identified (Figure 4).

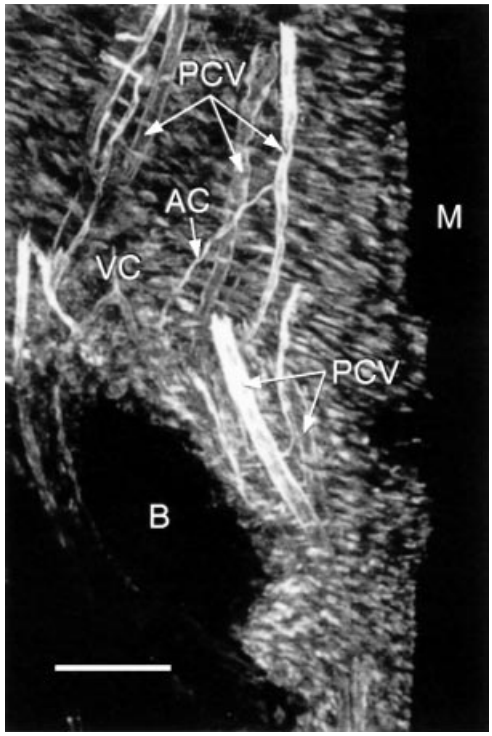

Figure 5 A region of presumed PDL shear loading adjacent to the mesial alveolar crest. Vessels of the microvascular bed revealed a more intense level of RECA-1 immunoreactivity in the endothelial cells. The change was most evident in vessels adjacent to the alveolar bone and tooth. Extended focus image of 49 slices totalling 96 μm. Experimental section. Distal of the first molar. AC, arterial capillary; VC, venous capillary; PCV, post-capillary-sized venule; B, alveolar bone; M, molar. Bar = 100 μm.

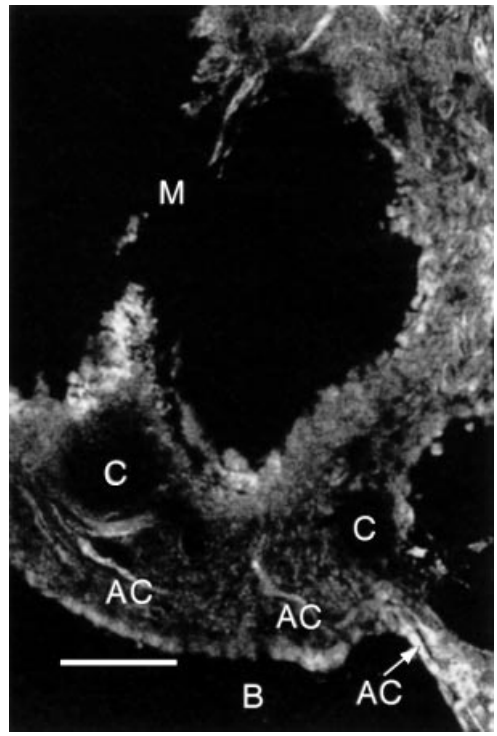

Figure 6 The PDL pressure area below the apex of the loaded first molar in Figure 5. RECA-1 immunofluorescence in the collecting venules has virtually disappeared. Arterial capillaries in the PDL, and a terminal arteriole in the alveolar bone, retain their strong immunofluorescence. Image number 6 from a stack of 25 slices totalling 48 μm. Experimental section. AC, arterial capillary; TA, terminal arteriole; C, collecting venule; B, alveolar bone; M, molar. Bar = 100 μm.

When subjected to occlusal loading the molar teeth transmitted compression, tension and shear effects to the PDL. These stresses caused enhanced RECA-1 immunolabelling of many vessels (Figures 5 and 6). In regions judged to be affected by tension and shear stress, immunoreactivity to RECA-1 in the endothelial cells of AC, VC, and PCV was enhanced (Figure 5). Compression in the apical region resulted in a loss of RECA-1 in the endothelium of VC, PCV and also in the large CV. By contrast, the AC in the apical PDL and bone retained the intense immunoreactivity of their endothelium and subjacent smooth muscle layers (Figure 6). These immunofluorescence changes were consistent across all experimental PDL sections.

Figures 4 and 5 are multiple layered images, and their relative thickness could create the false impression of a lack in RECA-1 immunospecificity, since noise is additive, but the signal is not. The true noise to signal ratios were demonstrated by comparison of all the single 1 μm optical slices (Figures 7–10). Figures 8 and 9, where the background noise was not amplified by layering, are taken from the multilevel stacks in Figures 4 and 5. Thus the RECA-1 immunostaining was highly specific. There was noticeable axial attenuation of the laser beam with scattering at

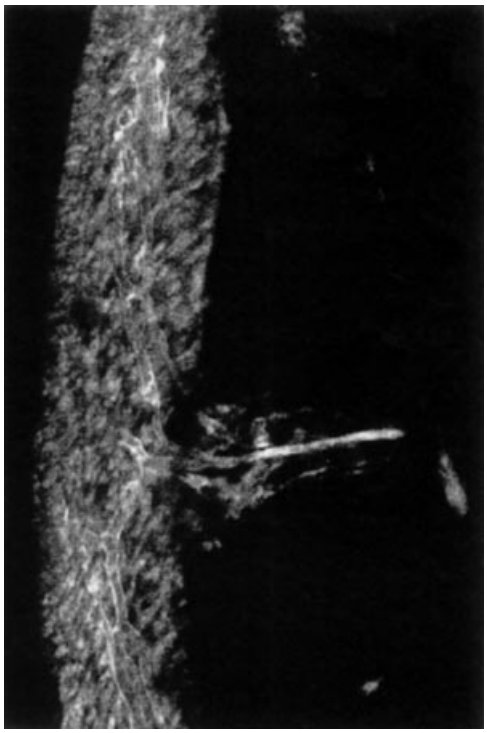

Figure 7 The single optical slice of Figure 3 illustrating the signal to noise ratio at the 40-μm level.

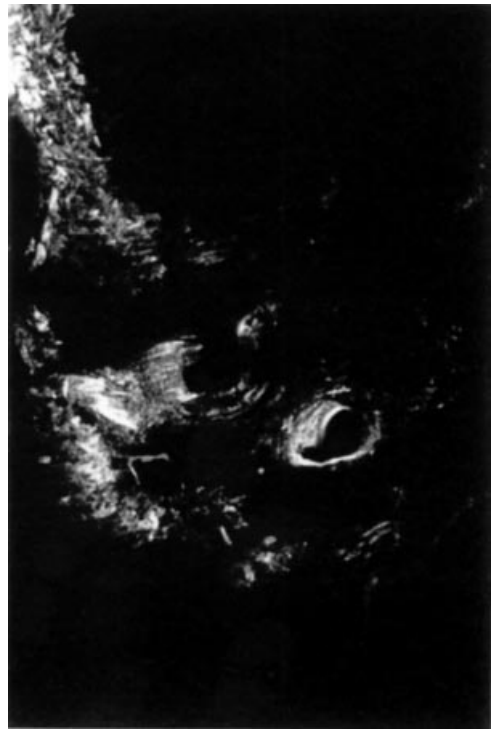

Figure 8 A single optical slice from Figure 4 at the 8-μm level.

depth (Guilak, 1994) as demonstrated in Figure 5, which comprised a 96 μm stack from a jaw section 160 μm thick.

Statistical findings

A preliminary analysis showed no significant correlation between the wall and background measurement. The overall result demonstrated very significant differences between the vessel wall raw means ($P < 0.001$). The individual means, both the simple means and the predicted means adjusted for the background, were then compared using Fisher's protected least significant differences (Table 1). Any two means not sharing the same letter differ at the $P < 0.05$ level. For example, the AC and the CV in Figure 4. Similarly, the PCV in Figures 3 and 5, and the CV in Figures 4 and 6. On a similar basis, comparison of the arterial wall immunoreactivity showed no significant difference at the $P < 0.05$ level.

Within the control tooth there was no significant difference between the coronal and apical endothelium immunoreactivity. There was a very significant difference ($P < 0.001$) in the loaded tooth, where overall the crown had a much higher value. The treatment had opposite effects in the crown and apex regions, where these effects effectively negated each other, so that averaged across positions there was no effect. Because the findings proved to be definitive for the initial data set of 190 control and experimental pairs, additional section analyses were not deemed necessary.

Discussion

As with other microvascular beds in the rat, RECA-1 antibody recognized an endothelial cell

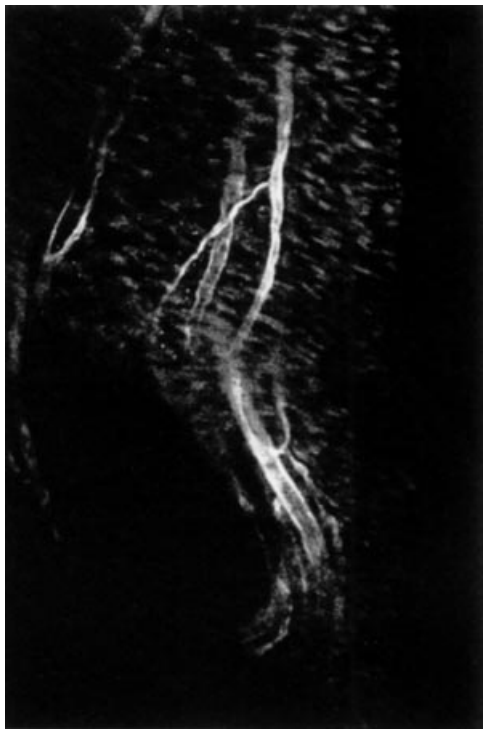

Figure 9 A single optical slice from Figure 5 at the 40-μm level.

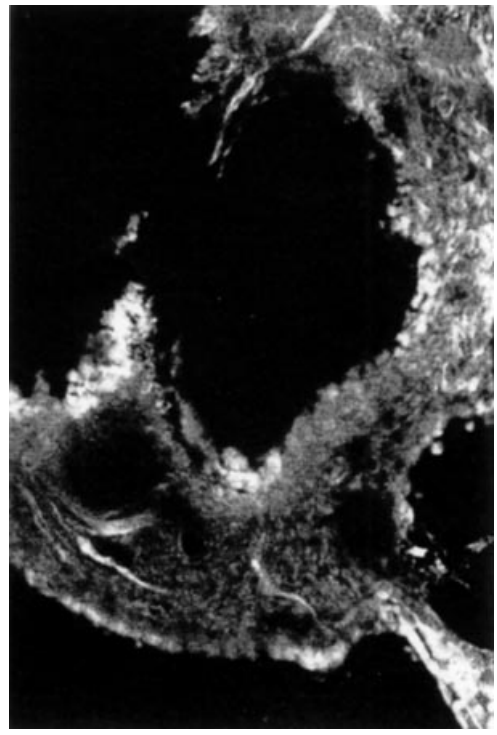

Figure 10 The single optical slice of Figure 6 illustrating the signal to noise ratio at the 12-μm level.

Table 1 Comparison of the mean values of endothelial wall immunoreactivity. Any two means not sharing the same letter differ at the $P < 0.05$ level. For example, AC and CV in Figure 4.

Figure no.	Vessel type	Replicates	Raw means	Predicted means	Letters
3	AC	1	1707	1836	bc
4	AC	2	2098	2125	c
5	AC	1	2181	2110	bc
6	AC	2	1783	1858	bc
3	CV	0			
4	CV	2	1476	1481	ab
5	CV	0			
6	CV	2	483	559	
3	PCV	4	1254	1223	a
4	PCV	0			
5	PCV	4	2270	2204	c
6	PCV	0			
3	TA	0			
4	TA	1	2229	2190	c
5	TA	0			
6	TA	0			

AC, arterial capillary; CV, collecting venule; PCV, post-capillary-sized venule; TA, terminal arteriole.

antigen and reacted with the normal PDL blood vessel endothelium in all the different categories of micro-vessels, as has been reported for other rat tissues (Duijvestijn *et al.*, 1992). These authors, who examined vascular endothelium in a variety of tissues including liver, kidney, tongue, gut, and thymus, reported that the strongest immunofluorescence occurred in the large blood vessels. By contrast, in the PDL, the greatest immunofluorescence was observed in the small AC and TA, whereas the VC, PCV, and notably the large CV showed a reduced intensity, but comparable endothelial distribution of immunofluorescence.

Duijvestijn *et al.* (1992) attributed the antibody reactivity to occur with an endothelial cell surface molecule. The nature of the antigen protein expressed at the surface of the endothelial cells is not known. Nevertheless, it is evident that with occlusal loading of the tissues an up-regulation in the intensity of RECA-1 expression occurs. One can speculate that the applied load results in additional protein being created *de novo* in the endothelial cell or that the protein is already present on the luminal endothelial surface, and is activated or deactivated by stress changes. Why the expression of RECA-1 is so intense in the arterial walls of both normal and loaded PDL and bone remains to be determined.

Experimental loading caused regional alterations in the patterns of immunostaining, the alteration depending on the nature of the physical force; that is, the regions of presumed compression, tension or shear, and the type of vessel profile affected. However, the RECA-1 did not reveal any evidence of vessel or site specificity of epitope location. Nevertheless, RECA-1 was established as an excellent endothelial cell probe for both normal and stressed rat PDL. Modulation of endothelial cells by loading, as indicated by their changing expression of RECA-1 antibody, suggests that an alteration in the endothelial cell expression of surface antigen is a small blood vessel effect.

The RECA-1 reactivity with the vascular endothelium signified that all the blood vessel endothelium shared specific sites to RECA-1. Nevertheless, like other peripheral vessel systems (Duijvestijn *et al.,* 1992; Haynes, 1995), the PDL microvascular bed has a heterogeneous structure and distribution (Sims, 1995) and the endothelial wall is sensitive to tensional changes, which result in significant alterations in permeability (Cooper *et al*., 1990; Tang and Sims 1992; Tang *et al.*, 1993). A co-existing pattern of endothelial phenotypic complexity could be related to specific regional physiological functions and the influence of local micro-environmental factors on endothelial cell differentiation (Gumkowski *et al*., 1987). Pries *et al.* (1995) maintain that blood flow induced endothelial shear stress levels are much lower in veins and venules than in arterioles. Such structural and functional heterogeneity suggests that investigations with other endothelial cell-specific markers may be fruitful in the study of the physiological responses of endothelium in normal and stressed PDL.

In other tissues, endothelial and smooth muscle cells can be triggered to function in response to alterations in micro-environmental stimuli and physical factors, such as tissue stress, by the enhanced production of cytokines (Nicola, 1994; Webb, 1997). In the present study, RECA-1 immunoreactivity also showed stress-induced alterations in the endothelial cells of the PDL microvascular bed. This investigation confirmed the hypothesis that RECA-1 antibody was a suitable probe to identify the endothelium of blood vessels in normal and stressed rat molar PDL. Furthermore, it demonstrated a significant alteration in endothelial cell expression of a surface antigen, RECA-1, in the rat PDL microvascular bed as a response to short-term tooth loads. RECA-1, however, did not prove to be entirely endothelium specific in the rat PDL, since it also revealed arterial smooth muscle cells. It is noteworthy that, unlike the CV, the apical AC and TA were prominent for maintaining their immunoreactivity in apical PDL compression regions.

At present no explanation can be provided for the endothelial changes demonstrated in this immunohistochemical study. The findings refer specifically to the load and time span involved. The desirability of ongoing studies for differing load and time regimes is apparent, but outside

the scope of the present report. It has been stated that when cells are subjected to shear stress or mechanical stretching, a diverse set of responses is evoked; some of which are extremely fast, while others develop over many hours (Davidovitch, 1995). A continuous occlusal load applied for 10 minutes is beyond the norm of intermittent physiological function. Therefore, the activation of endothelial cells, indicated by the up-regulation in RECA-1 antibody expression, suggests a possible functional alteration that may arguably reflect the view that endothelial cell activity is an important early consequence of tooth loading.

Current empirical concepts of 'biological' clinical orthodontic loads are not based on a comprehensive understanding of force transduction and its relationship to the generation of biomolecular signals in mechanically-stressed PDL blood vessels (Chang *et al*., 1997). The use of immunological probes to identify physiological responses of endothelial cells to applied stress could eventually lead to a determination of optimal orthodontic loading for tooth movement.

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