



AN IMMUNOHISTOCHEMICAL STUDY OF MARMOSET PERIODONTAL LIGAMENT MICROVASCULATURE

A CONFOCAL LASER SCANNING MICROSCOPIC STUDY

A research project submitted in partial fulfilment of the requirements for the degree
of Master of Dental Surgery

by

JONATHAN F. ASHWORTH

B.D.S. (Adel.)



Orthodontic Unit
School of Dentistry
Faculty of Health Sciences
The University of Adelaide
South Australia

1999

TABLE OF CONTENTS

	Page No
List of Figures	vii
List of Tables	x
List of Abbreviations	xi
Summary	xii
Signed Statement	xiv
Acknowledgements	xv
Chapter 1	
INTRODUCTION	1
Chapter 2	
AIMS AND HYPOTHESES	3
Chapter 3	
REVIEW OF THE LITERATURE	4
3.1 The Morphology of the Periodontal Ligament (PDL)	4
3.2 The PDL Microvascular Bed	5
3.2.1 PDL Vascular Supply	5
3.2.2 PDL Vessel Sizes/Classification	6
3.2.3 PDL Vessel Distribution	7
3.2.4 PDL Vessel Volume	9
3.2.5 Monkey PDL Microvasculature	10
3.2.6 Functions of the PDL Microvasculature	11
3.2.7 Regulation of PDL Blood Flow	12
3.3 Periendothelial Cells	12
3.3.1 Periendothelial Cells in PDL Microvasculature	13
3.4 Alpha Smooth Muscle Actin	14
3.5 Mechanical Stimulation of PDL Tissues	15
3.5.1 Cellular Biology	15
3.5.2 Vascular Changes	16
3.6 Angiogenesis	19
3.6.1 Control of Angiogenesis	20
3.6.2 Pericyte Involvement in Angiogenesis	21
3.7 Cytokines	22

3.8 Endothelin-1	23
3.8.1 Biosynthesis	24
3.8.2 Actions	25
3.8.3 Receptors	26
3.8.4 The Physiological Role of Endothelin-1	27
3.9 Endothelin-Related Investigations	28
3.9.1 Endothelin-Related Investigations in Non-Human Primates	28
3.9.2 Immunohistochemical Labelling for Endothelin-1 in Dental Tissues	28
3.10 Immunofluorescence	30
3.10.1 Fluorescence Microscopy	32
3.10.2 Confocal Laser Scanning Microscopy	32
3.11 The Marmoset as an Experimental Animal	33
3.11.1 The Marmoset Dentition	33
Chapter 4	MATERIALS AND METHODS
4.1 Summary	36
4.2 Ethics Approval	36
4.3 Research Colony	36
4.4 Summary of Pilot Studies	37
4.5 Pilot Study No 1 (RECA-1)	38
4.5.1 The Experimental Animal	38
4.5.2 Tissue Preparation and Immunohistochemistry	38
4.6 Pilot Study No 2 (ET-1)	39
4.6.1 The Experimental Animal	40
4.6.2 Tissue Preparation and Immunohistochemistry	40
4.7 Pilot Study No 3 (QB-END-10)	41
4.8 Pilot Study No 4 (JC-70A)	41
4.9 Pilot Study No 5 (α -SMA)	41
4.10 Main Experiments	42
4.10.1 The Experimental Procedure	42
4.10.2 Loading Device and Intra-Oral Pad	42
4.10.3 Tissue Preparation	44
4.10.4 Immunohistochemistry	44
4.10.5 Examination of Mandibular Sections and Data Collection	47

Chapter 5	FINDINGS	48
5.1	Summary of Pilot Study Findings	48
5.1.1	RECA-1	48
5.1.2	Endothelin-1	48
5.1.3	Suitability of QB-END 10 as a Pan-endothelial Immunolabel	49
5.1.4	Suitability of JC-70A as a Pan-endothelial Immunolabel	49
5.1.5	Suitability of α -SMA as a Periendothelial Cell Immunolabel	49
5.2	Pilot Study No 1 (RECA-1)	49
5.3	Pilot Study No 2 (ET-1)	50
5.3.1	Experiment A	50
5.3.2	Experiment B	50
5.3.3	Experiment C	50
5.3.4	Experiment D	51
5.3.5	Experiment E	51
5.3.6	Experiment F	52
5.3.7	Experiment G	52
5.4	Pilot Study No 3 (QB-END 10)	53
5.5	Pilot Study No 4 (JC-70A)	53
5.6	Pilot Study No 5 (α -SMA)	53
5.7	Summary of Findings of Main Experiments	58
5.7.1	Endothelin-1	58
5.7.2	JC-70A	58
5.7.3	α -SMA	58
5.8	Experiment No 1 (ET-1)	58
5.8.1	Experiment 1	58
5.8.2	Experiment 2	59
5.8.3	Experiment 3: Unloaded and Loaded Mandibular Sections	60
5.9	Experiment No 2 (JC-70A)	60
5.9.1	Vascular Morphology	60
5.9.2	Antigen Expression	61
5.10	Experiment No 3 (α -SMA)	62
Chapter 6	DISCUSSION	77
6.1	Material	77

6.2	Load Application	77
6.3	Immunohistochemical Methodology	78
6.3.1	Fixation	78
6.3.2	Immunohistochemical Controls	78
6.3.3	Refinement of the Laboratory Methodology	79
6.3.4	Limitations of the Current Laboratory Methodology	80
6.4	Immunohistochemical Labelling with RECA-1	81
6.5	Immunohistochemical Labelling for ET-1	81
6.5.1	General Discussion	81
6.5.2	Pilot Study for ET-1 Immunolabelling	83
6.5.3	Secondary Antibody for Anti-ET-1	85
6.5.4	ET-1 Antibody Cross-Reactivity	85
6.5.5	Immunolabelling for ET-1 in Dental Tissues	85
6.5.6	Production of ET-1 in the MVB of the Unloaded and Loaded PDL	87
6.6	Immunohistochemical Labelling for Endothelium	87
6.6.1	Pilot Study for Pan-endothelial Immunolabel	87
6.6.2	Endothelial Cells in Dental Tissues	88
6.6.2.1	Microvascular Morphology	88
6.6.2.2	Clinical Implications	89
6.6.2.3	Antigen Expression	90
6.7	Immunohistochemical Labelling for Periendothelial Cells	91
6.7.1	Pilot Study for Periendothelial Cell Immunolabel	91
6.7.2	Periendothelial Cells in Dental Tissues	91
6.8	Suggestions for Future Research	92
6.8.1	Endothelin-1	92
6.8.2	Morphological Studies	93
6.8.3	Angiogenesis	93
Chapter 7	CONCLUSIONS	95
Chapter 8	APPENDICES	97
8.1	Anaesthetic	97
8.2	Zamboni's Fixative	97
8.3	Phosphate Buffered Saline (PBS)	98
8.4	PBS / 30% Sucrose / 0.1% Sodium Azide	98

8.5 Dimethyl Sulphoxide (DMSO)	98
8.6 Coating Slides	99
8.7 Drying Slides with Tissue Sections	99
8.8 Antibody Diluent (0.02M)	99
8.9 Donkey Normal Serum	100
8.10 RECA-1 Antibody	100
8.11 Rabbit Anti-Endothelin-1 Antibody	100
8.12 Rabbit Anti-Big Endothelin-1 Antibody	100
8.13 Rabbit Anti-Pre-proendothelin Antibody	101
8.14 Monoclonal Anti-Endothelin Antibodies	101
8.15 Endothelial Cell Marker: QB-END 10 Antibody	101
8.16 Endothelial Cell Marker: Anti-PECAM-1 Antibody	101
8.17 Mouse Monoclonal Anti-Human Alpha-Smooth Muscle Actin Antibody	102
8.18 Cy3™ Conjugated Donkey Anti-Mouse IgG Antibody	102
8.19 Cy3™ Conjugated Donkey Anti-Rabbit IgG Antibody	102
8.20 Cy5™ Conjugated Donkey Anti-Rabbit IgG Antibody	103
8.21 Dichlorotriazinyl Amino Fluorescein (DTAF) Antibody	103
8.22 Biotin-SP-Conjugated Affinipure Donkey Anti-Rabbit IgG	104
8.23 Cy3™ Conjugated Streptavidin	104
8.24 Immunohistochemical Labelling Procedure – Soft Tissues. Indirect Technique	105
8.25 Immunohistochemical Labelling Procedure – Soft Tissues. Streptavidin-Biotin Amplification Technique	105
8.26 Immunohistochemical Labelling Procedure – Mandibular Sections. Indirect Technique	106
8.27 Chequerboard for Pilot Study No 1	106
8.28 Chequerboards for Pilot Study No 2	107
8.29 Chequerboard for Pilot Study No 3	108
8.30 Chequerboard for Pilot Study No 4	108
8.31 Chequerboard for Pilot Study No 5	108
8.32 Quantification of PECAM-1 Expression in PDL Vessels in Unloaded and Loaded First Molars	109
Chapter 9	
REFERENCES	110

LIST OF FIGURES

Figure	Subject	Page No.
1	The periodontal ligament and surrounding structures	4
2	Luminal volume across PDL circumferential thirds	8
3	Blood volume of vessel groups as a percentage of the PDL	8
4	Structure and amino acid sequence of endothelins	23
5	Factors that alter endothelin-1 synthesis and the pathway for endothelin-1 generation	25
6	The direct, indirect and streptavidin-biotin immunofluorescent techniques	31
7	Marmoset permanent dentition	34
8	Sagittal sections of marmoset permanent mandibular dentition	35
9	Loading device and intra-oral pad	43
10	Marmoset with loading device and intra-oral pad <i>in-situ</i>	46
11	Marmoset mandibular body undergoing sagittal sectioning	46
12	Method of quantifying antigen expression	47
13	Immunolabelling of RECA-1 antigen in rat kidney	54
14	Immunolabelling of artery endothelium for Big ET-1 in kidney	54
15	Immunolabelling of capillary loops for Big ET-1 in glomerulus of kidney	55
16	Immunolabelling of peritubular capillaries for Pre-pro ET-1 in kidney	55
17	Immunolabelling of epithelial cells for ET-1 in kidney	56
18	Immunolabelling of endothelial cells for ET-1 in kidney	56
19	Immunolabelling of endothelial cells with JC-70A in stomach	57
20	Immunolabelling of α -SMA in proximal small intestine	57
21	Immunolabelling of vascular ET-1 within PDL	63
22	Immunolabelling of postcapillary-sized venule within PDL for ET-1	63
23	Immunolabelling of collecting venule within PDL for ET-1	64
24	Immunolabelling of collecting venule bordering PDL and bone for ET-1	64

25	Immunolabelling of terminal arteriole within alveolar bone for ET-1	65
26	Pan-endothelial immunolabelling in dental tissues	66
27	Immunolabelling of blood vessel traversing cortical plate of tooth socket	66
28	Immunolabelling of gingival capillary loops	67
29	Intercellular pattern of immunolabelling of endothelial cells in PDL	67
30	Confocal image of pan-endothelial immunolabelling within dental tissues	68
31	Confocal image of immunolabelled blood vessels traversing cortical plate of tooth sockets	68
32	Magnified confocal image of immunolabelled blood vessels traversing cortical plate of tooth socket	69
33	Confocal image of immunolabelled blood vessels in subapical region of mandible	69
34	Merged confocal image of immunolabelled endothelial cells of collecting venule in apical PDL of mandibular first molar	70
35	Merged confocal image of immunolabelled endothelial cells of collecting venule in PDL	70
36	Confocal images of apical vessels of unloaded molar selected for PECAM-1 quantification	71
37	Confocal images of apical vessels of loaded molar selected for PECAM-1 quantification	72
38	Confocal images of interradicular vessels of unloaded and loaded molars selected for PECAM-1 quantification	73
39	Immunolabelled α -SMA within periendothelial cells in dental tissues	74
40	Immunolabelling of α -SMA within pericyte of collecting vein in alveolar bone showing cell body, nucleus and cytoplasmic processes	74
41	Immunolabelled α -SMA within pericytes of collecting vein in alveolar bone	75
42	Confocal image of immunolabelled α -SMA within vascular smooth muscle cells of terminal arterioles in PDL	76

43	Magnified confocal image of immunolabelled α -SMA within vascular smooth muscle cells of terminal arterioles in PDL	76
----	--	----

LIST OF TABLES

Table	Subject	Page No.
1	Summary of studies on total vascular volume of the total PDL volume	9
2	Summary of pilot studies	37
3	Summary of main experiments	45

LIST OF ABBREVIATIONS

CLSM	Confocal laser scanning microscope
DMSO	Dimethyl sulphoxide
DNS	Donkey normal serum
DTAF	Dichlorotriazinyl amino fluorescein
ET-1	Endothelin-1
FITC	Fluorescein isothiocyanate
GCF	Gingival crevicular fluid
IHC	Immunohistochemistry
IL-1	Interleukin-1
MVB	Microvascular bed
PBS	Phosphate buffered saline
PDL	Periodontal ligament
TGF β	Transforming growth factor β
VEGF	Vascular endothelial growth factor
VSMC	Vascular smooth muscle cells
α -SMA	Alpha smooth muscle actin
I1	Central incisor
I2	Lateral incisor
C	Canine
PM1	First premolar
PM2	Second premolar
PM3	Third premolar
M1	First permanent molar
M2	Second permanent molar
TA	Terminal arteriole
AVA	Arteriovenous anastomosis
AC	Arterial capillary
p-VC	Pericytic venous capillary
a-PC	Apericytic venous capillary
p-PCV	Pericytic postcapillary-sized venule
a-PCV	Apericytic postcapillary-sized venule
CV	Collecting venule

SUMMARY

The biological response to the application of an orthodontic force to a tooth involves various cell types of the periodontium assuming altered functional states (Davidovitch, 1995). The vasculature of the periodontium undergoes functional and morphological alterations simultaneously along with other cellular elements. A 21-amino-acid peptide, endothelin-1 (ET-1, Yanagisawa *et al.*, 1988) is a cytokine with potent vasoactive properties that may play a role in the response of the PDL microvascular endothelium to tooth loading.

A custom designed device was constructed for the application of an intrusive occlusal load to the buccal segment teeth of four anaesthetised marmosets for 1.5 hours. The contralateral teeth served as controls. For immunohistochemical investigations, the jaws, and samples of kidney, skeletal thigh muscle and gut, were extirpated and fixed immediately after the animals were euthanased.

Sagittal sections of each mandibular body were prepared using a diamond wafering blade mounted on a low speed sectioning saw. Each section was 150-200 μ m thick and incorporated bone, periodontium and teeth. Immunohistochemistry (IHC) was performed on the undemineralised mandibular sections using laboratory methodologies established with pilot studies.

The pilot studies comprised systematic chequerboard tests of indirect immunohistochemical labelling for the identification of ET-1, endothelial cells and periendothelial cells on cryostat-cut sections of marmoset kidney, skeletal muscle and gut. The primary antibodies were anti-ET-1, JC-70A and alpha smooth muscle actin (α -SMA). Immunoglobulin G secondary antibodies conjugated with the fluorochromes DTAF, FITC, Cy3™ and Cy5™ were tested for optimum signal-to-noise ratios. Streptavidin-biotin amplification immunolabelled vascular endothelium for ET-1 but did not prove as satisfactory as the indirect IHC technique.

Fluorescence and confocal laser scanning microscopy were used to ascertain the location of immunolabelled vascular ET-1, endothelial and periendothelial cells. Antibody penetration routinely occurred to a depth greater than 85 μ m from either side of the mandibular sections.

Endothelin-1 demonstrated a heterogeneous distribution within the microvasculature of the PDL and alveolar bone with the anti-ET-1 primary antibody and the IgG/Cy5™ secondary antibody at dilutions of 1:50. Vascular ET-1 was located within the walls of terminal arterioles, postcapillary-sized venules and collecting venules. Immunolabelling for ET-1 occurred within the cytoplasm of endothelial cells and vascular smooth muscle cells. The presence of this potent vasoconstrictor suggests that the microvascular beds of the PDL and alveolar bone might possess contractile properties. The hypothesis that vascular ET-1 expression varies within the PDL in unloaded versus loaded conditions could not be established as insufficient data were generated for quantification. A method for future quantification of antigen expression was examined.

Endothelial cell, pericyte and vascular smooth muscle cell location and morphology was examined with the JC-70A (1:40) and α -SMA (1:100) primary antibodies respectively in combination with IgG/Cy3™ secondary antibody (1:50). The intercellular distribution of endothelial cell immunolabelling enabled illustration of cellular size and shape. Immunolabelling of intricate actin fibrils within pericytes and vascular smooth muscle cells provided further insight into periendothelial cell morphology. The confocal laser scanning microscope (CLSM) and CoMOS software (Bio-Rad, version 7.0a) enabled the construction of detailed three-dimensional cellular images by computer stacking of the optical sections.

The results of immunohistochemical investigations of vasoactive peptides such as ET-1 may eventually lead to pharmacological targeting of the PDL microvasculature to assist orthodontic tooth movement. Endothelin-1 may play a significant role during angiogenesis within the periodontium concomitant with orthodontic tooth movement. The administration of agents that influence the function of PECAM-1, the antigen to JC-70A, and their effect on the rate of tooth movement might also be investigated. Further research utilising the marmoset experimental and control maxillary sections and the laboratory methodology established in the current study are warranted.

SIGNED STATEMENT

This report contains no material that has been accepted for the award of any other degree or diploma in any other university. To the best of my knowledge and belief, it contains no material previously published except where due reference is made in the text.

I give consent to this copy of my thesis, when deposited in the University Library, being available for loan and photocopying.

Jonathan F. Ashworth

B.D.S. (Adel.)

ACKNOWLEDGEMENTS

I would like to thank the following people for their support during the last two years:

Professor M. R. Sims, Visiting Professor, The University of Sydney; Visiting Research Fellow, The University of Adelaide, for his guidance, patience and perseverance with this project, particularly during the times in which progress was slow.

Associate Professor B. J. Gannon, Department of Anatomy and Histology, Flinders University, for willingly sharing his expertise and for the use of his laboratory.

Professor W. J. Sampson, P. R. Begg Chair in Orthodontics, The University of Adelaide, for his encouragement and suggestions in the preparation of this thesis.

Dr C. W. Dreyer, Senior Lecturer in Orthodontics, The University of Adelaide, for his suggestions and direct laboratory assistance.

Dr. P. Kolesik, Manager, Confocal Facility, Department of Horticulture, Viticulture and Oenology, The University of Adelaide, for operation of the confocal microscope.

Dr. J. Clark, Manager of Animal Services, CSIRO Human Nutrition, for her expert handling of the marmosets.

Bev Manthey, Bone and Joint Research Laboratory, Institute of Medical and Veterinary Science, for assistance with the bone-cutting machine and stereomicroscope.

My friends and colleagues, Drs. C. Lapidis and P. Greatrex for their assistance and encouragement during the experiments.

My wife, Donna, for the ongoing love, patience and support she has demonstrated during the course of this research project.

I also wish to gratefully acknowledge the support for this project provided by the Australian Dental Research Fund and the Australian Society of Orthodontists Foundation for Research and Education.