The Cerebrovascular Response to Metastatic Melanoma and Clostridium perfringens Type D Epsilon Toxin

Kimberley Mander
BHlthSc (Hons)
BPychSci

Discipline of Anatomy and Pathology
School of Medicine
The University of Adelaide

A thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy
Dedication

For Peter John & Patricia May Mander
Declaration

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name, in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission in my name, for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint-award of this degree. I give consent to this copy of my thesis when deposited in the University Library, being made available for loan and photocopying, subject to the provisions of the Copyright Act 1968. I acknowledge that copyright of published works contained within this thesis resides with the copyright holder(s) of those works.

I also give permission for the digital version of my thesis to be made available on the web, via the University’s digital research repository, the Library Search and also through web search engines, unless permission has been granted by the University to restrict access for a period of time.

I acknowledge the support I have received for my research through the provision of an Australian Government Research Training Program Scholarship.

Kimberley Anne Mander

Date
# Table of Contents

Dedication ii  
Declaration iii  
Table of Contents iv  
Publications and Presentations xii  
Acknowledgments xiv  
Abbreviations xvi  
Figures and Tables xviii  
Thesis Format xxi  
Abstract xxii  

## General Introduction 2

1.1 Background 2  
1.1.1 The vascular system in health and disease 2  
1.1.2 Endothelial injury 5  
1.1.3 Special features of the cerebral vasculature 6  

1.2 Blood–Brain-Barrier 8  
1.2.1 Cerebral endothelial cells 9  
1.2.2 Cerebral Endothelial Tight Junction Proteins 10  
1.2.3 Transport across the blood-brain barrier 13  
1.2.4 Cellular Transcytosis 16  
1.2.5 Transcytosis in the Brain 17  

1.3 Caveolae 18  
1.3.1 Structure and Function of Caveolae 21  
1.3.2 Caveolae and cancer 23  

1.4 Pathobiology 24  
1.4.1 The Metastatic Process 25  
1.4.2 Dissemination 27  
1.4.3 Colonisation 29
1.4.4 Tumour associated angiogenesis and barrier permeability 30

1.5 Modeling Cerebrovascular Response to Metastatic Melanoma 33

1.5.1 In-Vitro Blood-Brain Barrier Model 34

1.5.2 In-Vivo models 36

1.6 Conclusion and Aims 36

Chapter 2: Materials and Methods 39

2.1 General 39

2.2 Experimental Procedures 39

2.2.1 Human Tissue 39

2.2.2 In Vitro Experiments: Cell Culture 40

2.2.3 A-375-MA1 Human Melanoma Cell line 41

2.2.4 Labelling A-375-MA1 for transmigration assay 41

2.2.5 Blood-Brain Barrier hCMEC/D3 cell line 42

2.2.6 Transwell Assay 42

2.2.7 Permeability Assay 44

2.2.8 In vitro immunofluorescence and immunohistochemistry 45

2.2.9 Transmission electron microscopy 46

2.2.10 Cell Viability Assessment 47

2.2.11 Western Blotting 48

2.2.12 Substance P ELISA 49

2.3 Epsilon toxin (ETX) 49

2.4 Animal Care 50

2.4.1 Anaesthesia 51

2.4.2 In Vivo Experiment 51

2.4.3 Intraperitoneal ETX inoculation 53

2.4.4 Tissue Processing 54
### Chapter 3: Vascular Patterns in Human Cerebral Metastatic Melanomas

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Background</td>
<td>57</td>
</tr>
<tr>
<td>Introduction</td>
<td>58</td>
</tr>
<tr>
<td>3.2.1 Malignant Melanoma</td>
<td>59</td>
</tr>
<tr>
<td>3.2.2 Cerebral Vasculature &amp; Malignancy</td>
<td>60</td>
</tr>
<tr>
<td>Materials and Methods</td>
<td>67</td>
</tr>
<tr>
<td>3.3.1 Experimental Design</td>
<td>67</td>
</tr>
<tr>
<td>3.3.2 Histological Analysis</td>
<td>68</td>
</tr>
<tr>
<td>3.3.3 Statistical Analysis</td>
<td>72</td>
</tr>
<tr>
<td>Results</td>
<td>73</td>
</tr>
<tr>
<td>3.4.1 Melanoma Classification</td>
<td>73</td>
</tr>
<tr>
<td>3.4.2 Blood vessel morphology</td>
<td>76</td>
</tr>
<tr>
<td>3.4.3 Cerebral metastatic melanoma have decreased BBB claudin-5 expression</td>
<td>88</td>
</tr>
<tr>
<td>Discussion</td>
<td>92</td>
</tr>
<tr>
<td>Conclusion</td>
<td>99</td>
</tr>
</tbody>
</table>

### Chapter 4: Role of Caveolin-1 and NK-1R in Human Cerebral Metastatic Melanoma

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Background</td>
<td>101</td>
</tr>
<tr>
<td>Introduction</td>
<td>102</td>
</tr>
<tr>
<td>Experimental Design</td>
<td>105</td>
</tr>
<tr>
<td>4.3.1 Immunohistological Analysis</td>
<td>105</td>
</tr>
<tr>
<td>4.3.2 Statistical Analysis</td>
<td>107</td>
</tr>
<tr>
<td>Results</td>
<td>108</td>
</tr>
<tr>
<td>4.4.1 Cerebral metastatic melanoma-associated blood vessels have marked reduction in CAV-1 immunopositivity</td>
<td>108</td>
</tr>
<tr>
<td>4.4.2 Cerebral metastatic melanocytes overexpress CAV-1</td>
<td>111</td>
</tr>
</tbody>
</table>
4.4.3 Cerebral metastatic melanoma have decreased BBB NK-1R expression 111

4.5 Discussion 114

4.6 Conclusion 117

Chapter 5: Characterisation of in-vitro Blood-Brain Barrier Model 119

5.1 Background 119

5.2 Introduction 120

5.3 Materials and Methods 122

5.3.1 Experimental Design 122

5.3.2 Transendothelial Electrical Resistance 123

5.3.3 Statistical Analysis 125

5.4 Results 126

5.4.1 hCMEC/D3 cells in vitro maintain morphological characteristics of cerebral microvascular endothelial cells found in normal brain tissue. 126

5.4.2 Cultured hCMEC/D3 cells retain expression of key endothelial markers 126

5.4.3 Cultured hCMEC/D3 cells express key membrane receptors and transport channels 127

5.4.4 hCMEC/D3 Barrier Function 128

5.4.5 Transmission electron microscopy images of hCMEC/D3 cells grown on permeable transwell membrane 131

5.5 Discussion 133

5.6 Conclusions 137

Chapter 6: The Effects of Targeted Caveolae and NK1 Antagonist Treatment on Tumour Cells transmigration In Vitro. 139

6.1 Background 139

6.2 Introduction 140

6.3 Methods 143

6.3.1 Experimental Design 143
6.3.2 Statistical Analysis

6.4 Results

6.4.1 Substance P secretion by human melanoma cell line A375-M1

6.4.2 Treatment with Filipin III and NK-1R antagonists at high concentration reduce hCMEC/D3 and A375-M1 cell viability in vitro

6.4.3 Transmigration Assay Characterisation

6.4.4 Treatment efforts failed to reduce A375-M1 melanocyte transmigration across hCMEC/D3 in vitro barrier.

6.4.5 Pre-treatment with substance P increased melanocyte transmigration capability at 5 hr.

6.4.6 Migrated A375-M1 melanocytes showed conserved phenotypical properties at 24 hrs.

6.5 Discussion

6.6 Conclusion

Chapter 7: Characterisation of the Intra-carotid Inoculation Model of Secondary CNS Metastases.

7.1 Background

7.2 Introduction

7.3 Materials and Methods

7.3.1 Experimental Design

7.3.2 Histological Analysis

7.3.3 Clinical Assessment

7.4 Results

7.4.1 Pilot study determined $10^6$ A375-M1 cell density is optimal for intra-carotid tumour inoculation.

7.4.2 Histopathology

7.5 Discussion
Chapter 8: Part B. The Role of the microvasculature in Clostridium perfringens type D epsilon toxin (ETX) neurotoxicity 174

8.1 Background 174

8.2 Introduction 175

8.3 Conclusion 188

Chapter 9: The Effect of Clostridium perfringens type D epsilon toxin (ETX) on cerebral microvasculature endothelial cells in vitro 190

9.1 Introduction 190

9.2 Materials and Methods 191

9.2.1 Experimental Design 191

9.2.2 Epsilon toxin (ETX) 191

9.2.3 Morphologic studies 191

9.2.4 Cell viability 191

9.3 Results 192

9.3.1 Human microvascular endothelial cells are susceptible to ETX-induced cytotoxicity. 192

9.3.2 Human microvascular endothelial cells undergo pyknosis and organelle swelling following ETX exposure 195

9.4 Discussion 197

9.5 Conclusion 200

Chapter 10: Characterisation of ETX-induced microvascular endothelial damage and attendant increased vascular permeability in vivo 202

10.1 Background 202

10.2 Introduction 202

10.3 Materials and methods 204

10.3.1 Experimental Design 204

10.3.2 Immuno histochemical Analysis 205
10.4 Results
   10.4.1 Albumin extravasation
   10.4.2 Aquaporin-4 (AQP-4)
   10.4.3 Endothelial barrier antigen (EBA)

10.5 Discussion

Chapter 11: Retinal microvascular damage produced by *Clostridium perfringens* type D epsilon toxin 216
   11.1 Introduction
   11.2 Materials and methods
      11.2.1 Experimental Design
      11.2.2 Transmission Electron Microscopy
   11.3 Results
      11.3.1 Albumin Extravasation
   11.4 Discussion
   11.5 Conclusion

Chapter 12 Concluding Discussion 229

ADDENDUM 236
   13.1 Scanning electron microscopy (SEM) of human brain microvascular endothelial cells in vitro exposed to *Clostridium perfringens* type D epsilon toxin 236
   13.2 Materials and methods
      13.2.1 In vitro cell culture
      13.2.2 Epsilon Toxin (ETX)
      13.2.3 Scanning Electron Microscopy
   13.3 Results
   13.4 Discussion

Appendix 248
   14.1 Mechanisms of new blood vessel formation in brain tumours
   14.2 Clinical Diagnosis
<table>
<thead>
<tr>
<th>Section</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>14.3</td>
<td>Histological Descriptions</td>
<td>279</td>
</tr>
<tr>
<td>14.4</td>
<td>Clinical Record Sheet</td>
<td>286</td>
</tr>
<tr>
<td>14.5</td>
<td>Reference List</td>
<td>287</td>
</tr>
</tbody>
</table>
Publications and Presentations

Publications


Wardill HR, **Mander KA**, Van Sebille YZ, Gibson RJ, Logan RM, Bowen JM, Sonis ST (2016) Cytokine-mediated blood brain barrier disruption as a conduit for cancer/chemotherapy-associated neurotoxicity and cognitive dysfunction. *International Journal of Cancer*.


**Mander, KA**, Williams, R., Finnie, JW (2016) Clostridium perfringens type D epsilon toxin produces a rapid and dose-dependent cytotoxic effect on cerebral microvascular endothelial cells in vitro. *Anaerobe (in review)*


transgenic (B6C3-Tg(APPswe, PSEN1dE9)85Dbo/Mmjax) mouse model of Alzheimer’s disease. Journal of Comparative Pathology

Abstracts/Presentations

**Mander, K, Thornton, E, Harford-Wright, E, Blumbergs, P, Vink, R (2014)**
Determining the mechanisms of cancer cell entry in the development of secondary brain tumours; an *in vitro* approach to blood-brain barrier research. *Neurosurgical Research Foundation Annual General Meeting.*

**Mander, K, Thornton, E, Harford-Wright, E, Blumbergs, P, Vink, R (2015)**
Characterising tachykinin NK-1 receptor and caveolin-1 expression in cerebral metastases. *Aust. Society for Neuroscience, 35th Annual meeting.*

**Mander, K, Williams, R, Finnie, J (2016)**
**Acknowledgments**

This PhD project was made possible by the support and expertise of a number of people, and it is with sincere gratitude that I take the opportunity to formally acknowledge and thank them for their contribution.

To my primary supervisor Professor Robert Vink, thank you for the opportunity to undertake this PhD and for the introduction to research as an honours student in your laboratory.

To my co-supervisors, Dr Emma Thornton and Dr Elizabeth Harford-Wright for their unwavering support, encouragement and optimism. This project certainly would not have been possible without your patience and dedication, particularly during the dark days of troubleshooting the world of cell culture. I thank you both for your valuable guidance and treasured friendship.

To Dr John Finnie, not only are you a diligent and curious pathologist, but also a delightful person. Thank you for taking an interest and adopting a student, for the indispensible wisdom, teaching and motivation you have provided, and above all else, your friendship.

In addition, I would like to thank Dr Fiona Bright. It has been an absolute privilege to share this experience with you. Thank you for the countless ‘wine & whines’, the international skype dates, book club recommendations, and infinite selfless carpooling. You’re the right kind to be stuck in the trenches with.

Dr Viythia Katharesan, for always being the voice of reason and my moral compass.
Additionally, I would like to thank the members of the IMVS Centre for Neurological Diseases;

- Sofie, Kathryn and Teresa for their time, attention and technical expertise. It was a pleasure to share lab space and conversation with these wonderful women.
- Professor Peter Blumbergs, for many thought-provoking meetings and neuropathological expertise.
- The one and only, Jim Manavis. Need I say more?

I would also like to acknowledge the assistance, advice and support of fellow members of the lab and research groups;

Associate Professor Corinna Van Den Heuvel, Dr Renée Turner, Dr Frances Corrigan, Dr Anna Leonard, Dr Joshua Burton and Josh Woenig.

Wilhelm Lab (Hungarian Academy of Sciences), in particular, Dr Imola Wilhelm and Dr Csilla Fazakas for a wonderful learning experience in Szeged.

Dr Sanam Mustafa, Dr Karlea Kremer and Dr Hannah Wardil.

To my incredible family and friends, I am eternally grateful for your belief and support. Thank you for your tolerance and eagerness, but most importantly, thank you for knowing when not to ask, “how’s it all going”.

To my brilliant mother, who unwittingly imparted resilience and courage.

To Dad, for teaching me the value of curiosity.

And to my best friend, Julia, for everything else.
## Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABC</td>
<td>ATP-Binding Cassette</td>
</tr>
<tr>
<td>AMT</td>
<td>Absorptive-Mediated Transport</td>
</tr>
<tr>
<td>AQP4</td>
<td>Aquaporin-4</td>
</tr>
<tr>
<td>BBB</td>
<td>Blood-Brain Barrier</td>
</tr>
<tr>
<td>CAV-1</td>
<td>Caveolin-1</td>
</tr>
<tr>
<td>CNS</td>
<td>Central Nervous System</td>
</tr>
<tr>
<td>CSF</td>
<td>Cerebrospinal Fluid</td>
</tr>
<tr>
<td>Da</td>
<td>Dalton</td>
</tr>
<tr>
<td>DAB</td>
<td>3,3’-diaminobenzidine</td>
</tr>
<tr>
<td>DAPI</td>
<td>4’,6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>EBA</td>
<td>Endothelial Barrier Antigen</td>
</tr>
<tr>
<td>EC</td>
<td>Endothelial Cell</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular Matrix</td>
</tr>
<tr>
<td>EEL</td>
<td>External Elastic Lamina</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epithelial Growth Factor Receptor</td>
</tr>
<tr>
<td>EGM</td>
<td>EndoGRO-MV Complete Media</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-Linked Immunosorbent Assay</td>
</tr>
<tr>
<td>ETX</td>
<td>Clostridium perfringens type D</td>
</tr>
<tr>
<td>GBM</td>
<td>Glioblastoma Multiforme</td>
</tr>
<tr>
<td>GLUT1</td>
<td>Glucose Transporter 1</td>
</tr>
<tr>
<td>hCMEC/D3</td>
<td>Human Cerebral Microvascular Endothelial Cells</td>
</tr>
<tr>
<td>HER2</td>
<td>Human Epidermal Growth Factor Receptor 2</td>
</tr>
<tr>
<td>HIF</td>
<td>Hypoxia Inducible Factor</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>Intercellular Adhesion Molecule-1</td>
</tr>
<tr>
<td>ICP</td>
<td>Intracranial Pressure</td>
</tr>
<tr>
<td>IEL</td>
<td>Internal Elastic Lamina</td>
</tr>
<tr>
<td>IL-1</td>
<td>Interleukin-1</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilodalton</td>
</tr>
<tr>
<td>MTT</td>
<td>3-[(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide</td>
</tr>
<tr>
<td>MRI</td>
<td>Magnetic Resonance Imaging</td>
</tr>
<tr>
<td>NHS</td>
<td>Normal Horse Serum</td>
</tr>
<tr>
<td>NK-1R</td>
<td>Tachykinin Receptor 1</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>--------------------------------------</td>
</tr>
<tr>
<td>NSCLC</td>
<td>Non-Small Cell Lung Carcinoma</td>
</tr>
<tr>
<td>PAS</td>
<td>Periodic Acid-Schiff</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffer Solution</td>
</tr>
<tr>
<td>PECAM-1</td>
<td>Platelet Endothelial Cell Adhesion Molecule-1</td>
</tr>
<tr>
<td>PET</td>
<td>Positron Emission Tomography</td>
</tr>
<tr>
<td>Pgp</td>
<td>P-Glycoprotein</td>
</tr>
<tr>
<td>rh</td>
<td>Recombinant Human</td>
</tr>
<tr>
<td>RMT</td>
<td>Receptor-Mediated Transport</td>
</tr>
<tr>
<td>SP</td>
<td>Substance P</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris-Buffered Saline</td>
</tr>
<tr>
<td>TEER</td>
<td>Transendothelial Electrical Resistance</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission Electron Microscope</td>
</tr>
<tr>
<td>TJ</td>
<td>Tight Junction</td>
</tr>
<tr>
<td>VAM-1</td>
<td>Vascular Cell Adhesion Molecule-1</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular Endothelial Growth Factor</td>
</tr>
<tr>
<td>ZO-1</td>
<td>Zona Occludens</td>
</tr>
</tbody>
</table>
**Figures and Tables**

Figure 1.1: Schematic depiction of cerebrovascular specialisation of the BBB. 9
Figure 1.2: Schematic depicting tight junction and adherens junction complex. 11
Figure 1.3: Routes of transport across the BBB. 14
Figure 1.4: Morphological appearance of caveolae structure. 20
Figure 1.5: Caveolae mediated transcytosis. 21
Figure 1.6: Metastatic cascade. 26
Figure 1.7: Stages of extravasation. 29
Figure 1.8: Transmigration assay. 35
Figure 2.1: Transparent PET hanging permeable support membrane. 43
Figure 2.2: Intracarotid artery surgery. 53
Figure 2.3: Animal handling for intraperitoneal injection. 54
Figure 3.1: Metastatic pigmented malignant melanoma. 58
Figure 3.2: Semi-quantitative grading system representative images. 70
Figure 3.3: Melanoma subtypes H&E. 75
Figure 3.4: Normal Morphology. 76
Figure 3.5: Microvessels in cerebral metastatic melanoma. 77
Figure 3.6: Cerebral metastatic melanoma microvessel angioectasia and telanglectasis. 78
Figure 3.7: Cerebral metastatic melanoma ateriolar-sized blood vessels. 79
Figure 3.8: Cerebral metastatic melanoma ectatic venules. 80
Figure 3.9: Cerebral metastatic melanoma blood vessels with markedly tortuous lumina. 82
Figure 3.10: Melanocyte aggregation and infiltration of blood vessels. 84
Figure 3.11: Perivascular cuffing/vasculitis. 85
Figure 3.12: Thrombosis. 86
Figure 3.13: Intra-tumoural haemorrhage. 87
Figure 3.14: Blood vessel invasion by melanoma. 87
Figure 3.15: Intratumoural vessels demonstrate decreased claudin-5 immunoreactivity. 89
Figure 3.16: Glioma. 90
Figure 3.17: Glioblastoma Multiforme (GBM). 91
Figure 4.1: Representative images of SP immunoreactivity in human control tissue. 106
Figure 4.2: Semi-quantitative grading system representative images of melanocyte staining. 107
Figure 4.3: Immunohistochemical analysis of CAV-1. 108
Figure 4.4: Intratumoural vessels of cerebral metastatic melanoma demonstrate reduced Caveolin-1 immunopositivity. 110
Figure 4.5: Intratumoural vessels and melanocytes of cerebral metastatic melanoma are positive for NK-1R. 112
Figure 4.6: Metastatic melanocytes demonstrate NK-1R immunopositivity. 113
Figure 4.7: Immunohistochemical analysis of NK-1R. 113
Figure 4.8: Substance P immunoreactivity. 114
Figure 5.1: Permeable transwell support apparatus. 123
Figure 5.2: hCMEC/D3 human microvascular endothelial cells in vitro.

Figure 5.3: Immunofluorescent staining of hCMEC/D3 cells in culture.

Figure 5.4: Western Blot results demonstrating NK-1R and CAV-1 expression in confluent hCMEC/D3.

Figure 5.5: Transendothelial electrical resistance (TEER) development over 7 days in hCMEC/D3 cell line.

Figure 5.6: Tacer permeability of hCMEC/D3 transwell monolayer.

Figure 5.7: Transmission electron microscopy of hCMEC/D3 in experimental culture conditions.

Figure 6.1: Substance P secretion ELISA.

Figure 6.2: Treatment effects on hCMEC/D3 cell viability.

Figure 6.3: Treatment effects on A375-M1 cell viability.

Figure 6.4: Transmission electron microscopy images of the hCMEC/D3 transwell system for the investigation of A375-M1 migration.

Figure 6.5: A375-M1 melanocyte transmigration across the hCMEC/D3 transwell system at 5 hrs.

Figure 6.6: Treated transmigrated A375-M1 cells show no morphological alterations when compared to control tumour cells (inset).

Figure 7.1: Intracarotid A375-M1 injection.

Figure 8.1: ETX-intoxicated sheep.

Figure 8.2: Bilaterally symmetrical necrotic foci (arrows).

Figure 8.3: Bilaterally symmetrical and haemorrhagic necrotic foci (arrows).

Figure 8.4: Perivascular desposition of abundant extravasated proteinaceous material (P).

Figure 8.5: TEM of an ETX-injured capillary.

Figure 8.6: ETX-induced severe vasogenic cerebral oedema.

Figure 9.1: Untreated hCMEC/D3 cells.

Figure 9.2: ETX-treated hCMEC/D3 cells.

Figure 9.3: ETX-treated hCMEC/D3 cells.

Figure 9.4: Effect of ETX on cell viability.

Figure 9.5: TEM image of untreated hCMEC/D3 cells.

Figure 9.6: TEM image of ETX-treated hCMEC/D3 cells.

Figure 10.1: ETX expose increases blood-brain barrier permeability to albumin.

Figure 10.2: ETX damaged microvascular endothelium.

Figure 10.3: Perivascular pooling of strongly immunostained albumin, with less intense staining of the surrounding neuropil.

Figure 10.4: AQP4 immunoexpression.

Figure 10.5: EBA immunoreactivity in an EXT-treated brain.

Figure 11.1: Non-treated control retina, all layers are compact, there is no discernible albumin extravasation, and microvessels are inconspicuous.

Figure 11.2: In an ETX-treated retina, there is diffuse albumin immunopositivity, microvessels are prominent (arrows) due to strong and diffuse mural albumin immunoreactivity, and there is nuclear pyknosis in the inner nuclear layer (arrow).
Figure 11.3: ETX-Exposed capillary endothelium. 224
Figure 13.1: Scanning Electron Microscopy Images. 241
Figure 13.2: ETX exposed hCMEC/D3 cells. 246
Thesis Format

The format of my thesis includes two main themes and is arranged as follows: a general introduction, five research chapters, a second background chapter, three research chapters, a general discussion and references.

Broadly, my thesis is focused on the, fundamental and important role of the cerebral vasculature in two disease processes, namely cerebral metastatic melanoma and Clostridium perfringens type D epsilon neurotoxicity. The first theme aims to characterise the extent of vascular alteration following metastatic progression in human tissue, giving rise to the first two research chapters (chapter 3 and 4). Together, these chapters form the scope of the remaining research chapters pertaining to the development of reliable in vitro and in vivo models for the study of malignant transmigration of the blood-brain barrier and targeted therapeutic approaches. This is reported in chapters 5, 6 & 7). The second theme relates to the investigation of several vascular features of the neurological disorder produced by Clostridium perfringens type D epsilon toxin (ETX), giving rise to an additional three primary research chapters (chapters 8, 9 and 10).
Abstract

The principal focus of this thesis is the cerebral vasculature and, more specifically, its fundamental and important role in two disease processes, namely cerebral metastatic melanoma and *Clostridium perfringens* type D epsilon neurotoxicity. Firstly, blood vessels are critical for both impeding and facilitating the penetration, colonisation, and spread of metastatic tumours such as melanomas in the brain and, secondly, the microvasculature is the major target of the potent bacterial neurotoxin, *Clostridium perfringens* type D epsilon toxin, which causes a severe, and frequently fatal, naturally-occurring, neurological disorder in domestic livestock and is a potential bioterrorism agent for human populations.

There are important structural and functional differences between blood vessels in the brain and other tissues and the regional distribution is inhomogeneous. These features also have consequences for patterns of disease expression, for example lodgement of tumour emboli. Moreover, the dynamic microvascular interface between blood and brain parenchyma, termed the blood-brain barrier (BBB), differs in important structural detail from capillaries elsewhere and is critical in maintaining homeostasis in the central nervous system.

In Part A of this thesis, the different patterns of neovascularisation in archival, human melanomas metastatic to the brain were characterised, given that
acquisition of a new vascular supply is essential for these neoplasms to survive, proliferate, and disseminate. These new blood vessels are frequently structurally and functionally aberrant and those examined in the metastatic melanoma cohort herein were classified using histological and immunohistochemical techniques. It was also determined whether there was any correlation between vascular subtype and histological category of melanoma, mitotic index, extent of tumour necrosis, and intratumoural haemorrhage.

Since the substance P (SP)/NK-1 receptor (NK-1R) system plays an important role in tumour survival, proliferation, and progression, its distribution was examined immunohistochemically in these metastatic melanomas, both in tumour-associated blood vessels and melanocytes. The NK-1 receptor was expressed by most melanocytes and endothelium in a small subset of tumour blood vessels, but there was no detectable immunoreactivity of the tachykinin peptide, SP, in tumour cells or blood vessels. The distribution of caveolin-1, the main structural component of caveolae, was also examined in these melanomas. Its immunoreexpression was reduced in tumour-associated blood vessels, concordant with increased neoangiogenesis, and CAV-1 was commonly expressed in melanocytes, particularly in cell membranes, reflecting its important role in both tumour progression and suppression.

Since melanomas generally metastasise via the haematogenous route and finally encounter the BBB when they reach the brain, it was decided to
examine the transendothelial migration of melanocytes using *in vitro* and *in vivo* models. In a culture system, the migration of melanocytes from a melanoma cell line across a membrane representing a “blood-brain barrier” was quantified and the manner of their passage across this endothelial barrier examined by light and electron microscopy, the ultrastructural assessment being one of the very few studies of this type conducted to date. In order to examine how melanocytes in the systemic circulation enter the brain, a melanoma cell line was injected into rat carotid arteries and the distribution of melanocytes in the brain assessed at different time intervals post-injection. Unfortunately, very few tumour cells penetrated into the brain parenchyma and this technique proved to be unsatisfactory for examining transendothelial migration of metastatic melanocytes and evaluation of drugs that might impede this process.

In Part B of this thesis, several vascular features of the neurological disorder produced by *Clostridium perfringens* type D epsilon toxin (ETX) were studied. In the principal, and novel, study, the aim was to determine whether ETX produced a direct and damaging effect on cerebral microvascular endothelial cells *in vitro*. While previous histological and ultrastructural studies suggested that the fundamental lesion in this neurotoxicity was ETX-induced microvascular injury, with subsequent BBB breakdown, increased vascular permeability and severe, generalised cerebral vasogenic oedema, the effect of ETX on brain-derived endothelial cells in culture had not been examined. The present study found, for the first time, that EXT produces a dose-
dependent cytopathic effect on cultured human brain microvascular endothelial cells, confirming the importance of microvascular endothelial damage in the pathogenesis of this neurological disorder.

In an animal model of ETX neurotoxicity using Sprague-Dawley rats, extravasation of endogenous albumin was used as a surrogate immunohistochemical marker of increased vascular permeability; loss of endothelial barrier antigen was evaluated after exposure to ETX as it is a marker of an intact BBB in this species; and the role of the major water channel protein in the brain, aquaporin-4, in the development/resolution of EXT-induced cerebral oedema was studied. Since the BBB is a prime target for ETX-induced brain damage and the blood-retinal barrier (BRB) resembles the BBB in many respects, the action of ETX on the BRB was also examined in rats using albumin immunohistochemistry to assess enhanced vascular permeability and electron microscopy to study retinal blood vessels. Retinal microvascular endothelial damage resembled that found in ETX-disrupted BBB and there was widespread retinal oedema as indicated by diffuse albumin extravasation.

Studies carried out in this thesis aimed to better characterise the cerebral microvasculature alterations and the associated mechanisms, in response to two distinct insults; metastatic melanoma, and ETX. A range of investigative modalities facilitated the detailed exploration of vascular reactions in these 2
neuropathological states and findings from this thesis will direct further research in the field of cerebrovascular pathology.
Chapter 1

General Introduction
1 General Introduction

1.1 Background

While cerebral microvascular endothelial cells (EC) are highly labile and respond to a dynamic environment in the brain and variety of potentially injurious insults, the capacity of the mature brain to form new vessels is limited. In contrast, the malignant transformation of a primary or secondary tumour within the brain incites the generation of new vascular supply via a number of means, revealing a delicate interplay between constituents of cerebral vessel walls and their altered surrounding milieu.

The principal focus of this thesis is to study cerebrovascular alterations in response to (1) the colonisation and progression of metastatic melanoma, and (2) Clostridium perfringens type D (ETX) neurotoxicity.

As a prelude, the normal structure of the different components of the vascular system and pathological reactions will be briefly reviewed.

1.1.1 The vascular system in health and disease

The vascular system can be divided into the delivery arm - arteries, the exchange bed - microvessels, and the removal arm - veins and lymphatics.
Arteries may be subdivided into large elastic arteries, small muscular arteries, and arterioles, although there is a gradual transition between these divisions as one vessel type merges into another, with concomitant structural alterations.

The arterial wall can be divided into three layers: tunica intima, tunica media, and tunica adventitia. The intima is composed of endothelial cells (EC), which abut the lumen, subendothelial connective tissue, and the internal elastic lamina (IEL), which is the outer limit of the intima. The media is comprised of concentric layers of smooth muscle cells, elastic fibres and the external elastic lamina (EEL). The adventitia is composed of a meshwork of collagen and elastic fibres that are in continuity with the surrounding connective tissue; the proportion of elastic fibres diminishes as vessel size decreases.

In arterioles, the ratio of mural thickness: lumen is 1:2, with the intima comprised of EC and IEL, the media of circularly arranged smooth muscle, and the adventitia composed of collagen and elastic fibres.

Veins can be categorised as small venules, medium collecting veins and large great veins and all have a large lumen relative to wall (mural) thickness. Venules > 30 µm have an incomplete muscular media and thin adventitia, while medial thickness increases in medium and large veins and the IEL becomes more prominent. The adventitia is the thickest layer in veins, whereas the media predominates in arteries. In venules, the lumen is lined by
EC, deep to which is the basement membrane and a small amount of loose connective tissue. As venules transition into small veins smooth muscle becomes a prominent feature.

The microcirculation is the exchange portion of the vascular system and is represented by vessels < 100 µm in diameter. It includes arterioles, terminal arterioles, capillaries, post-capillary venules, and venules. Capillaries are low pressure vessels ~ 8 µm in diameter, the lumen being lined by 2 EC, which are wrapped in a basement membrane (Abbott et al. 2010). Endothelial cells are connected by tight junction complexes and are the principal component of blood vessel walls thus recognised as a central candidate in all types of vascular pathology.

Endothelial cells form the thromboresistant monolayer lining the vascular system and mechanically isolate the circulating blood from highly thrombogenic subendothelial elements. Endothelial cells also contribute to the modulation of blood flow and vascular reactivity, regulation of inflammation and immunity by producing interleukin-1 (IL-1) and various adhesion molecules, intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), selectins, immunoglobulins, and integrins, to mediate adhesion and emigration of leukocytes, aid in the regulation of cell growth, and engender contractility (Cardoso et al. 2012).
1.1.2 Endothelial injury

Endothelial injury leads to reactive changes of cellular enlargement or hypertrophy, and proliferation or hyperplasia. When ECs are separated from the underlying basement membrane, there is insudation of plasma proteins, into the subendothelial region and resulting development of vascular hyalinosis (Baluk et al. 2003). This pathological change can impede the diffusion of metabolites into the intima and inner media, thus impairing the viability of smooth muscle cells and resulting in degeneration of vessel walls termed fibrinoid necrosis (Cines et al. 1998). Alteration to the EC monolayer therefore results in exposure of circulating blood to subendothelial collagen, a potent inducer of coagulation, platelet aggregation, and thrombosis (Baluk et al. 2003). The resulting thrombosis can lead to ischemia/hypoxia and infarction of tissue supplied by compromised perfusion (Dvorak 2002).

Vasculitis is characterised by the presence of inflammatory cells within and around blood vessel walls. There is concordant mural damage comprised of fibrin deposition, collagen degeneration and necrosis of the EC and smooth muscle cells. Conversion of the wall to an eosinophilic, homogenous, amorphous appearance is termed hyaline degeneration (when PAS-negative) and fibrinoid degeneration (when PAS-positive). Fibrinoid necrosis is associated with endothelial damage resulting in the entry and accumulation of serum proteins followed by fibrin polymerisation in the vessel wall. This manifests as an intensely eosinophilic collar in histological examination which
completely obliterates any cellular detail. The eosinophilic material referred to as “fibrinoid” consists of fibrin, complement, immunoglobulin, platelets and degenerate collagen. Endothelial defects can be repaired to some extent, by proliferation and migration of viable EC at the margins of the denuded area.

1.1.3 Special features of the cerebral vasculature

Anatomically, the blood supply to the brain is derived from the internal carotid and vertebral arteries, which anastomose under the brainstem. The vertebral arteries merge to form the basilar artery which contributes as the 3rd “feeder” to the circle of Willis. These vessels anastomose freely in the pia-arachnoid but, once an artery or arteriole penetrates the brain parenchyma, it tends to become an end-artery, although there are some anastomoses at the capillary level. Cerebral veins, by contrast have abundant and advantageous anastomoses. If a given cerebral blood vessel is occluded, some collateral circulation may develop, but it does not perfuse more than the periphery of the area of supply. Arteries entering the brain parenchyma are relatively small and arise at right angles from parent vessels in the pia-arachnoid; there are abrupt changes in calibre when meningeal vessels divide, providing an entrapment mechanism for tumour and bacterial emboli. Blood vessels that enter the brain are progressively attenuated to capillaries, but many capillaries loop back into the cortex near the grey-white matter (corticomedullary) junction and it is here that circulating tumour cells tend to
lodge and proliferate, predisposed by sudden alterations in vessel flow rate and luminal diameter. Cerebral capillaries are the smallest vessels of human circulation at 3-7µm in diameter (Pardridge 2003).

Cerebral arteries and veins, but not capillaries, have an outer adventitia and perivascular (Virchow-Robin) space, the inner aspect lined by adventitia and the outer by pia mater. Thickening of the adventitia by infiltrating mononuclear cells is a common reaction to cerebral neoplasms, attended by endothelial proliferation.

The muscle coat of intracerebral arteries is thinner than that in extracerebral arteries of similar size, the EEL is lacking, and the adventitia is very thin. Cerebral arterioles and venules are thin-walled, especially venules, the latter being composed mainly of thin layers of fibrous tissue with scant elastin and no muscle. Accordingly, these vessels are prone to injury and haemorrhage, especially white matter venules (Hawkins et al. 2005). Capillary density is higher in grey than white matter and varies from one grey matter region to another. There is a vast degree of heterogeneity across the vessel structures of the brain however cerebral vasculature is seen to be unified by the presence of the highly specialised blood-brain barrier.
1.2 Blood–Brain-Barrier

The BBB represents the tightest endothelial barrier of any organ and in this way is a key component for maintaining cerebral homeostasis by regulating the traffic of both solutes and cells between the peripheral vasculature and brain parenchyma (Wilhelm et al. 2013). The permeability properties of the BBB are those of the capillary endothelium, which differs in important structural detail from endothelia elsewhere. The effectiveness of the BBB depends largely on the presence of tight junctions (TJs), but also on a paucity of micropinocytotic vesicles, and an enhanced basement membrane (Sedlakova et al. 1999). These endothelial characteristics are abetted by the inductive influence of astrocytic end-feet that cover the capillary surface. Traditionally, the BBB was thought to prohibit the passage of molecules and cells in a size dependent manner, for example substances with a molecular weight <500 Daltons could gain entry however, larger molecules were excluded (Abbott et al. 2010, Caffo et al. 2013). It is now understood that this systematic method of regulation is much more complex and as such, the BBB represents a dynamic and vital structure which is now at the forefront of current research for a number of neurological pathologies.

Endothelial cells, astrocytes and pericytes comprise the crucial cellular elements of the BBB, however, additional cell types such as neurons, glial cells and smooth muscle cells of the vascular wall ultimately contribute to the function of the BBB and modulate the composition of the perineuronal fluid in
a highly localised manner. This system is often referred to as the neuro-glial-vascular unit and constitutes a complex signalling system that permits neuronal activity to be signalled to the BBB to regulate blood flow and maintain optimum ionic composition for synaptic and axonal function (Figure 1.1) (Abbott et al. 2006, Abbott et al. 2010, Nag et al. 2011).

![Diagram of cerebrovascular specialisation of the BBB](image)

Figure 1.1: Schematic depiction of cerebrovascular specialisation of the BBB.

(Abbott et al. 2010).

1.2.1 Cerebral endothelial cells

Whilst all ECs exhibit similar morphology – a flat, polygonal shape that elongates in relation to the direction of blood flow, they show significant heterogenic diversity dependent on their location. Cerebral ECs, much like their nonneural counterparts not only express glycoproteins, caveolae,
adhesion molecules and integrin receptors, but also contain specialised circumferential tight junctions which restrict the passage of certain ions and solutes, and completely block others from traversing via this route (Arshad et al. 2010).

1.2.2 Cerebral Endothelial Tight Junction Proteins

Tight junctions are present at the apical end of the interendothelial junction and closely rely on the integrity of the corresponding cadherin junction, which is located at the basolateral region of the interendothelial space (Figure 1.2). Ultrastructural studies of cerebral endothelial cells indicate the circumferential arrangement of the TJ’s provide the barrier formation required to actively restrict the movement of small hydrophilic molecules such as sodium and particular permeability tracers (Ballabh et al. 2004). The major components of the BBB TJ complex are zonula occludans (ZO-1); occludin and claudin, and zonula adherens or cadherins (Nag 2011).
Figure 1.2: Schematic depicting tight junction and adherens junction complex.

(Polakis 2008)

1.2.2.1 Occludin

Occludin (65kDa) represents the first major protein identified within the zonula occludan complex, whereby two occludin molecules interact within the interendothelial space to form a restrictive bond (Furuse et al. 1999, Huber et al. 2001). The expression of occludin has been well documented in both the rodent and adult human brain, with the added distinction of higher expression recognised in brain ECs when compared to non-neuronal tissues, thus giving further weight to its specialised function for the BBB (Wilhelm et al. 2013). Furthermore, increased occludin expression levels correlate with an improved transendothelial electrical resistance (TEER) across the BBB – a known marker of strong BBB integrity, thereby suggesting that the presence of occludin confers low paracellular permeability depending on its phosphorylation status (Liebner et al. 2000, Wolburg et al. 2002, Nag 2011).
Additionally, a loss of occludin expression in the tissue surrounding some brain tumour types is correlated with tumour associated BBB breakdown (Papadopoulos et al. 2001). Finally, Saitou et al (2000) demonstrated occludin knockout mice are capable of forming interendothelial TJ’s with normal morphology and barrier function, despite the absence of this protein, suggesting it may have more of a complementary rather than essential role in barrier function (Saitou et al. 2000).

1.2.2.2 Claudin

The claudin family was first described by Furuse et al. (1998) with subtypes 1 and 2 initially acknowledged as the integral components of TJs (Van Itallie et al. 2006). To date, 24 subtypes (22kDa) have since been identified in both mouse and human tissue, with claudin-1, 2, 3, 5, and 11 localised to human brain ECs (Huber et al. 2001, Ballabh et al. 2004). Claudin-5 is endothelial-specific in the brain and thought to be crucial for BBB function and is currently accepted as the main structural component of intramembrane strands responsible for recruiting occludin (Nag 2011). Claudin-5 knockout animals offer further confirmation demonstrating an increase in paracellular permeability to molecules <800Da, and thus suggesting a primary role in regulating size dependent selective diffusion of small molecules (Nag et al. 2011). Finally, a loss of claudin-1 expression in cerebral vessels is correlated with increased BBB permeability associated with stroke, inflammation and some tumour types (Wilhelm et al. 2013).
1.2.2.3 Junctional adhesion molecules (JAMs)

Junctional adhesion molecules (40kDa) are a separate group belonging to the immunoglobulin superfamily and are involved in cell-cell or cell-ECM binding (Jia et al. 2013). Human brain endothelial cells express JAM-1, JAM-3, and JAM-C, with JAM-1 known to play an important role in TJ regulation and assembly (Abbott et al. 2010). As such, the arrangement and status of JAMs will directly influence cellular permeability and resistance to macromolecules to that shown in a number of physiological and pathological events (Liu et al. 2000). Specifically, JAM-1 has been implicated in the extravasation of leukocytes during inflammatory conditions, particularly in the process of adhesion and transmigration through the BBB, angiogenesis and platelet activation (Nag 2011, Jia et al. 2013). As such, it has been suggested that metastatic cells may exploit a similar interaction as described for migrating leukocytes during transmigration via the adhesion molecules of the JAMs (Ballabh et al. 2004).

1.2.3 Transport across the blood-brain barrier

The movement of substances and cells across the BBB occurs via two main routes; the transcellular passage within cells or via lateral movement between cells, the latter termed paracellular transport (Figure 1.3) (Wilhelm et al. 2013).
Figure 1.3: Routes of transport across the BBB.

(a) Passive diffusion through the cell membrane (b) Active efflux carriers (ABC transporters) (c) Carrier mediated influx via solute carriers determined by the substrate concentration gradient (d) RMT requires receptor binding of ligand and can transport a variety of macromolecules such as peptides and proteins across the cerebral endothelium (transcytosis). AMT is non-specific and transports positively charged macromolecules across the endothelium. (e) Leukocytes cross the BBB either by a process of diapedesis through the endothelial cells or via modified tight junctions (Abbott et al. 2010).

The continuous cell membrane established by the TJ barrier of the ECs effectively limits paracellular diffusion, therefore polar nutrients such as glucose and amino acids remain isolated from the brain (Banks 1999). In this way, cerebral ECs are highly specialised and contain a number of membrane transporters that enable the bidirectional transcellular passage of essential molecules and the crucial efflux of potentially neurotoxic substances and waste products (Cecchelli et al. 2007). Known transporters exist for a variety of molecules, such as amino acids, glucose, insulin, ferritin, micronutrients, electrolytes, hormones and peptides. These transporters use a series of different mechanisms including carrier-mediated transport, ion pumps, low density lipoprotein receptors and receptor mediated transcytosis (Luissint et al. 2012). The characteristic polarized arrangement of ECs creates a distinct luminal and abluminal membrane and, therefore the specific expression of
transporters at either aspect of the cell membrane can be seen to direct the movement of certain molecules and ions (Daneman 2012). Blood-brain barrier transporters work in the direction of either blood-to-brain or brain-to-blood however some may operate more efficiently in one direction depending on the molecule being transported (Banks 2010). The efficacy of transmembrane movement, more specifically, will be influenced by molecular weight, charge and lipid solubility, with uni-directional systems known to be energy dependent in contrast to ATP-absent bi-directional transport (Banks 2010). The majority of these transporter proteins are polarized in their expression either at the luminal or abluminal membrane, thereby facilitating preferential unidirectional flux of certain nutrients either from blood to brain or vice versa. For example, glucose will utilise the presence of glucose transporter-1 (GLUT1-) carriers localised both luminally and abluminally at the BBB, while transport originating from the blood to brain is recognized as the dominant direction (Abbott et al. 2010).

The transport of larger molecules such as proteins, peptides and viruses will likely employ vesicular dependent, or transcytotic mechanisms to cross the restricted BBB and enter the CNS unaltered. Such vesicular transport mechanisms include podocytosis, clatherin-dependent, caveolae and adsorptive endocytosis and fall under two broad categories of either receptor-mediated (RMT) or absorptive-mediated (AMT) transcytosis (Banks 2010). Receptor-mediated transport denotes the binding of macromolecular ligands to defined receptors on the EC surface in order to induce endocytotic
activity. This is followed by the formation of a cavelous, which facilitates the establishment of a vesicle ready to transport the contents across the cytoplasm to be exocytosed at the opposite cell membrane. Conversely, AMT relies on a positive charge on the molecule to facilitate an interaction with the cell surface binding site and induce endocytosis and subsequent transcytosis (Abbott et al. 2010). For both mechanisms, the transporting vesicle is routed away from lysosomal degradation which achieves the unique process for delivery of the intact protein or peptide. This appears to be a specialised process occurring only within the BBB and is not widely demonstrated within the peripheral endothelia where the contents of an endocytotic vesicle are more readily directed to acidic lysosomes to undergo enzymatic degradation (Tuma et al. 2003).

1.2.4 Cellular Transcytosis

Transcytosis is a process used by many cell types as a means of transporting macromolecules within a membrane-bound carrier from one side of a cell to the other (Tuma et al. 2003). It is a specialised process which enables the selective movement of material between two environments, for example blood and brain, and importantly, allows for the trafficking of substances without instigating disruption to the differing compositions of the two locations (Broadwell 1993).

The study of transcytosis across ECs demonstrates that movement of substances occurs in both directions - apical to basolateral or vice versa and
is dependent on the substance in transport (Tuma et al. 2003). Variation in transcytotic mechanisms across a number of organs indicate that multiple processes have evolved specific to the cellular needs of a particular tissue type, for example, intestinal cells exhibit receptor-mediated internalisation of specific cargo which is then segregated intracellularly (Tuma et al. 2003). Intestinal cells, for example, exhibit receptor-mediated internalisation of a specific cargo, which is then segregated intracellularly as it reaches its destination (21). Conversely, transcytotic transport within endothelial cells of blood capillaries utilises a direct route to traverse the cell and, in doing so, circumvents degradation typical of the commonly described endocytotic pathway (Caffo et al. 2013).

1.2.5 Transcytosis in the Brain

Cerebral endothelial cells are equipped with an endocytic system, including clatherin-coated vesicles, caveolae, endosomes, and lysosomes (Banks 1999). Specific receptors localised on the plasma membrane of ECs are said to be responsible for distinguishing between substances directed to transcytosis versus degradation via endocytosis, particularly, the point of entry is known to determine the processing of the substance (Tuma et al. 2003).

The brain exhibits a tightly regulated environment in order to maintain required chemical parameters (Banks 1999). Cerebral capillary endothelium and epithelial cells of the choroid plexus are specialised for the passage of
substances from the blood (Ermisch et al. 1985). The choroid plexus supplies nutrients that are required in lower quantities and less frequently, such as folate and other vitamins, whereas ECs move nutrients such as glucose and amino acids, which are rapidly required in high concentration. Importantly, the observation that certain macromolecules will traverse from blood to the cerebral environment via capillary ECs, but not across the epithelial cells of the choroid plexus confirm the localisation of specific receptors and transport systems (Tuma et al. 2003). Recently, human melanotransferrin, a cell-surface glycoprotein localized to human melanoma cells was demonstrated to cross brain ECs in vivo and accumulate in mouse brain at high quantity without altering the integrity of the BBB, and at a higher rate compared with the transport of transferrin or albumin (Tang et al. 2007). The molecular events underlying the transport of melanotransferrin across the BBB remain unclear (Demeule et al. 2002). One potential avenue yet to be explored may involve endothelial caveolae ‘pits’. Caveolae are recognised vesicular transport systems which have been shown to mediate transcytosis in cerebral ECs (Tang et al. 2007).

1.3 Caveolae

The cellular biology of caveolae is now a growing area of research despite the first identification of caveolae microdomains almost 60 years ago (Bastiani et al. 2010). Recently, focus has been directed to the formation, regulation, and function of caveolae in different cell types and, particularly, their role in a
number of human diseases, including cancer and diabetes (Frank et al. 2003). It is widely understood that the physiological action of caveolae will vary depending on the cell type and organ system in question (Bastiani et al. 2010). As such caveolae have been implicated in a number of cellular processes, including endocytosis, transcytosis/trafficking, compartmentalisation of signalling molecules, cholesterol and lipid metabolism and mechanosensation (Anderson 1998).

Caveolae are flask-shaped pits present on, and continuous with, the apical and basal plasma membranes of all ECs (Figure 1.4) (Couet et al. 2001). They have a mean diameter of approximately 70nm which can open to both the luminal and abluminal plasma membrane through a neck of 10-40nm in diameter (Nag 2011). Intracerebral vessels have been shown to contain a mean of 5 caveolae/μm² in arteriolar and capillary endothelium and as such, cerebral endothelium is shown to contain 14-fold fewer vesicles as compared with endothelium of non-neural vessels (Nag 2011). Interestingly, capillaries of circumventricular organs such as the area postrema, where the BBB is absent, are reported to exhibit significantly higher numbers of endothelial caveolae (Nag 2011).
For many years, caveolae were considered to predominantly serve as an endocytotic device. However, more recently, it has been confirmed that these structures constitute an entire membrane system enabling the formation of unique endocytotic and exocytotic compartments (Anderson 1998). During transcytosis, caveolae “pinch off” from the plasma membrane to form vesicular carriers that rapidly and efficiently shuttle to the opposite membrane of ECs, fuse and release their contents via exocytosis (Figure 1.5) (Predescu et al. 2007).
Figure 1.5: Caveolae mediated transcytosis.

Caveolae mediated transcytosis demonstrating process of budding from the plasma membrane, followed by vesiculation of caveolae from plasma membrane as a means of transporting contents across endothelial cell (Predescu et al. 2007).

The identification of three essential caveolin proteins as key structural components of caveolae, and the subsequent generation of knockout mice further clarified the contribution of caveolae in a range of physiological processes (Hansen et al. 2010).

1.3.1 Structure and Function of Caveolae

Caveolae located within ECs have a striated coat which is comprised of the three integral membrane proteins; caveolin-1, 2 and 3 (Anderson 1998). Caveolin-1 (CAV-1) was the first and most abundant protein to be identified as a prominent resident of caveolae and is considered to be the crucial component necessary for the unique formation of caveolae in BBB endothelium (Hansen et al. 2010). Caveolin-1 is formed by two isoforms α and β, with α being the most highly expressed within the brain (Scherer et al. 1994,
Lisanti et al. 1995, Ikezu et al. 1998a, Ikezu et al. 1998b). Caveolin-2 (CAV-2) has been suggested to play a role in the formation of invaginations, but evidence suggests that the characteristic flask-like morphological appearance of caveolae is conserved only in cells expressing CAV-1. Accordingly, the role of CAV-2 remains inconclusive (Bastiani et al. 2010). Caveolin-3 knockout mice confirm a role for this protein in endocytosis, budding and intracellular trafficking of vesicles (McMahon et al. 2009).

In addition to the crucial caveolin proteins, a number of signalling molecules are localised on the caveolae, including specific receptors (Sowa 2012). Receptor tyrosine kinase, G-protein coupled receptors, transforming growth factor-beta (TGF-β) type 1 and 2, certain steroid receptors, G-proteins of low molecular weight and enzymes have a confirmed presence within endothelial caveolae and, as such, may play a role in facilitating macromolecular transcytosis (Cameron et al. 1997). Of particular note, tachykinin NK-1 receptor associated with the potent neuropeptide substance P is localised within endothelial caveolae (D'Alessio et al. 2005). While it is well established that both classic and neurogenic inflammatory mediators play a role in compromised barrier function, it remains unclear whether the localisation of these specific receptors within the caveolae pits implicate caveolae in the development of barrier changes. If proven, caveolae offer an alternative target in understanding and manipulating barrier biology. Caveolae have been shown to mediate the transport of molecules such as albumin, iron-transferrin, insulin, low-density lipoproteins, epidermal growth
factor, leptin, tumour necrosis factor (TNF) and a range of additional cytokines and chemokines through the specific localisation of the aforementioned corresponding receptors within caveolae (Ge et al. 2008, Sowa 2012).

1.3.2 Caveolae and cancer

Understanding the role of caveolae in malignancy is in its infancy (Lloyd et al. 2011). *In vitro* studies investigating the CAV-1 protein suggest a dual role in tumour promotion and suppression, depending on cell and cancer type (Burgermeister et al. 2008).

A number of malignant neoplasms demonstrate a reduction in CAV-1 expression which subsequently leads to downstream displacement of the caveolae structure (Goetz et al. 2008, Quest et al. 2008, Shatz et al. 2008, Freeman et al. 2012, Mercier et al. 2012). Such alterations correlate with increased cell proliferation in primary prostate and breast cancer, suggesting that a loss of CAV-1 may be a crucial component in the development of cancer (Gould et al. 2010). In contrast, Thompson et al (2010) demonstrated that elevated levels of CAV-1 correlate with metastatic progression of prostate cancer, but the precise mechanisms remain to be elucidated (Thompson et al. 2010). Similarly, Goetz et al. (2008) demonstrated increased CAV-1 expression localised to metastatic tumour cells of primary prostate origin, despite there being no elevated expression at the primary site (Goetz
et al. 2008). Nevertheless, the role of CAV-1 in cancer progression is dependent on tumour type and stage and may have an integral role in regulating crucial cell signalling pathways which underpin tumour progression or suppression. Taken together, the role of CAV-1 in tumour cell growth, migration or suppression requires further investigation.

1.4 Pathobiology

It has been proposed that six fundamental elements in cellular physiology are required to initiate tumour growth and enable metastatic dissemination (Hanahan et al. 2011). Cellular events such as unlimited replication potential, self-sufficient growth signals, insensitivity to growth inhibitors, evasion of programmed cell death, establishment of a new blood supply, and the capacity for tissue invasion and cancer spread lead the discussion about the central characteristics of cancer (Hanahan et al. 2011).

Cellular growth and division under normal physiological conditions are directed by a number of specific proteins which regulate signals via the promotion and suppression of certain genes throughout the cell cycle. These, in turn, maintain normal cellular homeostasis (Kumar V 2009). However, in the case of malignant tumours, these regulatory genes are vulnerable to hijacking. An accumulation of mutations in genes encoding for regulatory proteins compromise the homeostatic balance and enable cancer cells to assume control of their own growth and development, culminating in
unlimited replication (Hanahan et al. 2011). Among these genetic alterations, a small number of malignant cells may acquire the ability to deviate from the original site, breach tissue boundaries and invade, relocate to distant sites and ultimately contribute to the establishment of secondary sites of malignancy (Hanahan et al. 2000, Rahmathulla et al. 2012). This complex multifactorial process of invasion and metastasis is not well understood and is recognised as the ‘last great frontier for exploratory cancer research’ (Hanahan et al. 2011).

1.4.1 The Metastatic Process

The process of metastasis is multifaceted, but is broadly conceived in two main phases. Firstly, the initial migration of tumour cells from the primary tumour (dissemination) followed by the secondary colonisation of the new location (Rahmathulla et al. 2012). Metastasis is heavily dependent on characteristics of the original tumour site and the new location, and will develop as a series of highly selective sequential events (Caffo et al. 2013). The cells from brain metastases show a slower growth rate and exhibit lower metastatic potential than cells from visceral metastases, suggesting once again, a unique interplay is required to support and develop metastasis in the brain (Goulart et al. 2011). The relationship between the tumour cell and cerebral microenvironment was first described in 1889 by Paget as the ‘seed and the soil’ principle (Postovit et al. 2006). Recently three microenvironments have been identified within the brain to actively contribute to metastatic
colonization; the perivascular niche, the brain parenchyma and the cerebrospinal fluid (Steeg et al. 2011).

Genetically, heterogeneous cancer cells within a primary tumour have a heightened potential to metastasize due to their mutagenic differentiation. This capacity develops via the well-described metastatic cascade (Eichler et al. 2011). Initial genetic mutations enable tumour cells to enter a proliferative phase, which sees the establishment of a ‘fit’ colony of cells that are more readily able to acquire the genetic alterations that facilitate carcinogenesis and spread (Caffo et al. 2013) (Figure 1.6).

![Diagram of the metastatic cascade]

Figure 1.6: Metastatic cascade.

Metastatic cascade represented schematically dictating the events involved in progression of metastasis from the normal cell acquiring DNA mutation, altered neoplastic growth, clonal expansion coupled with angiogenesis. Tumour cells enter circulation and travel to and colonize distant organs, and their subsequent growth at the secondary sites constitutes metastases (Francia et al. 2011, Faltas 2012).
1.4.2 Dissemination

Metastatic cancer cells initially spread from the primary site through the local degradation of the primary basement membrane (BM) (Fein et al. 2013). Expansion of the tumour tends to compress the surrounding ECM, leading to a pathological change in lymphatic and blood vessel flow (Fokas et al. 2013). Further changes to the BM, and molecular and cellular alterations such as a critical loss of intercellular ‘glue’ proteins (E-cadherins), facilitate cancer dissemination (Rahmathulla et al. 2012). Similarly, tumour cells secrete proteolytic enzymes, such as metalloproteinases, which facilitate cleavage of epithelial and vascular basement membrane collagen components and release autocrine motility factors to further enhance tumour advancement (Nguyen et al. 2009). Furthermore, compression forces instigated by the developing tumour can pathologically alter cytokine and mediator action known to drive cell signalling (Kumar et al. 2009). Cleavage products of the ECM such as collagen and laminin have been shown to act as chemo-attractants for tumour cells and enhance their progression through the damaged membrane and zones of proteolysis (Kumar V 2009). While it is evident that a myriad of interrelated factors are at play during the initial transformation of a primary tumour, their relative involvement and time-points of action remain unclear. Once a cancer cell has traversed the ECM and microenvironment of the primary organ, it is in a position to gain entry to the vasculature or lymphatic system.
Tumour cells undergo additional morphological changes such as cytoskeletal reinforcement and increased adherence ability in response to altered signalling pathways, mechanical forces, high sheer forces and stress patterns encountered upon exiting the initial microenvironment (Kumar et al. 2009). Initial arrest of the tumour cells within capillary beds will result from mechanical entrapment due to size discrepancy between tumour cells and the diameter of vessels; the relative contributions of these two elements will vary depending tumour cell type and target organ (Steeg et al. 2008). Tumour cells reside within the vasculature and grow along pre-existing vessels using them as scaffolding for the spread of metastasis to the new location in a blood-supply dependent manner (Nguyen et al. 2009). Furthermore, elements within the vasculature such as protein CD82 are involved in facilitating tumour cell adherence to endothelium and encouraging senescence. Loss of CD82 has also been associated with metastatic spread (Nguyen et al. 2009). Additionally, circulating tumour cells are known to enlist the assistance of platelets as a protectant against stressors within the vasculature and in the evasion of an immune response (Bambace et al. 2011, Gay et al. 2011).

Once a tumour cell has negotiated the vasculature and made contact with the secondary target tissue, it is required to adhere and once again penetrate the endothelial barrier (Kumar et al. 2009). Endothelial cell retraction is a common feature associated with tumour cell extravasation whereby tumour cells induce withdrawal via the release of bio-active lipids such as arachidonic acid metabolites. Alternatively, the release of such
substances may also instigate EC injury or death which in turn may facilitate tumour cell entry through the induction of endothelial gaps (Steeg et al. 2008, Steeg et al. 2011). In this way, products of the microenvironment such as resident and transient growth factors, chemokines, cytokines and proteases are shown to facilitate crucial metastatic processes such as ECM alteration and angiogenesis at the secondary site (Steeg et al. 2008).

1.4.3 Colonisation

Colonisation is understood to occur via two pathways. The first is mediated by cellular diapedesis, extravasation and proliferation (Figure 1.7), whereas the second involves tumour cell accumulation and subsequent proliferation and angiogenesis (Rahmathulla et al. 2012).

Figure 1.7: Stages of extravasation.

Schematic demonstrating the main stages of extravasation beginning with transient rolling interactions of tumour cells with endothelial cells lining the vasculature, followed by adhesion and lateral migration across endothelium and culminating in tumour cell diapedesis (Carman 2009).
Often this process is discussed in relation to the better-characterised technique of leukocyte adhesion and extravasation in inflammatory conditions (Entschladen et al. 2004). However, there are fundamental differences between mechanisms underlying tumour cell extravasation and leukocyte migration. For example, the diapedesis of leukocytes is a biologically driven process which enables leukocytes to transmigrate through endothelium without cellular disruption and within minutes of activation. In contrast, tumour cells are shown to migrate over a period of days, suggesting a more laborious and complex process is required for the specialised migration of tumour cells (Steeg et al. 2008).

Regardless of their mode of invasion, tumour cells as a rule follow one of three processes once they have gained entry to the secondary tissue; cell dormancy, cell death, or colonization via proliferation (Steeg et al. 2008). The composition of the target tissue will largely determine which pathway will dominate and hence, whether metastatic colonisation will be successful.

1.4.4 Tumour associated angiogenesis and barrier permeability

Once established, tumours must acquire and maintain a reliable blood supply to facilitate further growth. The ability to acquire a new vascular supply is widely researched, but is still incompletely understood. Moreover, it is now evident that cerebral tumours use a number of mechanisms to form new blood vessels and circumvent anti-angiogenic therapies; angiogenesis,
vasculogenesis, co-option, intussusception and vasculogenic mimicry (Eichler et al. 2011), although these blood vessels are often structurally and functionally abnormal. The pattern of neovascularisation is largely driven by intra-tumoral hypoxia and tightly regulated by pro- and anti-angiogenic growth factors.

The early stages of angiogenesis are facilitated by the tightly regulated balance between inducers and inhibitors of angiogenesis. When the former predominate, there is BM degradation of the ECs and surrounding ECM, followed by the directed migration of ECs into the surrounding tissue in response to angiogenic stimuli (Caffo et al. 2013). While a number of molecules have been shown to act as angiogenic inducers, including SP, TGF-α and β, TNFα, and interleukin-8 (IL-8), vascular endothelial growth factor (VEGF) and angiopoietin factors contribute a central role (Xie et al. 2011).

Substance P is a potent neuropeptide known to stimulate endothelial cell proliferation and mediate vasodilation and vascular permeability through binding preferentially to the NK-1R. Application of an NK-1R agonist has been shown to enhance tumour neoangiogenesis and growth, while administration of an NK-1R antagonist arrested tumour growth (Harford-Wright et al. 2013, Munoz et al. 2013). It is suggested that an increased level of NK-1R expression will support and drive angiogenesis, favouring rapid growth and metastasis of highly malignant tumour types (Arshad et al. 2010). As a result, patients overexpressing NK-1R are expected to be more susceptible to SP-mediated
vascular changes (Harford-Wright et al. 2013, Munoz et al. 2013, Munoz et al. 2014).

New tumour-associated blood vessels tend to be immature, structurally disorganised and functionally compromised. This has implications not only for the migration of tumour cells, but also for the unregulated movement of blood-borne components via increased vascular permeability, leading to common neurological complications such as peritumoural oedema and raised ICP. Evaluation of BBB status in the presence of human cerebral metastases and malignant gliomas via MRI demonstrate a compromised BBB, depending on the specific tumour type (Nduom et al. 2013). Findings from experimental animal models confirm that BBB integrity in cerebral neoplasms is complex and can fluctuate depending on the size, type and location of the tumour (Hasegawa et al. 1983). However, small metastatic tumours, as well as those exhibiting a diffuse growth pattern, have been shown to maintain an intact BBB, suggesting differences in BBB stability between some cerebral metastases and primary CNS neoplasms (Harford-Wright et al. 2011). Indeed, the relationship between BBB permeability and metastases has been further explored in a number of experimental models which indicate a correlation between tumour growth and changes to BBB integrity (Chen et al. 2012).

Despite BBB disruption in late-stage tumour growth, it appears that, during early metastatic tumourgenesis, invading tumour cells have a discrete effect on BBB integrity. The BBB may temporarily ‘open’ to metastatic cells and then
reconstitute itself after tumour cell entry (Kang 2005, Harford-Wright et al. 2011). Furthermore, the BBB may enhance angiogenesis, growth and the proliferation of tumour cells through local endothelial cell release of various cytokines; TNF–α, IL-1, and IL-6 in response to the tumour-induced altered biochemical environment (Banks et al. 1995).

1.5 Modeling Cerebrovascular Response to Metastatic Melanoma

In vitro and in vivo models have been invaluable in replicating and elucidating the genetic, molecular and physiological processes governing pathological progression of metastatic tumours. They also permit screening of potential therapeutic interventions (Tuma et al. 2003).

Due to the complex and multistep process known to dictate the establishment of cerebral metastasis, it is useful to study these processes in a controlled and replicable in vitro system, such as the triple cell culture model of an artificial in vitro BBB (Geldenhuys et al. 2012). However, it is recognised that there are limitations in extrapolating in vitro results to human oncology and they need to be validated with in vivo experimentation (Myklebust et al. 1994).

Traditionally, rodent and murine models are the mainstay for brain tumour and associated vascular research, for example using genetically engineered
mice and immune system modulation, the latter permitting the inoculation of human cell lines to better represent the human oncogenesis.

1.5.1 *In-Vitro* Blood-Brain Barrier Model

Many *in vitro* models have been developed, most notably, co-culture models, which use rodent, bovine, porcine or human-derived primary cells to establish an endothelial monolayer representing the BBB. Human tissue presents the most reliable and optimal model, but ethical consideration and availability of viable samples are limiting factors. Thus, rodent tissue is commonly used for primary endothelial cells, especially as bovine and porcine cells are more costly and produce a lower yield. Rat-derived brain ECs have been shown to have comparative permeability values (TEER) to porcine and human tissue, as well as being well-characterised in terms of antibody use (Saxena et al. 2013).

An *in vitro* model of the BBB must demonstrate specific barrier properties of low paracellular permeability, functional transporters, and expression of junctional properties. The most common BBB model is the Transwell apparatus or Boyden Chamber (Figure 1.8) (Naik et al. 2012). It is a microporous semipermeable membrane that separates a luminal (vascular) and abluminal (parenchymal) compartment. It was developed as a simplified model of the BBB, having a monolayer of highly specialised brain microvascular ECs. Endothelial cells from various sources (bovine, rodent, porcine, non-human primate and human) can be grown to confluence on the upper (luminal)
surface of the membrane, immersed in their specific growth media. Although
a number of BBB co-culture models have been developed in recent years,
the combination of ECs and astrocytes (or astrocytic conditioned media) is
sufficient to replicate the BBB in terms of structure and permeability function
(Naik et al. 2012).

Figure 1.8: Transmigration assay.

Transmigration assay using the Boyden Chamber method (Reymond et al. 2013).

Reliable in vitro models that closely mimic the human BBB microenvironment
are essential to understand the cellular/molecular basis of brain microvessel
endothelial physiology, thus facilitating the identification and characterisation
of BBB regulatory mechanisms involved in pathological changes.

Recently, an immortalized brain microvascular endothelial cell line,
hCMEC/D3, was derived from isolated human primary BBB ECs by lentiviral
vector-mediated co-expression of human telomerase and SV40 T antigen
(Weksler et al. 2005). This stable cell line exhibits robust proliferation, while
retaining the morphological and known biochemical phenotype of
differentiated human BBB ECs over many passages (Weksler et al. 2005). As
such, this cell line has been extensively characterised for its utility as a model
of human BBB for CNS drug delivery and translational neurovascular research focusing on BBB function (Sajja et al. 2014).

1.5.2 In-Vivo models

1.5.2.1 Intra-carotid Innoculation

Haematogenous spread of tumour cells provides the most common route of entry for metastasis to the brain (Nguyen et al. 2009). Accordingly, injection of tumour cells into the internal carotid artery has been suggested to mimic tumour cell spread to, and colonisation of, the brain. As such, the final stages of metastases, that is extravasation and colonisation are able to adequately be assessed. The internal carotid model is documented to produce secondary brain metastasis, as the first capillary bed tumour cells will encounter upon injection is located within the brain. Despite this, the model is known to be challenging to perform, limited in tumour yield, producing variable and often disappointing results, and highly dependent on the cell lines used.

1.6 Conclusion and Aims

There are important structural and functional differences between blood vessels in the brain and other tissues and the regional distribution is inhomogeneous. These features also have consequences for patterns of disease expression, for example lodgement of tumour emboli. Moreover, the
dynamic microvascular interface between blood and brain parenchyma, termed the BBB, differs in important structural detail from capillaries elsewhere and is critical in maintaining homeostasis in the central nervous system.

Current understanding of the pathological compromise of the barrier leading to permeability alterations remains unclear, and as such a number of targeted therapeutic options are largely ineffective. Notwithstanding, the role, regulation and activation of endothelial caveolae within the brain remains an underappreciated contributor to permeability regulation. Given the identified location of NK-1R within the caveolae apparatus, and NK-1R ligand SP's known role in vascular alteration and barrier permeability associated with cerebral tumour development, this relationship needs to be investigated. The current thesis hypothesises that caveolae may provide a novel adjunct target for BBB alteration associated pathologies. As such, the broad aims of Part A of this thesis are to:

1. Define the vascular patterns in a cohort of human metastatic melanoma to the brain

2. Investigate the contribution of NK-1R and CAV-1 to establishment and dissemination of metastatic melanomas in the brain

3. Establish an experimental in vitro and in vivo model of the human BBB to study microvascular endothelial transmigration of human melanocytes.
Chapter 2

Materials & Methods
2 Chapter 2: Materials and Methods

2.1 General

For the investigation of cerebrovascular changes resulting from metastatic melanoma, archived human surgical tissue was utilised. All animal experimental work was performed on male, Balb/c nude mice, aged 10-12 weeks, with a minimal pre-surgical weight of 20g due to the invasive nature of the procedure. For the epsilon toxin (ETX) studies in Part B of this thesis, Sprague-Dawley rats were used at a weight range of 270-350g. All experiments were performed under the NH&MRC guidelines and approved by the Royal Adelaide Hospital (RAH) human ethics committee (RAH-140308), animal ethics committee of the University of Adelaide (M-2014-140) and SA Pathology/CALHN (43-13; 26-14). Archived human surgical brain tumour tissue was accessed via the SA Pathology, Centre for Neurological Disease and the RAH.

2.2 Experimental Procedures

2.2.1 Human Tissue

Cases were selected based on a clinical diagnosis performed by a neuropathologist. Tumour samples were then cut into 5 µm sections for immunohistological assessment. Sections were initially de-waxed in xylene and dehydrated through two washes of 100% ethanol. Non-specific
peroxidase activity was quenched by immersion of sections in methanol containing 0.05% H$_2$O$_2$ for 30 mins. Slides were then washed twice in PBS (three mins) before submersion in appropriate retrieval buffer at 96°C for 10 mins and allowed to cool to below 50°C before washing in PBS (twice for three mins) and incubated with 3% NHS/PBS for 30 mins in a humidified chamber. Slides were then incubated overnight in the primary antibody serum diluted in NHS at room temperature. On day two, slides were washed with PBS (twice for three mins) followed by 30 min incubation with a biotinylated immunoglobulin (1:250), before a 60 min incubation with streptavidin peroxidase conjugate (SPC) (1:1000). A further two PBS washes (three mins) were completed and the immunocomplex was visualised with DAB containing 0.01% H$_2$O$_2$ for six9 mins. Sections were then washed in water and counterstained with haematoxylin. Slides were allowed to dry overnight and viewed under light microscopy or scanned and visualised with the “NanoZoomer” digital slide scanner.

2.2.2 In Vitro Experiments: Cell Culture

All culture procedures were carried out under aseptic conditions within a routinely sterilised laminar flow hood. Equipment and solutions used were commercially purchased sterile, sterile filtered, or autoclaved. Cell cultures were maintained at 37°C with 5% CO$_2$ and routinely passaged when culture monolayers reached approximately 80% confluence at subculture ratios between 1:3 and 1:6 in fresh growth medium.
2.2.3 A-375-MA1 Human Melanoma Cell line

Human metastatic melanoma cell line (A-375-MA1, ATCC #CRL-3222) was derived using in vivo selection of highly metastatic cells from A-375 melanoma cell line, (ATCC #CRL-1619) obtained from the skin of a 54 year old female patient with malignant melanoma. Thus, the A-375-M1 cell line was used throughout this thesis due to its highly metastatic properties. Cryopreserved cells were recovered via rapid thawing in a 37°C water bath and subsequent suspension in complete culture media containing Dulbecco’s modified Eagle’s medium (DMEM) (Sigma-Aldrich, NSW, Australia; #D6546), with 5% fetal bovine serum (FBS) (Sigma-Aldrich, NSW, Australia; 12003C), and 1% penicillin and streptomycin (Sigma-Aldrich, NSW, Australia; 10,000 units penicillin and 10mg of streptomyocin/mL, P4333). The contents then underwent centrifugation at 300 G for five mins before resuspension in complete culture medium and transfer to 75cm² tissue culture flask (Corning Life Sciences, MA, USA; #430725U) for expansion.

2.2.4 Labelling A-375-MA1 for transmigration assay

For visualisation of transmigrated tumour cells, the cells were loaded with a fluorescent CellTracker™ Green CMFDA (ThermoFisher Scientific, Victoria, Australia; #C2925). The cancer cells were incubated with the compound at 10µM in serum free media for 45 mins at 37°C. The media was then removed and replaced with complete media for 45 mins before experimental use.
2.2.5 Blood-Brain Barrier hCMEC/D3 cell line

Cryopreserved hCMEC/D3 cells (passage 1-28) derived from human temporal lobe microvessels were commercially obtained from Merck Millipore (Massachusetts, USA, #SCC066). Cells were thawed in a 37°C water bath. The contents of the vial were then resuspended in complete media for centrifugation at 300 G for three min to generate a pellet. Supernatant containing cryopreservative was discarded and resulting pellet resuspended in complete culture media. The cell suspension was then transferred to a Collagen-Type 1 coated 75cm² tissue culture flask (Corning Life Sciences, MA, USA #430725U) with 10mL of EndoGRO-MV complete media (Merck Millipore #SCME004) supplemented with 1ng/mL of Fibroblast Growth Factor-basic (Merck Millipore #GF003). EndoGRO-MV complete media (EGM) consisted of basal medium, EndoGRO-LS Supplement 0.2%, rh EGF5 ng/mL, L-GLutamine 10 mM, Hydrocortisone Hemisuccinate 1.0 μg/mL, Heparin Sulfate 0.75 U/mL, Ascorbic Acid 50 μg/mL and FBS 5% (Merck Millipore).

2.2.6 Transwell Assay

Experimental cultures were routinely passaged when culture monolayers reached approximately 80% confluence at subculture ratios between 1:3 and 1:6 in fresh growth medium. Cells were detached under sterile conditions by aspirating growth medium from flask, washing twice with phosphate buffer solution (PBS; pH 7.4, Sigma-Aldrich, NSW, Australia; #D8662), and incubating
with 3mL trypsin-EDTA for five mins at 37 ºC (Sigma-Aldrich, NSW, Australia; #59417C). The reaction was then quenched by the addition of growth medium. Cells were collected and centrifuged at 300 g for five min, supernatant was discarded and cells resuspended in fresh, complete media. Cell counts were conducted using an automated cell counter (BioRad, NSA, Australia) and were seeded into either 8µm pore pre-coated transwell hanging inserts (Figure 2.1) (BD falcon, CA; #353182) or Nunc Lab-Tek II Chamber Slide System (ThermoFisher Scientific, Victoria, Australia; #154453). A375-M1 cells were used in transmigration studies at 1x10^5 density. hCMEC/D3 cells were seeded at the following densities; 5x10^4/cm^2; 1x10^5/cm^2; 2x10^5/cm into the apical chamber. The cell culture media in both the apical and basolateral chambers was changed at 48hr. All experiments were performed in triplicate and repeated twice.

Transendothelial electrical resistance (TEER) was measured daily using an EVOM2 volt-ohm meter with chopstick electrodes (World Precision Instruments, Sarasota, FL, USA) for 5 days during the growth period and area adjusted for analysis using the following formula:

\[
\text{TEER monolayer (}\Omega/\text{cm}^2) = \frac{\text{[raw TEER (}\Omega) - \text{TEER blank (}\Omega)]}{\text{area of membrane (cm}^2)\text{).}}
\]

Figure 2.1: Transparent PET hanging permeable support membrane.
2.2.7 Permeability Assay

Functional assessment of the transwell system was further supplemented by permeability evaluation of the established monolayer. Human endothelial hCMEC/D3 monolayers grown on filter membranes were exposed to two fluorescently labelled compounds of varying molecular size (4-70kDa). The selected tracers (Sigma-Aldrich, NSW, Australia: Na-F; MW 376.27Da #F6377, FITC-Albumin; MW 70kDa #A9771) were added to the upper compartment. At time points 15, 30, 60 mins, filters were placed in a new well for sampling. Initially 200µL was pipetted from the abluminal compartment and used to determine a maximum intensity value for each compound and quantified using the BioTek Synergy™ Mx microplate reader (BioTEk, Vermont, USA) and Gen5 version 2.00.18 software. At specified time points, a 100µL sample was taken from the abluminal compartment of each well for analysis relative to a standard curve (range 0-20µg/mL). Diffusion of each compound across the monolayer was assessed as volume (µL) as a function of time. Changes in concentration over the given time period allow for the calculation of the permeability coefficient (cm/s) of the monolayer. Filter permeability (for insert collagen-coated only) and insert plus endothelial cell permeability (for insert containing both collagen and cells), as well as surface area of the insert were taken into consideration.
2.2.8 *In vitro* immunofluorescence and immunohistochemistry

Immunofluorescence and immunohistochemistry for key hCMEC/D3 characteristics were performed to determine if the chosen cell line would support barrier investigation studies. Once monolayers had reached confluence, cells were fixed in 4% paraformaldehyde or 100% acetone at -20°C for 10 mins. Cells were rinsed with 1X PBS and permeabilised with 0.1% (v/v) Triton-X-100/PBS for 10 mins. After 2 x 5 mins washes in 1XPBS, cells were blocked in normal horse serum (NHS) (Sigma-Aldrich, NSW, Australia; #H0146). The blocking solution was aspirated and cells were then incubated with primary antibodies as detailed in relevant chapters. All primary antibodies were diluted in 3% NHS/PBS and incubated for 1 hr at room temperature. The primary antibody was then aspirated and cells washed 2 x 5 mins in 1xPBS+0.05% tris-buffered saline (TBS). After washing, cells were incubated with either the fluorescent-conjugated secondary antibody (anti-mouse 488; anti-rabbit 568 Alexa Fluor; invitrogen, VIC, Australia, #A21207, #A21202) or biotinylated secondary antibody (Vector Laboratories, anti-mouse #BA-9200, anti-rabbit #BA-1000). All secondary antibodies were diluted in 3% NHS and incubated for one hr at room temperature. Cells were washed for a final time in 1XPBS + 0.05% TBS 2 x 5 mins and either incubated with streptavidin peroxidase conjugate (SPC) for one hr at room temperature followed by staining with 3,3’-diaminobenidine (DAB) (Sigma-Aldrich, NSW, Australia; D-8001) or mounted with medium containing 4’,6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, #H-1200) for nuclear staining, or DePex (DPX)
 Cells were visualised using fluorescent and light microscopy or scanned using the NanoZoomer (Hamamatsu, Hamamatsu City, Japan) digital slide scanner and viewed with its associated software (NDPI View 2, Hamamatsu). Negative controls had primary antibody omitted.

2.2.9 Transmission electron microscopy

For ultrastructural studies, confluent hCMEC/D3 cells under experimental conditions were prepared for transmission electron microscope (TEM) analysis. Adherent cells were removed with trypsin-EDTA (Sigma-Aldrich, NSW, Australia; #59417C) and centrifuged at 300g for five mins to obtain a pellet of cells. The pellet was then washed in PBS (PBS; pH 7.4, Sigma-Aldrich, NSW, Australia; #D8662), fixed in 2.5% glutaraldehyde in 0.1M phosphate buffer (pH 7.3) overnight in an Eppendorf microfuge tube. It was then postfixed in 1% osmium tetroxide, dehydrated in graded alcohols, Epon embedded, and ultrathin sections cut and collected on 200 mesh copper/palladium grids and stained with uranyl acetate and lead citrate. Grids were visualised using the Philips CM200. Cells were then carefully assessed for any fine structural morphological changes.

Similarly, transmigration of A375-MA1 cells was confirmed using TEM. The hCMEC/D3 cells were cultured on 8µm pore pre-coated transwell hanging inserts (BD Falcon, CA; #353182). The cell culture media was changed every
48 hrs and TEER was measured daily using an EVOM2 endothelial volt-ohmmeter. Once TEER was maintained within optimal range, 1x10^5 A375-MA1 were plated into the endothelial monolayer and left for 5 and 24 hrs. Monolayers were washed with 1XPBS before being fixed in 2.5% glutaraldehyde in 0.1M phosphate buffer (pH 7.3) overnight. Monolayers were post-fixed in 1% osmium tetroxide and dehydrated in graded alcohols before removal from the transwell system for resin mounting and polymerisation at 70°C for 24 hrs. Sections were cut and analysed as previously described.

2.2.10 Cell Viability Assessment

An MTT assay was used to determine treatment effects on hCMEC/D3 and A375-MA1 cell lines. Metabolically viable cells convert assay tetrazolium salts into formazan that produces a quantifiable colour change by spectrophotometry. Cells were seeded into 96 well plate at a cell density of 1x10^5 cells/well. MTT labelling reagent (10µL) was added to wells and incubated for 4 hrs, after which solubilisation solution was added for overnight incubation. A microplate reader was used to read the samples at 540nm, including a reference wavelength of 650nm.
2.2.11 Western Blotting

Protein extraction and quantification

The hCMEC/D3 were cultivated under standard conditions and culture samples were immersed in 200 µL ice cold Radio-Immunoprecipitation Assay (RIPA) buffer containing 150mM NaCl, 0.1% NP-40/IGEPAL, 0.1% SDS, 0.5% sodium deoxycholate, 0.1% protease inhibitor cocktail and 50mM Tris, pH 7.4 (Sigma-Aldrich #P8340). Samples were briefly sonicated before centrifugation at 13,000g for 10 mins at 4°C. Supernatants were collected, aliquoted and stored at -80°C until use. Total protein was quantified using the Peirce BCA Protein Quantification Kit (ThermoFisher Scientific, VIC, Australia #23225) relative to a 5 point standard curve (0.04-1mg/mL).

Total protein lysates were quantified and 15µg supplemented with 4µL Bolt reducing agent (ThermoFisher Scientific, VIC, Australia #B0008) and 10µL Bolt lithium dodecyl sulfate (LDS) sample buffer (ThermoFisher Scientific, VIC, Australia #B0007). The total volume was then adjusted to 40µL with milliQ water. Samples were denatured for 10 mins at 95°C before being loaded into precast Bolt 4-12% Bis Tris Plus, SDS-PAGE Gels (12-well) (ThermoFisher Scientific, VIC, Australia; #NW04122BOX). Samples were separated using the Bolt mini gel tank (Thermo Fisher Scientific, VIC, Australia #A25977) at 100V for 1.5 hrs. Proteins were transferred to a polyvinylidene difluoride membrane using the iBlot transfer stacks (ThermoFisher Scientific, VIC, Australia #IB24002) and compatible iBlot 2 gel transfer device (ThermoFisher Scientific, VIC, Australia
The membrane was washed 3x 5 mins with 1Xtris-buffered saline and Tween20 (TBST) before blocking and probing with primary antibody overnight at 4°C. Following overnight incubation, the membrane was washed and incubated with secondary antibody for 2 hrs at 4°C. Western blots were assessed using ImageStudio Lite version 4.0 and signal intensity determined relative to local background. Data are presented relative to loading control (GAPDH). Antibody details can be found in relevant chapter.

2.2.12 Substance P ELISA

For the quantitative determination of SP secretion by A375-MA1 cells in culture an enzyme-linked immunosorbent assay (ELISA) was performed. Either mono or co-cultures of A375-MA1 and hCMEC/D3 cells were analysed using Quantikine ELISA kits (R&D systems, Minneapolis, MN, USA) SP according to the manufacturer’s protocol.

2.3 Epsilon toxin (ETX)

The ETX (Commonwealth Serum Laboratories (CSL), Melbourne) used in experiments in Part B of this thesis, was produced by a highly toxigenic Clostridium perfringens type D vaccine strain. Culture supernatant containing inactive prototoxin was activated at pH 7.5 by trypsin (0.08% weight/volume) for 30-45 min at 37°C to form the active epsilon toxin, which was then sterile filtered. Assay by a commercial facility (CSL) showed that the batch of toxin
used in the present study contained 386L+/ml (where 1 L+ = the amount of toxin neutralised by 1 International Unit of antitoxin) and 69,000 mouse minimal lethal doses (MLD)/ml. The toxin was then frozen at –60°C, at which temperature it is very stable (CSL, personal communication). The trypsin-activated toxin was used at a dose rate of 1:100, 1:200 and 1:800, diluted in complete cell culture medium.

2.4 Animal Care

All animal experimental work was performed on immunodeficient, male, Balb/c nude mice or male Sprague-Dawley rats. The Balb/c mouse strain is critical for use in xenograft models of cancer as it enables the study of human cancer cell lines in a controlled animal system.

Mice were derived from generation F25 stock obtained from the Animal Resource Center (Canning Vale, Western Australia) and acclimated for 5-7 days prior to experiments. Once a pre-surgical weight threshold was reached, animals were randomly allocated to tumour inoculation, vehicle, or control (all surgical and anaesthetic component without injection) groups and group housed behind a barrier at 24°C on a 12 hr day/night cycle. Animals were given access to standard rodent pellets and water ad libitum, and supplemented with Nutella™ post-surgery.
Rats were acclimated for 4-6 days prior to experiments. Following this animals were allocated to control or toxin group and co-housed in a conventional facility at 24°C on a 12 hr day/night cycle. Animals were given access to standard rodent pellets and water ad libitum.

2.4.1 Anaesthesia

The animal was placed in a transparent induction box, and 3% Isoflurane in 1.5L/min oxygen (Independent Veterinary Supplies -IVS) was delivered via a calibrated vaporizer. Animals requiring early euthanasia were deeply anaesthetised with 5% Isoflurane in 100% oxygen for 5 mins and death confirmed through the pain reflex test.

2.4.2 In Vivo Experiment

2.4.2.1 Intra-carotid Inoculation A-375M1 Human Melanoma Cells

An initial pilot study was performed with animals injected with either A375-MA1 or complete culture media via intra-carotid injection. The surgical procedure produced a high mortality rate, with 9 animals succumbing to hypovolemic shock during carotid cannulation, 4 dying within 24 hrs of surgery, and 2 requiring early euthanasia due to post-surgical weight loss exceeding cut-off scores on the clinical record sheet. Following intra-carotid inoculation with tumour cells, animals were perfused at 24hr, 3, 7 and 14 days. Microscopic analysis of the brain revealed insufficient penetration of neural
tissue by melanoma cells to be useful for analysis and, accordingly, this study was discontinued.

2.4.2.2 Surgical Procedure

Once anaesthetised, the animal was placed in a supine position on a heat pad, prepared for surgery and anaesthesia maintained via nose cone at a dose rate of between 1.5-2% of Isoflurane. A longitudinal midline incision was performed from the hyoid bone to sternal notch in order to expose the right neurovascular bundle, using the trachea as a central landmark (Figure 2.2). The carotid vessels were isolated from the neurovascular bundle via gentle blunt dissection and the injection site was prepared by ligating both the pterygopalatine and external carotid arteries with 3-0 silk suture. Finally, the common carotid artery was temporarily (< 1 min) occluded using a silk sling. After preparation, a small incision was made using micro-scissors to allow a catheter to be inserted and threaded into the common carotid artery. Tumour cell suspension (1x10^6) or control media (200µl) was then slowly injected at a rate of eight seconds/µl, followed by the re-establishment of blood flow by removal of the temporary sling. The wound was then sutured and treated with lignocaine subcutaneously and the animal allowed to recover.
A longitudinal midline incision was performed from the hyloid bone (chin dimple) to sternal notch (upper sternum) to expose the right neurovascular bundle using the trachea as a central landmark. (B) The carotid vessels were isolated from the neurovascular bundle (C) Schematic of the intra-carotid cell injection method; CCA = common carotid artery, ECA = external carotid artery, ICA = internal carotid artery, and PPA = pterygopalatine artery (Cranmer et al. 2005).

2.4.3 Intraperitoneal ETX inoculation

Animals were handled using the two-handed method (Figure 2.3) for administration of intraperitoneal injection of 1ml of a 1:10 dilution of ETX.
2.4.4 Tissue Processing

Mice and rats were deeply anaesthetised (5% isoflurane, 1.5% O₂ L/min) at predetermined time points followed by perfusion fixation of the brain with 4% paraformaldehyde (PFA). The brain was then left in situ for 2 hr to permit adequate penetration of fixative and reduce mechanical artefacts associated with handling, removed, and immersion-fixed in 10% NBF for at least 7 days prior to processing.

Brains were placed in a rodent brain blocker (Kopf, PA -002) and sectioned into 4mm, consecutive, coronal slices and processed overnight in a Tissue-Tek VIP. Tissue was processed for 20 mins in each graded ethanol (50%, 70%, 80%, 95%, and 100%), followed by two 90 min xylene baths, and finally paraffin (30, 60, 60, and 90 mins). Brains slices were individually embedded in paraffin wax, serially sectioned at 5 µm or 6 µm on a rotary microtome (Leica, RM2245,) and mounted onto Superfrost® microscope slides (Menzel-Glsae, Braunschweig,
Sections were allowed to dry overnight at room temperature and then stored in an oven at 37°C until staining.

Three coronal sections of rat brain were selected, in accordance with the United States National Toxicology Programme for routine toxicological screening procedures: at the optic chiasm (level 1); mammillary bodies (level 2); and at the widest part of the cerebellum (level 3). At level 1, the cingulate and parietal cortices, caudate-putamen, and corpus callosum were examined; at level 2, the occipital and temporal cortices, thalamus and internal capsule; and, at level 3, the cerebellum and pons.

2.5 Statistical Analysis

Data were compared and analysed using Prism version 7.0 (Graphpad Software, San Diego, USA) and presented as mean ± standard error of the mean (SEM). A P value of less than 0.05 was considered significant. Test details are reported within each chapter as appropriate.
Chapter 3

Vascular patterns in human cerebral metastatic melanomas
3 Chapter 3: Vascular Patterns in Human Cerebral Metastatic Melanomas

3.1 Background

The diagnosis of cancer has an enormous personal, emotional and economic impact worldwide. Most critically, the majority of cancer related deaths (90%) will be the result of metastatic spread of primary tumour cells to distant organs, ultimately resulting in the establishment of secondary tumour(s) (Nguyen et al. 2009). This intricate and multifaceted process of metastasis represents a significant challenge with regard to treatment options and patient outcome. Consequently, uncovering mechanisms of cancer metastasis continues to be eagerly anticipated and at the forefront of current research (Kefford 2012).

A review of the literature failed to find any previous study classifying the different types of blood vessels in metastatic melanomas in the brain. The only paper to address this topic was an in vivo model of brain melanoma metastasis in mice and humans in which a melanoma cell line was injected directly into the brain (Amit et al. 2013). In this report, blood vessel calibre only was measured using CD31 immunostaining to define blood vessels. It found that blood vessels were less abundant at the tumour front compared to its core, but those in the former tended to be larger.
3.2 Introduction

Central nervous system (CNS) metastasis represents a common and particularly devastating extension of a primary tumour, and will occur in a quarter of all systemic malignancies (Denkins et al. 2004). The nature of cerebral brain metastases cause patients to experience a number of neurological disturbances, including cognitive and behavioural changes, which impact greatly on their quality of life and the lives of those around them (Figure 3.1).

Figure 3.1: Metastatic pigmented malignant melanoma.

Note the midline shift (arrows) and distortion of the brain.

However perhaps most distressing for both clinicians and their patients is the realisation that the presence of secondary metastasis may signal tumour resurgence and an ability of the primary cancer to evade treatment. In the case of adult brain tumours, the prevalence of secondary cerebral
metastases significantly outweighs the diagnosis of a primary cerebral neoplasm, occurring 5-10 times more frequently and, as such, is responsible for the bulk of fatal intracranial neoplasms in adults (DeAngelis 2001, Bafaloukos et al. 2004). Devastatingly, the diagnosis of metastatic brain tumours will consistently align with poor prognosis, offer limited treatment options, and is a known contributor to increasing mortality associated with a number of primary tumours (Norden et al. 2005). Not surprisingly, treatment plans are aggressive, limited, and often unsuccessful, with many patients succumbing to their secondary brain tumour(s) within months of diagnosis (Gavrilovic et al. 2005). Additionally, studies conducted at autopsy indicate 25% of terminal cancer patients develop undiagnosed brain metastases prior to their death (Deeken et al. 2007). For primary cancers such as melanoma, the incidence of cerebral metastasis is reported to be in excess of 55%, with a further 30-40% of primary melanoma patients diagnosed with secondary brain tumours at autopsy (Soffietti et al. 2002, Deeken et al. 2007, Goulart et al. 2011).

3.2.1 Malignant Melanoma

Melanoma represents one of the more aggressive and commonly fatal forms of skin cancer, accounting for more than 80% of skin cancer related deaths worldwide (Postovit et al. 2006). In Australia, melanoma is ten times more common when compared to other countries and is responsible for the premature death of many young Australians each year (AIHW, 1999). Most alarmingly, malignant melanoma has the highest propensity to colonise the
brain of all primary adult neoplasms, closely followed by both lung and breast cancer (Fazakas et al. 2011). In fact, patients diagnosed with malignant melanoma demonstrate a 40-60% risk of forming secondary cerebral metastasis throughout the course of their condition, suggesting that properties of circulating melanoma cells indicate a predilection to spread to the brain.

The incidence of brain metastasis is reportedly increasing, seemingly as a consequence of improved treatments for systemic disease and increased patient survival rates (Eichler et al. 2011, Steeg et al. 2011). Unfortunately, patients deemed to be in remission of their primary malignancy have a high risk of developing secondary cerebral metastases. In the absence of treatment, mean survival time for a patient diagnosed with cerebral metastasis is estimated to be 1 month, which is extended to just 3.5 months in the presence of basic treatments (Bindal et al. 1993).

3.2.2 Cerebral Vasculature & Malignancy

In both primary and metastatic brain tumours, exploitation of existing, and generation of new, cerebral vasculature is critical, not only to facilitate initial dissemination and colonisation, but also to nurture tumour growth. To date, the ability of tumours to acquire a new vascular supply has been widely investigated, but remains incompletely understood. Moreover, it is now evident that these neoplasms may employ a number of different mechanisms to form new blood vessels and circumvent anti-angiogenic therapies.
Cerebral microvascular endothelial cells are highly labile and respond to a variety of insults by proliferation, however the capacity of the brain to form new capillaries is very limited (Baluk et al. 2005). Despite this, brain tumours are able to acquire and generate a new vascular supply by several means, although these blood vessels are often structurally and functionally abnormal (Sedlakova et al. 1999, Abbott et al. 2010).

The blood brain barrier (BBB) is maintained by the presence of tight junctions between ECs, a paucity of micropinocytotic vesicles, and a thickened basement membrane, abetted by the inductive influence of surrounding astrocytic end feet (foot processes) (Nag 2011). The structure of the interendothelial junctions that form an important component of the BBB is highly complex and, as previously mentioned, the two most critical proteins in these junctions are occludin and the claudins, including claudin-5. Tumours of the CNS exhibit characteristic traits such as altered BBB tight junction structure, increased intraendothelial vesicles, modified endothelial cell morphology and defects in basement membrane integrity, which are thought to facilitate migration of malignant cells (Ballabh et al. 2004, Arshad et al. 2010, Nag 2011, Nag et al. 2011). Furthermore, the altered cohesiveness of interendothelial tight junctions leads to increased vascular permeability and leakage of fluid and plasma proteins into the extracellular space (Harford-Wright et al. 2013). This oedematous fluid can add significantly to the space-occupying volume of the tumour mass, resulting in increased intracranial pressure and its deleterious sequelae, such as shift and distortion of the brain.
and herniation, with attendant severe neurological dysfunction and even death.

Cellular remodelling of cerebral endothelial cells is apparent in the early extravasation stage of metastasis, with tumour cells shown to promote a rearrangement of the endothelial cytoskeleton, resulting in an overt phenotypic change to cells which, in turn, has downstream effects on their function (Huber et al. 2001, Polakis 2008). These structural alterations result in the formation of a structurally and functionally distinct vascular complex which is referred to as the ‘blood-tumour barrier’ (BTB). Small metastatic tumours, as well as those exhibiting a diffuse growth pattern, have been shown to maintain an intact BBB but large, rapidly growing tumours can have a deleterious effect on the BBB (Arshad et al. 2010). Moreover, the pattern of new blood vessel formation in human cerebral tumours appears to be largely driven by local influences, such as intra-tumoral hypoxia, and tightly regulated by pro- and anti-angiogenic growth factors (Sceneay et al. 2013).

Mechanisms of neovascularisation of brain tumours – (See complete review in submitted paper; appendix 11.1.)

Two phases of tumour growth are recognised. Firstly, the avascular or dormant phase, which occurs in tumours up to 1-2 mm³ in diameter, and is characterised by a balance between proliferation and apoptosis of endothelial and tumour cells (Liu et al. 2000). In these tumours, diffusion from
pre-existing host blood vessels supplies the required oxygen and nutrients. The diffusion coefficient of oxygen in tissues is in the order of 150-200 µm and, accordingly, tumour cells located < 100 µm from a blood vessel are viable, whilst more distant cells undergo apoptosis (Banks 1999, Cecchelli et al. 2007, Luissint et al. 2012). Consequently, rapidly growing tumours readily become hypoxic and necrotic due to rapid proliferation and vascular insufficiency, precipitating a vascular phase. This phase is characterised by an “angiogenic switch” to support new growth and is induced when a tumour produces sufficient angiogenic growth factors and/or suppresses the expression of inhibitors (Banks 1999, Tuma et al. 2003, Daneman 2012). Commonly, the maximal vascular concentration is observed in more centrally located areas of the tumour, as well as the periphery at the interface with surrounding parenchymal tissue (Broadwell 1993, Banks 1999, Tuma et al. 2003, Daneman 2012). However, in some high-grade gliomas, the avascular phase may be fleeting or even largely absent (Ermisch et al. 1985).

New blood vessel development usually commences at the periphery of a tumour, and in the adjacent parenchyma, occurring concomitantly with tumour cell infiltration and alterations to the stroma. The latter changes, termed stromatogenesis, result in a less compact and more oedematous microenvironment to facilitate tumour cell infiltration, and their attendant ECs. The process of stromatogenesis is orchestrated by paracrine communication between endothelial, stromal and tumour cells. However, as a tumour grows and the invasion front becomes internalised, this milieu becomes more
hypoxic and acidic, altering the balance from proliferation towards apoptosis. The unfavourable microenvironmental changes cause the tumour to respond by activating glycolytic anaerobic pathways and upregulation of angiogenic and anti-apoptotic factors (Banks 1999, Daneman 2012). If, however, these responses prove to be inadequate, tumour necrosis and vascular regression ensue.

The vascular pattern in a given brain tumour is often heterogeneous, with areas of high microvascular density interspersed with those of sparse vascularity. As discussed, tumour-associated capillaries are structurally impaired and characterised by altered endothelial-pericyte interactions, abnormal blood flow, increased permeability, and delayed maturation. Moreover, these new vessels are often irregularly shaped, dilated, tortuous and prone to haemorrhage (Predescu et al. 2007). Blood vessels supplying the often hypoxic, central regions of tumours tend to be more abnormal than those supplying the tumour periphery (Banks 2010). In the context of brain tumours, enhanced vascular permeability leads to peritumoral vasogenic oedema, which can be associated with a life-threatening rise in intracranial pressure (ICP), subsequent distortion of the brain, or in severe cases, herniation (Hansen et al. 2010).

Until recently, neovascularisation of brain tumours was considered to be largely achieved by endothelial sprouting, or angiogenesis, but it is now recognised that a number of different processes may occur concurrently or
sequentially (Sowa 2012). Nevertheless, in most brain tumours, the contribution of processes other than angiogenesis to the formation of new tumour-associated blood vessels is probably relatively minor and some of the newly described modes of neovascularisation remain controversial. In normal tissues, modes of vascular formation are limited to vasculogenesis, sprouting, and intussusception, whereas in tumours, co-option of pre-existing blood vessels, vascular mimicry, and endothelial transdifferentiation are also available (Table 3.1).

<table>
<thead>
<tr>
<th>Table 3.1: Mechanisms of Brain Tumour Neovascularisation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vascular co-option</td>
</tr>
<tr>
<td>Sprouting angiogenesis</td>
</tr>
<tr>
<td>Vessel intussusception</td>
</tr>
<tr>
<td>Vascular mimicry</td>
</tr>
<tr>
<td>Bone marrow-derived endothelial progenitor cell vasculogenesis</td>
</tr>
<tr>
<td>Cancer stem-like cell-derived and bone marrow-derived monocyte/macrophage-mediated vasculogenesis</td>
</tr>
</tbody>
</table>

Initial contact between circulating metastatic tumour cells and the brain is often at the level of the BBB. Despite its highly selective properties, the BBB does not prevent invasion by circulating metastatic tumour cells and, in fact, may harbour them from circulating chemotherapeutic agents in some cases. Once a primary brain tumour or metastases reach more than 1-2 mm in diameter, and neovascularisation is induced, the BBB becomes structurally and functionally compromised, as previously mentioned. Although elements of the BBB are disrupted in experimental brain metastases that exceed 0.2 mm², it has been reported to be largely maintained following passage of solitary metastatic tumour cells into the brain. What remains unclear is
whether tumour cell emigration always disturbs BBB integrity, with or without recovery (Faltas 2012, Fein et al. 2013).

Neovascularisation of brain tumours is essential for their continued growth and progression. Furthermore, tumour vascularisation can be diagnostically useful for histological grading, a prognostic indicator, and is a frequent target for anti-tumour therapies. As such, the pattern of neovascularisation in cerebral neoplasms warrants further study in order to better understand and target these aggressive tumours.

The present chapter aims to characterise the different intra-tumoral vascular phenotypes expressed by brain metastatic melanomas in human tumour biopsies in order to determine whether the different blood vessel types correlate with melanoma morphological sub-types, mitotic index, presence and distribution of non-surgical haemorrhage, or areas of tumour necrosis. Additionally, as tumour-associated blood vessels are known to be structurally and functionally aberrant, it was decided to determine whether the immunoexpression of claudin-5 was altered in these blood vessels as a marker of blood vessel maturity and BBB status.
3.3 Materials and Methods

3.3.1 Experimental Design

Ten, de-identified human surgical cases of metastatic melanoma to the brain were used to assess the correlation between the different types of blood vessels found in these metastatic tumours and the morphological features of these tumours. Central nervous system (CNS) biopsy tissue was obtained from the SA Pathology, Centre for Neurological Diseases and approved by the Royal Adelaide Hospital (RAH-140308) ethics committee. Experiments were performed under the NH&MRC guidelines for use of human tissue. Cases receiving a pathological diagnosis of cerebral metastatic lesion originating from a primary malignant melanoma (n=10) were selected for immunohistological evaluation. Additionally, a cohort of glioblastoma multiforme (GBM) (astrocytoma, WHO grade IV) were included (n=5) to serve as positive controls for neoplastic vascularisation patterns. Melanoma cases had a mean age of 54 and a split of females (n=4) to males (n=6) (Table 3.2). All human tissue was classified according to the pathological diagnosis performed by the RAH associated pathologist at the time of surgery.
3.3.2 Histological Analysis

Following collection of the tumour biopsies at surgery, samples were immediately fixed in 10% neutral buffered formalin and processed to paraffin wax using standard protocols. For each case, 6 µm sections were cut, mounted and dried overnight at room temperature before being stored at 37°C until staining. All cases underwent standard haematoxylin and eosin (H&E), specialised staining, or immunohistochemistry. A panel of antibodies (Table 3.3) was used to characterise the aberrant structure of the different blood vessel types, particularly EC, subendothelial and smooth muscle components. Periodic acid-Schiff (PAS) staining of vascular basement membranes was also conducted to better define the structure of these vessels. Evaluation and diagnosis for each selected tumour case was performed by a clinical neuropathologist (Appendix 11.3 - PB/CS/BK/SE/SO/CB/JMW) and reviewed by two independent examiners (Appendix 11.4 - KAM/JWF).

<table>
<thead>
<tr>
<th>Site</th>
<th>Number of cases</th>
<th>Age (years)</th>
<th>Males</th>
<th>Females</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temporal</td>
<td>3</td>
<td>34, 36, 52</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Parietal</td>
<td>2</td>
<td>59, 82</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Frontal</td>
<td>1</td>
<td>52</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Occipital</td>
<td>1</td>
<td>73</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Undefined</td>
<td>3</td>
<td>60, 53, 39</td>
<td>2</td>
<td>1</td>
</tr>
</tbody>
</table>
**Table 3.3: Panel of Immunohistochemical Antibodies**

<table>
<thead>
<tr>
<th>Antibody to</th>
<th>Clone</th>
<th>Dilution</th>
<th>Positive Control</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-SMA</td>
<td>Mouse Monoclonal 1A4</td>
<td>1:30,000</td>
<td>Human Hippocampus</td>
<td>Dako M0851</td>
</tr>
<tr>
<td>CD31</td>
<td>Mouse Monoclonal JC70A</td>
<td>1:100</td>
<td>Human Hippocampus</td>
<td>Dako M0823</td>
</tr>
<tr>
<td>CD34</td>
<td>Mouse Monoclonal QBEnd/10</td>
<td>1:100</td>
<td>Human Lymph Node</td>
<td>Novacastra 6025272</td>
</tr>
<tr>
<td>Claudin-5</td>
<td>Mouse Monoclonal 4C3C2</td>
<td>1:750</td>
<td>Human Hippocampus</td>
<td>Invitrogen 352500</td>
</tr>
<tr>
<td>PAS</td>
<td>Periodic acid-Schiff</td>
<td>N/A</td>
<td>Human Illeum</td>
<td>Australian Biostain 160057</td>
</tr>
<tr>
<td>VEGF</td>
<td>Mouse Monoclonal</td>
<td>1:500</td>
<td>Human GBM IV</td>
<td>Abcam 1316</td>
</tr>
</tbody>
</table>

H&E and PAS staining were used to analyse the vascular architecture which was categorised as outlined in Table 3.4. Each immunohistochemical stain was analysed in order to determine vessel type, morphology and maturity. Expression at both peri and intra-tumoural vasculature was assessed. Qualitative analysis was performed by two independent assessors. Staining intensity for tight junction protein claudin-5 was analysed using a validated semi-quantitative grading system (Al-Azri et al. 2015) from 0-3; where 0 = no staining, 1 = mild staining, 2 = moderate staining and 3 = intense staining (Figure 3.2).
Figure 3.2: Semi-quantitative grading system representative images.

Representative images of cerebral metastatic melanoma microvasculature showing immunohistochemical staining of claudin-5 within the endothelial lining of varying intensities. Staining is graded on a scale of 0-3, where 0 = no staining (A), 1 = mild staining (B), 2 = moderate staining (C) and 3 = intense staining (D).

In addition to defining the different intra-tumoral vascular types (Table 3.4), the frequency of occurrence of each vascular profile in the respective melanomas was graded (+ to +++), and attendant vascular pathology (thrombosis, perivascular lymphocytic cuffing/vasculitis, and haemorrhage). In each melanoma, the mitotic index (number of mitotic figures in 10, randomly selected high power (x40) fields) and percentage area of tumour necrosis was also calculated.
### Table 3.4: Intratumoral Vascular Parameters

<table>
<thead>
<tr>
<th></th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Microvessels* with normal-sized or mildly distended lumina and often endothelial proliferation</td>
</tr>
<tr>
<td>2</td>
<td>Microvessels* with conventional endothelial lining but marked luminal distention (angioectasia), often found in clusters (telangiectasis)</td>
</tr>
<tr>
<td>3</td>
<td>Arteriolar-type vessels with thick walls relative to luminal size</td>
</tr>
<tr>
<td>4</td>
<td>Large venule with markedly distended (ectatic) lumen</td>
</tr>
<tr>
<td>5</td>
<td>Clusters of thick-walled vessels with narrow, very tortuous lumina</td>
</tr>
<tr>
<td>6</td>
<td>Venules with marked perivascular melanocytic cuffing</td>
</tr>
<tr>
<td>7</td>
<td>Vascular thrombosis</td>
</tr>
<tr>
<td>8</td>
<td>Perivascular lymphocytic cuffing</td>
</tr>
<tr>
<td>9</td>
<td>Intratumoral haemorrhage</td>
</tr>
</tbody>
</table>

*Microvessels = capillaries and small venules

Additionally, an investigation was made to determine whether blood vessel morphology is correlated with (1) melanocyte cell type; (2) the mitotic index; (3) the degree of tumour necrosis; or (4) intra-tumoral haemorrhage.
3.3.3 Statistical Analysis

To determine whether there was any correlation between the severity of 9 vascular profiles and mitotic index, necrosis and haemorrhage, Kruskal-Wallis tests were used. For significant Kruskal-Wallis tests, pair-wise Wilcoxon Rank Sum tests were used to identify which comparisons were significant. A $p$-value of less than 0.05 was considered to be significant. All non-parametric data were assessed using a Kruskal-Wallis test with Dunn’s multiple comparison.
3.4 Results

3.4.1 Melanoma Classification

In H&E sections of melanomas in this cohort, 3 subtypes were evident: epithelioid, spindle cell, and small cell variant (Figure 3.3). Their distribution among the 10 cases studied is shown in Table 3.5.

<table>
<thead>
<tr>
<th>Case no.</th>
<th>Predominant melanocyte cell type</th>
<th>Mitotic Index</th>
<th>% area necrosis</th>
<th>% area haemorrhage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient A</td>
<td>Epithelioid in sheets</td>
<td>5</td>
<td>0</td>
<td>60</td>
</tr>
<tr>
<td>Patient B</td>
<td>Spindle cell, often whorling around vessels</td>
<td>50</td>
<td>20</td>
<td>10</td>
</tr>
<tr>
<td>Patient C</td>
<td>Spindle cell, often whorling &amp; interlacing bundles</td>
<td>30</td>
<td>15</td>
<td>10</td>
</tr>
<tr>
<td>Patient D</td>
<td>Epithelioid, with few areas of whorling spindle cells</td>
<td>10</td>
<td>20</td>
<td>10</td>
</tr>
<tr>
<td>Patient E</td>
<td>Small melanocytes in sheets with irregularly shaped nuclei and scant cytoplasm</td>
<td>2</td>
<td>20</td>
<td>10</td>
</tr>
<tr>
<td>Patient F</td>
<td>Epithelioid, highly pleomorphic and sometimes pseudopalsading around blood vessels</td>
<td>35</td>
<td>25</td>
<td>10</td>
</tr>
<tr>
<td>Patient G</td>
<td>Epithelioid, often whorling around vessels</td>
<td>5</td>
<td>80</td>
<td>10</td>
</tr>
<tr>
<td>Patient H</td>
<td>Small melanocytes in sheets, irregularly shaped nuclei and scant cytoplasm</td>
<td>1</td>
<td>5</td>
<td>80</td>
</tr>
<tr>
<td>Patient I</td>
<td>Epithelioid</td>
<td>1</td>
<td>60</td>
<td>10</td>
</tr>
<tr>
<td>Patient J</td>
<td>Epithelioid</td>
<td>5</td>
<td>15</td>
<td>40</td>
</tr>
</tbody>
</table>
Figure 3.3: Melanoma subtypes H&E.

**Epithelioid (A-B)** Solid sheets of pleomorphic, polygonal-shaped melanocytes of varying size, without evidence of melanin pigmentation (Scale bar = 75µm). (C) Higher power showing melanocytes with generally round to ovoid nuclei, often containing one or more prominent nucleoli, and ample eosinophilic cytoplasm with distinct cell boundaries. An occasional bi-nucleated melanocyte is present (black square). A few nuclei contain clear vacuoles (black arrows). (D) Pleomorphic population of epithelioid-type melanocytes with an occasional megalocytic melanocyte showing a bizarre nucleus (arrow). (E) Melanocytes in mitotic division, some mitotic figures being abnormal (arrows) (Scale bar C-E = 50µm). **Spindle cell (F)** Spindle-shaped melanocytes disposed in a whorling pattern. Nuclei tend to be elongated and usually contain one or more nucleoli; moderate amount of eosinophilic cytoplasm, with often indistinct cell boundaries. Melanin pigmentation is scant (Scale bar = 60µm). (G) Spindle-shaped melanocytes whorling around a central blood vessel (Scale bar = 75µm). **Small cell variant (H)** Melanocytes are irregularly shaped with a high nucleus to cytoplasmic ratio. Nuclei tend to be hyperchromatic and variably shaped with a small to moderate amount of deeply eosinophilic cytoplasm and generally distinct cell borders. A few melanocytes are pigmented (Scale bar = 50µm).
3.4.2 Blood vessel morphology

In order to characterise the different blood vessel types found in these melanomas, routine H&E staining was used in conjunction with PAS – to demonstrate intimal thickening, CD31 – to identify endothelial proliferation, and α-SMA – to determine the pattern of mural smooth muscle organisation. The pattern of normal staining with these markers is shown in Figure 3.4.

![Image of blood vessel morphology](image)

Figure 3.4: Normal Morphology.

Normal H&E appearance of microvessels in perfusion-fixed mammalian brain of (A) small venule –type, with lumina free of plasma protein, erythrocytes and leukocytes. (B) Endothelial lining of the intima shown by CD31 immunostaining (arrows). (C) PAS-positive basement membrane staining of a venule (V) and capillaries (c). (D) A normal artery showing circularly arranged smooth muscle cells indicated by α-SMA immunostaining (Scale bar = 50µm).
In the metastatic melanomas studied, 5 different vascular subtypes were identified (Table 3.4); Type 1 (Figure 3.5) were microvessels, with lumina of normal dimension, but often appearing to have been collapsed by pressure from surrounding melanocytes. CD31 immunostaining revealed marked endothelial proliferation with resultant intimal thickening.

![Figure 3.5: Microvessels in cerebral metastatic melanoma.](image)

**Figure 3.5** Microvessels in cerebral metastatic melanoma.

**(A-B)** Microvessels show marked intimal thickening due to endothelial proliferation (CD31 immunostaining) **(C)** Venule [V] showing luminal dilatation and intimal thickening due to endothelial proliferation. Capillary sized vessels with similar endothelial hyperplasia are shown at the top right (CD31 immunostaining). **(D)** Abnormal venules showing marked intimal thickening by PAS-positive material. Endothelial cells are hypertrophied and hyperplastic (Scale bar A-C = 75 µm; D = 50µm).
Type 2 (Figure 3.6) microvessels showed marked luminal distention and were often collected in clusters (telangiectasis). These vessels were invariably congested and it was unclear whether they represented new blood vessel formation (neovascularisation) or were pathologically altered, pre-existing vessels.

Figure 3.6: Cerebral metastatic melanoma microvessel angioectasia and telangiectasis.

(A-C) Clusters of severely congested microvessels with marked luminal dilatation at the periphery of the melanoma H&E. (D) Similar area showing microvessels with prominent intima due to endothelial proliferation (CD31 immunostaining) (Scale bar = 75 µm).
Type 3 (Figure 3.7) blood vessels were arteriolar-sized and often showed hyaline degeneration characterised by an amorphous, homogenous mural appearance. They also showed endothelial proliferation and the smooth muscle layers were poorly organised and haphazardly disposed as shown by α-SMA immunostaining.

Figure 3.7: Cerebral metastatic melanoma arteriolar-sized blood vessels.

(A) Arteriolar-sized blood vessels (a) with thickened walls showing hyaline degeneration (arrows) H&E.
(B) A cluster of arteriolar-sized blood vessels with adjacent epithelioid melanocytes containing melanin pigmentation (black square). (C-D) Tumour-associated blood vessels immunostained with α-SMA showing disorganised, and sometimes segmentally disposed, medial smooth muscle cells (Scale bar = 50µm).
Type 4 (Figure 3.8) were large venules with markedly distended (ectactic) lumina, the walls being relatively thin in relation to luminal size. The intima was thickened due to deposition of excess PAS-positive material and endothelial proliferation. Mural smooth muscle was disorganised with α-SMA immunostaining.

Figure 3.8: Cerebral metastatic melanoma ectactic venules.

(A–B) Tumour-associated venules with markedly distended (ectactic) lumina H&E (Scale bar = 50µm).
(C) A venule with markedly distended lumen shows intimal thickening (PAS), and (D) endothelial proliferation (CD31) and (E) disorganised arrangement of mural smooth muscle (α-SMA) (C–E Scale bar = 75 µm).
Type 5 (Figure 3.9) blood vessels were thick-walled with endothelial hyperplasia and tortuous (serpentine) and narrowed (stenotic) lumina. Mural smooth muscle was again disorganised and haphazardly arranged.
(A) Thick-walled blood vessels with tortuous and stenotic lumina (H&E). (B) These vessels immunostained with CD31 show intimal thickening due to endothelial proliferation. (C) The basement membrane is poorly defined by PAS staining and PAS-positive material extends deep into the vessel wall. (D) α-SMA immunostaining shows a haphazardly arranged, poorly organised medial smooth muscle layer. (E-H) A cluster of thick-walled, abnormal blood vessels with stenotic lumina. PAS staining (F) shows an ill-defined basal lamina and abundant mural PAS positive material. (G) CD31 immunostaining shows intimal thickening due to endothelial proliferation and (H) the poorly organised mural smooth muscle (α-SMA immunostaining) (Scale bar = 75 µm).

Several other vascular pathologies were identified;

**Melanocyte cuffing:** a pattern of melanocytic perivascular cuffing of vessels (Figure 3.10), with some melanocytes appearing to infiltrate the vessel wall, although the direction of movement for these tumour cells is unclear.
Figure 3.10: Melanocyte aggregation and infiltration of blood vessels.

(A) A dilated venule cuff by malignant melanocytes (H&E) with (B), intima marked by CD31 immunostaining. (C) Intimal thickening shown by PAS staining. (D) Vessel is partially effaced (arrow) by infiltrating melanocytes (E) in this melanoma, the predominant pattern is aggregation of melanocytes around a blood vessel (H&E). (F) Vessel is cuff by melanocytes with a stenotic lumen, thickened endothelium and poorly organised wall. (G) A blood vessel wall has been destroyed by invading melanocytes, with some visible within the lumen (L). (H) PAS-staining identifies a discontinuous basement membrane (arrows) (Scale bar = 25 µm). (Scale bar A-C = 75 µm; D = 25 µm).

Perivascular cuffing/vasculitis: Some tumour associated blood vessels showed perivascular lymphocytic aggregation (termed perivascular cuffing), while some additionally showed lymphocytic infiltration of, and damage to, the vessel wall (lymphocytic vasculitis) (Figure 3.11).
Figure 3.11: Perivascular cuffing/vasculitis.

(A) Blood vessels showing perivascular lymphocytic aggregation (perivascular cuffing) and mural lymphocytic infiltration (vasculitis). (B) Perivascular lymphocytic cuffing with marked mural deposition of PAS-positive material and (C) intimal thickening due to endothelial proliferation (CD31). (D) Blood vessels showing a lymphocytic vasculitis with mural hyalinisation (H) and endothelial hypertrophy (arrows). (E) Severe lymphocytic vasculitis with marked mural degeneration and splitting (PAS) (Scale bar A-C = 75 µm; D-E = 50 µm).
Vascular thrombosis: blood vessels within melanomas often demonstrate thrombosis (Figure 3.12). The fibrin clot well demonstrated by PAS staining Partial or complete luminal occlusion in these vessels was associated with adjacent tumour necrosis due to ischaemia/hypoxia.

Figure 3.12: Thrombosis.

(A) Thrombosed intra-tumoural blood vessel wall within a cerebral metastatic melanoma (H&E). (B) The vessel stained with PAS shows marked fibrin deposition in the occluding thrombus. (C) A dilated venule contains a thrombus, the lumen being totally occluded by deposited PAS-positive fibrin (D) Blood vessels within a cerebral metastatic melanoma are occluded by thrombi (T), while a vessel at top right shows a partial luminal thrombotic occlusion (arrow). There is massive necrosis of melanoma cells around these thrombosed vessels with loss of cellular detail. (E) A large area of necrosis within metastatic melanoma, not apparently blood vessel associated (Scale bar A-B = 50 µm; D = 100 µm; E= 75).
Many melanomas showed, to varying degrees, haemorrhage, which was focal, multifocal or confluent (Figure 3.13).

![Figure 3.13: Intra-tumoural haemorrhage.](image)

**(A-B)** Marked multifocal to confluent haemorrhage within a cerebral metastatic melanoma (Scale bar = 75 µm)

Blood vessel invasion by melanoma (Figure 3.14).

![Figure 3.14: Blood vessel invasion by melanoma.](image)

**(A)** H&E. A dilated blood vessel is surrounded, and infiltrated, by malignant melanocytes. Part of the wall is compromised by invading tumour cells (arrows) and the remainder is degenerate. **(B)** High power image of invading tumour cells (image A) (Scale bar A = 50 µm; B =25 µm).
3.4.3 Cerebral metastatic melanoma have decreased BBB claudin-5 expression

Claudin-5 expression was assessed in blood vessels within cerebral metastatic melanomas in order to assess the integrity of tight junctions. In non-pathological controls, there was strong claudin-5 immunostaining of microvessels and larger calibre veins and arterioles/arteries, corresponding to the complex pattern of endothelial tight junction arrangement in these mature vessels. By contrast, in most melanoma-associated blood vessels, there was a marked reduction in claudin-5 immunostaining, which was particularly evident when compared to blood vessels in contiguous brain parenchyma (Figure 3.15 – image B). The diminution in claudin-5 immunoreactivity was found in intra-tumoural blood vessels of all sizes (Figure 3.15 – image C-E). The decreased claudin-5 immunopositivity found in many melanoma-associated blood vessels is consistent with the intra- and peri-tumoural vasogenic oedema often accompanying these brain metastases, fluid extravasation being permitted by break down of inter-endothelial tight junctions.
Figure 3.15: Intratumoural vessels demonstrate decreased claudin-5 immunoreactivity.

(A) Vascular claudin-5 immunoreactivity in a control brain (B-D) Blood vessels within a melanoma show markedly reduced endothelial Claudin-5 immunopositivity. (E) Blood vessels of varying calibre show strong endothelial Claudin-5 immunopositivity in the brain parenchyma. The immunoreactivity declines at the melanoma/brain interface (dotted line). Within the tumour, immunoreactivity is markedly reduced or absent (arrows). (Scale bar A, B-D= 50 µm; E = 75 µm).
Since high grade glioblastoma multiforme (GBM), also termed high grade IV astrocytoma, is amongst the most vascular and oedematous of all solid tumours, a sample of was examined. One of the cardinal diagnostic features of GBM is the formation of ‘glomeruloid tufts’ or aggregation of abnormal blood vessels (Figure 3.16). The other characteristic histological feature of GBMs is the tendency of glioma cells to pseudopallisade around focal areas of tumour necrosis (Figure 3.16). Some GBM tumour associated vessels were immunopositive for VEGF (Figure 3.17).

Figure 3.16: Glioma.

(A) A glomeruloid tuft within a glioblastoma multiforme (GBM) showing a cluster of dilated and congested blood vessels with proliferating endothelium and poorly organised walls. The vessel at the bottom image is thrombosed (T). (B) Glioblastoma multiforme showing focal area of necrosis (n), surrounded by pseudopalisading glioma cells (black box) (Scale bar A = 50 µm; B = 100 µm).
Figure 3.17: Glioblastoma Multiforme (GBM).

(A) Strong endothelial VEGF immunopositivity in a GBM-associated blood vessel  (B) Higher power image A.  (C) GBM-associated vessel shows marked endothelial proliferation, which is marked by strong VEGF immunostaining  (Scale bar A & C = 50 µm; B = 25 µm).

Table 3.6 shows the graded frequency of the different vascular phenotypes in each of the 10 metastatic melanomas studied.
Table 3.6: Vascular types in metastatic melanoma

<table>
<thead>
<tr>
<th>Vascular morphology*</th>
<th>Melanoma Case</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>1</td>
<td>+++</td>
</tr>
<tr>
<td>2</td>
<td>++</td>
</tr>
<tr>
<td>3</td>
<td>+</td>
</tr>
<tr>
<td>4</td>
<td>+</td>
</tr>
<tr>
<td>5</td>
<td>+</td>
</tr>
<tr>
<td>6</td>
<td>-</td>
</tr>
<tr>
<td>7</td>
<td>++</td>
</tr>
<tr>
<td>8</td>
<td>+</td>
</tr>
<tr>
<td>9</td>
<td>++</td>
</tr>
</tbody>
</table>

*Frequency of occurrence graded + to +++

To compare mitotic index, necrosis and haemorrhage values between severity of 9 vascular profiles, Kruskal-Wallis tests were used. For significant Kruskal-Wallis tests, pair-wise Wilcoxon Rank Sum tests were used to determine significance.

3.5 Discussion

In this study, the morphological pattern of tumour-associated blood vessels was examined in a cohort of 10 archival human melanomas metastatic to the brain. They were categorised on the basis of their principal morphological
features, which were defined using routine histological staining and special staining and immunohistochemistry to delineate endothelial cell hyperplasia/hypertrophy (CD31), basement membrane deposition (PAS), and mural smooth muscle arrangement (α-smooth muscle actin or α-SMA).

Multiple blood vessel types were found within the metastatic melanomas, including (1) microvessels with a normal calibre or mildly distended lumen, which was often apparently collapsed from the pressure of surrounding tumour cells; (2) microvessels with dilated (ectatic) lumina, often found in clusters (telangiectasis), and frequently at the tumour periphery; (3) arteriolar-type, thick-walled vessels; (4) large venules with markedly distended lumina; and (5) clusters of thick-walled vessels with very tortuous (“serpentine”) and stenotic lumina. Importantly, in all vessel types, endothelial cell proliferation was almost invariably present, coupled with a marked decrease in tight junction claudin-5 expression. The latter finding is concordant with the increased vascular permeability, and the subsequent intra- and peri-tumoural oedema, found in many metastatic tumours (Papadopoulos et al. 2001). Diminution of claudin-5 immunoexpression and vasogenic oedema has also been described for a number of other neurological disorders, including traumatic brain injury, stroke, and inflammation, and degenerative conditions such as Alzheimer’s disease (Brooks et al. 2005, Hawkins et al. 2005, Bednarczyk et al. 2011). However, the precise regulation of claudin-5, and signalling pathways involved in internalisation and recycling, not only in tumour metastases, but most aforementioned neurological diseases, remains
incompletely understood (Jia et al. 2013, Jia et al. 2014). Other vessel-associated pathological features were also examined, including thrombosis, perivascular lymphocytic cuffing (which was often accompanied by vasculitis), and intratumoral haemorrhage, which were distinguished from peritumoral iatrogenic haemorrhage produced during surgical biopsy of the tumour.

In the metastatic melanomas assessed, there was a great diversity of intra- and peri-tumoral blood vessels and the structure was aberrant in many important respects. Luminal dilatation was a feature in both microvessels and large venules, with luminal dilation in microvessels observed predominately at the periphery of metastatic melanomas where they abutted brain parenchyma and were often arranged in clusters. Luminal distension could be caused, in part at least, by the higher viscosity of blood in tumour-associated blood vessels and turbulent flow (Chabner et al. 2005). The degree of endothelial proliferation has been shown to correlate with tumour grading, particularly in GBM and, in general, endothelial proliferation in tumour-associated vasculature is greater than in normal tissue (Sherbet 1989). Endothelial hyperplasia, and hypertrophy, was almost always present in these blood vessels and sometimes associated with thrombosis (with partial or complete luminal occlusion), often resulting in ischaemic-hypoxic tumour necrosis, as commonly occurs in gliomas (Baluk et al. 2005). Additionally, some blood vessels also exhibited markedly tortuous, narrowed lumina and, in larger vessels, the medial smooth muscle arrangement was severely
disordered. Furthermore, intratumoral haemorrhage was frequent, and sometimes extensive, manifesting either as multifocal or coalescing erythrocyte extravasations and highlighting the fragility of these abnormal vessels. Metastatic melanomas are particularly susceptible to bleeding, with approximately 50% of cases showing spontaneous intracranial haemorrhage. By contrast, haemorrhage is significantly lower in metastatic adenocarcinoma, anaplastic carcinoma and squamous carcinoma (Suzuki et al. 2003). Intra-tumoural haemorrhage is posited to be due, in part at least, to endothelial proliferation, vessel compression, vessel necrosis, vessel wall invasion and obliteration (Yoo et al. 2011). It has been suggested that the observed perivascular lymphocytic aggregation and mural lymphocytic infiltration found in some melanomas in the present study could represent a protective response to counter weakened vascular architecture. Macrophage and lymphocyte activity is also credited as a major contributor to the observed intratumoural inflammatory setting and this inflammatory microenvironment may drive tumour progression (Thun et al. 2002, Zbytek et al. 2008, Colotta et al. 2009, Buddingh et al. 2011, Algra et al. 2012).

The 10 human metastatic melanomas examined in the brain were classified morphologically as epitheliod, characterised by polygonal-shaped, epithelial-like melanocytes; spindle cell, in which melanocytes usually had elongated nuclei and indefinite cell boundaries and were frequently disposed in whorling and interlacing bundles; and a third melanoma pattern in which melanocytes showed irregularly-shaped, hyperchromatic nuclei and
usually scant cytoplasm. Tumour cell type is an important prognostic factor, with epithelioid melanomas being the most common variant and having the greatest malignant potential (Ascierto et al. 2000, Kaliki et al. 2015).

As the principal types of tumour-associated blood vessels had been categorised and morphological sub-types of melanoma defined, the correlation between melanoma type and blood vessel morphology was investigated. In addition, any correlation between attendant features such as mitotic index (as a measure of melanocyte proliferation), area of tumour necrosis (reflecting a tumour growth rate exceeding the ability of the vasculature to sustain further growth), intratumoral haemorrhage, and melanoma sub-type was analysed. Statistical analysis revealed no correlation between the different morphological subtypes of metastatic melanoma and the above blood vessel types identified in these tumours. There was a statistically significant difference in percentage of necrosis and blood vessels characterised by mildly distended lumina with endothelial proliferation (vascular profile 1, \(p\)-value=0.0446). Furthermore, in melanomas in which vascular changes characterised by aggregation of thick-walled vessels (vascular profile 5) were common, there was a statistically significantly (\(p\)-value=0.0485) increase in degree of tumour necrosis. No other statistical comparisons were significant. A number of histopathological features of melanomas have been investigated as a means of determining prognosis, including cell type, mitotic activity, diameter of nuclei, microvascular density, tumour-infiltrating lymphocytes and macrophages, and growth factor
expression. Poor prognosis tends to align with tumours of epithelioid type with high mitotic activity, higher nucleoli diameter means and microvascular density, and presence of inflammatory infiltrate (Kaliki et al. 2015).

From the perspective of blood vessel structure, it was remarkable how greatly these metastatic melanoma-associated blood vessels differed from normal blood vessels, and those found in other non-neoplastic disease states. Tumour-associated blood vessels were markedly aberrant in structure and this could reflect, in part, the ability of brain tumours to utilise a variety of different mechanisms to acquire a new vascular supply in order to sustain further growth and promote tumour infiltration. Folkman et al (2015) first described the relationship between the mitotic index of tumour cells and tumour vascularisation in metastases. For every increase in tumour diameter, there needs to be a corresponding increase in tumour vasculature and, as such, the mitotic index as a marker for tumour growth can be used to determine potential vascular expansion. Tumour volume increase is also a longstanding marker when evaluating clinical treatment options and tumour angiogenesis may be an important contributor to tumour volume (de Vries et al. 2012).

Furthermore, tumour microvascular density (TMD) is routinely used to anticipate tumour rate of growth and aggression and, more recently, used to evaluate the efficacy of anti-angiogenic therapies (Bertolini et al. 2006). However, this technique relies on determining tumour vascular ‘hot spots’ for calculation and, therefore, may not necessarily be representative of the tumour as a whole.
The diagnosis of a brain tumour presents a challenging and complex medical situation with regard to devising an appropriate and effective treatment protocol for patients (Drappatz et al. 2007). In terms of anti-tumour therapy, it is essential that both the primary and metastatic brain tumour-associated vasculature is better understood in order to develop interventions that are more accurately targeted to achieve improved clinical outcomes. Treatment regimens designed to impede tumour neovascularisation, such as the inhibition of growth factors such as VEGF and HIF that drive new blood vessel formation, have been developed. However, unfortunately, the resulting benefits have often been inconsistent, in part due to the ability of these neoplasms to circumvent the particular angiogenic mechanism being blocked and utilise other modalities to obtain a new vascular supply. Accordingly, it is likely that a multifaceted approach will be required to significantly impair neovascularisation and achieve a more durable clinical improvement. This will necessitate targeting the different mechanisms by which brain tumours can acquire a new vascular supply, including co-option of existing microvessels, formation of new blood vessels from pre-existing capillaries, development of vascular channels lined by tumour cells rather than endothelial cells, and incorporation of endothelial precursor and immune cells into existing vessels to expand the vascular network. Moreover, since tumour-associated blood vessels are structurally and functionally aberrant, targeting these defects could provide another avenue for anti-tumour treatment.
3.6 Conclusion

The complexity and diversity of the brain tumour-associated vasculature appears to be a double-edged sword with respect to successful treatment of primary and metastatic brain tumours. On the one hand, these neoplasms can use a number of different mechanisms to acquire the nutrients and oxygen supplied by blood vessels to sustain further growth and spread in host tissue. However, since a vascular supply is essential for tumour survival and dissemination, this vulnerability can potentially be exploited to impair brain cancer progression and alleviate associated symptoms. Understanding the dynamic relationship between tumour cells, brain vasculature and local factors at varying stages of tumour establishment, development and growth is essential to the development of effective targeted therapeutics. The present study has demonstrated a number of vascular sub-types in established cerebral metastasis of melanoma origin, but it is unclear from the literature whether such changes are the result of factors elaborated by neoplastic melanocytes themselves or those generated by the host microenvironment, or a combination of both. It is now accepted that targeting angiogenesis alone is insufficient and efforts to normalise tumour vasculature are expected to improve treatment response (Azzi et al. 2013).
Chapter 4

The Role of Caveolin-1 and NK-1R in Human Cerebral Metastatic Melanoma Tissue
4 Chapter 4: Role of Caveolin-1 and NK-1R in Human Cerebral Metastatic Melanoma

4.1 Background

Morphological alterations to the endothelial cells of the BBB in response to the presence of malignancy are well described throughout the literature (Papadopoulos et al. 2001, Ballabh et al. 2004, Daneman 2012, Luissint et al. 2012). A number of studies have investigated both primary and metastatic CNS tumours with regard to the integrity of the BBB and reported consistent alterations that differentiate tumour-associated vasculature from healthy parenchymal vessels (Jain et al. 2007, Martin et al. 2009, Wei et al. 2014). These alterations described in previous chapters include an increase in the perivascular space, thickened subendothelial basement membrane, varied tight junction structure, increased fenestrations, and altered caveolae/transporter expression. Such alterations can result from tumour-mediated cytokines and inflammatory mediators, chief among them, SP (Liu et al. 2000). While the involvement of abnormal TJ’s are credited in playing a central role in the progression of BBB alteration for a number of CNS pathologies, it may only partially account for the tumour-induced BBB breach, particularly in early metastatic development (Tuma et al. 2003, Banks et al. 2010).
The principal aim of this study is to determine whether CAV-1 and NK-1 receptor systems could play a role in the extravasation of metastatic tumours into the brain and foster their continued growth by stimulating tumour cell proliferation, dissemination and angiogenesis. As such, the present chapter is an immunohistochemical study of expression of CAV-1, NK-1R and SP in a cohort of human metastatic melanomas.

4.2 Introduction

The substance P (SP)/NK-1 receptor (NK-1R) system seems to play an important role in the progression of a number of different neoplasms. Substance P belongs to the tachykinin family of peptides and is widely distributed throughout the body (Mantyh 2002, Chappa et al. 2006). Its biological actions are principally mediated through the NK-1R. In the brain, SP is an excitatory neuropeptide that is mainly released from primary sensory nerve endings, but is also found in endothelial cells, from which it can be released by cytokine stimulation (Mantyh 2002). Since binding of SP to the NK-1R causes increased cerebral vascular permeability and alteration of expression of tight junction proteins, it has been posited to play a role in mediating metastatic tumour extravasation in the brain (Hasegawa et al. 1983, Munoz et al. 2010, Munoz et al. 2011, Munoz et al. 2014).
The cancer-related properties of SP include effects on tumour proliferation, neoangiogenesis, and tumour cell migration, all of which are required for tumour invasion, infiltration and metastasis (Munoz et al. 2005, Nowicki et al. 2007, Munoz et al. 2008, Munoz et al. 2010, Rodríguez et al. 2013). It also has an anti-apoptotic effect on tumour cells. Substance P is an important mediator of inflammation and increased cytokine production. In addition to its vasodilatory action on blood vessels, SP is mitogenic for vascular endothelial and mural smooth muscle cells (Pernow 1983, Stanisz 2001, Seegers et al. 2003). The NK-1R sustains tumour viability and, in addition to being overexpressed in tumour versus non-tumour cells, malignant tumours express more NK-1R than benign neoplasms. Moreover, the most malignant phenotypes have the highest NK-1R expression (Munoz et al. 2014). Several isoforms of NK-1R are expressed in tumour cells; one mediates slow growth, while another enhances tumour growth by stimulating the production of cytokines, which have growth-promoting properties (Bigioni et al. 2005). Many tumour cells overexpress NK-1R compared with non-tumour cells and SP and NK-1R have been found on intra- and peri-tumoural blood vessels. Although synthesised in the cytoplasm, SP is more strongly expressed in the nucleus, the latter suggesting that it could act as a genetic modulator (Mantyh 2002). The NK-1R is located in the plasma membrane, cytoplasm and, occasionally, the nucleus of tumour cells (Mantyh 2002).

Since NK-1R antagonists inhibit tumour cell growth and migration, and neoangiogenesis, they have become a useful target for anti-cancer

The role of caveolae in the progression of cancer progression and tumour metastasis is largely undefined, and much of the current literature is suggestive of a dual function in both tumour promotion and suppression (Razani et al. 2001, Quest et al. 2008, Shatz et al. 2008). Caveolin-1 (CAV-1), the main structural component of caveolae, is found predominantly in terminally differentiated cells, such as endothelium and smooth muscle, and has an important role in modulating cell signalling (Quest et al. 2008). Caveolin-1 also controls angiogenesis and its expression correlates with chemotherapeutic resistance (Shatz et al. 2008, Hehlgans et al. 2011, Parat et al. 2012). The recent finding that some caveolae contain the NK-1R suggests that there may be an interplay between caveolae-mediated transcytosis and signalling and the SP/NK-1R system (Covenas et al. 2014). Since CAV-1-mediated signalling pathways cause disruption of endothelial tight junction proteins in stroke and traumatic brain injury, it has been postulated that CAV-1 might also alter tight junctions in cerebral microvessels, thus facilitating breach of the BBB by metastatic tumour cells and the development of vasogenic oedema (Deng et al. 2012). Caveolae-dependent internalisation and recycling of critical tight junction protein claudin-5 has been linked to increased endothelial paracellular permeability during neuroinflammation.
(Jia et al. 2013). Furthermore, downregulation of stromal CAV-1 in tumours promotes tumour survival and is an indicator of a poor prognosis.

In an attempt to better understand the role of CAV-1 and the SP/NK-1R system in tumours, immunoexpression of these proteins was studied in 10 human melanomas metastatic to the brain.

4.3 Experimental Design

4.3.1 Immunohistological Analysis

Immunohistochemistry (IHC) was carried out on 5 μm sections of cerebral metastatic melanoma biopsies cut on a rotary microtome and mounted onto Superfrost® Plus microscope slides (Menzel-Glaser, New South Wales, Australia). Immunohistochemical analysis was performed for the neuropeptide SP and its receptor NK-1R, as well as CAV-1 (Table 4.1). Sections were immunostained with the DAB method to visualize the target protein.

<table>
<thead>
<tr>
<th>Antibody to</th>
<th>Clone</th>
<th>Dilution</th>
<th>Positive Control</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caveolin-1</td>
<td>Rabbit Monoclonal SP43</td>
<td>1:250</td>
<td>Human Hippocampus</td>
<td>LS Bio C210307</td>
</tr>
<tr>
<td>NK1R</td>
<td>Rabbit Polyclonal</td>
<td>1:4000</td>
<td>Human Hippocampus</td>
<td>Thermo Fisher PA3-301</td>
</tr>
<tr>
<td>Substance P</td>
<td>Mouse Monoclonal SP-DE4-21</td>
<td>1:5000</td>
<td>Human Midbrain</td>
<td>Abcam 14184</td>
</tr>
</tbody>
</table>
Figure 4.1: Representative images of SP immunoreactivity in human control tissue.

Representative images of SP immunoreactivity in human control tissue. (A) Several fibre tracts showed marked substance P immunopositivity in the form of immunopositive granules. (B) Sometimes the granules were larger and more intensely immunoreactive (arrows). (C) Some fibre bundles were strongly immunopositive. (D) Rarely a blood vessel showed apparent mural immunopositivity, possibly corresponding to substance P immunoreactive nerve endings, and (E) an occasional neuron and processes showed strong immunopositivity.
Slides were scanned using a NanoZoomer digital slide scanner (Hamamatsu, Hamamatsu City, Japan) and viewed with its associated software for analysis (NDPI View 2, Hamamatsu). Staining intensity was analysed using a semi-quantitative grading as previously described (Figure 4.2). Immunopositivity was graded on a scale of 0-3, where 0 = no staining, 1 = mild staining, 2 = moderate staining, and 3 = intense staining for both melanocytes and tumour associated blood vessels.

![Image](image_url)

**Figure 4.2**: Semi-quantitative grading system representative images of melanocyte staining.

Representative images of cerebral metastatic melanocytes showing immunohistochemical staining of varying intensities. **A-D** indicate NK-1R tumour cell staining intensities, while CAV-1 is shown in images **E-H**. Staining is graded on a scale of 0-3, where 0 = no staining (**A,E**), 1 = mild staining (**B,F**), 2 = moderate staining (**C,G**) and 3 = intense staining (**D,H**).

### 4.3.2 Statistical Analysis

All non-parametric data were assessed using a Kruskal-Wallis test with Dunn’s multiple comparison. Significance was determined at p-value <0.05.
4.4 Results

4.4.1 Cerebral metastatic melanoma-associated blood vessels have marked reduction in CAV-1 immunopositivity

In control brains, there was strong CAV-1 endothelial cell immunopositivity in all blood vessels, both endothelial and in mural smooth muscle cells. However, in the majority of melanoma-associated blood vessels, there was a marked reduction in immunopositivity (Figure 4.4). The results of the statistical analysis are shown in Figure 4.3.

![Figure 4.3: Immunohistochemical analysis of CAV-1.](image)

Immunohistochemical analysis membrane caveolae scaffolding protein (CAV-1) in a cohort of cerebral metastatic melanoma. **(A)** Histogram at left shows a marked reduction in vascular CAV-1 immunoreactivity, while, at right, there is marked increase in melanocyte CAV-1 expression, compared with normal brain tissue and immunonegative breast cancer cells, respectively (EC = endothelial cell, TC = tumour cell). Each point denotes individual rank of each case (*p<0.05, ++ p<0.0015; Scale bar B = 20 µm; C-D = 40µm).
Figure 4.4: Intratumoural vessels of cerebral metastatic melanoma demonstrate reduced Caveolin-1 immunopositivity.

**(A-B)** Blood vessels from a control brain showing strong endothelial and smooth muscle CAV-1 immunopositivity. *(C-F)* Venules within melanoma metastatic to the brain show markedly reduced CAV-1 immunoreactivity, however surrounding melanocytes show stronger CAV-1 immunopositivity. *(G-I)* Arteries/arterioles show markedly reduced CAV-1 immunoreactivity, the panel I showing significant disruption of the walls in these aberrant vessels. *(J)* Melanocytes showing marked CAV-1 immunostaining of cell membranes (arrows) and more modest cytoplasmic immunopositivity, a blood vessel at top left (bv) shows a marked reduction in CAV-1 immunostaining. *(K)* Melanocytes CAV-1 immunopositivity contrasted with immunonegative metastatic breast carcinoma cells *(L).* (Scale bar  A-B= 50 µm; C-D 75 µm; E = 65 µm, F = 45µm G = 50 µm; H-I = 65 µm; J-L = 25 µm).
4.4.2 Cerebral metastatic melanocytes overexpress CAV-1

Most melanocytes showed weak to moderate cytoplasmic CAV-1 immunostaining, with many of these tumour cells also showing strong membrane immunoreactivity (Figure 4.4, images K & L).

4.4.3 Cerebral metastatic melanoma have decreased BBB NK-1R expression

Most melanocytes showed moderate to strong cytoplasmic NK-1R immunopositivity, with a small subset in one melanoma showing coarsely granular cytoplasmic immunoreactivity, with an occasional melanocyte showing stronger peripheral immunostaining (Figure 4.5). In one melanoma, there also appeared to be nuclear NK-1R immunostaining (Figure 4.6). A small subset of blood vessels also showed endothelial NK-1R immunoreactivity.
Figure 4.5: Intratumoural vessels and melanocytes of cerebral metastatic melanoma are positive for NK-1R.

(A-B) There is vascular endothelial NK-1R immunopositivity (arrows), with melanocytes surrounding these vessels show strong NK-1R immunopositivity. (C) Some melanocytes showed weaker cytoplasmic NK-1R reactivity, while a subset showed coarse granular cytoplasmic immunopositivity (arrows) (D). An occasional melanocyte (inset) showed stronger peripheral NK-1R immunostaining (Scale bar A= 75 µm; B = 50 µm; C-D = 30 µm).
Figure 4.6: Metastatic melanocytes demonstrate NK-1R immunopositivity.

(A) A subset of melanocytes in one metastatic melanoma showed cytoplasmic and nuclear (arrows) immunopositivity (B) A small subset of melanocytes which showed coarse granular NK-1R immunopositivity. (Scale bar A = 45 µm; B = 25 µm).

Figure 4.7: Immunohistochemical analysis of NK-1R.

Immunohistochemical analysis of cerebral tachykinin receptor NK-1R, in a cohort of cerebral metastatic melanoma (A) Compared with a control immunonegative metastatic brain breast carcinoma, melanocytes showed greater NK-1R immunopositivity while the occasional vascular endothelial NK-1R immunoreactivity in melanomas was not significantly greater than in control brain tissue (****p=0.0001)
In SP positive control tissue from human midbrain (Figure 4.1), selected fibre tracts were robustly immunoreactive to varying intensity, while only occasional blood vessel and neuron was immunoreactive. (A-D) In all ten melanomas studied, melanocytes and blood vessels were uniformly SP immunonegative (Scale bar A = 35 µm; B = 25 µm).

4.5 Discussion

The present study sought to further our understanding of the role of the SP/NK-1R system and the principal caveolae protein, caveolin-1 (CAV-1), in the sustainability and progression of melanomas metastatic to the brain and the associated tumour vasculature.

In the metastatic melanomas studied herein, CAV-1 immunostaining of most tumour-associated blood vessels was reduced, as assessed by morphological
examination and histoquantification. This result is concordant with previous studies showing that vascular CAV-1 loss correlated with increased tumour angiogenesis (Chidlow et al. 2009, Tahir et al. 2009, Thompson et al. 2010). By contrast, the majority of melanocytes showed CAV-1 immunopositivity, which was moderate in the cytoplasm, but strong on plasma membranes. Previous studies have demonstrated that an increased expression of CAV-1 facilitates malignancy by inhibiting apoptosis, supporting attachment and adhesion, and promoting drug resistance associated with metastasis (Nwosu et al. 2016). The robust and consistent expression of CAV-1 by melanocytes suggests their important role in tumorigenesis, both as a promoter and suppressor of tumour growth and progression (Chatterjee et al. 2015, Nwosu et al. 2016), which is concordant with a number of previous studies in multiple tumour types, including liver, colon and breast (Burgermeister et al. 2008).

In this study, immunoexpression of NK-1R was widespread in all melanocytes, predominantly cytoplasmic but, occasionally, intranuclear. However, only a small subset of tumour-associated blood vessels exhibited endothelial NK-1 immunoreactivity. These morphological evaluations were supported by histoquantitation. Although the antibody used to detect SP expression in these metastatic melanomas was validated in positive control human brain tissue, there was no demonstrable SP immunoreactivity in any of the melanomas examined, either vascular or melanocytic.
It is well-established that NK-1R is immunoexpressed by the majority of tumour cells, particularly strongly in the more malignant forms; a reflection of its vital role in supporting tumour survival, growth and spread (Munoz et al. 2011). However, although SP has been implicated in tumour growth and dissemination, and angiogenesis, no immunoexpression was detected in any melanocytes or tumour-associated blood vessels in the present study. The reasons for this SP immunonegativity will require further investigation, but a number of possibilities are advanced. Following spinal cord injury, it has been shown that there is increased NK-1R immunoreactivity, but a decrease in SP expression, the latter attributed to release and subsequent depletion of SP stores (Leonard et al. 2014). Accordingly, it is possible that marked NK-1R upregulation found in the melanomas examined resulted in progressive, and substantial, diminution of SP in these tumours. Moreover, since we used archival brain material, it is possible that the nature of SP precluded it being expressed in these tumour biopsies, either due to its denaturation or altered antigenicity, leading to a failure of immunodetection. Furthermore, while SP increases the sensitivity of melanomas to radiotherapy, this therapy also induces loss of SP from these tumours and may underlie the radioresistance of some melanomas (Korcum et al. 2009). If all of the 10 melanoma cases examined in the present study had been subjected to radiotherapy, this could explain, in part at least, the lack of SP immunoexpression in the tumours examined herein. Unfortunately, we had limited information relating any radiotherapy or chemotherapeutic administration these patients may have received and, in addition to SP depletion by radiotherapy, some
chemotherapeutic agents have been shown to deplete SP levels in tumours, as well as the tight junction protein, claudin-5 (Korcum et al. 2009).

4.6 Conclusion

In summary, while CAV-1 was strongly expressed in blood vessels in control brain tissue, it was markedly depleted in melanoma-associated blood vessels, the latter being linked to robust neoangiogenesis in neoplasms. The majority of melanocytes expressed CAV-1, particularly on cell membranes, reflecting its important role in tumour growth and progression. While only a small subset of tumour-associated blood vessels showed endothelial NK-1R immunopositivity, most melanocytes showed strong immunoreactivity, concordant with its vital role in the sustainability of malignant neoplasms. There was no immunoexpression of SP in blood vessels or melanocytes in any of the 10 metastatic melanomas examined and a number of reasons are advanced to explain this lack of immunoexpression.
Chapter 5

Characterisation of an in-vitro Blood-Brain Barrier Model
Chapter 5: Characterisation of an in-vitro Blood-Brain Barrier Model

5.1 Background

Currently, a number of in vitro platforms are being trialled for the investigation of transendothelial migration of metastatic tumour cells into the brain. However, few enable functional and structural analyses of brain endothelium to be performed in parallel.

This chapter describes our attempt to determine whether an immortalised cell line derived from human microvascular endothelial cells (hCMEC/D3) seeded into 8 µm pore collagen-coated, hanging transwell inserts mimics phenotypical barrier properties sufficiently to enable further characterisation of tumour cell interaction with the cerebral vasculature. Transendothelial electrical resistance (TEER) was measured daily to assess barrier formation in real-time and permeability studies were also conducted to assess barrier integrity. Immunofluorescence for key tight junction proteins (claudin-5, ZO-1, occludin) and transmission electron microscopy were performed to assess tight junction integrity. Western blotting was used to determine CAV-1 and NK-1R expression.

In this BBB model, transparent polyester (PET) membrane inserts support an hCMEC/D3 endothelial cell phenotypical monolayer, which has functional barrier properties of tight junction formation with appropriate resistance and
permeability scores. Transmission electron microscopy indicated morphologically healthy endothelial cells housing occasional caveolae within the cell membrane and laterally-located tight junctions. As such, hCMEC/D3 exhibit acceptable characteristics of a BBB in the transwell system, making this an attractive platform to study mechanisms of human metastatic tumour cell endothelial transmigration.

5.2 Introduction

Whilst not all malignant cancers uniformly infiltrate distal organs, select subtypes will exhibit a predilection for certain sites. It is reported that approximately 20-40% of all adult patients with a systemic malignancy will present with a secondary symptomatic brain metastasis over the course of their disease, implicating the brain as a prominent tumorigenic refuge for migrating cells (Tsao et al. 2005). Malignant melanoma is widely acknowledged to preferentially metastasise and subsequently colonise the central nervous system (CNS) (Goulart et al. 2011). As such, the interplay between tumour cell characteristics and features of the host site are credited for regulating the destination and subsequent formation of metastases (Amit et al. 2013). Despite exhaustive genetic studies investigating the involvement of specific genes as a means of identifying a driving force for tumour development and progression, the fundamentals of cerebral metastatic formation remain largely undefined (Nguyen et al. 2007). Understanding the relationship circulating tumour cells establish with the specialised cerebral ECs
of the BBB may unveil novel targets for the prevention or control of cerebral dissemination in high risk melanoma patients, and ultimately improved outcomes. Although our understanding of metastasis, and specifically cerebral metastasis, is constantly evolving, the interpretation of many studies’ data are limited due to the inherent challenges in accessing the BBB (Vernon et al. 2011, Czupalla et al. 2014). As such, the development of reliable in vitro alternatives are required to further explore of BBB pathophysiology and permit novel drug development and screening.

For an in vitro model of the BBB to be valid, it must exhibit three main criteria; (1) high transendothelial resistance (TEER); (2) low permeability to solutes, and (3) functional expression of transporter mechanisms. Recently, the hCMEC/D3 cell line was generated by immortalising primary human brain endothelial cells from temporal lobe microvessels (Vu et al. 2009). The suitability of the hCMEC/D3 cell line as an appropriate candidate for the experimental in vitro model of a functional BBB model is supported by the expression and localisation of distinctive cerebral microvascular endothelial cell traits at the molecular level resembling those of primary microvascular cells. However, unlike primary cells, the hCMEC/D3 BBB cell line is more suitable for experimental purposes on account of the robust nature of the cells being maintained over a number of passages (Sajja et al. 2014). Furthermore, hCMEC/D3 cells exert the same TEER as primary cells, even in the absence of astrocytic foot processes (Cucullo et al. 2008).
The use of the hCMEC/D3 cell line in transport studies has been widely reported (Poller et al. 2008, Higuchi et al. 2015, Alemi et al. 2016). However, few studies exist detailing the use of this cell line in migration studies. This study, therefore, aimed to determine if a migratory pore size of 8 µm pore transwell membrane insert supports hCMEC/D3 monolayer establishment with phenotypical and functional characteristics of BBB formation. If successful, this model will be used to further explore malignant melanocyte interaction with cerebral microvascular endothelium in a reproducible, high-throughput system.

5.3 Materials and Methods

5.3.1 Experimental Design

5.3.1.1 Blood-Brain Barrier hCMEC/D3 Cell line

All cell culture procedures were carried out in sterile conditions within a certified laminar flow as described in Chapter 2 (section 2.2.2). For all experiments within this chapter, hCMEC/D3 cells were cultivated as per methods outlined in chapter 2 (section 2.2.5 & 2.2.6).

5.3.1.2 Transwell Apparatus

This system consists of an inner chamber of collagen-coated porous membrane, allowing the hCMEC/D3 monolayer to be suspended in supportive medium (Figure 5.1).
5.3.2 Transendothelial Electrical Resistance

An electric volt ohm-meter (World Precision Instruments, Sarasota, FL, USA) fitted with chopstick electrodes, was used to pass current and measure voltage to determine the transendothelial resistance (ohms) of the developing monolayer. It is critically important to note and maintain position and stability of the electrodes during readings to ensure reproducible results.

5.3.2.1 Permeability Assay

Initial permeability assessments of the transwell inserts were performed to obtain baseline permeability value. This score is derived from the transport of two labelled compounds of varying molecular size (4 and 70kDa) See chapter 2 (section 2.2.7) for tracer details. This information is used for comparison to experimental data containing cell monolayers.
5.3.2.2 Western Blotting

Total protein lysates were cultivated and quantified as detailed in chapter 2 (section 2.2.11). Antibody details are as follows; caveolin -1 (Cell Signaling Technology, #3238; 1:1000), neurokinin 1 Receptor (Abcam #ab183713; 1:5000), GAPDH (Abcam #ab83956; 1:2000), IRDye anti-rabbit 800CW, IRDye anti-chicken 680 CW. Western blots were assessed using ImageStudio Lite, version 4.0.

5.3.2.3 Transmission Electron Microscopy

Monolayers were assessed for distribution and attachment to collagen-coated membrane inserts in order to confirm optimal density and time-point for migration studies. Initially, it was important to determine whether permeable transwell membranes would support TEM processing. Once a stable, high TEER was achieved, monolayers were fixed and processed as detailed in chapter 2 (section 2.2.9).

5.3.2.4 Immunofluorescence

Immunofluorescence complemented Western blotting analysis and TEM studies to confirm the expression and distribution of hCMEC/D3 cell characteristics. Chapter 2 (section 2.2.8) details the immunofluorescence protocol used in all in vitro experiments. A number of antibodies were trialled, with the following selected for use in the in vitro chapters (Table 5.1).
Table 5.1: Antibodies for *in vitro* immunofluorescence

<table>
<thead>
<tr>
<th>Antibody to</th>
<th>Clone</th>
<th>Dilution</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pecam/CD31</td>
<td>Mouse Monoclonal JC70A</td>
<td>1:750</td>
<td>Dako M0823</td>
</tr>
<tr>
<td>Occludin</td>
<td>Rabbit Polyclonal</td>
<td>1:100</td>
<td>Abcam ab64482</td>
</tr>
<tr>
<td>ZO-1</td>
<td>Polyclonal Rabbit</td>
<td>1:100</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>Claudin-5</td>
<td>Mouse Monoclonal 4C3C2</td>
<td>1:100</td>
<td>Invitrogen 352500</td>
</tr>
</tbody>
</table>

5.3.3 Statistical Analysis

Data were compared using Prism version 7.0 (GraphPad® Software, San Diego, USA). Where necessary, a D’Agostine-Pearson omnibus test was used to access normality. When normality was confirmed, a paired t-test or two-way analysis of variance with appropriate post hoc testing were performed to detect statistical significance. In other cases, a Kruskal-Wallis test with Dunn’s multiple comparisons test and Dunnett correction was performed. A $p$-value of $<0.05$ was considered significant.
5.4 Results

5.4.1 hCMEC/D3 cells in vitro maintain morphological characteristics of cerebral microvascular endothelial cells found in normal brain tissue.

When grown to confluence with collagen type IV in optimal culture conditions, hCMEC/D3 cells form a contact-inhibited monolayer of elongated cells (Figure 5.2).

![Figure 5.2: hCMEC/D3 human microvascular endothelial cells in vitro.](image)

5.4.2 Cultured hCMEC/D3 cells retain expression of key endothelial markers

The hCMEC/D3 cells in culture immunostain positively for endothelial marker PECAM/CD31, confirming conservation of cerebral endothelial phenotype (Figure 5.3 – Image A). The border of each cell can be distinguished by immunocytochemical circumferential staining of the tight junction marker claudin-5).
Figure 5.3: Immunofluorescent staining of hCMEC/D3 cells in culture.

(A) hCMEC/D3 cells show cytoplasmic positivity for PECAM/CD31 endothelial cell marker, and (B) tight junction protein – claudin-5.

5.4.3 Cultured hCMEC/D3 cells express key membrane receptors and transport channels

The membrane expression of a number of receptors and transporters, both luminal and abluminal, have been confirmed for the hCMEC/D3 cell line. Identifying the presence and distribution of the caveolae structural protein, caveolin-1, and NK1 tachykinin receptor, in hCMEC/D3 cell line was sufficient for the purposes of the current study (Figure 5.4).

Figure 5.4: Western Blot results demonstrating NK-1R and CAV-1 expression in confluent hCMEC/D3.
5.4.4 The hCMEC/D3 Barrier Function

Monolayers exhibit high transendothelial electrical resistance and low permeability to sodium fluorescein and FITC-albumin in the transwell system at optimal seeding density (Figure 5.5). In conjunction with the formation of interendothelial junction proteins producing adequate resistance scores (TEER), hCMEC/D3 cells are capable of generating restrictive permeability to solutes. Transendothelial electrical resistance (TEER) permits an immediate measurement of monolayer resistance for assessment of tight junction functionality within the system. Transendothelial electrical resistance values were measured manually in each transwell system set up and monitored daily using an EVOM2. Optimal seeding density was determined at $1 \times 10^5$ cells/cm$^2$, with resistance achieved on day 5 post-seeding (Figure 5.5).

![Figure 5.5: Transendothelial electrical resistance (TEER) development over 7 days in hCMEC/D3 cell line.](image)

The hCMEC/D3 cells seeded at a density of $1 \times 10^5$/cm$^2$ achieved optimal resistance within 5 days ($p>0.05$). Seeding density of $1 \times 10^4$/cm$^2$ demonstrated an elevated trajectory and delayed maturation. However, at this density, resistance tended to plateau, without achieving optimal range. Data expressed as mean ± SEM.
Permeability assays using fluorescently-labelled tracers of varying molecular size (4-70kDa) enable definition of the relative contribution of paracellular and transcellular pathways of permeability under varying conditions in this model BBB system. The selected tracers (Na-F; MW 376.27Da, FITC-albumin; MW 70kDa, RITC 70kDa) normally have low penetration across the BBB and are not substrates for efflux transporters located on endothelial cell membranes. Importantly, permeability studies further confirm that hCMEC/D3 cells adequately exhibit physiological properties resembling those of the BBB. Under optimised culture conditions, barrier formation appeared to be successful at a seeding density of 1x10^5 cells/cm^2, as indicated by minor fluorescent signal detection in the basal chamber (Figure 5.6).
Figure 5.6: Tacer permeability of hCMEC/D3 transwell monolayer.

The hCMEC/D3 cells grown to confluence in transwell system for five days and cultured with NaF (A) or FITC-Albumin (B) in the apical chamber. Fluorescence was measured in basal chamber at given time points. Barrier integrity was achieved for seeding density $1\times10^5$ cells/cm$^2$ for both tracers, when hCEMC/D3 inserts were compared with blank inserts ($p=0.0001$). Data expressed as mean ± SEM.

Taken together, low permeability to solute tracers, coupled with high TEER measurements, indicated that optimal barrier integrity and tightness was being achieved at a day five post-seeding density of $1\times10^5$ cells/cm$^2$. 
5.4.5 Transmission electron microscopy images of hCMEC/D3 cells grown on permeable transwell membrane

Ultrastructural examination confirmed that hCMEC/D3 monolayers grown on transwell membranes maintain an endothelial phenotype and, critically, appropriate intercellular tight junction complexes at the lateral border of endothelial cells (Figure 5.7 –Image A). Occasional membranous caveolae were found (Figure 5.7 –Image C).
Figure 5.7: Transmission electron microscopy of hCMEC/D3 in experimental culture conditions.

Characterisation of transwell apparatus with hCMEC/D3 cells (A) Cultured endothelial cells exhibit close apposition with formation of tight junction complexes (arrows) (B) Cytoplasmic organelles are well-preserved, confirming cell viability. (C) Confirmation of correct endothelial morphological arrangement and expression of a caveolae invagination of the cell membrane (square) (D) Transmission electron microscopy images of hCMEC/D3 cells grown on filter membranes (E) Endothelial cell, with the cell nucleus (n) labelled.

5.5 Discussion

The application of an hCMEC/D3 cell line as a rudimentary scaffold for conducting exploratory investigations of BBB function and dysfunction has been widely validated and is considered to be a reliable replication of the many unique properties of cerebral microvascular endothelium. This study has clearly highlighted the potential application for this cell line in migration and barrier studies. Importantly, confluent hCMEC/D3 constitutively display a number of endothelial and BBB-specific markers, functional receptors, and
transport channels. Moreover, the immunolocalisation of these proteins remains consistent with their functional role within the dynamic constraints of the BBB. Junctional proteins were appropriately observed to be intercellular, demonstrating overall tight junction organisation consistent with that normally found in situ. No change in cell phenotype was detected with passage increase (not shown). This study also confirmed endothelial expression of CAV-1, a key contributor to both barrier permeability and metastatic tumour dissemination.

A number of optimisation points were trialled during the generation of this model, in order to confirm that key barrier features were expressed, consistent with its applicability as an in vitro model for the study of metastatic tumour transendothelial migration. Initial seeding densities were streamlined to include high – $2 \times 10^5$, medium – $1 \times 10^5$, and low- $1 \times 10^4$ and trialled in both collagen present (for multiple incubation times) and collagen-free conditions. It was determined that pre-coating membranes for one hr prior to seeding is sufficient to induce endothelial cell attachment, with further investigations determining a seeding density of $1 \times 10^5$/cm$^2$ is optimal for generation of a tight monolayer by day five.

Since the interaction between endothelial cells and astrocytes (reflecting the anatomical organisation of the BBB, in which cerebral endothelial cells are almost completely invested by the perivascular foot processes or end-feet of astrocytes) have been extensively studied, a number of in vitro barrier models
use co-culture models, incorporating both of these cell types (Abbott et al. 2006). As such, co-culture with SVG p12 (ATCC #CRL-8621), or SVG conditioned media, was trialled as it is generally accepted that cerebral glial cells confer barrier inducing properties and improved barrier function (Thomsen et al. 2015, Wang et al. 2015). However, results of these studies (data not shown) indicated suboptimal barrier performance. It is thought that the large pore size (8 µm) required for migration experiments encourages astrocyte processes to not only make contact with the endothelial monolayer, but also cause localised disruption, resulting in lower resistance and increased permeability scores. Furthermore, culturing with astrocyte-conditioned media often led to monolayer contamination and an altered endothelial phenotype.

Characterisation of the monoculture model confirmed its suitability for further investigation of metastatic melanoma transendothelial migration and study of involvement of caveolae and NK-1R-mediated barrier alterations. Ultrastructurally, the hCMEC/D3 monolayers displayed normal characteristics of cerebral endothelium, including lateral binding junctional complexes, typical cytoplasmic organelle distribution, and an elongated disposition across the transwell membrane. These findings also accord with previous reports (Eigenmann et al. 2013). Importantly, cultured endothelial filter inserts permitted subsequent TEM processing, as evidenced by preservation of mitochondrial cristae and other cytoplasmic organelle (e.g. endoplasmic reticulum) structure. Establishing that this model is suitable for TEM imaging is a
significant advantage for it enables the investigation of sequential cellular endocytosis, localisation and transcytosis, which are important sub-cellular events involved in melanoma cell transendothelial migration.

A wealth of evidence supports the notion that modification of tight junction proteins corresponds with altered barrier performance, both developmentally, and in response to pathological cues (Stamatovic et al. 2009). Kinases and inflammatory mediators occupy much of the current research space, as evidence suggests that they can induce post-translational degradation, phosphorylation, redistribution and internalisation of TJ proteins and, importantly, those responsible for BBB function such as ZO-1 and claudin-5 (Song et al. 2007). There was no evidence of internalisation of ZO-1, or claudin-5, in the hCMEC/D3 monolayers grown in the transwell system, suggesting that this monoculture system supports molecularly conserved, functional TJs that resemble the typical barrier formation found in vivo. Caveolin-dependent internalisation and recycling of claudin-5 has been implicated in increased endothelial barrier permeability in the presence of neuroinflammation (Jia et al. 2014). The fact that the hCMEC/D3 cell line expresses CAV-1 protein, and has morphologically demonstrable caveolae invaginations, further highlights the suitability of this model for the investigation of caveolae as a metastatic tumour cell promoter. Development of this in vitro model not only enables the study of barrier mechanisms associated with permeability changes and remodelling, but also acts as a suitable platform in
which to screen pharmacological agents that seek to exploit these modifications.

5.6 Conclusions

Based on the findings of the current chapter, the hCMEC/D3 cell line appears to be suitable candidate for the development of *in vitro* BBB models. This study has successfully shown that hCMEC/D3 endothelial cells grown in a transwell membrane apparatus are able to develop, and maintain, the functional barrier characteristics that required for the investigation of metastatic tumour cell interaction with the BBB.
Chapter 6
The Effects of Targeted Caveolae and NK1 Antagonist Treatment on Tumour Cell transmigration In Vitro.
6 Chapter 6: The Effects of Targeted Caveolae and NK1 Antagonist Treatment on Tumour Cell transmigration In Vitro.

6.1 Background

The BBB is a dynamic interface between blood and brain designed to restrict the passage of circulating substances into the parenchyma and maintain central nervous system homeostasis. However, in spite of this formidable “barrier” obstacle, some metastatic tumour cells are able to breach the BBB and colonise the brain, although the precise mechanisms by which this occurs are incompletely understood and pose a challenge to both researchers and clinicians.

Until recently, it was accepted that barrier regulation was primarily enforced through ‘gating’ properties of tight junction (TJ) proteins and that the ensuing molecular passage was largely size-dependent (Abbott et al. 2010). It is now apparent that a number of additional factors determine what is permitted to enter the brain and by which transport pathway this is achieved. In Chapter 5 of this thesis, a hCMEC/D3 endothelial cell line was assessed as being a suitable candidate for BBB in vitro modelling.
6.2 Introduction

The diagnosis of a secondary brain tumour signals the dissemination of a primary neoplasm, for which current treatment is limited and largely ineffective. As such, secondary brain tumours are responsible for majority of cancer-related deaths in Australian adults.

Secondary brain tumour formation is a complex process, heavily dependent on characteristics of both the primary tumour and the brain microenvironment. It involves two main phases: initial migration of tumour cells from the primary site, followed by the secondary colonisation of a new location. Cancer cells within a primary tumour acquire genetic alterations which enable them to migrate from their original tissue site and gain entry to surrounding blood vessels (and/or lymphatics), by which route(s) they can potentially spread widely via the systemic circulation. In order for cancer cells to reach the brain, and form a secondary tumour, they must first pass through the highly restrictive BBB (Wilhelm et al. 2011, Wilhelm et al. 2013).

Generally, the passage of cell entry into a tissue is understood to occur via two routes: movement between two ECs (paracellular) or transport across a single EC (transcellular). Paracellular or 'between-cell' migration is regulated, and largely limited by, the presence and stability of specialised proteins known as tight junctions. By contrast, transcellular movement involves the use of channels known as caveolae, which have been shown to play a role in the multi-step process of molecules moving from blood to brain. It is also
suggested that a low-grade inflammatory state often induced by the presence of a metastatic tumour favour will further drive transendothelial migration of tumour cells into the brain, either through upregulation of transcytotic mechanisms or caveolin-mediated tight junction remodelling (Fazakas et al. 2011, Molnar et al. 2016).

Substance P is a neuropeptide implicated in neurogenic inflammation involved in a number of neuropathological states, including BBB disruption, multiple sclerosis and cancer growth. Administration of a SP receptor NK-1R antagonist has been found to be beneficial in limiting tumour growth, stabilizing the BBB, and reducing complications such as tumour-associated oedema (Harford-Wright et al. 2013, Rodriguez et al. 2013). It has been suggested that targeting caveolae channels, along with the NK-1R for SP, may impede the early entry of cancer cells into the brain and potentially form the basis of a novel therapy to prevent the formation of secondary brain tumours in high-risk patients. Accordingly, this is a major impetus for the conduct of the present study.

*Filipin* is a macrolide, pentene polyene antibiotic which belongs to the sterol-binding class of pharmacological agents, which are known to disrupt caveolae in a number of cell types, including endothelial cells (Severs et al. 1986, Rothberg et al. 1990, Davis et al. 1992). Furthermore, *Filipin* is able to disrupt caveolae and exclusively inhibit their function, without altering other pathways such as clathrin-dependent endocytosis (Schnitzer et al. 1994, Frank
et al. 2003). Filipin is, therefore, a promising agent for investigation of caveolae-mediated processes associated with endocytosis, tight junction modification, and tumour cell transendothelial migration.

It has been documented in this thesis and briefly within the literature that SP/NK-1R and caveolae have unclear roles in the mechanisms of metastasis and tumour-associated cerebral vascular alterations (Rodriguez et al. 2013). It is evident that SP/NK-1R and CAV-1 are altered in the malignant setting, with much of the research focusing on a mitogenic and angiogenic action on both tumour and endothelial cells (Ziche et al. 1990, Munoz et al. 2013). However, little is understood about whether these systems act in synergy to bring about localized vascular changes, whether that is endothelial tight junction mediated, increased trancytotic capability, or a combination of the two. Consequently, administration of a NK-1R antagonist that inhibits the action of SP and Filipin III which disables caveolae internalisation may further elucidate the mechanism and potential relationship of these two systems. To investigate this, the present experiments first developed a transmigration system to mimic metastatic interaction with cerebral endothelium. Further, metastatic migration of the A375-M1 cell line was examined in the presence of both antagonist treatments, as well as exogenous SP, to ascertain inhibitory effects on rate of migration.
6.3 Methods

6.3.1 Experimental Design

6.3.1.1 Cell Culture

All cell culture procedures using endothelial (hCMEC/D3) and melanoma (375-M1) cell lines were carried out as described in Chapter 2 (section 2.2.2). For all experiments within this chapter, transwell assays were generated as per methods outlined in Chapter 5 (section 5.3.3).

6.3.1.2 A375-M1 and hCMEC/D3 Substance P secretion

Human melanoma A375-M1 and endothelial hCMEC/D3 cells lines were investigated for their substance P secretion within 24 hr of expansion, as described in Chapter 2 (section 2.2.12). Briefly, following 24 hrs of incubation in serum-free media, cell supernatants were collected, centrifuged, and analysed for SP content using Quantikine ELISA kit for substance P.

6.3.1.3 Treatments

The hCMEC/D3 and A375-M1 cells were grown to confluence in 96-well plate and treated with;

Filipin III (Sigma-Aldrich, NSW, Australia, #F9765) at the following doses: 1, 1.5, 2.5, 5 and 10 ng/mL.

Substance P (Sigma-Aldrich, NSW, Australia, #S6883) at the following doses: 1, 5 and 10 ng/mL.
NK-1R antagonist EUC001 (Eustralis Pharmaceuticals Ltd, Melbourne) at the following doses: 0.1, 0.5, 2.5, 3 and 5 µg/mL.

6.3.1.4 Cell Viability Assessment

Cell viability assays were performed to exclude possible toxic effects of inhibitors and treatments. Assays were performed in the presence of Filipin III, NK-1R antagonist and SP, as detailed in chapter 2 (section 2.2.10). Furthermore, a 0.01% DMSO vehicle control and 0.05% Triton X (TX)-100 positive control were included. TX-100 was selected as a positive control based on its ability to disrupt barrier integrity through breakdown of the plasma membrane.

6.3.1.5 Transmigration Assay

The hCMEC/D3 monolayers were grown to confluence as detailed in chapter 5 (section 5.3.3) and exposed to A375-M1 cells once a high, stable TEER was achieved.

The 1x10^5 A375-M1 human melanoma cells were plated into the apical chamber onto the hCMEC/D3 monolayer in serum-free media. Cells were left for 5 and 24 hr before analysis. All basal chambers contained complete media to generate a chemotactic gradient for migrating A375-M1 cells to follow.
6.3.1.6 Labelling A375-M1 cells for visualisation and counting

To quantify the number of transmigrated A375-M1 cells across the hCMEC/D3 transwell system, cells were labelled with a fluorescent marker – Cell Tracker™ Green CMFDA as detailed in chapter 2 (section 2.2.4). At the appropriate time points cells were fixed with ice-cold acetone and non-migrated cells from the apical chamber were removed with a cotton swab and migrated melanoma cells via the pores on the filter were counted. This setup enables the quantitative analysis of the transendothelial migration of tumor cells. The number of transmigrated A375-M1 cells was determined by averaging the total number of green fluorescent cells in 10 random fields at 200X magnification under a fluorescent microscope.

6.3.2 Statistical Analysis

Data were compared using Prism version 7.0 (GraphPad® Software, San Diego, USA). Where necessary a D’Agostine-Pearson omnibus test was used to access normality. When normality was confirmed, a paired t-test or two-way analysis of variance with appropriate post hoc testing were performed to detect statistical significance. In other cases, a Kruskal-Wallis test with Dunn’s multiple comparisons test and Bonferroni or Dunnetts correction was performed. A p-value of <0.05 was considered significant.
6.4 Results

6.4.1 Substance P secretion by human melanoma cell line A375-M1

Human melanoma cell line was analysed for SP content and secretion in isolation and co-culture with hCMEC/D3 (Figure 6.1). Cultured A375-M1 cells secreted high levels of SP within 24 hrs of growth. When co-cultured with hCMEC/D3 cells, SP levels were further elevated.

Figure 6.1: Substance P secretion ELISA.

Secretion of SP by A375-M1, hCMEC/D3 and co-culture of the two cell lines. A375-M1 contains high levels of SP when grown in isolation, and further increased under co-culture conditions. The results are presented as mean ± SEM (p=<0.0001).
6.4.2 Treatment with Filipin III and NK-1R antagonists at high concentration reduce hCMEC/D3 and A375-M1 cell viability in vitro

Assessment of hCMEC/D3 and A357-M1 cell viability following administration of the caveolae-inhibitor, Filipin III, an NK-1R antagonist (EUC001; gifted from Eustralis Pharmaceuticals, Melbourne), and exogenous SP was determined using an MTT assay.

Human cerebral microvascular endothelial cells (hCMEC/D3) treated with Filipin III treatment showed significant disruption to cellular metabolism at concentrations greater than 1ng/mL. Similarly, treatment with the NK-1R antagonist, at 3µg/mL and 5µg/mL, significantly impaired cell viability compared with non-treated controls. Conversely, administration of substance P at 10ng/mL was shown to significantly increase cellular metabolism and, by extension, the proliferative capacity of hCMEC/D3 cells in vitro (Figure 6.2).
Filipin at a dose rate of 1.5ng/mL, 2.5ng/mL, 5ng/mL and 10ng/mL decreased cell viability within 24 hr of treatment as indicated by decreased cellular metabolism detected in this assay. Similarly, high concentrations of NK-1R antagonist, at 3ug/mL and 5ug/mL impaired cell viability compared with untreated controls. Based on this assay, it is deduced that substance P shows no toxicity to hCMEC/D3 cells and, at 10ng/mL, there is increased viability compared to untreated controls. Data is expressed as mean ± SEM, where \( p<0.05 \) is considered to be statistically significant; \*\( p<0.05 \), **\( p<0.001 \), ***\( p<0.0001 \).

The human malignant melanoma (A375-M1) cell line demonstrated susceptibility to Filipin III toxicity at concentrations of 2.5ng/mL, 5ng/mL and 10ng/mL. Furthermore, administration of the NK-1R antagonist at 2.5 ug/mL, 3ug/mL and 5ug/mL was found to significantly reduce cell viability compared with non-treated controls. Finally, exposure to SP supported melanoma cells in vitro, with all concentrations showing robust levels of viability, and exposure to 10ng/mL significantly increasing cellular metabolism (Figure 6.3).
Filipin III administered at dosage 2.5ng/mL, 5ng/mL and 10ng/mL decreased A375-M1 cell viability within 24 hr of treatment. NK-1R antagonist elicited a similar toxic response at 2.5 µg/mL, 3µg/mL and 5µg/mL compared with untreated control. Administration of substance P stimulates proliferative effects to A375-M1 cells at all concentrations, with 10ng/mL significantly increasing cell viability compared with untreated controls. Data expressed as mean ± SEM where p<0.05 is considered to be statistically significant; *p<0.05, **0.001, ****p<0.0001).

Thus, the transmigration treatment regimen was capped at Filipin III - 1ng/mL, NK-1R at 2.5µg/mL and SP at 5ng/mL in order to eliminate any effects that may result from compromised cell viability.

6.4.3 Transmigration Assay Characterisation

The in vitro model of transendothelial tumour cell migration characterised in Chapter 5 of this thesis was here further evaluated.
The model, which consists of brain microvascular endothelial cells cultured on 8 µm pore filter inserts, was exposed to highly metastatic A375-M1 melanocytes to determine the migration capability of the latter. It was found that this cell line was able to breach the endothelial monolayer and traverse the insert pores within 5, and up to 24, hrs of administration.
Figure 6.4: Transmission electron microscopy images of the hCMEC/D3 transwell system for the investigation of A375-M1 migration.

(A-B) Filter insert sections showed A375-M1 melanocytes (m) closely apposed (white arrows) to hCMEC/D3 endothelial cell (e) membranes (arrows). They were also found to have breached monolayer prior to migration as demonstrated by the presence of melanocytes within the filter pore. (C) Following complete migration through the endothelial monolayer and filter pore, A375-M1 melanocytes appear to continue their movement across the basal length of the filter membrane (arrow).

6.4.4 Treatment efforts failed to reduce A375-M1 melanocyte transmigration across hCMEC/D3 in vitro barrier.

Fluorescently-labelled, A375-M1 melanoma cells were plated on to the apical aspect of the cultured hCMEC/D3 endothelial monolayer once barrier characteristics had been achieved, as previously described. In order to assess the potential efficacy of treatment regimens, monolayers were both pre- and post-treated to determine any inhibitory effect. Transmigration across the hCMEC/D3 endothelial monolayer was not significantly affected by
caveolae-targeted inhibition by Filipin III pre- or post-treatment, nor was it inhibited through pharmacological targeting of the NK-1R (Figure 6.5).

6.4.5 Pre-treatment with substance P increased melanocyte transmigration capability at 5 hr.

A substance P pre-treatment regime was applied 30 mins prior to A357-M1 melanocyte induction to investigate the tumour cell migration capacity in the presence of low-grade neuroinflammation. Substance P was found to promote A375-M1 melanocyte transmigration as assessed by increased A375-M1 green-labelled cells counted on the basal surface compared with control wells (p=0.001) (Figure 6.5).
Figure 6.5: A375-M1 melanocyte transmigration across the hCMEC/D3 transwell system at 5 hrs.

(A) Cell Tracker™ Green CMFDA-labelled A375-M1 melanoma cells have migrated through to the basal aspect of the membrane 5 hr. (B) Merged image of DAPI (blue) stained hCMEC/D3 transwell endothelial cell monolayer overlayed by transmigrated A375-M1 melanocytes (green) attached to the basal surface. (C) A375-M1 tumour cells were seeded over the monolayer in the presence or absence of treatments and allowed to transmigrate up to 5 hr. Green-labelled cells were then counted and expressed as a percentage of A375-M1 relative to a control. Treatment with Filipin III, NK-1R and SP at all concentrations were shown to have no significant effect in reducing A375-M1 melanocyte transmigration at 5 hrs. Pre-treatment of SP at 5ng/mL was shown to significantly increase A375-M1 cell migration at 5 hr, compared with control wells. Data shown as mean ± SEM (where p<0.05 is considered to be statistically significant; **p=0.001), and representative of at least three independent assays performed in triplicate wells.
6.4.6 Migrated A375-M1 melanocytes showed conserved phenotypical properties at 24 hrs.

Consideration was given to potential morphological alterations sustained during tumour cell migration and treatment effects over time. Transmigrated cells were examined at 24 hr post-seeding and demonstrated no overt morphological changes, displaying a mature phenotype resembling that of their non-migrated counterparts.

Figure 6.6: Treated transmigrated A375-M1 cells show no morphological alterations when compared to control tumour cells (inset).

Image representative of n= 27/group (Scale bar = 50µm).

6.5 Discussion

The metastastic process resulting in cerebral colonization is complex and multifaceted, and known to involve a number of genetic and molecular alterations to both the receiving cerebral endothelium and infiltrating tumour cells.
This study firstly aimed to characterise metastatic melanoma interaction and endothelial transmigration in a novel *in vitro* model of the BBB. Similar systems have been widely used to study the transmigration of leukocytes through endothelial barriers (Kalman et al. 1976, Kugler et al. 2007). Results obtained in chapter 5 of this thesis confirmed that hCMEC/D3 endothelial cells grown on transwell inserts display sufficient BBB-like characteristics to warrant their use as an experimental model of the BBB. The A375-M1 human melanoma cell line was confirmed to secrete substance P when unstimulated. Moreover, this secretion was elevated in the presence of hCMEC/D3 endothelial cells, suggesting an interplay between tumour cells and host environment, as has previously been reported in the literature (Munoz et al. 2013). Additionally, the growth trajectory, and morphology, of this cell line was unaffected by fluorescent labelling, which remained stable for 72 hrs following uptake and incubation with Cell Tracker™ Green CMFDA. This stability would also enable extended time-points to be further investigated, particularly by *in vivo* time-lapse imaging.

Both TEM and fluorescent microscopy herein confirmed the transmigratory capacity of the A375-M1 melanoma cell line in this *in vitro* system, suggesting that it would be useful as a suitable candidate for on-going *in vitro* investigation into metastatic-migratory mechanisms. This accords with observations of Fazakas, et al, (2013) using the A2058 human melanoma cell line, a study which also demonstrated transmigrated melanocytes moving along the basolateral side of the endothelial monolayer (Wilhelm et al. 2013).
Furthermore, in vivo studies have shown that human breast cancer cells injected intracardially into mice will align along the blood vessels following extravasation (Lu et al. 2007). Taken together, these studies suggest a “pseudo-pericytic” behavior pattern of tumour cells once they have breached the BBB.

The second aim of this study was to identify whether pharmacological inhibition of caveolae and/or NK-1R could reduce melanocyte transmigration and further assess the posited role of caveolae and neurogenic inflammation in metastatic tumour cell endothelial transmigration. This is particularly important in the setting of the cerebral vascular response to malignancy and results in this thesis have characterised, for the first time, significant remodeling and, frequently, resulting aberrant structure of intratumoural (metastatic melanoma) blood vessels (Chapter 3) and postulated involvement of both CAV-1 and NK-1R in this process (Chapter 4). In order to exclude the possibility that any of the treatment regimens, at any concentration, might have had a toxic effect on melanocytes before they migrated, a cell viability assay was conducted. No reduction in tumour cell viability was found. Thus, any failure to reduce migration of melanocytes by any of the treatments would not have been due to the viability of these cells being compromised before they commenced their migration. Similarly, endothelial cells were shown not to be adversely affected by these treatments.
In the present *in vitro* study, both *Filipin III* and the NK-1R antagonist (EUC001) failed to have any significant inhibitory effect on A375-M1 migration across the BBB. It is possible that the lack of efficacy shown by these drugs in tumour cell migration inhibition may have been due to the fact that the melanocytes used transmigratory mechanisms that were not impeded by these agents. Additionally, Chatterjee et al. (2015) showed *in vitro* that cholesterol chelator methyl-beta-cyclodextran (MβCD) disrupts caveolae and when applied to a malignant pancreatic cell line, results in reduced invasion and migration in a similar transwell system, supporting the proposition that CAV-1 may carry varied roles dependent on cell origin (Chatterjee et al. 2015).

It is recognised that a number melanoma cell lines are effective in breaking down the paracellular barrier. Indeed, time-lapse studies by Molnár et al. (2016) demonstrate a time-dependent disappearance of junctional proteins from endothelial cell membranes, induced by human melanoma cells (Molnar et al. 2016, Varga et al. 2016). It is postulated that melanoma cells catalyse a proteolytic cascade and, since junctional proteins such as claudin-5 are targets for proteolytic degradation, this may account for the persistent melanocyte migration shown by the present study. Accordingly, *in vitro* mechanistic study showed that CAV-1 overexpression induced known mediators of migration and invasion, namely matrix metalloproteinases 2 and 9, and vascular epidermal growth factor which have all been shown to modulate tight junction proteins (Nwosu et al. 2016). Furthermore, it is known that caveolae are involved in a number of cellular processes, including cell
signalling and tight junction regulation and, as such, inhibition of caveolae by filipin III may impact some of these processes. The use of additional caveolae inhibitors, such as dynamin, chlorpromazine, or nystatin, which target various stages of the process of internalisation of the transcytosed material that is performed by the invaginated caveolin pits of the plasma membrane, may clarify the role of caveolae in endothelial permeability and vascular modification more generally (Oh et al. 1998, Nabi et al. 2003). Finally, caveolae activation requires active phosphorylation, which is induced by kinases, and impedance of kinase activity is a possible mechanism by which to frustrate tumour cell endothelial transmigration (Gottlieb-Abraham et al. 2013). Caveolae are dependent on the Src family of protein kinases, which are known to mediate endosomal compartments, cell adhesions, cellular differentiation, and migration. Identification of the specific kinases and phosphatases involved in regulating phosphorylation of caveolae will help define the signalling pathways involved in upstream control of caveolae and may provide a novel therapeutic target for inhibition of Src-mediated regulation of endothelial penetration by metastatic tumour cells.

6.6 Conclusion

While metastatic tumour cells that are presented to the cerebral microvascular by the systemic circulation have to overcome the restrictive BBB, if this barrier is breached, survival of tumour cells is augmented by the BBB excluding entry of, and access to these tumour cells, by chemotherapeutic agents and the anti-tumoural immune response. In the
search for effective strategies to prevent the formation of brain metastases. An attractive target is the transmigration of neoplastic cells across the endothelial BBB, a process that remains incompletely understood. *In vitro* models of the BBB such as that described here are, therefore, essential for exploration of the efficacy of potential therapeutic drugs. Future studies would also benefit from *in vitro* time-lapse systems to elucidate tumour cell-endothelial interactions in real time.
Chapter 7

Characterisation of the Intra-carotid Inoculation Model of Secondary CNS Metastases.
Chapter 7: Characterisation of the Intra-carotid Inoculation Model of Secondary CNS Metastases.

7.1 Background

As described in previous chapters, the modified expression of CAV-1 and NK-1R has been implicated in tumour progression to metastases, however in vitro experiments failed to fully capture CAV-1/NK-1R regulation, function and interaction. An abundance of evidence exists to support the proposal that CAV-1 has a dual capability of tumour suppression in early stages of cancer and the switch to promotor in advanced/metastatic stage (Nunez-Wehinger et al. 2014), indicating that the action of CAV-1 is heavily dependent on cues from the surrounding tumour environment in addition to the neurogenic inflammatory drive mediated in part through NK-1R.

Generally, the precise mechanisms by which metastatic tumour cells are able to transmigrate across endothelial cells forming the BBB are incompletely understood. In an attempt to investigate this process, an intra-carotid artery injection model was trialed.
7.2 Introduction

While in vitro models have proven beneficial for the investigation of specific steps of the metastatic process, they remain limited in their application to real-world brain tumour metastasis, due to the complex and dynamic environment of the CNS. It is difficult to adequately recreate the myriad of changes that lead to the development of secondary tumours without the use of a complete animal model.

A number of in vivo experimental models have been developed in an attempt to further clarify the cellular and molecular interactions that take place during the course of transendothelial passage of neoplastic cells into the brain. A model is generally deemed to be successful if injected tumour cells consistently establish solid tumours within the brain and this modality has been applied to lung, breast and melanoma tumours. However, it is argued that such models are limited in precisely defining the mechanisms at play during the course of the human disease.

Currently, the field is led by rodent-synergistic and human-rodent xenotransplantation models and, depending on the route of inoculation, are further sub-typed as orthotopic or ectopic. Xenotransplantation or graft models utilise human cancer cell lines that are introduced into immunocompromised animals and allowed to grow as tumours. They have variable success, which is largely dependent on the tumour cell line selected and site of injection in the
body, the latter influencing the neuroanatomical distribution of metastasis (Table 7.1) (Khanna et al. 2005).

<table>
<thead>
<tr>
<th>Models of disease features</th>
<th>Inoculation routes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spontaneous dissemination</td>
<td>Subcutaneous</td>
</tr>
<tr>
<td></td>
<td>Tail vein</td>
</tr>
<tr>
<td>Haematogenous arterial dissemination</td>
<td>Intracardiac - Left ventricle</td>
</tr>
<tr>
<td></td>
<td>Intracarotid – internal carotid artery</td>
</tr>
<tr>
<td>Established parenchymal disease</td>
<td>Intracranial - Stereotactic</td>
</tr>
<tr>
<td>Leptomeningeal melanoma</td>
<td>Subarachnoid catheter for delivery to cerebrospinal fluid</td>
</tr>
<tr>
<td>Spinal cord involvement</td>
<td>Para-spinal catheter</td>
</tr>
</tbody>
</table>

The aim of the present study was to characterise an in vivo metastatic model, which would permit examination of the mechanisms operative in transendothelial migration of metastatic tumours cells and assessment of treatment strategies to impede brain penetration.

7.3 Materials and Methods

All experiments were performed on immune deficient male Balb/C nude mice (n_total = 39), weighing between 20-28 g (8-12 weeks of age). All studies were performed within the guidelines established by the NH&MRC and were approved by the animal ethics committee of the University of Adelaide.
7.3.1 Experimental Design

Mice were randomly allocated into the following groups: 14 tumour injected (highly malignant A375-M1 human melanoma cell line), 6 culture medium injected, and 4 sham mice given no injection but underwent all aspects of procedure). A total of 15 animals were excluded as they were humanely killed after attaining the cut-off score for euthanasia in keeping with ethical constraints on the clinical record sheets. Tumour cell injection was performed using the intra-carotid implantation method (as described in chapter 2 – Materials & Methods).

7.3.2 Histological Analysis

Mice were transcardially perfused with 10% PFA at 24 hr, 3, 7, or 14 days following tumour cell inoculation or control surgery. Brains were prepared for processing (as described in chapter 2 – Materials & Methods), and stained for histological examination.

In order to identify the tumour cells as melanocytic lineage, HMB-45 and Melan-A immunohistochemistry was performed. These antibodies are highly specific for melanocyte cell types, with sensitivity in the 60-80% range (Wick 2006).
7.3.3 Clinical Assessment

Mice were assessed four times daily for clinical parameters, these included post-surgical pain, stress, dehydration, and locomotive behaviour. Weight was measured daily to track loss/gain and mice were euthanized if they recorded > 15% weight loss, significant distress and clinical deterioration, in compliance with animal ethical requirements (Appendix 11.5 - Clinical Record Sheet).

7.4 Results

7.4.1 Pilot study determined $10^6$ A375-M1 cell density is optimal for intra-carotid tumour inoculation.

An initial pilot study was performed to determine optimal tumour cell density for inoculation, animals being injected with either $10^3$, $10^5$ or $10^6$ A375-M1, with the latter density selected for further investigation (Table 7.2). Animals injected with A375-M1 (n=14) or complete culture media (CCM) (n=6) via intra-carotid inoculation. A high surgical mortality rate attended this procedure, as has been the case in previously published studies, with 9 animals succumbing to uncontrolled surgical bleeding related to the catheterisation required for tumour cell injection. A further 4 animals died within 24 hrs of injection and 2 animals required early euthanasia due ethical constraints.
<table>
<thead>
<tr>
<th>Time Course</th>
<th>$10^3$</th>
<th>$10^5$</th>
<th>$10^6$</th>
</tr>
</thead>
<tbody>
<tr>
<td>D1</td>
<td>0/3</td>
<td>0/3</td>
<td>1/3*</td>
</tr>
<tr>
<td>D3</td>
<td>0/3</td>
<td>0/3</td>
<td>2/4*</td>
</tr>
<tr>
<td>D7</td>
<td>0/3</td>
<td>0/3*</td>
<td>3/5</td>
</tr>
<tr>
<td>D14</td>
<td>0/3*</td>
<td>0/3*</td>
<td>1/2</td>
</tr>
</tbody>
</table>

Brain metastases found/number of mice given injections.

*Animals euthanised or succumbing to an adverse event before reaching the designated time-point.
7.4.2 Histopathology

Figure 7.1: Intracarotid A375-M1 injection.

(A) Small population of individual melanocytes (arrows) have penetrated the brain parenchyma following intracarotid injection (n=4). (B) Higher power view showing melanocytes with ample basophilic cytoplasm with often shrunken hyperchromatic nuclei, suggesting that many of these tumour cells are likely to be non-viable (C) Infiltrating melanocytes show Melan A immunopositivity, an immunohistochemical marker for melanoma cells (D) focal aggregation of viable Melan A immunopositive melanocytes (E) Infiltrating melanocytes show strong cytoplasmic immunoreactivity to HMB45, another immunohistochemical marker of melanoma cells (Scale bar = µm).
In H&E sections, the small number of melanocytes that penetrated into the parenchyma were ovoid to polygonal in shape, with a strongly basophilic cytoplasrn and frequently shrunken, hyperchromatic, or pyknotic nuclei (Figure 7.1 – A/B). The melanocytes were generally disposed as individual cells, rather than being grouped in clusters. Occasionally, melanocytes with a large, viable-appearing nucleus and moderate amount of cytoplasm were found to be aggregated (Figure 7.1 - D). No mitotic figures were found in any infiltrating melanocyte.

The identity of the tumour cells as melanocytes was confirmed by cytoplasmic immunopositivity to HMB-45 and Melan-A (Figure 7.1 – C-E).

7.5 Discussion

The complex nature of metastasis demands the need for experimental systems that enable exploration of endogenous contributing factors. An enduring problem however is the failure to accurately mimic the multifaceted human disease in a reproducible, reliable form (Francia et al. 2011). The melanoma cell line used in this study failed to produce sufficient tumour cells penetrating into the brain and, the few that did enter the parenchyma, appeared often to be non-viable (on the basis of morphology) and were invariably non-proliferative (as assessed by no mitoses found). It was also unclear as to whether these tumour cells were extravasated into neural tissue.
or entered via the choroid plexus, across the ependymal or by another route, as melanocytes were not observed in the process of actually entering the brain by any of these routes at any of the time-points examined.

The principal aim of this study was to examine extravasation of injected melanoma cells into the brain and ascertain the route(s) of endothelial transmigration in a complete system. It was then envisaged that the site of transmigration could then be excised from the paraffin block, re-embedded in epon and studied in 1µ toluidine blue and ultrathin sections. However, since the number of melanocytes penetrating the brain was low, these cells often appeared to be non-viable, and the site of entry into the brain was unclear, it was decided to discontinue this study.

In general, xenograft models of tumours are challenging to develop and in most cases, fail to generate sufficient metastatic tumour cell populations to be useful for investigation (Iorns et al. 2012, Brodaczewska et al. 2016). Sharkey and Fogh (1987) found that of 106 malignant human tumour cell lines tested, only 1% generated metastasis in mice. Importantly, studies have suggested that the behaviour of some tumour cells lines in vitro is poorly correlated with oncogenesis in vivo (Mierke et al. 2008, Muller et al. 2008).

Several reasons have been advanced to explain the lack of success with this model. Since malignant cells undergo an number of important steps early in the metastatic process to select a subset capable of metastasis,
circumventing the oncogenic state through injection may limit the ability of circulating tumour cells to bind to the cerebral endothelium by expressing, for example, adhesion molecules (selectins and integrins) (Fazakas et al. 2011, Wilhelm et al. 2013). Furthermore, Kienast et al (2010) noted that melanoma cells that had extravasated, but lacked intimate association with the abluminal side of the microvasculature, failed to proliferate and thus regressed (Kienast et al. 2010). Other explanations for failure of this model include size differences between human and rodent vasculature and non-expression of specific human signalling pathways in rodents that are required for metastasis (Iorns et al. 2012). Enzyme solutions, such as trypsin, used in preparation of tumour cell lines may also alter the expression of surface molecules essential for tumour cell attachment to microvessels (Rahmathulla et al. 2012, Daphu et al. 2013).

Although the M1 variant of the A375 cell line is a subpopulation of melanocytes selected for their metastatic capability, they were not able to colonise the brain in useful numbers. In order to confirm cell viability at time of inoculation, cell counts were doubled to allow a tandem vial of cells to undergo all procedural elements culminating in further incubation rather than injection, which consistently produced viable subcultures confirming cell mitogenic capability at injection. In light of this, it was concluded that despite the technical challenges of the procedure, elements of the designated cell line were insufficient for tumour establishment by way of the intra-carotid inoculation model. However, direct stereotactic injection of this melanoma
cell line into the brain has produced valuable tumour growth (Harford-Wright et al. 2013, Simonsen et al. 2015), suggesting that bulk administration of tumour cells directly into the brain, and provision of a microenvironment conducive to tumour cell survival and proliferation, can result in establishment of successful cerebral metastases.

7.6 Conclusion

While animal models continue to be useful for the investigation of the many facets of oncogenesis, the current study failed to produce beneficial results. It was concluded that the in vivo investigation of metastatic melanocyte extravasation to the brain would require further piloting.
Chapter 8

Part B. The Role of the microvasculature in *Clostridium perfringens* type D epsilon toxin (ETX) neurotoxicity
Chapter 8: Part B. The Role of the microvasculature in *Clostridium perfringens* type D epsilon toxin (ETX) neurotoxicity

8.1 Background

In Part B of this thesis, the cerebrovascular theme is continued by examining the effect of *Clostridium perfringens* type D epsilon toxin (ETX) on brain microvasculature *in vitro* (Chapter 7) and *in vivo* (Chapter 8), the latter also including a section on ETX-induced blood-retinal barrier (BRB) injury, since the BRB resembles the BBB in many important respects.

The *in vitro* study is novel, while the *in vivo* investigations provide further confirmational support to the limited published experimental findings and extend the expertise of the candidate in toxicological pathology.

These studies are warranted for 2 main reasons: (1) the complete neuropathogenesis of ETX toxicity is still not fully understood, although microvascular damage is a critical part of the disease process, and (2) ETX is on most international lists of potential bioterrorism agents and its effect on humans is unknown. Moreover, there are currently no treatment measures should human populations be exposed.
8.2 Introduction

Clostridium perfringens (welchii) type D neurotoxicity is an important worldwide disease (Wioland et al. 2013). Although vaccination has reduced the prevalence of this disease in sheep, it still, nevertheless, occurs commonly and is frequently fatal (Uzal et al. 2015). While the principal clinical manifestation is severe neurological dysfunction, the pathogenesis of central nervous system lesions is incompletely understood (Finnie 2003, Finnie 2004, Stiles et al. 2013). The high stability of ETX, in concert with the possibility of its expression as a recombinant protein in E. coli and the dearth of relevant therapeutics for humans, had led to the recognition of ETX as a potential biological weapon (Mantis 2005, Wioland et al. 2013).

The anaerobic, spore-forming, Gram-positive bacillus, Clostridium perfringens, is classified into 5 strains (A, B, C, D, and E), these designations based on their production of one or more of the 4 main lethal exotoxins, namely alpha, beta, epsilon (ETX), and iota (Freedman et al. 2016). The principal toxin produced by the type D strain is ETX, although smaller amounts of alpha toxin may be elaborated. ETX is the third most potent clostridial toxin, the LD$_{50}$ in mice being ~ 100ng/kg, with only botulinum and tetanus toxins being ranked higher (Popoff 2011). The very high lethality of ETX also ranks it amongst the 4 most potent poisonous substances so far discovered. Clostridium perfringens type D also produces an alpha toxin, a 42.5 kDa, single polypeptide, but its role is minimal when Clostridium perfringens causes disease originating in the
intestine (Stiles et al. 2013). Its LD$_{50}$ in mice is 3 μg/kg (Popoff 2011, Stiles et al. 2013, Prescott et al. 2016).

Type D bacteria are produced as a ~33kDa, single polypeptide precursor or prototoxin, which is rapidly digested by proteolytic enzymes in the intestine (particularly trypsin and chymotrypsin, and probably Clostridium perfringens lambda toxin) to yield the fully activated ~29 kDa ETX. The activation by proteases removes both N- and C-terminal amino acids, the latter being most important for ETX activation. Activated ETX is at least 1000-fold more neurotoxic than the minimally toxic prototoxin and is encoded by a gene carried on a large plasmid in type D strains (Finnie 2003, Freedman et al. 2016, Uzal et al. 2016).

Clostridial diseases generally require predisposing conditions to produce clinical disease which, in the case of Clostridium perfringens type D, is a change to the intestinal microflora balance in favour of this bacterium due to alterations in the feed presented to the intestine. Many ruminant livestock normally harbour Clostridium perfringens in their intestine, but bacterial numbers are usually small and, accordingly, toxin production is low and relatively harmless. However, if the microbial balance in the intestine is disrupted by the ingestion of large quantities of starch, these saccharolytic bacteria use this substrate to produce large quantities of ETX. The conditions that predispose to this altered intestinal milieu include lambs grazing abundant, starch-rich, lush pasture or access to, and ingestion of, large
amounts of cereal grains, for example in feedlots. However, it has recently been suggested that, while starch favours proliferation of this microorganism, an absence of glucose stimulates ETX production (Freedman et al. 2016, Uzal et al. 2016).

When a high intestinal luminal content of ETX persists for several hours, the toxin increases mucosal permeability, thus enhancing its own absorption (apparently traversing the intestine without producing intestinal injury, suggesting it may use paracellular permeability routes to reach the systemic circulation), and sufficient quantities may enter the systemic circulation to cause severe neurological disturbance. Thus, the disease is a pure toxaemia as there is no extra-intestinal bacterial invasion (Finnie 2003, Freedman et al. 2016, Uzal et al. 2016).

While ETX-induced disease in sheep, cattle and goats is generally similar, the more chronic intoxication in goats is usually restricted to the intestine and manifests as a generally fibrinonecrotising enterocolitis and probably due to a local damaging effect of the toxin. The clinical disease produced by ETX mainly affects young, rapidly growing lambs, although older sheep can be affected. Morbidity rates are typically up to 10%, but mortality can approach 100% in fully susceptible animals. In the absence of any protective antibody, for example from vaccination, the clinical course can be very brief (peracute), often < 2 hours and usually not exceeding 12 hours. Some lambs are found dead, without premonitory signs, or succumb after a brief period of
violent convulsions. With a more prolonged clinical course, there may be neurological signs of staggering gait, hyperaesthesia (increased sensitivity to stimuli), opisthotonus (retraction of the head over the dorsum, as shown in Figure 8.1), convulsions, and coma. There may also be diarrhoea, tenesmus (straining) and ptyalism (excessive salivation). While clinical signs are similar in susceptible calves, young goats generally present with abdominal pain and dysentery rather than neurological signs, possibly because intestinal absorption of ETX is slower in this species (Uzal et al. 1997, Uzal et al. 1998, Uzal et al. 2016).
Figure 8.1: ETX-intoxicated sheep.

(A) demonstrating terminal paddling convulsions excavating the sandy soil and (B) showing paddling convulsions and opisthotonus.
The acute clinical syndrome that occurs when large doses of ETX are absorbed from the intestine, and the resulting neurotoxicity, is frequently rapidly fatal. However, when lower doses of ETX are absorbed from the intestine, or animals are partially immune, sheep may survive for 1-2 weeks and this more chronic syndrome is characterised by neurological signs of blindness, aimless wandering, incoordination (ataxia), bruxism (grinding of the teeth), head pressing (reflecting increased intracranial pressure or ICP), nystagmus (oscillating movement of the eyeballs), posterior paresis (muscle weakness), lateral recumbency, opisthotonus, and paddling convulsions. The neuropathological presentation of this chronic ETX neurotoxicity is that of bilaterally symmetrical foci of necrosis (Figure 8.2 & 8.3) in certain brain regions, particularly basal ganglia, internal capsule, thalamus, and substantia nigra (Hartley et al. 1956). Other macroscopic changes include pulmonary oedema, often fibrin clots in excess pericardial fluid, subendocardial and epicardial haemorrhages, and soft, “pulpy’ kidneys, the latter often attributed to rapid autolysis (Uzal et al. 2016).
Figure 8.2: Bilaterally symmetrical necrotic foci (arrows).
In the brain of a sheep dying from chronic ETX intoxication.

Figure 8.3: Bilaterally symmetrical and haemorrhagic necrotic foci (arrows).
In the brain of a sheep dying from chronic ETX intoxication.
After absorption from the intestine, circulating ETX accumulates preferentially in the brain and, to a lesser degree, kidneys, the latter possibly affording some neuroprotection by reducing the amount of ETX presented to the brain (Finnie 2003, Uzal et al. 2016).

The luminal surface of the cerebral microvasculature appears to be the principal site of ETX binding and the major target for toxin-induced brain damage. Moreover, ETX can be prevented from exerting its endotheliotoxic action, albeit transiently, by prior administration of the prototoxin, probably by competitive inhibition of binding sites (Buxton 1976). The relatively short duration of this protection by the prototoxin has been suggested to be due to its internalisation by the endothelial cell, enabling the receptor sites to become free to bind ETX again (Nagahama et al. 1991).

The ETX is a member of a large group of cytolysins belonging to the aerolysin family, the cytotoxicity of which is believed to be due to their ability to bind to target cells, assemble into oligomers, and form large transmembrane pores. However, ETX lethality is 100-fold greater than that of similar pore-forming toxins. One ETX domain interacts with receptors on host cells, while another inserts into membranes during pore formation. ETX first binds to a receptor, then uses lipid rafts and caveolins to oligomerise into a hepatameric pre-pore on the host cell membrane surface, for example a cerebral microvascular endothelial cell. It then extends a β-hairpin loop into the lipid bilayer to form an active pore, which results in a rapid decline in cytoplasmic K⁺ levels, with a
corresponding influx of Na\(^+\) and Cl\(^-\). Cellular necrosis is believed to be caused by this rapid loss of K\(^+\) and involves ATP depletion, but the process is not as yet fully elucidated ([Freedman et al. 2016, Li et al. 2016]).

After binding to its receptor, a double receptor composed of a protein and glycoprotein, ETX oligomerises to form a large membrane complex, corresponding to ETX heptamers, which are formed by progressive addition of monomer to oligomer of smaller size. It is debated as to whether ETX monomers inserted into a membrane assemble very rapidly to form heptamers or heptamers are inserted into a membrane as a whole. ETX seems to assemble as a pre-pore complex onto the membrane surface before heptamers are inserted into the bilayer, similar to many other pore-forming toxins. The ETX oligomer incorporated into the plasma membrane is likely to remain attached to its receptor. When inserted into a membrane, ETX heptamers form general diffusion pores, permitting the passage of large compounds ~ 1 kDa. ETX pores also appear to remain open for long periods of time. Expression of caveolins also greatly potentiates ETX-induced cytotoxicity and caveolae (caveolin-dependent invaginations of the plasma membrane) permit confinement of ETX to specific regions of the membrane, thus favouring ETX oligomerisation. However, since ETX can form pores in artificial membrane bilayers devoid of a specific ETX receptor, ETX binding to its receptor appears not to be always required for pore formation. There is no evidence to date that ETX must enter target cells to produce cytotoxicity. Rather, the flux of ions and leakage of small molecules through ETX-induced
pores appears to be responsible for cell death. However, there is emerging evidence that some of the early deleterious effects of ETX may not be due to ETX pore formation (Wioland et al. 2013).

Some of the neurological signs of ETX toxicity may be due to hyperexcitability caused by excessive glutamate release from glutamatergic neurons, probably also abetted by dopamine, noradrenaline and adrenaline. ETX has been shown to produce release of large amounts of glutamate by binding to receptors on a subset of neurons (for example cerebellar granular microneurons) and this excitatory amino acid transmitter can induce neuronal cell death. However, ETX receptors seem not to be ubiquitously expressed on the neuronal surface, primarily targeting somata and dendrites, but not axons or nerve terminals. ETX has also been shown to co-localise with myelin markers and binds to oligodendrocytes, but apparently not astrocytes (Nagahama et al. 1993, Miyamoto et al. 2000, Wioland et al. 2013).

When ETX injures the cerebral capillary endothelium, there is disruption of the BBB and increased vascular permeability. An important diagnostic histological finding in the brains of ETX-exposed animals is perivascular deposition of a plasma protein-rich, extravasated fluid (Figure 8.4). At the ultrastructural level, injured endothelial cells initially show swelling and loss of cytoplasmic organelles, with blebbing of the luminal surface, the cytoplasm eventually appearing as an attenuated, electron-dense band with nuclear pyknosis (Figure 8.5). The cerebral vasogenic oedema produced by ETX is severe and
widely distributed (diffuse, generalised) in the brain, leading to brain swelling, increased ICP, and death after a brief clinical course of marked neurological disturbance. Because the brain is encased in a rigid, bony skull, any volumetric change to one of the intracranial contents (brain, cerebrospinal fluid, blood) must be compensated for by a reciprocal and equivalent volume reduction in other constituents (the Monro-Kellie doctrine); when these compensatory mechanisms are exhausted, the ICP will rise. Moreover, protein-rich oedema fluids are cleared slowly from the brain and resolution of such a vasogenic oedema commences only when the protein macromolecules are degraded and removed by the brain extracellular space (Ohata et al. 1990). If brain swelling is less severe, there may be distortion, shift and herniation of the brain, especially protrusion of the cerebellar tonsils through the foramen magnum into the spinal canal (termed “cerebellar coning”) (Figure 8.6) (Griner 1961, Uzal et al. 1997, Finnie 2003, Garcia et al. 2015, Freedman et al. 2016).
Figure 8.4: Perivascular deposition of abundant extravasated proteinaceous material (P).

In the brain of an acutely ETX-intoxicated sheep.

Figure 8.5: TEM of an ETX-injured capillary.

Cerebellar granular layer. TEM of an ETX-injured capillary showing attenuated, electron-dense endothelium (arrow), nuclear pyknosis (n), and swelling of perivascular astrocytic end-feet (as). Granular layer neurons (g) are also shown. Inset shows a normal capillary in a perfusion-fixed brain with compact neuropil embracing the external surface of the vessel. Bar = 5µm.
Figure 8.6: ETX-induced severe vasogenic cerebral oedema.

(A) “Cerebellar coning” with protrusion of the cerebellar tonsils through the foramen magnum due to high intracranial pressure resulting from ETX-induced severe vasogenic cerebral oedema. (B) Similar lesion showing haemorrhagic necrosis of herniated portions of brain.

The cerebral lesions produced by ETX may be regionally selective or more widely expressed, depending on the dose of toxin and the time between toxin exposure and death. In general, higher doses of ETX tend to expand the distribution of brain lesions, although still favouring certain neuroanatomical areas of predilection, while lesions in more subacutely intoxicated animals exposed to lower doses of circulating toxin are smaller, well-circumscribed, and restricted to fewer selectively vulnerable brain regions. As the time from toxin exposure to death increases, so does the distribution of brain lesions. There are also interspecies differences in response to ETX for, while young goats and lambs appear to be equally sensitive to this toxin, brain lesions occur infrequently in the former, although occasional perivascular accumulations of extravasated plasma protein are sometimes observed (Griner 1961, Uzal et al. 1997, Finnie 2003, Finnie 2004, Garcia et al. 2015, Freedman et al. 2016).
8.3 Conclusion

The fundamental brain lesion produced by ETX appears to be microvascular endothelial damage and the resulting cerebral lesions occur in a seemingly dose- and time-dependent manner. Extensive BBB breakdown after high dose exposure to ETX results in severe, diffuse, vasogenic cerebral oedema, a marked rise in ICP, and an acute or peracute clinical course to death. Blood-brain barrier disruption also permits ETX to traverse this barrier and enter the brain parenchyma where, additionally, it has been shown to produce a direct cytotoxic effect on neurons (Finnie et al. 1999), thus potentiating its potent neurotoxicity. By contrast, subacute intoxication tends to cause focal, bilaterally symmetrical necrosis in selectively vulnerable brain regions after a more protracted clinical course. As microvascular endothelial cells form the frontline of the BBB, an investigation into their role in barrier breakdown subsequent to ETX exposure is warranted. As such, Part B of this thesis aims to;

1. Determine whether Clostridium perfringens type D epsilon toxin has a direct and damaging effect on human cerebral microvascular endothelial cells in vitro and in vivo

2. Investigate the effect of epsilon toxin on the blood-retinal barrier in vivo
Chapter 9

The Effect of \textit{Clostridium perfringens} type D epsilon toxin (ETX) on cerebral microvasculature endothelial cells \textit{in vitro}
Chapter 9: The Effect of *Clostridium perfringens* type D epsilon toxin (ETX) on cerebral microvasculature endothelial cells *in vitro*

9.1 Introduction

While the cerebral capillary endothelium seems to be the major target of ETX, a direct, deleterious effect of this toxin on cultured cerebral endothelial cells *in vitro* has not previously been demonstrated. The only study to examine the effect of ETX on cultured endothelial cells was unable to show any resulting endothelial injury, but the target endothelium was derived from ovine, caprine and bovine aorta (Uzal et al. 1997), rather than brain microvascular endothelium. Cerebral endothelium differs from endothelia elsewhere by possessing tight interendothelial junctions, a paucity of micropinocytotic vesicles, and surrounding astrocytic end-feet and these anatomical features, and possibly a lack of ETX-specific receptors, may account for the failure of ETX to damage endothelium of non-neural origin *in vitro*.

This study was designed to determine whether ETX has a direct and damaging, dose-dependent effect on cerebral endothelial cells *in vitro* and is the first to investigate this ETX-microvascular interaction on target endothelial cells of brain origin. The cultured endothelial cell line used in this study were also of human origin, which is pertinent due to the potential for use of this potent neurotoxin as a bioterrorism agent.
9.2  Materials and Methods

9.2.1  Experimental Design

9.2.1.1  Cerebral endothelial cells

All cell culture procedures using the endothelial (hCMEC/D3) cell line were carried out as described in Chapter 2 (section 2.2.5).

9.2.2  Epsilon toxin (ETX)

For all experiments within this chapter, ETX toxin was generated and prepared as per methods outlined in Chapter 2 (section 2.3).

9.2.3  Morphologic studies

For light and electron microscopy analysis samples were prepared in accordance with protocols detailed in Chapter 2 (section 2.2.8 and 2.2.9).

9.2.4  Cell viability

The MTT assay was used to determine cytotoxic effects of ETX on hCMEC/D3 cell line and prepared as detailed in Chapter 2 (section 2.2.10).
9.3 Results

9.3.1 Human microvascular endothelial cells are susceptible to ETX-induced cytotoxicity.

In control cultures of brain endothelium examined by light microscopy, the cell line was confirmed as endothelial by CD31 immunopositivity. Nuclei were round to ovoid, and occasionally slightly elongate in shape, with generally evenly distributed coarse to finer chromatin clumps. The cytoplasm was markedly elongated and palely eosinophilic in H&E-stained sections and cytoplasmic boundaries were sometimes indistinct (Figure 9.1).

Figure 9.1: Untreated hCMEC/D3 cells.

Untreated cerebral capillary endothelial cells in culture with round to ovoid nuclei and a variable amount of palely eosinophilic cytoplasm, which was sometimes (inset) markedly elongated. H&E.
A few binucleated cells were present and an occasional endothelial cell was found in mitosis. Endothelial cells exposed to ETX were more sparsely distributed than in control cultures and became conspicuously more depleted with increasing concentrations of ETX (Figure 9.2 & 9.3). Endothelial cell depletion was confirmed by cell viability data (Figure 9.4). Injured endothelial cells at higher concentrations frequently contained a pyknotic nucleus and is shrunken, hypereosinophilic cytoplasm; at lower concentrations of ETX, nuclei were degenerate with chromatin condensation and the cytoplasm less intensely eosinophilic.

Figure 9.2: ETX-treated hCMEC/D3 cells.

ETX-treated cerebral capillary endothelial cells in culture, most of which have condensed nuclear chromatin and shrunken, hypereosinophilic cytoplasm (arrows). H&E.
Figure 9.3: ETX-treated hCMEC/D3 cells.

Cultured cerebral capillary endothelial cells exposed to ETX showing (A) a mix of necrotic (arrows) and less degenerate cells and (B) necrosis of all endothelial cells with a higher dose of ETX, the cytoplasm shrunken and hypereosinophilic cytoplasm and nuclei pyknotic. H&E.

Figure 9.4: Effect of ETX on cell viability.

Exposure to ETX for cultured hCMEC/D3 cell indicates dose dependent cell death. (A) Viability of cultured hCMEC/D3 cells exposed to high, intermediate and low concentration of ETX is significantly reduced compared with control as detected via cell viability assay (B) and cell counts, both graphically expressed as mean ± SEM **** p <0.0001 as compared with control, ANOVA.
9.3.2  Human microvascular endothelial cells undergo pyknosis and organelle swelling following ETX exposure

At the ultrastructural level, control endothelial cells (Figure 9.5) were usually polygonal in shape and sometimes in close apposition to neighbouring cells, with adherens-type junctions occasionally seen at sites of cell contact. The oval nucleus was more or less centrally disposed with one or more nucleoli; the fine chromatic granules were uniformly distributed throughout the nucleus and the nuclear envelope was smooth and well-defined. The cytoplasm contained large quantities of rough and smooth endoplasmic reticulum, prominent Golgi complexes, many vesicles, mitochondria, numerous bundles of fine filaments, occasional microtubules; microvilli projected from the cell surface. Endothelial cells exposed to ETX (Figure 9.6) showed condensation of nuclear chromatin (hyperchromasia) or nuclear pyknosis. The cytoplasm was either shrunken and more electron dense than control endothelium or showed organelle swelling and degeneration. In a few endothelial cells, vacuoles contained condensed nuclear remnants and degenerate cytoplasm, suggesting that they had phagocytosed effete endothelial cells.
Figure 9.5: TEM image of untreated hCMEC/D3 cells.

TEM image of untreated, cerebral capillary endothelial cells in culture. Cells have surface microvillous projections and adherens-type junctions (arrowed in the inset) between closely apposed endothelial cells.
TEM image of cerebral capillary endothelial cells in culture exposed to ETX. Endothelial cells show marked condensation of nuclear chromatin and either shrunken, more electron-dense cytoplasm or swelling of cytoplasmic organelles.

9.4 Discussion

The present findings have shown, for the first time, that ETX causes a direct and dose-dependent, cytotoxic effect on cultured cerebral endothelial cells. This in vitro study complements previous studies in sheep and laboratory rodents in which ETX produced marked extravasation and perivascular deposition of plasma protein, and more widely distributed vasogenic edema, in routine histologic sections of brain (Griner 1961).
Many sheep harbour Clostridium perfringens type D in their alimentary tract, but only small, harmless amounts of ETX are normally produced. This bacterium elaborates a relatively inactive prototoxin that is converted to the potent ETX by removal of N- and C-terminal peptides by proteolytic digestive enzymes. However, a severe and frequently fatal neurologic disorder can be induced, particularly in young thriving lambs, when copious quantities of lush pasture or young cereal crops are introduced to the small intestine. This starch overload can also occur after heavy grain feeding in feedlots. Under these conditions, C. perfringens proliferates rapidly and produces large amounts of ETX. The toxin then increases intestinal mucosal permeability, thus facilitating its absorption into the systemic circulation. The disease is, therefore, a pure toxemia, with no bacterial invasion of tissues, and is not transmissible. Circulating ETX accumulates preferentially in the brain and, to a lesser extent, in the kidneys (Uzal et al. 1997, Uzal et al. 1998).

Clostridial perfringens type D EXT is a member of the aerolysin family of pore-forming toxins. One domain of ETX binds to endothelial receptors, where it utilizes lipid rafts and caveolin to oligomerize into a pre-pore on the surface of the cell membrane. Another ETX domain then inserts into the membrane lipid bilayer and forms an active pore. Clostridial perfringens type D EXT can be transiently prevented from exerting its deleterious effect by prior administration of the prototoxin, presumably by competitive inhibition of endothelial receptors. The ETX-induced endothelial cytotoxicity appears to be due, in part at least, to a rapid decline via pores in cytoplasmic potassium.
levels, with a concomitant influx of sodium and chloride ions. This process, which seems to involve ATP depletion, leads to cell necrosis (Freedman et al. 2016). Cerebral microvascular endothelial injury produced by ETX results in a severe, generalised, vasogenic oedema, leading to a marked rise in intracranial pressure. Peracutely affected lambs are often found dead without premonitory signs or die after a few minutes of violent convulsive activity; if the brain swelling is less severe, there may be attendant distortion, shift, and herniation (Finnie 2004, Uzal et al. 2016).

The ETX-cerebral microvascular interaction requires further investigation to better define pathogenetic mechanisms and develop more accurately targeted, therapeutic intervention strategies, particularly if humans are exposed. Vaccination of sheep and calves, but not goats, has substantially reduced the prevalence of ETX neurotoxicity, but the disease still occurs commonly (Finnie 2004). While Clostridium perfringens types B and D that produce ETX are rarely isolated from humans, there is no report of human death attributable to ETX (Greenfield et al. 2002), and only a few reports of disease in humans (Popoff 2011), it is, nevertheless, the most potent clostridial toxin after botulinum and tetanus neurotoxins (Ciottone et al. 2005). Moreover, due to its high potency and potential for use as a bioterrorism agent, ETX has been classified as a category B bioterrorism agent by the United States Government Centres for Disease Control and Prevention (Yim et al. 2009).
9.5 Conclusion

This novel study has shown that Clostridium perfringens type D ETX produces a direct and dose-dependent cytotoxic effect on cerebral microvascular endothelial cells in vitro and complements previous light microscopic and ultrastructural studies suggesting that capillary endothelial damage in the brain is a major target of ETX and responsible for much of the ensuing neurological disturbance. This study provides a framework for further investigation of the cellular injury mechanism by which microvascular endothelial cells become vulnerable to ETX induced death. In essence, it is a critical step from the extrapolation of experimentally infected animals to better understanding a potential human response.
Chapter 10

Characterisation of ETX-induced microvascular endothelial damage and attendant increased vascular permeability \textit{in vivo}
10 Chapter 10: Characterisation of ETX-induced microvascular endothelial damage and attendant increased vascular permeability 
in vivo

10.1 Background

Tracer study of ETX-induced cerebral microvascular permeability changes and immunoexpression of the water channel protein, aquaporin-4, and endothelial barrier antigen.

10.2 Introduction

While ETX causes microvascular endothelial damage in the brain, leading to increased vascular permeability, severe vasogenic oedema, increased intracranial pressure and severe neurological signs often resulting in death, the degree and neuroanatomical distribution of this cerebral oedema is often difficult to appreciate in routine haematoxylin and eosin-stained sections.

Accordingly, immunohistochemistry for endogenous albumin was used as a surrogate marker of increased vascular permeability and immunoexpression of the water channel protein, aquaporin-4 (AQP-4) examined as another indicator of vasogenic oedema. Moreover, as rats were used as an animal model of ETX neurotoxicity, it was decided to study endothelial barrier antigen (EBA) expression. Endothelial barrier antigen is a membrane protein expressed by microvessels (capillaries and venules) in this species and its presence is regarded as a marker of an intact and competent BBB (Saubamea et al. 2012). Endothelial barrier antigen immunoreactivity is markedly diminished in
pathological conditions in which there is BBB disruption and, accordingly, is an appropriate marker to confirm ETX-induced BBB damage.

Aquaporins are a family of at least 13 membrane channel proteins that facilitate movement of water in the brain. AQP-4 is the most abundant AQP in the brain and is expressed in astrocytes at fluid-parenchyma interfaces in blood-brain and subependymal/subpial-cerebrospinal fluid (CSF) barriers (Papadopoulos et al. 2004). Cells expressing AQP’s on plasma membranes have up to 50-fold higher osmotic water permeability than membranes devoid of these proteins (Badaut et al. 2014). AQP-4 is found in high concentration in astrocytic perivascular end-feet, suggesting that this protein is involved in regulation of water movement at blood-brain and CSF-brain barriers (Zador et al. 2007). The function of AQP-4 in the pathogenesis of vasogenic and cytotoxic brain oedema seems to be controversial. In vasogenic oedema, extra water enters the extracellular space independently of AQP-4 (Zador et al. 2007). However, this excess water is removed from the brain through AQP-4 channels in astrocytic foot processes. By contrast, the AQP-4 water channels in astrocytic foot processes have an active role in the formation of cytotoxic oedema (Papadopoulos et al. 2004, Zador et al. 2007, Badaut et al. 2014).

Endothelial barrier protein (EBA) is a membrane protein expressed almost exclusively on the luminal aspect of all rat brain microvessels and is regarded as a specific marker of an intact and functionally competent BBB, although its
precise function is unknown. Its specificity for the BBB in this species is attributed to the fact that (1) EBA is lacking in microvessels in those brain regions devoid of a BBB and (2) it is downregulated in pathological disorders in which BBB integrity or function is compromised, for example in stroke, traumatic brain injury, exposure to neurotoxins, and experimental allergic encephalomyelitis (Badaut et al. 2014). It is still debated, however, as to whether decreased EBA immunoexpression has a causative role in opening of the BBB or is a consequence of this disruption, although the former role is supported by the finding that intravenous administration of an anti-EBA antibody results in opening of the BBB and subsequent fluid extravasation (Saubamea et al, 2012).

10.3 Materials and methods

10.3.1 Experimental Design

A group of six Sprague-Dawley rats was given an intraperitoneal injection of 1ml of a 1:10 dilution of ETX. The details of this ETX preparation were outlined earlier in the section on in vitro effects of ETX on cerebral microvascular endothelium. At 1 hour post-injection, rats were depressed, huddled and unresponsive to external stimuli and, at this time, were anaesthetised with isoflurane and killed by perfusion fixation of the brain with 4% paraformaldehyde. The brain was then immersion fixed in 10% neutral buffered formalin, paraffin-embedded, and coronal, 6 µm sections of brain cut and immunostained. By using ventral landmarks, 3 coronal sections of
brain were selected, these being levels used by the National Toxicology Programme in the United States for routine toxicological screening: at the optic chiasm (level 1); mammillary bodies (level 2); and at the widest part of the cerebellum (level 3). At level 1, the cingulate and parietal cortices, caudate-putamen, and corpus callosum were examined; at level 2, the occipital and temporal cortices, thalamus and internal capsule; and, at level 3, the cerebellum and pons.

10.3.2 Immunochemical Analysis

Immunohistochemical analysis was performed as per methods outlined in Chapter 2 (Section 2.2.8) for albumin, AQP4 and EBA (Table 10.1). Sections were immunostained with the DAB method to visualize the target protein.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Clone</th>
<th>Dilution</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Albumin</td>
<td>goat anti-rat</td>
<td>1:20 000</td>
<td># 0113-0341, Cappel Laboratories</td>
</tr>
<tr>
<td>AQP4</td>
<td>rabbit polyclonal anti-rat</td>
<td>1:4000</td>
<td>AB3068, Chemicon, Temecula</td>
</tr>
<tr>
<td>EBA</td>
<td>mouse monoclonal anti-EBA</td>
<td>1:5000</td>
<td># SMI-71R, Covance</td>
</tr>
</tbody>
</table>

10.4 Results

10.4.1 Albumin extravasation

Light microscopic examination of the brain revealed extravasated albumin to be widely distributed in the brain (Figure 10.1), without apparent regional
predilection. Leakage was either multifocal, particularly in the cerebral cortex or, more commonly, coalescing to diffuse in distribution.
Figure 10.1: ETX exposure increases blood-brain barrier permeability to albumin.

Multifocal, vasculocentric albumin extravasation (arrows) in the cerebral cortex. (A) More diffuse parenchymal albumin immunopositivity in the hippocampus (right of image) and thalamus (left of image) (B); caudate-putamen (C); and cerebellar cortex, particularly the molecular layer (m) (D).
Albumin was frequently present in the walls of microvessels, apparently flooding endothelial cells after ETX-induced injury (Figure 10.2).

![Figure 10.2: ETX damaged microvascular endothelium.](image)

Strong albumin immunopositivity of the damaged microvascular endothelium (arrow). The surrounding neuropil is also diffusely immunostained for albumin.

Albumin was also often pooled immediately around these damaged vessels and extended into the surrounding neuropil, the immunoreactivity decreasing as the distance from the affected blood vessel increased (Figure 10.3).
10.4.2 Aquaporin-4 (AQP-4)

In control brains, microvascular AQP-4 immunopositivity was expressed as fine granularity of astrocytic processes of both grey and white matter, especially the former, and particularly where their foot processes (end-feet) were in contact with the abluminal surface of capillaries and post-capillary venules (Figure 10.4 - A), and in subpial sites – outer (pia mater) and inner (ependymal) surfaces of the brain (glia limitans). In many microvessels exposed to EXT, AGP-4 immunopositivity was markedly increased and expressed as coarse and more numerous APP-immunoreactive granules (Figure 10.4 - B), particularly in astrocytic foot processes surrounding microvessels. The ependyma was strongly AQP-4 immunopositive.
Figure 10.4: AQP4 immunoexpression.

(A) Control brain. Fine granular AQP-4 immunopositivity in perivascular astrocytic end-feet, with unstained surrounding neuropil. (B) ETX-treated brain. Increased AQP-4 immunopositivity expressed as numerous, coarse granules in astrocytic foot processes and strong, diffuse albumin immunoreactivity of the surrounding neuropil.
10.4.3 Endothelial barrier antigen (EBA)

In control rat brains, microvessels (capillaries and venules) were uniformly immunolabelled with EBA whereas, by contrast, in ETX-exposed brains, there was often partial, or even complete, loss of EBA immunopositivity in many microvessels (Figure 10.5). Loss of EBA immunoreactivity appeared to be randomly distributed throughout the brain, without regional predilection, and vessels depleted of EBA immunoreactivity were interspersed with strongly EBA immunolabelled, unaffected microvessels.
Figure 10.5: EBA immunoreactivity in an EXT-treated brain.

Many microvessels show complete loss of EBA immunoreactivity (arrows), while other vessels resemble control brains with uniform EBA immunopositivity.
10.5 Discussion

Albumin extravasation was widely distributed in the brain of rats that were clinically moribund, 1 hour post-ETX injection, but there appeared to be no discernible regional neuroanatomical susceptibility to this microvascular leakage of serum albumin. Albumin immunoreactivity was particularly strongly expressed perivascularly, reflecting ETX-induced microvascular damage and subsequent leakage of plasma proteins, particularly albumin, and the albumin immunostaining usually decreased as the distance from the damaged vessel increased. In some brain areas, however, the parenchymal staining was more uniformly albumin immunopositive. In some ETX-injured microvessels, endothelial cells were strongly albumin immunopositive, ETX-induced cell membrane disruption apparently leading to entry and diffuse flooding of albumin into these cells.

The increased expression of AQP-4 in brain microvessels of rats exposed to ETX correlated with marked albumin leakage from these blood vessels and suggested that this water channel protein plays an important role in the clearance of oedema fluid from the brain by astrocytic uptake, moving water from the extracellular space into these glial cells, and reducing osmotic stress on surrounding neurons.

Loss of EBA immunopositivity in ETX-exposed brain microvessels confirmed the important action of this neurotoxin in disrupting BBB integrity. EBA is
considered to be a specific marker of an intact and functionally competent BBB in this rats and it is likely that any loss of EBA is involved in the ensuing BBB breakdown, increased vascular permeability, and extravasation of plasma proteins such as albumin.
Chapter 11

Retinal microvascular damage produced by Clostridium perfringens type D epsilon toxin
11 Chapter 11: Retinal microvascular damage produced by *Clostridium perfringens* type D epsilon toxin

11.1 Introduction

The retina is embryologically part of the central nervous system and retinal microvessels possess a blood-retinal barrier (BRB) resembling the blood-brain barrier (Cunha-Vaz et al. 2011). An intact BRB plays an important role in homeostasis of the retinal microenvironment and when BRB breakdown occurs, the resulting vasogenic oedema and retinal damage can affect vision, often markedly.

The major functions of the BRB are to: (1) control delivery of nutrients and oxygen (the retina consuming oxygen more rapidly than any other tissue) to, and removal of metabolic waste products from, the retina; (2) exclude large molecules (macromolecules) from the transparent tissues of the eye, particularly the vitreous humor, in order to maintain optical transparency, and (3) maintain optimal retinal hydration to preserve the close relationship between photoreceptors and the retinal pigment epithelium (RPE) and the choriocapillaris. Normally, ocular blood flow is maintained over a wide range of perfusion pressures induced by changes in intraocular pressure and blood pressure.

Blood vessels in the retina are distributed in 2 plexuses: an inner plexus located in nerve fibre and ganglion layers and an outer plexus situated at the junction
of the inner nuclear and outer plexiform layers. Retinal capillary endothelial cells are not fenestrated and have a paucity of micropinocytotic vesicles.

The BRB is comprised of inner and outer components. The inner part is formed by tight junctions (zonula occludens) between adjacent microvascular endothelial cells, abetted by ensheathing pericytes between the endothelium and basal lamina (the ratio of pericytes to vascular endothelial cells being higher in the retina than in any other vascular bed) and foot processes of astrocytes and Muller cells (the latter forming the principal glia of the retina). The outer BRB is formed by tight junctions between retinal pigment epithelial cells, which rest on Bruch’s membrane and separate the retina from the choriocapillaris, and plays an important role in transporting blood-borne nutrients to the outer retina (Sivakumar et al. 2008, Cunha-Vaz 2011, Cunha-Vaz et al. 2011, Runkle et al. 2011).

Since ETX-induced microvascular damage in the brain is well-established, we wished to determine whether similarly ETX-exposed eyes also showed retinal microvascular injury, particularly since blindness occurs in clinical cases of ETX in sheep (Uzal et al. 1998) and fluorescently-tagged ETX has been shown to co-localise to mouse retinal microvessels (Rumah et al. 2013).
11.2 Materials and methods

11.2.1 Experimental Design

Studies were conducted in 4, 6-week-old, Sprague-Dawley rats. EXT was prepared and administered as previously described in chapter 2 (section 2.3). At 3 hours post-injection of toxin, rats were euthanased by perfusion fixation of the brain with 4% paraformaldehyde via a needle inserted in the beating left ventricle; the right auricle was incised to permit escape of the perfusate. The eyes and optic nerves were collected and immersed in Davidson’s fixative and one eye was also fixed in Karnovsky’s paraformaldehyde-glutaraldehyde fixative for electron microscopy. Brains were also collected in 10% buffered formalin for light microscopy.

Immunohistochemical analysis was performed as per methods outlined in Chapter 2 (Section 2.2.8) for albumin, AQP4 and EBA (Table 11.1). Sections were immunostained with the DAB method to visualize the target protein.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Clone</th>
<th>Dilution</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Albumin</td>
<td>goat anti-rat</td>
<td>1:20 000</td>
<td># 0113-0341, Cappel Laboratories</td>
</tr>
</tbody>
</table>

11.2.2 Transmission Electron Microscopy

For ultrastructural examination, small blocks of glutaraldehyde-fixed retina were postfixed in 2% osmium tetroxide buffered in 0.1 M cacodylate (pH 7.3)
for 1 hr. The tissue pieces were then dehydrated in increasing concentrations of ethanol, cleared in propylene oxide, and embedded in Epon.

11.3 Results

11.3.1 Albumin Extravasation

No albumin extravasation was detected in control retinas (Figure 11.1) while, in EXT-exposed eyes, there was diffuse albumin immunopositivity in all retinal layers (Figure 11.2). BRB breakdown apparently resulted in extravasated albumin being translocated intracellularly. Similar neuronal albumin uptake has also been found in the retinas of animal models of other ocular diseases characterized by albumin extravasation (Carter-Dawson et al. 2010). Ultrastructurally, there was severe microvascular endothelial damage characterised by marked attenuation and increased electron density of endothelial cells, some of which showed endothelial discontinuity and nuclear hyperchromasia (Figure 11.3). For internal positive control purposes, brains of ETX-treated rats showed multifocal to coalescing areas of albumin extravasation (see Chapter 10), albumin immunopositivity being strongest around damaged microvessels and within the walls of these injured vessels, apparently flooding the damaged endothelial cells with albumin.
Figure 11.1: Non-treated control retina, all layers are compact, there is no discernible albumin extravasation, and microvessels are inconspicuous.

Figure 11.2: In an ETX-treated retina, there is diffuse albumin immunopositivity, microvessels are prominent (arrows) due to strong and diffuse mural albumin immunoreactivity, and there is nuclear pyknosis in the inner nuclear layer (arrow).
Figure 11.3: ETX-Exposed capillary endothelium.

There is marked attenuation and electron density of the ETX-exposed capillary endothelium (arrows), with endothelial discontinuity (*) and an endothelial nucleus showing marked hyperchromasia (n), some endothelial cells showed blebbing (G- arrows) and an occasional platelet (pl) was adherent to the damaged endothelium (F)

11.4 Discussion

This study showed that EXT causes similar retinal microvascular damage to that found in ETX-damaged brain capillaries and thus may contribute to the visual impairment found in EXT-intoxicated sheep in the naturally-occurring disease. Moreover, the similarity in vascular injury to EXT-exposed cerebral microvessels suggests that comparable mechanisms of injury are operating, but this needs to be further investigated. While this study in rats has shown ETX-induced BRB damage, morphological retinal changes produced by ETX
should also be investigated in species, especially sheep, in which the naturally-occurring disease occurs.

11.5 Conclusion

Histopathological and ultrastructural studies in sheep and laboratory rodents have suggested that the fundamental lesion underlying the severe, and frequently fatal, naturally-occurring, neurological disorder produced by Clostridium perfringens type D epsilon toxin (ETX) in domestic ruminants, and potentially exposed humans, is damage to the cerebral microvasculature (capillaries and post-capillary venules). This notion is supported by the fact that ETX produces a severe, generalised, vasogenic oedema in the brain and that highest concentration of extravasated fluid and plasma proteins is found as perivascular lakes due to increased vascular permeability following BBB disruption. After compensatory mechanisms have been exhausted, the diffuse cerebral oedema causes a marked rise in intracranial pressure, leading to shift, distortion, and sometimes herniation, of the brain, severe neurological disturbance, including convulsions, coma and, often, death. However, the interaction between ETX and the cerebral microvasculature is still incompletely understood.

Thus, while the cerebral microvasculature appears to be the primary injurious target of EXT neurotoxicity, it has never previously been shown that EXT causes a direct and damaging effect on brain-derived microvascular endothelial
cells in vitro. Accordingly, we wished to study this aspect of EXT toxicity in the present thesis. We showed, for the first time, that ETX produces a direct and dose-dependent cytopathic (endotheliotoxic) effect on an immortalised human cerebral microvascular-derived cell line, adding substantial new evidence that microvessels in the brain are the major target of ETX. For, even if ETX has secondary effects on neurons, glia and other neural elements, for example by releasing excessive quantities of the excitatory neurotransmitter, glutamate, penetration of the BBB would still be required in order for ETX to gain entry to the brain parenchyma.

Since microvascular damage with increased vascular permeability is a hallmark neuropathological finding in brains exposed to ETX, we also investigated this pathological event using the vascular tracer, endogenous albumin, and the major water channel protein in the brain, aquaporin-4. There was marked, and widely distributed, immunostaining of the brain parenchyma for extravasated plasma albumin, the immunopositivity being strongest in perivascular sites and progressively decreasing in intensity as the distance from an injured blood vessel increased. This albumin-rich oedema fluid leaking from EXT-damaged microvessels also correlated with increased expression of AQP-4, especially in pericapillary astrocytic foot processes, but also in subpial and subependymal locations and, less abundantly, in astrocytic processes in the neuropil. The latter finding suggests that AQP-4 has a significant role in the development and resolution of the cerebral oedema produced by ETX. In the rat, endothelial barrier antigen (EBA) is considered to
be a reliable indicator of an intact BBB and, accordingly, we studied the immunoexpression of this BBB marker in rats administered ETX. BBB breakdown was confirmed by substantial loss of EBA immunoreactivity in widely distributed brain microvessels, particularly as it is now generally believed that reduced levels of EBA in microvessels have a causative role in increasing vascular permeability.

Although the blood-retinal barrier (BRB) resembles, in most structural and functional respects, the BBB, it had not, until very recently, been shown that ETX could produce similar damage to the retinal microvasculature. A confirmatory study was conducted in rats exposed to ETX and, using immunohistochemical and ultrastructural techniques, it was demonstrated that ETX did produce retinal microvascular damage and ensuing severe retinal oedema, which, almost certainly, would have resulted in visual disturbance. Using albumin as a surrogate marker of increased vascular permeability, diffuse albumin immunostaining of the retina was observed, together with albumin flooding of damage vascular endothelial cells. At the ultrastructural level, retinal microvessels were reduced to an attenuated, electron-dense band, similar to the fine structural damage found in ETX-injured brain capillaries and small venules.
Chapter 12

Concluding Discussion
12 Chapter 12 Concluding Discussion

The cerebral vasculature differs in important structural and functional respects from blood vessels in other tissues, an important, but by no means sole, discriminator being the microvascular blood-brain barrier (BBB). Moreover, the brain is the most inhomogeneous organ in the body, the vascular architecture varying, not only between grey and white matter, but the diverse neuroanatomical regions that comprise the central nervous system. The concept of the neurogliovascular unit at the cellular level also emphasises the complex interplay between blood vessels, neurons, and glia to ensure maintenance of homeostasis by supplying essential nutrients and oxygen, and removing the waste products of metabolism, in a tightly regulated manner. These distinctive morphological features play an important role in the pattern of disease expression in the brain.

The unifying theme in this thesis is the cerebral vasculature and, more specifically, its role in two neuropathological processes. Firstly, the nature and response of brain blood vessels in metastatic tumours was studied, these neoplasms being represented here by disseminated melanomas, which are a major cause of morbidity and mortality in neurooncology. Secondly, the involvement of blood vessels in the pathogenesis of the neurological disorder produced by Clostridium perfringens type D epsilon toxin (ETX) was examined. ETX is one of the most potent toxins so far discovered and is responsible for a severe, and frequently fatal, neurological disease in ruminant livestock.
Moreover, ETX is on most international lists of potential bioterrorism agents, reflecting the fact that, despite its proven neurotoxicity in many animal species, its potential deleterious effects on humans are largely unknown and there is a dearth of available therapeutic interventions.

A range of investigative modalities was applied to better characterise the vascular reactions in these 2 neuropathological states: diagnostic histopathology, immunohistochemistry, immunofluorescence, ultrastructural pathology (TEM and SEM), cell culture, and histoquantitation. It is acknowledged that in addition to these techniques, further molecular investigation by way of in situ hybridisation (ISH) would give greater validity to the studies throughout this thesis.

Part A of this thesis aimed to assess the effects of melanoma on the cerebrovasculature. Ten archival, human melanomas metastatic to the brain were studied in order to categorise the different types of intra-tumoural blood vessels and determine whether there was any correlation between the 5 vascular subtypes identified (microvessels with normal-sized luminal calibre; microvessels with markedly distended and congested lumina (angioectasia); arteriolar-sized vessels; venules with markedly distended (ectatic) lumina; and thick-walled blood vessels with tortuous (serpentine) and narrowed (stenotic) lumina) and melanoma subtype (epithelioid, spindle cell and small, irregular cell), mitotic index, extent of tumour necrosis, or tumoural haemorrhage. The morphological characterisation of these tumour-associated blood vessels was
assisted by the application of CD31 and α-smooth muscle actin (SMA) immunohistochemistry and periodic acid-Schiff (PAS) staining to define endothelial hyperplasia/hypertrophy, disposition of mural smooth muscle, and deposition of basement membrane material, respectively. No statistical correlation between vascular type and any of these parameters was detected, or with other vascular pathologies found in the metastatic melanomas (thrombosis; perivascular cuffing by malignant melanocytes; and lymphocytic perivascular cuffing/vasculitis). Furthermore, a marked reduction in claudin-5 immunoreactivity in these structurally aberrant, tumour-associated blood vessels was demonstrated, the diminution of this critical tight junction component being consistent with loosening of interendothelial tight junctions, extravasation of fluid and plasma proteins, and resultant diffuse, vasogenic cerebral oedema.

The role of caveolin-1 (CAV-1), the principal structural component of caveolae or plasma membrane invaginations, and the SP/NK-1 receptor system was examined in the structurally and functionally often anarchic, melanoma-associated blood vessels present in the ten case cohort. CAV-1 immunoexpression in these blood vessels was often decreased, a finding in concert with other studies correlating diminished vascular CAV-1 immunoreactivity with robust neoangiogenesis in many tumours. Disruption of tight junctions in CAV-1-depleted blood vessels has also been found in traumatic brain injury and stroke and, in the context of oncology, could assist breaching of the BBB by metastatic tumour cells and the formation of intra-
and peri-tumoural vasogenic oedema. By contrast, CAV-1 was widely, and strongly, expressed on melanocyte cell membranes (and to a lesser degree in the cytoplasm) in these melanomas, reflecting its role as both tumour promoter and suppressor. The NK-1R was immunoexpressed by a subset of tumour blood vessels in this study, a finding also reported in cutaneous melanomas, and immunopositivity was almost always found in the cytoplasm, and occasionally nucleus, of melanocytes. The NK-1R is overexpressed in many types of neoplasms, more robustly in the more malignant variety, and has been assigned a vital role in tumour growth and progression. However, in all 10 metastatic melanomas studied, melanocytes were invariably immunonegative to SP and there was no SP immunoexpression in tumour-associated blood vessels.

In order to simulate the transendothelial migration of malignant melanocytes into the brain parenchyma and test its inhibition by anti-cancer agents, two models systems were used, one in vitro (the transwell apparatus) and the other in vivo (intracarotid injection of a melanoma cell line). The former is a porous membrane covered with collagen, this scaffolding replicating the BBB, and movement of melanoma cells across the membrane, and potential inhibition by drugs, can be quantified. While no inhibition by the agents tested (filipin III and NK-1R antagonist – EUC001) could be demonstrated, movement of melanocytes through the membrane was studied ultrastructurally. The injection of abundant melanoma cells into the carotid artery seems a feasible method of presenting sufficient tumour cells to the brain microvasculature to
permit study of their transendothelial penetration into the parenchyma, unfortunately however, very few of these administered melanoma cells were found in the brain. Moreover, those that were present were generally observed as individual melanocytes, rather than aggregates, and many showed nuclear shrinkage and hyperchromasia, or pyknosis, indicating that they were probably destined to die or were already necrotic. Mitotic figures were never found, even in apparently viable tumour cells, suggesting that they were not capable of proliferating in this new milieu. While this result was disappointing, the scientific literature to date suggests that this model is usually unsatisfactory with currently available melanoma cell lines, very few injected tumour cells reaching the brain and even fewer penetrating into neural tissue.

In Part B, the role of the cerebral microvasculature in the pathogenesis of _Clostridium perfringens_ type D ETX neurotoxicity was further investigated. While histopathological and ultrastructural studies have suggested that damage to the brain microvascular endothelium is the fundamental lesion in the development of neurological dysfunction after ETX exposure, a direct and damaging cytotoxic effect of ETX on cultured cerebral microvascular endothelium had not previously been demonstrated. The present study was the first to show that ETX causes a dose-dependent injury to brain microvascular endothelial cells _in vitro_, which was quantified and verified by histopathological and ultrastructural examination. Moreover, BBB disruption was confirmed by loss of endothelial barrier antigen (EBA) immunoreactivity in
ETX-exposed rat brains, this protein being a recognised marker of an intact BBB in this species. Furthermore, the important role of the major water channel protein in the brain, aquaporin-4 (AQP-4), in the development and resolution of the vasogenic oedema produced by ETX-induced BBB damage, and subsequent increased vascular permeability, was confirmed by its increased immunoexpression in ETX-injured microvessels.

While the blood-retinal barrier (BRB) is similar in many structural and functional respects to the BBB, its vulnerability to ETX has only recently been demonstrated. Accordingly, a confirmational study was conducted, which showed that exposure of the retinal microvasculature to ETX caused BRB disruption and a marked increase in vascular permeability, leading to severe retinal oedema and likely visual decrement. Endogenous albumin was used as a vascular tracer and surrogate immunohistochemical marker of increased vascular permeability and flooding of endothelial cells with albumin confirmed their ETX-induced injury. It is now proposed to determine whether there is, as in the BBB, loss of EBA in retinal microvessels exposed to ETX.

This binary study of the cerebral vasculature highlights the remarkable versatility, and sometimes vulnerability, of these blood vessels. On the one hand, the microvascular endothelium in brain and retina was very susceptible to ETX-induced injury, with resultant increased vascular permeability, extravasation of copious amounts of fluid and plasma protein, and severe, generalised, vasogenic oedema. However, in response to the demands of
metastatic brain tumours for nutritional support to foster growth and progression, neovascularisation can be achieved by a number of different mechanisms and often frustrate chemotherapeutic interventions. Nevertheless, these tumour-associated blood vessels are often structurally aberrant, their increased permeability ("leakiness") resulting in vasogenic oedema, which may substantially increase the space-occupying tumour volume, resulting in increased intracranial pressure, which then frequently precipitates a lethal cascade of ensuing detrimental pathological events.
These experimental results will be added as an appendix as the date of thesis submission precluded them from being included in the main body of work. They are presented here as they are germane to my consideration of epsilon toxin (ETX) neurotoxicity, especially the endotheliotoxic action.

13.1 Scanning electron microscopy (SEM) of human brain microvascular endothelial cells in vitro exposed to Clostridium perfringens type D epsilon toxin

My thesis previously examined the effect of this clostridial epsilon toxin (ETX) on cultured brain microvascular endothelial cells by light and transmission electron microscopy and I now extend this analysis by the application of SEM.

13.2 Materials and methods

13.2.1 In vitro cell culture

The hCMEC/D3 cell line was used in accordance with methods previously reported in chapter 2 (section 2.2.5) of this thesis.

13.2.2 Epsilon Toxin (ETX)

The ETX toxin was generated and prepared as per methods outlined in Chapter 2 (section 2.3).
13.2.3 Scanning Electron Microscopy

Exposed hCMEC/D3 are collected and maintained on filter membrane for fixation (4% PFA/1.25% Glutaraldehyde in PBS, + 4% sucrose, pH 7.2). Filters are then washed in washing buffer (PBS + 4% sucrose) 1 x 5 mins, followed by post-fixing in 2% OsO₄ for 1 hr. Samples then undergo dehydration though graded ethanol (70%-100%), before application of HMDS (hexamethyldisilazane) 1:1 with 100% ethanol for 10 mins. Samples are passed through 2 x 10 mins of 100% HMDS and allowed to air dry before mounting on stubs and coating.

Samples were imaged using the FEI Quanta FEC 450 and visualised using xT® microscope control software.

13.3 Results

In control cerebral microvascular endothelial cultures, the endothelial cell nucleus (n) was sometimes rounded in outline, with varying degrees of cytoplasmic spreading (arrow) evident. Some endothelial cells were more elongated in profile, but the nucleus was still prominent and raised (Figure 13.1, A-D).
Figure 13.1: Scanning Electron Microscopy Images.

(A-D) hCMEC/D3 control cells

In ETX-treated endothelial cultures, the endothelium tended to be shrunken and more irregular in outline, with numerous pits or craters (arrows) evident, and the nucleus was often less visible. These features were more apparent at higher resolution and, sometimes, the endothelial cell cytoplasm appeared to be attenuated (“stretched”) (E), with multiple defects (arrows) present (A-E).
Figure 13.2: ETX exposed hCMEC/D3 cells.

13.4 Discussion

An examination of ETX-treated endothelial cultures revealed degenerative features characterised by cellular shrinkage and more irregular contours, with cytoplasmic pitting or crater formation. The cytoplasm was also sometimes markedly attenuated, appearing to have been stretched.

These degenerative changes were consistent with the injured appearance of ETX-treated endothelial cell cultures examined earlier in this thesis by light
microscopy and transmission electron microscopy, while the morphology of untreated, control endothelial cells resembled that described by others (Bowman et al. 1983, Kirkpatrick 1995).

In summary, we have shown for the first time, that ETX produces a direct and damaging, dose-dependent cytopathic effect on brain-derived microvascular endothelial cells in vitro and examined the morphological changes produced by this potent neurotoxin in exposed endothelium by light microscopy and TEM/SEM, together with quantitation of this endotheliotoxic action.
14 Appendix

14.1 Mechanisms of new blood vessel formation in brain tumours

K.A. Mander\textsuperscript{a}, R. Vink\textsuperscript{b}, JW Finnie\textsuperscript{ac}

\textsuperscript{a}Schools of Medicine and Veterinary Science, University of Adelaide, Adelaide SA, Australia; \textsuperscript{b}Sansom Institute for Health Research, University of South Australia, Adelaide SA, Australia; \textsuperscript{c}SA Pathology Centre for Neurological Diseases, Adelaide SA, Australia

Corresponding Author:

Dr John Finnie

SA Pathology Centre for Neurological Diseases, Adelaide, S.A. 5000 Australia

Phone: 61-08-8222 3370/ MOB 0427862084

e-mail: john.finnie@sa.gov.au
Abstract

Mechanisms of new blood vessel formation (neovascularisation) of tumours are a major focus of study in cancer biology and of particular relevance for targeted cancer therapy. The intent of this review is to describe the different modes by which tumours can acquire a new blood supply to sustain continued growth and dissemination, with special features pertaining to brain tumours being highlighted. A better understanding of the development of various types of tumour-associated vasculature is important as it serves as a basis for devising novel anti-angiogenic intervention strategies that are of considerable potential benefit for the treatment of neoplasms, including those arising in domestic animal species. In neuro-oncology, a major impetus for blood vessel-targeted therapies is the fact that many primary brain tumours are largely refractory to treatment and have a poor prognosis, while tumours metastatic to the brain are similarly often fatal after a brief clinical course.

Keywords

Brain tumours; Neovascularisation; Mechanisms

Introduction

A fundamental tenet of tumour biology is that in order to sustain continued growth and invasiveness, a neoplasm must acquire a new blood supply (Fidler, 2011). The induction of neovascularisation is one of the hallmarks of cancer, which also includes sustaining proliferative signalling, evading growth suppressors, activating invasion and metastasis, enabling replicative
immortality, resisting cell death, reprogramming energy metabolism, and evading immune destruction (Hanahan and Weinberg, 2011). In this review, the generic term “neovascularisation” (from the Latin “neo” = new and “vasculum” = a vessel) is used to denote the formation of any new blood vessel, by whatever means this is enacted.

Two phases of tumour growth are recognised. In the avascular or dormant phase, which is found in tumours up to approximately 1-2 mm in diameter, there is a steady state between proliferation and apoptosis of tumour cells. In tumours of this size, diffusion from pre-existing host blood vessels supplies the required oxygen and nutrients and eliminates the waste products of tumour metabolism (Bergers and Benjamin 2003; Shahneh et al., 2013). However, in some tumours such as high grade gliomas, the avascular phase may be fleeting or even largely absent (Fischer et al., 2005). The diffusion coefficient of oxygen in tissues is of the order of 150-200 µm and, while tumour cells located < 100 µm from a blood vessel are viable, more distant cells undergo apoptosis. Growing tumours easily become hypoxic and necrotic due to rapid proliferation and vascular insufficiency, precipitating a vascular phase, which is characterised by an “angiogenic switch” in favour of new growth. This stage is induced when a tumour produces sufficient angiogenic growth factors and/or suppresses the expression of inhibitors. New blood vessel development usually commences at the periphery of a tumour, and in the contiguous parenchyma, and occurs concomitantly with tumour cell infiltration (Bergers and Benjamin 2003; Shahneh et al., 2013).
Mechanisms of brain tumour neovascularisation

Until recently, neovascularisation of brain tumours was considered to be largely achieved by endothelial sprouting, a process termed angiogenesis, but it is now recognised that a number of different mechanisms may be operative and they may occur concurrently or sequentially (Döme et al., 2007; Plate et al., 2012). Nevertheless, in most brain tumours, the contribution of processes other than angiogenesis to the formation of new tumour-associated blood vessels is probably relatively minor and some of the newly described modes of neovascularisation are still controversial.

The mechanisms of vascular formation which will be discussed are co-option of pre-existing blood vessels, angiogenesis (capillary sprouting), vascular intussusception, vascular mimicry, and vasculogenesis (in which bone marrow-derived endothelial progenitor cells and cancer stem-like cells contribute to the tumour-associated vasculature). A schematic representation of these different types of new blood vessel formation in tumours is shown in Figs. 1 and 2.
Figure 1: Schematic representation of the different mechanisms of neovascularisation. (A) vascular co-option, with tumour cells growing along pre-existing blood vessels; (B) angiogenesis, with proliferation and migration of endothelial cells (EC) from the parent vessel towards an angiogenic stimulus, forming new capillary sprouts; (C) vascular mimicry, where tumour cells invade and line capillaries and assume EC functions; (D) vasculogenesis, where bone marrow-derived EC progenitors and cancer stem-like cells are recruited, and incorporated into, the EC lining to form new blood vessels.
Figure 2: Vascular intussusception, with internal partitioning and division of pre-existing capillaries to form new blood vessels.

Vascular co-option

Since the brain, in common with lung and liver, is one of the most highly vascularised tissues, the cerebral capillary network is a good basis for vessel co-option and this mode of new blood vessel formation appears to be operative in a large proportion of brain tumours. Temporally, vascular co-option is the first mechanism by which gliomas, for example, acquire a new blood supply. When tumours infiltrate the brain, either as primary tumours or metastases, they initially grow preferentially along pre-existing microvessels, advancing between the basal lamina and the endothelial lining, and evoking endothelial proliferation. Co-option may be maximised by tumour cell pseudopodia, permitting these cells to interact with a larger vessel surface area, thus obtaining more oxygen and nutrients from the host and stimulating non-angiogenic growth. Tumour cells often grow as cuffs around co-opted
blood vessels. However, as the tumour cells migrate along these vessels, they tend to compress and destabilise them. A robust host defence mechanism is activated, in which co-opted vessels initiate an apoptotic cascade, leading to vascular regression and impaired perfusion which, in turn, causes hypoxia and even tumour necrosis, both of which are potent inducers of neovascularisation (Donnem et al., 2013; Plate et al., 2012; Ribatti et al., 2011).

**Angiogenesis**

Angiogenesis, or sprouting of capillaries from pre-existing blood vessels, is the principal method of neovascularisation and recapitulates the formation of blood vessels during embryogenesis, the latter termed vasculogenesis (Vallon et al., 2014). The orderly formation of these vessels involves a complex interplay between pro- and anti-angiogenic factors, tumour cells, glia, endothelial cells, the extracellular matrix (ECM), and immune cells (Jain et al., 2007). Angiogenesis can be initiated at different stages of tumour progression, depending upon the nature of the tumour and its microenvironment. However, while angiogenesis is an effective mechanism for new blood vessel formation in tumours, it is, nevertheless, less robust than that occurring under physiological conditions such as granulation tissue formation (Giatromanolaki et al., 2004).

In quiescent endothelium, proliferation and migration are minimal and, once formed, endothelial cells are amongst the longest living, their turnover time being measured in months or years. However, in tumours, new blood vessel
formation continues as long as the tumour keeps growing, with hypoxic and necrotic areas being especially potent inducers of angiogenesis (Plank and Sleeman, 2003).

When a capillary near a brain tumour receives a net angiogenic stimulus, there is a loosening of inter-endothelial tight junctions, vasodilatation, increased vascular permeability, pericyte detachment, and separation of astrocytic foot processes or end-feet which, in cerebral capillaries, almost completely cover the external surface of the vessel and contribute to blood-brain barrier (BBB) function. Pericytes normally stabilise the vessel wall, controlling endothelial proliferation, and the balance between endothelial cells and pericytes is tightly controlled by paracrine signalling. The basement membrane is then degraded to liberate endothelial cells from their surface anchors (integrins), which normally maintain the blood vessels in a stable state (Kalluri, 2013). Extravasated plasma protein forms a matrix upon which activated endothelial cells can migrate and the initial endothelial response is migratory rather than proliferative. Endothelial migration is governed mainly by a chemotactic response to a concentration gradient of different growth factors produced by the tumour and other cellular elements, creating a potent directional stimulus (Giatromanolaki et al., 2004; Jain et al., 2007; Plank and Sleeman, 2003).

Proteolytic enzymes such as matrix metalloproteinases and the plasminogen activator/plasmin system then degrade the ECM to permit migration of
endothelial cells towards angiogenic stimuli. However, there must be a balance between these proteases and their inhibitors to prevent excessive matrix degradation and loss of endothelial attachment to the underlying substratum. In normal capillaries, the basal lamina provides growth arresting cues but, when endothelium is exposed to the ECM, it receives proliferative signals (Jain et al., 2007; Plank and Sleeman, 2004). At this stage, the neuropil of the brain becomes a less compact and more oedematous microenvironment to facilitate penetration by tumour, and their attendant endothelial cells, especially since most tissues present, at least to some degree, a barrier to neoplastic invasion. However, brain parenchyma differs from most tissues in that the ECM is ill-defined and scant, except around blood vessels and at the pial surface (glia limitans), where there is a more conventional basement membrane and collagen fibres. Moreover, the composition of the brain parenchymal ECM differs from that of other solid organs, lacking fibronectin and collagen, but rich in proteoglycans, tenascin, laminin, hyaluronic acid, and heparin, chondroitin and dermatan sulphates. These neuropil changes are orchestrated by paracrine (cell-to-cell) communication between endothelial, stromal and tumour cells.

Endothelial sprouts initially form solid migration columns, led by a filopodia-rich tip cell, and followed by a group of stalk cells that divide and promote elongation of the sprout. The newly formed vessel then remodels, with endothelial cells changing shape and adhering to each other to construct a lumen; inter-endothelial tight junctions are then re-established. Proliferating
pericytes from the parent vessel migrate along the basement membrane of the capillary bud, resulting in complete coverage of the new blood vessel. Pericytes at the growing front of endothelial sprouts may also guide newly formed vessels or they can invade tissue ahead of endothelial cells and form a tube within which endothelial cells subsequently penetrate (Bergers and Song, 2005).

Capillary sprouts commence by growing almost parallel to each other but, at a certain distance from the parent vessel, begin to incline towards other sprouts, then fuse and form closed loops or anastomoses to permit circulation of blood into the newly vascularised area. In some tumours, capillary density and looping can become exaggerated, resulting in a dense network of vessels (telangiectasis). New capillaries thus reach and penetrate the tumour, substantially improving its blood supply and permitting more rapid growth (Dome et al., 2007).

Many tumour-associated capillaries do not form stable, mature vessels with a continuous basement membrane because of the constant production of angiogenic factors. Rather, they are characterised by an aberrant structure, altered endothelial-pericyte interactions, abnormal blood flow, increased permeability, and delayed maturation. Moreover, these new vessels are often irregularly shaped, dilated, tortuous, sometimes blind-ended, and prone to haemorrhage (Plank and Sleeman, 2004). Blood vessels supplying the often
hypoxic, central regions of tumours tend to be more abnormal than those supplying the tumour periphery (Giatromanolaki et al., 2004).

**Control of angiogenesis**

As a tumour grows, the invasion front becomes internalised, this milieu becoming more hypoxic and acidic and altering the balance from proliferation towards apoptosis. This unfavourable microenvironment, particularly hypoxia, causes the tumour to respond by activating glycolytic anaerobic pathways and upregulation of angiogenic and anti-apoptotic factors. If, however, these responses prove to be inadequate, tumour necrosis and vascular regression ensue (Furuya et al., 2005; You and Stallcup, 2015).

With respect to the regulation of angiogenesis, tumour hypoxia is the major driver of new blood vessel formation, with signalling pathways initiated from numerous growth factor receptors playing pivotal roles in tumour-mediated neovascularisation (Plank and Sleeman, 2004).

The hypoxia-inducing family coordinates a transcriptional programme that ensures metabolic and vascular adaptation to low oxygen tension. In the presence of oxygen, the α-subunit of hypoxia-inducible factor-1 (HIF-1α) is hydroxylated and rapidly degraded. Hypoxia inhibits this hydroxylation and, as a result, constitutively expressed HIF-1β can bind to HIF-1α. HIF-1 accumulates, is translocated to the nucleus, and binds to hypoxia-responsive elements to activate the transcription of numerous different hypoxia-inducible genes, which represent about 2-3% of all genes. Hypoxia induces the
secretion of growth factors, which include vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), interleukin-8 (IL-8), and stromal-derived growth factor-1 (SDF-1) (Jain et al., 2007).

The interplay between hypoxia, necrosis and angiogenesis is well illustrated by glioblastomas, the high grade types being amongst the most vascular of all solid tumours. Neovascularisation of these gliomas correlates directly with their biological aggressiveness, degree of malignancy, and frequency of clinical recurrence (Brat et al., 2001; 2004; Dong et al., 2005; Leon et al., 1996). In glioblastoma multiforme (GBM), also termed glioblastoma or grade IV astrocytoma, the cardinal diagnostic features are microvascular proliferation and necrosis. Small necrotic foci often develop in these rapidly growing tumours when metabolic demand exceeds the vascular supply, while thrombosis may contribute to the development of larger necrotic areas. Hypoxia seems to upregulate migration-associated genes, leading to movement of glioma cells away from the hypoxic region, thus clearing a central region, which is more vulnerable to necrosis. This selection pressure may favour the survival of more malignant clusters of glioma cells and necrosis in the highest grade malignant gliomas is a potent predictor of a poor prognosis (Louis et al., 2008). Densely packed glioblastoma cells tend to palisade around necrotic foci and VEGF is abundantly upregulated by these tumour cells in response to hypoxia. VEGF may then bind to endothelial VEGF receptors and stimulate angiogenesis by a paracrine mechanism. Vascular proliferation occurs in two forms – a diffuse increase in microvascular density
and the distinctive “glomeruloid” (named for its similarity to renal glomeruli) microvascular proliferation. Glomeruloid vessels are a feature of high grade gliomas and are complex structures composed of proliferating endothelial cells, pericytes and smooth muscle cells, with the latter predominating (Haddad et al., 1992). They tend to form garlands or festoons around necrotic areas, but can also be found at the invading edge of glioblastomas as a result of angiogenic factors secreted by migrating glioma cells (Hardee and Zagzag, 2012; Fischer et al., 2005; Wesseling et al., 1993).

A number of different factors are involved in the activation and inhibition of angiogenesis. Stimulation of endothelial cell chemotaxis, proliferation, protease secretion, survival, differentiation and permeability is performed by VEGF, angiopoietin-1, basic fibroblast growth factor (bFGF), SDF-1, IL-8, and tumour necrosis factor - alpha (TNF-α), while inhibition of these endothelial functions is executed by angiopoietin-2, angiostatin and endostatin (by-products of plasminogen and collagen proteolysis, respectively), interferon α and β and interleukins, TNF-β (and sometimes TNF-α), and thrombospondins. Platelet-derived growth factor (PDGF) secreted by activated endothelial cells recruits pericytes to sprouting vessels (Jain et al., 2007).

VEGF is the principal angiogenesis factor in tumour growth. Its receptors are expressed almost exclusively on endothelial cells and VEGF is a survival factor for endothelium by inhibiting apoptosis. VEGF is the ligand for vascular endothelial growth factor receptor-2 (VEGFR-2) on endothelial cells and this
receptor is essential for mediating endothelial proliferation, migration and permeability (Plank and Sleeman, 2003).

During angiogenesis, VEGF causes loosening of inter-endothelial tight junctions and vessels initially dilate, probably due to downregulation of cell-cell adhesion molecules such as cadherins. Pericyte detachment also promotes abnormal luminal dilatation (angioectasia). VEGF causes increased vascular permeability, producing marked vasogenic oedema. It induces upregulation of cell-substratum adhesion molecules such as integrins, shifting the balance towards cell-matrix rather than cell-cell adhesion, thus promoting an invasive endothelial phenotype. Hypoxic tumour cells express large amounts of VEGF and excess production for prolonged periods of time produces a tumour-associated vasculature that is tortuous, leaky and hyperperfused (Jain et al., 2007; Plank and Sleeman, 2003).

Intratumoral levels of VEGF and its receptor are well correlated with the histological grade of gliomas and high levels in these neoplasms portend a poor prognosis. The highly upregulated VEGF in GBM is mainly driven by hypoxia and its expression in perinecrotic palisading glioma cells can be increased up to 50-fold, attended by a similar increase in VEGFR-2 on intratumoral endothelial cells. Anti-VEGF therapies tend to slow angiogenesis, leading to “normalisation” of the vascular network, and enhanced drug delivery. However, this pharmacological intervention may also cause malignant glioma cells to find alternative mechanisms of neovascularisation,
stimulating further aggressive tumour behaviour (Takano, 2012; Tate and Aghi, 2009).

The angiopoietin family is also a key player in the balance between quiescent and activated endothelium, particularly ANG-1 and ANG-2. The endothelium-specific receptor, tyrosine kinase (Tie-2), binds ANG-1 and ANG-2 ligands to the same receptor site and the ANG-1-ANG-2, agonist-antagonist relationship allows the Tie-2 signalling pathway to regulate with a high degree of spatiotemporal precision the transition between endothelial quiescence and angiogenesis. Tie-2 appears to be constitutively expressed in adult vasculature, suggesting that it is important for continued vessel homeostasis (Holash et al., 1999; Plank and Sleeman, 2003).

ANG-1 stabilises blood vessels, while ANG-2 destabilised them. ANG-1, produced by pericytes and smooth muscle cells, maximises interactions between endothelial cells and pericytes, inhibits apoptosis, and is expressed behind the leading edge of angiogenic vessels, consistent with its role in vessel maturation. By contrast, ANG-2 is expressed by endothelial cells at the forefront of proliferating vessels. ANG-1 acts directly on endothelial cells and maintains their quiescent state, decreasing vascular permeability and promoting vascular integrity. However, in the presence of the ANG-1 antagonist, ANG-2, cell-cell and cell-matrix connections are weakened and the basement membrane and pericytes become dissociated from the endothelium. If VEGF is also present, endothelial cells begin to sprout and
angiogenesis follows. ANG-2 can, therefore, lead to either angiogenesis or vessel regression, depending upon whether a VEGF signal is detected (Jain et al., 2007).

**Vascular intussusception**

Vascular intussusception, literally meaning “growth within itself”, describes the remodelling and expansion of tumour blood vessels by the insertion of interstitial tissue columns or pillars into the lumen of pre-existing blood vessels. Initially, contact between opposing capillary walls is established by formation of a transluminal endothelial bridge. Inter-endothelial junctions are reorganised and there is perforation of the vessel wall, through which cytoplasmic extensions of myofibroblasts, pericytes and interstitial fibres invade the lumen to complete partitioning of the vessel lumen, splitting the pre-existing capillary into two new, independent vessels. Accordingly, intussusception is also known as “splitting angiogenesis” (Gianni-Barrera et al., 2011; Styp-Rebowska et al., 2011).

Intussusception is a response to local changes in haemodynamics, especially high levels of shear stress, whereas capillary sprouting is stimulated more by angiogenic factors released in response to hypoxia. Sprouting angiogenesis may switch to vascular intussusception as the latter process develops new vessels more rapidly, thus increasing the complexity and density of the tumour microvascular network. Intussusception is economical in terms of metabolic activity and energy consumption for, unlike angiogenesis, extensive
endothelial proliferation, basement membrane degradation, and invasion of surrounding tissue is not required. This process also permits continuous blood flow during remodelling, in contrast to capillary sprouting. However, the molecular mechanisms underpinning intussusception are currently poorly understood, although VEGF signalling is necessary for this mechanism to be instituted. After anti-angiogenic treatment, tumours can rapidly recover by switching from sprouting to intussusception (Kurz et al., 2003).

**Vascular mimicry**

Vascular mimicry refers to the formation of fluid-conducting, basement membrane-containing, tumour (not endothelial)-lined channels by highly invasive and genetically deregulated tumour cells. These channels are not true blood vessels, but mimic their function, with tumour cells assuming some phenotypic traits of endothelial cells, including luminal incorporation. Aggressive melanoma cells, in particular, have an ability to express an endothelial cell phenotype and form vessel-like networks, but other neoplasms, including carcinomas, sarcomas, highly angiogenic GBM’s, and astrocytomas, can also participate in vascular mimicry. This process, which mimics an embryonic vascular network, exposes tumour cells directly to flowing blood, thus permitting them to enter the microcirculation and metastasise (Folberg and Maniotis, 2004; Maniotis et al., 1999).

These tumour-lined, ECM-rich, periodic acid-Schiff (PAS) and laminin-positive, fluid-containing channels provide nutrient exchange for aggressive, rapidly
dividing tumours and help to prevent ischaemic-hypoxic necrosis, especially in the early stages of malignant tumour growth. PAS-positive, CD31-negative staining is commonly used to define these tumour-lined vessels. The intratumoral, fluid-conducting “vascular channels” contain plasma and erythrocytes and may eventually be relined by conventional endothelium from adjacent blood vessels or connect to blood vessels, thus increasing overall tumour perfusion (Folberg et al., 2000; Liu et al., 2016).

Vascular mimicry, which is largely driven by tumour hypoxia, seems to represent an early survival mechanism for nutrient exchange and involves deregulation of the tumour-specific phenotype and expression of endothelium-associated genes. Tumour cells exhibiting vascular mimicry show phenotypic plasticity similar to embryonic stem cells. Clinically, vascular mimicry, although probably uncommon, is associated with a poor prognosis, suggesting that it confers certain advantages for tumour progression (Hallani et al., 2010; Sun et al., 2016).

Two types of vascular mimicry are recognised. The tubular type is morphologically similar to endothelium-lined blood vessels, while the patterned matrix type does not at all resemble blood vessels, either morphologically or topologically. In the latter, the “vascular channels” are comprised of septa of connective tissue and ECM encircling clusters of tumour cells and fluid flows in the interstices between matrix and tumour (Qiao et al., 2015).
Vascular mimicry thus describes the functional ability of aggressive tumour cells to express a multipotent, stem cell-like phenotype and it can be directly induced by cancer stem cells in many malignant tumours, including glioblastomas. When endothelium-dependent blood vessels are inhibited by anti-angiogenic therapies, the resulting hypoxia promotes vascular mimicry to ensure selective perfusion (Hendrix et al., 2003; Mao et al., 2015).

However, the concept of vascular mimicry is controversial, with some contending that the channels are, in fact, lined by endothelium, not tumour cells, or that this phenomenon represents instead intratumoral vascular regression (McDonald et al., 2000).

**Bone marrow-derived endothelial progenitor cell vasculogenesis**

This mode of neovascularisation describes the de novo formation of vascular networks from endothelial precursor or progenitor cells (EPC). There is a close relationship between the development of blood and endothelium, with haematopoietic and endothelial cells arising from a common progenitor, the haemangioblast. In vasculogenesis, circulating bone marrow-derived EPC are recruited to the tumour, integrated into the vessel wall, and differentiate into endothelial cells. EPC incorporation into the tumour vasculature has been reported in many such vessels, but most consider this mode of neovascularisation to be a very minor contributor to new blood vessel formation (Ribatti et al., 2004). A contrary view asserts that EPC are not
incorporated into blood vessels to any useful degree, but rather reside perivascularly and support angiogenesis in a paracrine manner.

In cancer stem-like cell-derived vasculogenesis, GBM-derived cancer stem-like cells may contribute to the vasculature by integrating into the vessel wall and transdifferentiating into endothelial cells, while still retaining the genetic alterations typically found in glioblastoma cells. However, this mode of new blood vessel formation is controversial, with some proposing that these stem cells are, in fact, confined to the perivascular space or vessel wall, rather than lining the vessel lumen, and are present in very small numbers (Priya et al., 2016).

Bone marrow-derived monocytes/macrophages may be recruited in response to tumour-derived chemokines and growth factors and drive tumour angiogenesis by secreting a variety of pro-tumorigenic and pro-angiogenic factors. Tumour-associated macrophages can express endothelial markers and may contribute to the tumour vasculature directly, although there is scant evidence of luminal incorporation and any effect on angiogenesis may be directed from a perivascular site (Priya et al., 2016).

Special features relating to neovascularisation of brain tumours
The vascular pattern in a given brain tumour is often heterogeneous, with areas of high microvascular density interspersed with those of sparse vascularity. In low-grade astrocytomas, for example, the vascular density is
somewhat greater than that in brain parenchyma, but markedly elevated in high-grade gliomas (Vartanian et al., 2014). These tumour-associated blood vessels are also structurally anarchic and functionally aberrant, with the lumen dilated and the endothelium proliferative. Microvessels, particularly those in the innermost, more challenging, pro-inflammatory microenvironment, tend to be disorganised, being typically tortuous (“serpentine”), highly permeable, and having abnormalities in their wall, basement membrane (basal lamina), and pericytes. Every layer of the tumour vessel wall is abnormal. Endothelial cells lack a cobblestone appearance, are poorly interconnected, and often hyperplastic, resulting in intimal thickening; the basement membrane is irregular in thickness and composition; and there is a disorderly arrangement of mural smooth muscle cells. These vessels often have a distended lumen and blood flow is irregular, moving more slowly, and sometimes even oscillating. However, while these vessels are usually more permeable, this leakiness varies from one tumour to another, at different sites within a given tumour, and temporally. Enhanced vascular permeability leads to peritumoral vasogenic oedema, expanding the size of the space-occupying tumour mass, and causing a rise in intracranial pressure, which can result in shift and distortion of the brain, and even herniation. There is also a decreased density of astrocytic foot processes in tumour-associated microvessels. Tumour endothelial cells were once assumed to be genetically normal, even though they were structurally and functionally abnormal, but it has been suggested that tumour endothelial cells are, in fact, inherently genetically unstable and may acquire cytogenetic abnormalities while
present in the tumour microenvironment (Bergers and Benjamin, 2003; Fidler 2011; Furuya et al., 2005; Shahneh et al., 2013; You and Stallcup 2015).

This dysfunctional tumour vasculature can also restrict the circulation to, for example gliomas, and reduce drug delivery, thus contributing to chemotherapeutic resistance. Moreover, since chemotherapy relies upon rapid cancer cell proliferation, the lower proliferative capacity of hypoxic neoplastic cells may afford some protection and some tumour cells undergo adaptive changes in an hypoxic milieu, enhancing their survival (Vartanian et al., 2015).

Most tumours metastatic to the brain occur via the haematogenous route, with tumour cells becoming trapped where capillaries and post-capillary venules branch at the corticomedullary junction and watershed zones, at which sites the vessel diameter approaches the size of the tumour cell. The neuroanatomical distribution of metastases seems to be proportional to the blood flow to different regions in humans, with 80% found in the cerebral hemispheres, 10-15% in the cerebellum, and 1-5% in the brainstem. When metastatic tumours in the systemic circulation reach the brain, they first encounter the BBB which, however, is a double-edged sword. While the BBB provides a physical barrier to entry of metastatic tumours into the brain, it can also sometimes enhance brain penetration by actively participating in the transendothelial migration process, shielding tumour cells from immune surveillance and chemotherapeutic drugs, releasing tumour growth factors,
and assisting neovascularisation. Although the BBB may be disrupted at or near the core of high grade brain tumours, it seems to be intact at the growing edge of the tumour mass. Thus, even with aggressive treatment, recurrence of brain metastases is almost inevitable as large regions of the tumour survive behind an intact BBB. Moreover, most cancer therapies do not achieve therapeutic levels in the brain (Blecharz et al., 2015; Long, 1979; Weidle et al., 2015; Wilhelm et al., 2013).

While the mechanisms involved in migration of tumour cells across the BBB are poorly understood, transmigration can occur by 2 routes: the paracellular pathway through weakened interendothelial tight junctions, which largely determine the effectiveness of the BBB (Ballabh et al., 2004), and transcellular passage. Some tumour cells also appear to be able to mimic the molecular mechanisms of the leucocyte-endothelial interaction during inflammation to assist BBB penetration. Extravasation of tumour cells can take several days and metastatic tumours with a high affinity for the brain often proliferate intravascularly preceding transendothelial migration. In general, while the process of transendothelial migration of tumour cells into an organ is usually rapid, they require substantially more time to extravasate in the brain and, accordingly, need to survive for a longer period of time. Specific gene expression profiles appear to determine the interaction of tumour cells with the endothelium, with overexpression of genes associated with invasion, metastasis, adhesion, angiogenesis, and cell migration. During transmigration, there is attenuation of the vessel wall and emigration seems to occur through
discontinuities in the endothelium; tumour cells may also induce apoptosis. The degree to which the endothelium repairs after tumour transmigration and becomes intact again is still debated (Blecharz et al., 2015; Weidle et al., 2015; Wilhelm et al., 2013).

When a primary brain tumour or metastases grow beyond about 1-2 mm in diameter and neovascularisation is induced, the BBB tends to become structurally and functionally compromised. While the BBB is often not intact in experimental brain metastases that exceed 0.2 mm², it can apparently repair after passage of metastatic tumour cells into the brain and tumour cell emigration does not always disturb BBB integrity (Blecharz et al. 2015; Kim and Lee, 2009).

Tumour cells enter a generally supportive environment in the brain parenchyma, protected from chemotherapeutic drugs and the anti-tumour immune response, and being exposed to soluble factors favouring survival and proliferation. While astrocytes form part of the BBB and assist endothelial cells to impede tumour penetration of the brain, reactive astrocytes localise early to metastatic tumour cells and support them by providing protection from chemotherapy, upregulating survival genes in neoplastic cells, and secreting growth factors that stimulate tumour growth. Microglia also promote invasion of tumour metastases into the brain and may even guide them, thus promoting colonisation (Blecharz et al., 2015; Weidle et al., 2015; Wilhelm et al., 2013).
In conclusion, tumour-associated blood vessel formation (neovascularisation) is required to sustain continued growth of solid tumours beyond approximately 2 mm in diameter and this can be achieved by a number of different mechanisms, which can operate concurrently or sequentially. Hypoxia is the principal driver of this new blood vessel formation, particularly hypoxia-ischaemia-induced tumour necrosis. Co-option of pre-existing host microvessels is generally the initial phase of tumour infiltration, while sprouting angiogenesis from pre-existing brain microvessels is the principal mode of neovascularisation of brain tumours. These new tumour-associated blood vessels arising by angiogenesis are structurally and functionally abnormal, being characterised by loose inter-endothelial and endothelial-basement membrane and pericyte interactions, luminal dilatation, increased permeability, and detachment of microvascular astrocytic foot processes. Angiogenesis involves a complex interaction between pro- and anti-angiogenic factors produced by tumour, vascular, glial, ECM, and immune cells. VEGF is the principal angiogenic factor in tumours, regulating endothelial cell proliferation, migration, and permeability, but other factors such as angiopoietins, FGF, SDF-1, interleukins, and TNF also participate in this process. While anti-angiogenic therapy may temporarily impede tumour growth, many malignant tumours find alternative mechanisms of neovascularisation and become behaviourally more aggressive. The network of existing microvessels can sometimes also be rapidly expanded by splitting of these vessels (vascular intussusception), while maintaining continuity of blood flow, and some aggressive, rapidly-dividing tumours can form tumour
(not endothelial)-lined vascular channels, which is designated vascular mimicry. In addition, bone marrow-derived endothelial progenitor cells (sometimes abetted by cancer stem-like cells and circulating monocytes/macrophages) can also expand the tumour vasculature by sometimes incorporating themselves into the lining of existing vessels, a process termed vasculogenesis.

References


14.2 Clinical Diagnosis

Clinical diagnosis as performed by clinical neuropathologist(s) for the purpose of selecting and classifying appropriate cases

<table>
<thead>
<tr>
<th>Initials</th>
<th>Reporting Pathologist</th>
</tr>
</thead>
<tbody>
<tr>
<td>CS</td>
<td>Dr C. Smith</td>
</tr>
<tr>
<td>PB</td>
<td>Dr P. Blumbergs</td>
</tr>
<tr>
<td>BK</td>
<td>Dr B. Koszyca</td>
</tr>
<tr>
<td>SE</td>
<td>Dr S. Ernsting</td>
</tr>
<tr>
<td>SO</td>
<td>Dr S. Otto</td>
</tr>
<tr>
<td>JMW</td>
<td>Dr J. Ma Wyatt</td>
</tr>
<tr>
<td>CB</td>
<td>Dr C. Brennan</td>
</tr>
</tbody>
</table>

14.3 Histological Descriptions

Histological descriptions of human tissue as performed by two assessors (KAM & JWF)

Patient A

In H&E-stained sections, the tumour was characterised by solid sheets of melanocytes, sometimes separated into groups of varying size by thin connective tissue septa. The border between the tumour biopsy and contiguous brain parenchyma was generally well-defined, with only
occasional individual, infiltrating melanocytes. Melanocytes were a highly pleomorphic cell population of greatly varying size, but generally polygonal (epithelioid) in shape. The nucleus:cytoplasmic ration was sometimes high, but often there was abundant eosinophilic cytoplasm. Nuclei were very variable in size, but usually had one or more prominent nucleoli and coarse chromatin clumps. Some nuclei contained one or more, clear, non-staining vacuoles, resembling cytoplasmic invaginations into the nucleus, and sometimes occupied almost the entire nuclear volume. Bi- and multinucleated melanocytes were frequent. A few individual tumour cells were degenerate, appearing shrunken and hypereosinophilic with nuclear hyperchromasia. The tumour was mostly amelanotic, with only a small number of melanocytes exhibiting dark brown, cytoplasmic melanin pigment. The mitotic index was low, with ~5/10 randomly selected high power (x40) fields. Foci of tumour necrosis were not found, but a few lymphocytic aggregates were present.

Patient B

While some melanocytes were disposed in loosely arranged bundles, the predominant pattern was a tendency to aggregate around a central blood vessel in a whorling pattern. Nuclei were ovoid to more elongated in shape with a variable amount of cytoplasm and usually indistinct cell boundaries. Some nuclei showed marked karyomegaly and were bizarrely-shaped. Some nuclei contained one or more prominent nucleoli and a minority of melanocytes had cytoplasmic melanin pigmentation. The mitotic index was
high, with ~50/10 HP fields, and many were abnormal. Individual cell necrosis of tumour cells was observed.

Patient C
This neoplasm was highly cellular, with closely apposed nuclei, and was well delimited with compression of adjacent brain parenchyma. Melanocytes were generally arranged in whorling and interlacing bundles, with a high nucleus:cytoplasmic ratio and a variable amount of amphophilic cytoplasm with indistinct cell borders. Nuclei were of variable size and round to ovoid to more elongated in shape, with one or more prominent nucleoli. The mitotic rate was moderate to high, with ~30/10 HP fields, and some were abnormal. Multifocal areas of tumour necrosis and haemorrhage were found.

Patient D
The areas of brain present were largely effaced by copious haemorrhage. The majority of melanocytes were ovoid (epithelioid) in shape with round nuclei of varying size (occasionally marked karyomegaly with nuclei sometimes vacuolated, bean- or more bizarrely-shaped) containing one or more prominent nucleoli, a moderate amount of eosinophilic (sometimes very pale-staining) cytoplasm, and cell boundaries sometimes well-defined, but more commonly less distinct. The mitotic index was ~10/10HP, randomly selected fields. Some melanocytes contained a small amount of dark brown melanin pigment and heavily pigmented tumour cells sometimes palisaded around blood vessels. In a few areas, groups of melanocytes were
aggregated into nests by fine connective tissue septa. Tumour cells were sometimes disposed in whorling, interlacing bundles with more elongated nuclei (spindle cells). Areas of haemorrhage were common.

Patient E
In this tumour, melanocytes were generally organised into groups and nests by thick (but sometimes less well-developed) connective tissue septa (scirrhous or desmoplastic reaction). Nuclei were usually small, round to ovoid to more irregularly-shaped and sometimes eccentrically placed in the tumour cell, the cytoplasm was scant and eosinophilic, with variably well-defined cell boundaries. A few tumour cells were degenerate, appearing shrunken and hypereosinophilic with hyperchromatic nuclei. The mitotic index was low (~2/10 HP fields). An occasional large area of tumour necrosis was found.

Patient F
The tumour-brain boundaries were few, but appeared irregular with some infiltration evident. The cytology of this tumour was highly pleomorphic with melanocytes of greatly varying size and shape, some being very large (megalocytic). Similarly, nuclei varied markedly in size and shape (round to ovoid to more irregular ad sometimes bizarre), many tumour cells were bi- or multinucleated and nuclei contained one or more prominent nucleoli. The mitotic index was high (~35/10HP fields) and many were abnormal. Melanocytes were organised into loose or more tightly arranged sheets and sometimes showed pseudo-palisading around blood vessels.
Perivascular lymphocytic cuffing was frequent and there were substantial, multifocal areas of tumour necrosis.

Patient G
The 2 principal features of this tumour were: (1) the markedly vascular border between tumour and brain parenchyma and (2) the very large areas of tumour necrosis. The former was characterised by densely-packed blood vessels of greatly varying luminal diameter and mural thickness (capillary to venular to arteriolar), attended by fibroplasia/glial proliferation (GFAP warranted), and the type of vessel predominating varied from one area to another. These intensely vascular areas were loosely infiltrated by inflammatory cells, predominantly lymphocytes. The major pattern of tumour growth was a tendency for melanocytes to whorl around central blood vessels. These tumour cells contained round to ovoid to generally more elongated nuclei, with usually finely clumped chromatin and devoid of nucleoli. Nuclei were occasionally vacuolated and binucleated and sometimes showed significant karyomegaly; the cytoplasm was modest in amount and cell boundaries indistinct. Mitotic rate ~5/10HP fields.

Patient H
This tumour was characterised by diffuse, highly cellular sheets of melanocytes admixed with abundant microvessels, many showing erythrocyte diapedesis. Nuclei were closely apposed with scant discernible cytoplasm. They were small and greatly variable in shape from round to often
more elongated and irregular in outline. Nuclei were hyperchromatic with few discernible chromatin clumps and no nucleoli. Mitotic figures were rare.

Patient I
The tumour was composed of a highly pleomorphic population of melanocytes, some of which contained melanin pigment, and multifocal areas of tumour necrosis, in approximately equal proportion. Melanocytes varied greatly in size and shape, but generally had ample to voluminous, eosinophilic cytoplasm with often somewhat indistinct, but generally discernible, cellular boundaries. Nuclei were very variable and often either eccentrically placed in the tumour cell or disposed as a peripheral crescent. They varied in size and shape (occasionally very large) and usually contained coarse chromatin clumps and sometimes one or more nucleoli. The mitotic rate was very low (1 or </10 HP fields). There was mild perivascular lymphocytic cuffing and modest intra-tumoural haemorrhage.

Patient J
This tumour was characterised by highly cellular, solid sheets of melanocytes of diverse morphological appearance. Nuclei were generally rather small and, in a given area, of very variable shape, ranging from round to oval to elongated. They were generally hyperchromatic with little discernible individual chromatin clumps and no visible nucleoli or, less commonly, hypochromatic with a single nucleolus. In most areas, nuclei were closely apposed and cytoplasm modest in quantity, with cell boundaries not
discernible; in a few areas, the melanocyte cytoplasm was more abundant and nuclei more open with sometimes a visible nucleolus.
### LABORATORY ANIMAL SERVICES

**CLINICAL RECORD SHEET (Rat and Mouse)**

**RESEARCHER NAME:**

**DEPARTMENT:**

**ID NUMBER:**

**ANIMAL ETHICS NUMBER:**

<table>
<thead>
<tr>
<th>DATE</th>
<th>RUFFLED COAT</th>
<th>POOR POSTURE / HUNCHED</th>
<th>PALE OR SUNKEN EYES</th>
<th>CHANGE IN BEHAVIOUR</th>
<th>REDUCED FOOD INTAKE</th>
<th>DEHYDRATION</th>
<th>DIARRHOEA</th>
<th>SQUEALING WHEN HANDLED</th>
<th>RELUCTANT TO MOVE</th>
<th>WEIGHT LOSS</th>
<th>OTHER COMMENTS</th>
<th>TOTAL SCORE</th>
<th>SIGNED</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**YES = 1** Scores of 3 require the animal to be checked 3 times per day

**NO = 0** Scores of higher than 3 require Animal Welfare / Veterinary advice

Scores of higher than 4 require Euthanasia

*Where known / in single housed animals*
14.5 Reference List


