Mechanisms underlying hypoxic ischemic injury to the developing brain: The significance of matrix metalloproteinase 2 and 9

Himani Sumudumalee Ranasinghe

A thesis submitted in fulfilment of the requirements for the degree of Doctor of philosophy in Biomedical Science, University of Auckland, 2009

ABSTRACT

Perinatal hypoxic ischemic (HI) injury is a leading cause of long-term neurological complications in newborn babies. Matrix metalloproteinases (MMPs) are a family of endopeptidases that are capable of degrading the extracellular matrix (ECM) components. They are considered to be integral in many physiological processes. However, recently it has been demonstrated that the inappropriate activity of these proteases, particularly MMP-2 and 9, contribute to the pathogenesis of cerebral ischemia in the adult brain. Given that ECM disruption is frequently observed following injury to the developing brain, it is possible that MMPs play an important role in HI injury processes in the developing brain. Therefore, this thesis evaluated the hypothesis that MMP-2 and 9 participate in the pathophysiology of HI injury to the developing brain. Since ECM remodelling is a fundamental process during brain development it was important to first characterise the MMP-2 and 9 profiles in the normal developing forebrain. We demonstrated that MMP-2, which mainly was observed in cortical plate neurons, declined with age, thus indicating a potential role in the development and differentiation of the cortical plate. Conversely, MMP-9 was increased with age, particularly during active myelination, indicating that it may contribute in myelination. Secondly, we showed an upregulation of MMP-9 within the ischemic core during the early hours following HI injury, suggesting that MMP-9 may be involved in the development of delayed injury processes following hypoxic ischemia. On the contrary, MMP-2 was strongly upregulated during a later stage following injury surrounding the ischemic core possibly suggesting that it plays a role in wound repair processes. Thirdly, the profiles of tissue (tPA) and urokinase (uPA) plasminogen activators were characterised following HI injury since they are known to be major upstream activators of MMPs. uPA upregulation paralleled that of MMP-2 suggesting a function for uPA in wound repair processes following HI injury to the developing brain through activation of MMP-2. In contrast with uPA, tPA activity remained unaffected following injury at both ages. Finally, MMP-9 activity was inhibited using a very specific MMP-2/9 inhibitor, SB-3CT, to determine if the MMP-9 deficiency protects the developing brain from HI

injury. The elevated MMP-9 activity following HI injury was attenuated by the SB-3CT treatment. Although SB-3CT failed to confer any significant neuroprotection, we recommend that further investigations are needed before discounting the role of MMP-9 during HI injury to the developing brain. In conclusion, we suggest that MMP-9 is induced following an insult to the developing brain potentially contributing to the delayed neuronal death whilst MMP-2 is involved in essential developmental, differentiation and wound repair processes.

ACKNOWLEDGEMENTS

Completing this thesis has undoubtedly been an extraordinary journey. Throughout the past years I have learnt not only an abundance of scientific skills, but remarkable life skills too. It has not been an easy journey and it certainly would not have been possible without the invaluable support of many people.

I am greatly indebted to my current primary supervisor Dr Mhoyra Fraser, who went out of her way to ensure that I finish this thesis to a high standard. Thank you very much for lending a helping hand at the most difficult of times. I will always appreciate your guidance, support and interest in my research and all other aspects of my PhD. I am equally grateful to, Dr Arjan Scheepens, my former primary supervisor. I am deeply gratified to you for your invaluable contribution in designing this research project, bringing me up to speed in the lab and giving me constant encouragement throughout my PhD. I will fondly remember the great instances we shared as a group. I would also like to sincerely thank my former co-supervisor, A/Professor Chris Williams, for his scholarly advice in every aspect of this thesis from designing to writing.

I would also like to express my earnest gratitude to many people who were indirectly involved in making this thesis possible. To my advisor, Dr Hannah Gibbons, for being an extraordinary mentor as well as proof reading my thesis amongst her busy schedule. To Mr Ernest Sirimanne without whose magnanimous support in animal surgeries it would not have been possible to complete this research the way I envisaged. To Mr Eric Thorstensen for his generous help with the mass spectrometry experiments. To Dr Sam Mathai for his assistance with CSF collection. And, to Dr Mohan Kumar for teaching me numerous laboratory techniques amidst his own PhD research.

I would like to express my heartfelt gratitude to the fellow PhD students of the Perinatal Brain Injury Group. I am extremely grateful to Mrs Larissa Christophidis for providing me the cDNA that I needed to complete the studies, on top of innumerable other favours

she has done for me. Your input has greatly improved the quality of this PhD thesis. Above all, thank you for being an awesome friend, who I could always count on. Thank you, Ms Praneeti Pathipati for your assistance with countless tasks throughout the course of my PhD. Thank you for being there, as a great friend, to share some of the strenuous career experiences. Thank you, Dr Tanja Needham (nee Möderscheim) for giving us hope that there is light at the end of the tunnel. I will always cherish the wonderful moments we all had together. I would also like to acknowledge everyone in the Developmental Neuroendocrinology Research Group.

I would like to very warmly thank my good friend, Ms Vinthiya Paramanathasivam, although thank you would certainly be an understatement for her support during the past years from proof reading various reports to hearing my PhD horror stories almost all of the times we met. Also a gigantic “thank you” to, Dilini Hennayake, Kushil Ginige, Movin Kulathilaka, Nirosha Weerasinghe, Nishan Alahakoon and Ruchira Ginige for putting up with my PhD syndrome while helping in whichever way possible. And a very special “Winnie the Pooh Hug” to Baby Yeshika for being my biggest stress reliever through the last years without having the fainest idea of what I do. Above all, I love you all for making life outside PhD absolutely beautiful.

Last but certainly not the least, a very special mention to Mum, Dad and Bro. The warmth that you have given me throughout is beyond any measure that I know of. You truly are the inspiration of my strength. I love you.

TABLE OF CONTENTS

ABSTRACT ..... **i**

ACKNOWLEDGEMENTS ..... **iii**

LIST OF FIGURES ..... **viii**

LIST OF TABLES ..... **xi**

LIST OF ABBREVIATIONS ..... **xii**

CHAPTER ONE: GENERAL INTRODUCTION **1**

1.1 INTRODUCTION 1

1.2 DEVELOPMENT OF CENTRAL NERVOUS SYSTEM 3
1.2.1 Neurulation 3
1.2.2 Prosencephalic Development 4
1.2.3 Neuronal Proliferation 5
1.2.4 Neuronal Migration 7
1.2.5 Organization 9
1.2.6 Myelination 10
1.2.7 Summary 10

1.3 PERINATAL HYPOXIC ISCHEMIC BRAIN INJURY 11
1.3.1 Neuropathology 11
1.3.2 Mechanisms of Cell Death following Hypoxic Ischemia 20
1.3.3 Animal Models of Hypoxic Ischemia in Brain 28

1.4 MATRIX METALLOPROTEINASES 33
1.4.1 Introduction 33
1.4.2 Matrix Metalloproteinase Family 34
1.4.3 Structure 35
1.4.4 Regulation of Matrix Metalloproteinase Activity 38
1.4.5 Matrix Metalloproteinases in Brain Development 43
1.4.6 Matrix Metalloproteinases in Cerebral Ischemia 47

1.5 PLASMINOGEN ACTIVATORS 55
1.5.1 Introduction 55
1.5.2 Plasminogen to Plasmin 56
1.5.3 Tissue Plasminogen Activator 57
1.5.4 Urokinase Plasminogen Activator 58
1.5.5 Plasminogen Activators in Cerebral Ischemia 59

1.6 AIMS 63

CHAPTER TWO: MATERIAL AND METHODS **66**

2.1 HYPOXIC ISCHEMIC INJURY 66
2.1.1 Postnatal Day 21 66
2.1.2 Postnatal Day Three 67

2.2 PARAFFIN EMBEDDING 68

2.3 PROTEIN EXTRACTION 69

2.4 RNA EXTRACTION 70
2.5 CDNA SYNTHESIS 72
2.6 ACID FUCHSIN AND THIONIN STAINING 73
2.7 IMMUNOHISTOCHEMISTRY 74
2.7.1 Single Immunohistochemistry 74
2.7.2 Double Immunohistochemistry 76
2.8 GELATIN ZYMOGRAM 76
2.9 PLASMINOGEN ZYMOGRAPHY 80
2.10 QUANTITATIVE REAL -TIME PCR 82
2.11 STATISTICAL ANALYSIS 86

CHAPTER THREE: MMP-2 AND MMP-9 EXPRESSION IN THE NORMAL DEVELOPING RAT FOREBRAIN 88
3.1 INTRODUCTION 88
3.2 MATERIALS AND METHODS 89
3.3 RESULTS 94
3.4 DISCUSSION 98

CHAPTER FOUR: MMP-2 AND MMP-9 RESPONSE AFTER HYPOXIC ISCHEMIA IN THE DEVELOPING BRAIN 103
4.1 INTRODUCTION 103
4.2 MATERIALS AND METHODS 104
4.3 RESULTS 111
4.4 DISCUSSION 120

CHAPTER FIVE: tPA AND uPA RESPONSE AFTER HYPOXIC ISCHEMIA IN THE DEVELOPING BRAIN 126
5.1 INTRODUCTION 126
5.2 MATERIALS AND METHODS 128
5.3 RESULTS 134
5.4 DISCUSSION 138

CHAPTER SIX: EFFECT OF INHIBITION OF MMP-9 ACTIVITY AFTER HYPOXIC ISCHEMIA IN THE DEVELOPING BRAIN 142
6.1 INTRODUCTION 142
6.2 MATERIALS AND METHODS 144
6.3 RESULTS 148
6.4 DISCUSSION 161

CHAPTER SEVEN: GENERAL DISCUSSION 168
7.1 OVERVIEW 168
7.2 MAJOR FINDINGS AND THEIR IMPLICATIONS 170
7.3 LIMITATIONS 177
7.4 FUTURE DIRECTIONS 178
7.5 SUMMARY 183
7.6 CONCLUSIONS 184
APPENDIX 185
BIBLIOGRAPHY 189

LIST OF FIGURES

CHAPTER ONE

Figure 1. 1: Scanning electron micrographs of transverse sections through the chick embryo at the neuraxial levels of neurulation.....**4**

Figure 1.2: The choreography of cell proliferation in ventricular zone. **7**

Figure 1.3: Schematic illustration of the various modes of neuronal migration in the developing cerebral cortex.....**9**

Figure 1.4: Coronal section of cerebrum from an infant who died at 8 weeks after birth, featuring PVL. **13**

Figure 1.5: Coronal view (T2-weighted MRI image) of a eight year old brain that suffered asphyxia at term showing bilateral parasagittal lesion. **17**

Figure 1. 6: T2-weighted (a) and DWI (b) MRI scans of a term neonate brain showing infarction (arrows) in the left middle cerebral artery.....**20**

Figure 1.7: A systematic diagram demonstrating the cellular events occurring during excitotoxicity. **23**

Figure 1.8: Fluoro-jade B staining at 3 days following severe and moderate unilateral HI injury at P21.....**30**

Figure 1.9: Fluoro-jade B staining of P4 rat brain following HI injury at P3. .....**32**

Figure 1.10: Protein structure of MMPs.. .....**36**

Figure 1.11: Ribbon stereo plot of a representation of the catalytic domain of MMPs.**37**

Figure 1.12: Ribbon stereo plot of a representation of the haemopexin-like domain of MMPs.....**38**

Figure 1.13: Stepwise activation of proMMPs. **41**

Figure 1.14: Ribbon stereo plot of a representation of TIMP-2 illustrating the N (NTD) and C (CTD) terminal domains. **43**

Figure 1.15: MMP-2 immunoreactivity in a rat brain with 90 min MCAO and 3 h of reperfusion showing astrocytic immunoreactivity.....**51**

Figure 1.16: MMP-9 immunohistochemistry in an ischemic cortex at 48 h after 90 min MCAO and 3 h of reperfusion **52**

Figure 1.17: Schematic diagram of protease cascade that leads to ECM modulation...**57**

CHAPTER TWO

Figure 2.1: Denaturing agorose gel illustrating the 28S and 18S ribosomal RNA bands in 1μg of a representative RNA sample. **72**

Figure 2.2: Validation of the gelatin zymography analysis of the gelatinase activity...**79**

Figure 2.3: Representative gelatin zymogram illustrating the inhibition of gelatinolytic activity by 20mmol of EDTA in the developing buffer.....**80**

Figure 2.4: Representative gelatin zymogram illustrating the elimination of proteolytic activity in the absence of plasminogen. **81**

Figure 2.5: The rat brain GAPDH gene expression 1 day following HI injury at P21 and P3. .....**84**

Figure 2.6: Validation plots of ΔC_t against the long input amount of RNA for MMP-9 (a) MMP-2 (b) and uPA (c) with GAPDH. **85**

CHAPTER THREE

Figure 3.1: MMP-2 and 9 activities in the normal developing forebrain of the rat.....**92**

Figure 3.2: MMP-2 and 9 gene expression in the normal developing rat brain E18 to P21 .....**95**

Figure 3. 3: MMP-2 Immunoreactivity in the normal forebrain **97**

CHAPTER FOUR

Figure 4.1: MMP-2 and 9 activities after HI injury in P21 rat brain.. .....**107**

Figure 4.2: MMP-2 and 9 activities after HI injury in P3 rat brain **109**

Figure 4.3: MMP-2 and 9 gene expression after HI injury in P21 rat brain.....**113**

Figure 4.4: MMP-2 and 9 gene expression after HI injury in P3 rat brain.....**114**

Figure 4.5: MMP-2 and 9 Immunoreactivity after HI injury to the developing rat brain **116**

Figure 4.6: MMP-2 and MMP-9 immunoreactivity after HI in P21 brain. **118**

CHAPTER FIVE

Figure 5.1: tPA and uPA activities after hypoxic ischemia in P21 rat brain.. .....**130**

Figure 5.2: tPA and uPA activities after hypoxic ischemia in P3 rat brain. **132**

Figure 5.3: uPA gene expression after hypoxic ischemia in P21 and P3 rat brain respectively at 5 days and 6 hours after injury. **136**

Figure 5.4: uPA Immunoreactivity after HI injury to the P21 rat brain. **137**

CHAPTER SIX

Figure 6.1: Effect of MMP-2/9 specific inhibitor, SB-3CT, on the MMP-2 and 9 activities on gelatin zymography. **149**

Figure 6.2: MMP-9 activity after IP administration of MMP-2/9 inhibitor SB-3CT at 25mg/kg BW in 10% DMSO, 90% Saline. **151**

Figure 6.3: MMP-9 activity after IP administration of MMP-2/9 inhibitor SB-3CT at 25mg/kg BW in 25% DMSO, 75% PEG. **152**

Figure 6.4: MMP-9 activity after IP administration of MMP-2/9 inhibitor SB-3CT. .**153**

Figure 6.5: Detection of SB-3CT in blood and CSF after the IP treatment of SB-3CT by mass spectrometry.....**155**

Figure 6.6: BWs from the day of the injury until the sacrifice of the animals. **159**

Figure 6.7: Effect of IP administration of MMP-2/9 inhibitor SB-3CT on the severity of the HI injury to the P21 brain. **160**

Figure 6.8: Effect of IP administration of MMP-2/9 inhibitor SB-3CT on the severity of the HI injury to the P21 brain. **161**

Figure 6.9: SB-3CT (Parent) (a) mainly get metabolised via hydroxylation of the terminal phenyl ring producing a metabolite (M4) (b). **164**

LIST OF TABLES

CHAPTER ONE

Table 1.1: Peak time of occurrence of major development events in the human CNS .**10**

Table 1.2: Major class of MMP and their substrates. **35**

Table 1.3: Various activators of pro-MMPs. **40**

CHAPTER TWO

Table 2.1: Processing cycle used to process P21 rat brains in the processing machine **69**

Table 2.2: Processing cycle used to process P3 rat brains in the processing machine ..**69**

Table 2.3: Nanodrop results of several representative samples.....**71**

Table 2.4: Component composition for one reaction of cDNA synthesis **72**

Table 2.5: Summary of cDNA synthesis procedure **73**

Table 2.6: Descending alcohol series for hydrating paraffin embedded sections.....**74**

Table 2.7: Primary antibodies used in the study and their optimal conditions **75**

Table 2.8: Reagent composition for casting 10% gelatin zymograms.....**77**

Table 2.9: Reagent composition for casting 10% plasminogen zymograms **80**

Table 2.10: Applied Biosystems (New Zealand) TaqMan® gene expression assays ...**82**

Table 2.11: Reaction mix for qRT-PCR **82**

Table 2.12: qRT-PCR cycle steps.....**83**

Table 2.13: An example of $\Delta\Delta C_t$ method illustrating the stepwise calculations involved in determining the fold difference in MMP-9 gene expression in an injured (I) sample compared to the uninjured (U) sample. **86**

LIST OF ABBREVIATIONS

ADAM – A disintegrin and metalloproteinase family

AMPA - α-Amino-3-hydroxy-5-Methyl-4-Proprionate

ANOVA – Analysis of variance

APS - Ammonium persulfate

BBB – Blood brain barrier

BCA – Bicinchoninic acid

BSA – Bovine serum albumin

BW – Body weight

CC – Corpus callosum

CNS – Central nervous system

CSF - Cerebrospinal fluid

DEPC – Diethylpyrocarbonate

DMSO - Dimethyl sulfoxide

cDNA – Complementary deoxy ribonucleic acid

DG – Dentate gyrus

DPX - Dibutyl-phthalate-xylene

ECM – Extracellular matrix

EDTA - Ethylene diamine tetra acetic acid

GAPDH - Glyceraldehyde-3-phosphate dehydrogenase

GAP-43 – Growth associated protein – 43

GFAP – Glial fibrillary acidic protein

HCl - Hydrochloric acid

HI - Hypoxic ischemic

ICV - Intracerebro ventricular

IP – Intraperitoneal

KPBS - Potassium phosphate buffered saline

M - Molar

MAP-2 – Microtubule associated protein -2

MCAO – Middle cerebral artery occulsion

MMP – Matrix metalloproteinase

mRNA - Messenger ribonucleic acid

NO - Nitric oxide

NOS - Nitric oxide synthase

NMDA - N-methyl-d-aspartate

NeuN - Neuronal specific nuclear protein

dNTP - Deoxyribonucleotide triphosphate

OD – Optical Density

PA – Plasminogen activator

tPA – Tissue plasmingen activator

uPA – Urokinase plasminogen activator

Postnatal Day 7 – P7

Postnatal Day 2 – P3

Postnatal Day 21 – P21

PBS – Phosphate buffered saline

PCR – Polymerase chain reaction

PVL - Periventricular leukomalacia

ROS - Reactive oxygen species

RT - Room temperature

qRT-PCR – quantitative real time polymerase chain reaction

SDS – Sodium dodecyl sulphate

SEM – Standard error of the mean

SOD - Superoxide dismutase

SNN – Selective neuronal necrosis

TEMED - N,N,N,N-tetramethylethylenediamine

TIMP – Tissues inhibitor of matrix metalloproteinase