PROTEIN HYPERCITRULLINATION, A BASIC MECHANISM IN DEMYELINATING DISEASES

By

Haolan Lei

A thesis submitted in conformity with the requirements for the degree of Master of Science
Department of Laboratory Medicine and Pathobiology
University of Toronto

© Copyright by Haolan Lei 2010
Protein hypercitrullination, a basic mechanism in demyelinating diseases

Haolan Lei
Master of Science
Department of Laboratory Medicine and Pathobiology
University of Toronto
2010

Abstract

Multiple sclerosis (MS) is a complex disease of the human central nervous system (CNS) involving the patchy destruction of the myelin sheath. Previous studies have found a consistent biochemical change in MS normal appearing white matter (NAWM) i.e. the increased citrullination of myelin basic protein (MBP) resulting in decreased myelin compaction. This process is facilitated by the enzyme family of peptidylarginine deiminases (PADs), of which PAD2 and PAD4 are expressed in mouse and human brain white matter. Therefore, we propose the inhibition of PAD enzymes would reverse protein hypercitrullination and represents a potential treatment for MS. Treatment with 2-chloroacetamidine (2CA), an active site inhibitor of PAD, attenuated diseases in four independent mouse models of MS associated with decreased PAD activity level, normalized peptidylecitrullination, and improved myelin morphology. Therefore, protein hypercitrullination may be a basic mechanism implicated in both neurodegenerative and autoimmune models of MS.
Acknowledgments

Firstly I would thank my supervisor Dr. Moscarello. I am very grateful for that he introduced me to the world of research, and guided me during my research and study at the University of Toronto since he accepted me as an undergraduate research student. His lifelong dedication and enthusiasm in scientific research must have motivated all his advisees.

Dr. Adeli and Dr. Yeger deserve a special thank as my thesis advisory committee members. I am grateful for their valuable insight and suggestion during my committee meetings. They also set examples of world-class researchers for their attention to details and their rigor.

I would also like to thank everyone in my lab for their support and friendship throughout my years in Dr. Moscarello’s lab. I treasure all precious moments we shared. A special thank goes to our lab manager Teresa who is always so friendly and organized. I will remember her constant technical support and suggestion when I encountered difficulties in the lab and her companionship during my stay in the lab as we shared our happiness and tears together. I will remember, of course, all the delicious lunches we had together. I also thank Denise for her kindness and companionship during my stay. I am thankful to my colleague Zhen as well for his support during my trouble shooting and his wonderful sense of humor. A special thank goes to Fabrizio, for his guidance and insights. Besides his scientific knowledge, I will always remember his enthusiasm in Mac products and his amazing typing skills that enabled him to complete all the manuscript drafts with two index fingers only.

The generous funding support from the Multiple Sclerosis Society of Canada is greatly appreciated. I hope my research will eventually help finding a cure for this disease. I would like to acknowledge member’s in Dr. Dosch’s lab for our collaborative effort. I appreciate technical support from Aina, Yumen, Michael, and Cameron Division of Pathology, Department of Physiology & Experimental Medicine, The Hospital for Sick Children during the preparation of tissue samples for histological examination and imaging.

This thesis is dedicated to all my beloved friends who helped me overcome obstacles in every stage of my life. Life would not be so precious and wonderful without you.
# Table of Contents

ABSTRACT ............................................................................................................. ii

ACKNOWLEDGEMENT ......................................................................................... iii

TABLE OF CONTENTS ............................................................................................. iv

LIST OF FIGURES .................................................................................................... ix

LIST OF TABLES ....................................................................................................... viii

ABBREVIATIONS .................................................................................................... xi

CHAPTER ONE: INTRODUCTION ............................................................................. 1

A. Multiple Sclerosis (MS) .................................................................................... 1

   I. Clinical features ............................................................................................. 1

   II. Pathophysiology ........................................................................................... 4

   III. Etiology ......................................................................................................... 6

   IV. Current treatments ....................................................................................... 8

B. Myelin Basic Protein (MBP) .............................................................................. 10

   I. Deimination of myelin basic protein ............................................................. 15

C. Peptidylarginine deiminase (PAD) .................................................................. 17

D. 2-Chloroacetamide, a PAD inhibitor .............................................................. 24
CHAPTER TWO: MATERIALS AND METHODS

A. Materials ................................................................. 38

B. Animal Models ........................................................... 38
   I. Neurodegenerative models ......................................... 38
   II. Autoimmune models ................................................. 39

C. Experimental Autoimmune Encephalomyelitis (EAE) induction on ND4 mice ...... 40

D. Therapeutic protocol and clinical scoring ........................................ 40
   I. Transgenic mice ....................................................... 40
   II. EAE mice ............................................................ 41
   III. EAE induced ND4 mice ........................................... 42

E. Histopathology ........................................................... 42

F. Immunohistochemistry ................................................... 43

G. Electron microscopy .................................................... 43

H. PAD activity assay ........................................................ 44
I. Immuno slot blot .............................................................. 45
J. DNA methylation analysis ...................................................... 46
K. Statistics ................................................................. 47

CHAPTER 3: INHIBITION OF PAD IN HUMAN BRAIN TISSUE ................. 48

A. PAD activity in normal and MS white matter ....................... 48
B. Inhibition of PAD by 2-CA in MS normal appearing white matter ........ 52

CHAPTER 4: INHIBITION OF PAD IN NEURODEGENERATIVE MOUSE MODELS OF MS ......................................................... 54

A. ND4 mouse model ........................................................... 54
   I. Clinical scores after treatment ......................................... 55
   II. PAD activity in brain ...................................................... 59
   III. Morphological changes in central nervous system ................. 61
   IV. DNA methylation in cortical white matter .......................... 65
B. PAD2 overexpressor mouse model ......................................... 68
   I. Clinical scores and PAD activity in brain after treatment .......... 68
CHAPTER 5: INHIBITION OF PAD IN AUTOIMMUNE MOUSE MODELS OF MS …… 72

A. Chronic relapsing experimental autoimmune encephalomyelitis (CREAE) mouse model …………………………………………………………………………………………… 72
   I. PAD activity during disease progression ………………………………………. 73
   II. EAE scores during treatment with 2CA……………………………………….. 75
   III. PAD activity and peptidylcitrulline in brain after treatment ………………… 77

B. Acute (Progressive) EAE mouse model ……………………………………………………………… 79
   I. EAE score and survival rate during treatment ……………………………………. 79
   II. PAD activity in brain, spleen, and spinal cord ……………………………….. 83

CHAPTER 6: INDUCTION OF EAE IN A NEURODEGENERATIVE MOUSE MODEL OF MS ……………………………………………………………………………………………………… 87

A. EAE scores after induction with pertussis toxin …………………………………….. 88

B. Treatment with 2CA and measurement of PAD activity ………………………….. 89

C. Morphological changes in CNS …………………………………………………….. 90

CHAPTER 7: DISCUSSION ……………………………………………………………………………… 100
List of Tables

Table 1-1. Summary of current disease-modifying treatments for relapse-remitting MS 9
Table 1-2. Composition of human CNS myelin ................................................................. 11
Table 1-3. Comparison of five isozymes of peptidylarginine deiminases (PADs) ............ 18
Table 1-4. Percentage of homology between primary human peptidylarginine deiminases (PAD) amino-acid sequences, theoretical isoelectric point (pI), and molecular weight … 19
Table 1-5. Amino acids involved in peptidylarginine deiminase active site cleft are conserved between 5 PAD isozymes .................................................................................................................. 19
Table 1-6. Failed translation of experimental therapies from EAE models to clinical practice ................................................................. 33
Table 3-1. PAD activity of white matter from normal and MS brain samples .......... 50
Table 4-1. Methylation analysis on selective CpG sites of mouse Padi2 gene .......... 66
List of Figures

Fig. 1-1. Four clinical subtypes of MS .................................................................2

Fig. 1-2. Organization of (A) murine classic MBP gene and (B) human classic MBP gene12

Fig. 1-3. A BLASTP/CLUSTALW alignment of sequences of 18.5 kDa MBP from various species .................................................................14

Fig. 1-4. Deimination of arginyl residues within peptide bonds.................................16

Fig. 1-5. Proposed reaction mechanism of protein citrullination by PAD4 ....................21

Fig. 1-6. Structure of 2-chloroacetamidine (2CA) ...................................................24

Fig. 1-7. Time and concentration dependent inactivation of PAD4 by 2CA .................25

Fig. 1-8. Inhibition of recombinant PAD2 and PAD4 activity by 2CA .........................26

Fig. 1-9. Identification of the binding site of acetamidine by mass spectrometry sequencing ........................................................................................................27

Fig. 1-10. Mechanism of reaction of 2CA with PAD .................................................28

Fig. 1-11. Immuno-electron microscopy of optic nerve cryo-sections from non-treated (NT) and 2CA treated PAD2 transgenic mice .........................................................29

Fig. 1-12. A schematic of the induction of active EAE and adoptive transfer on mouse...31

Fig. 3-1. PAD activity in normal appearing white matter from MS brain compared with normal ...............................................................................................51

Fig 3-2. Inhibition of PAD activity by 2CA with standard deviation in normal appearing white matter from human brain tissue .........................................................53

Fig. 4-1. Mean clinical scores of non-treated and treated ND4 mice ..........................58

Fig. 4-2. PAD enzyme activity in treated, untreated ND4 and normal mouse brain homogenate ..............................................................................................60

Fig. 4-3. Luxol fast blue hematoxylin histological stain of cerebellum from normal (Normal), non-treated ND4 (ND4 no treatment), 2CA treated ND4 (ND4 2CA treatment), and 2CA + B12 treated ND4 (ND4 2CA+B12 treatment) at 6.5 months of age ....................62

Fig. 4-4. Morphological evidence of improved myelination after 2CA and 2CA plus B12CN treatments .........................................................................................64

Fig. 4-5. RT-PCR of PAD 2 mRNA from ND4 mouse brain white matter .........................67
Fig. 4-6. Attenuation of clinical signs and PAD activity in the PAD2 overexpressing transgenic mice by 2CA treatment .......................................................... 69

Fig. 5-1. Mean clinical scores and PAD enzyme activities of non-treated mice during CREAE progression ................................................................. 74

Fig. 5-2. Attenuation of disease in CREAE by PAD inhibition .............................................. 77

Fig. 5-3. Normalization of citrullinated proteins in 2CA treated chronic relapsing EAE mice ..................................................................................................... 78

Fig. 5-4. Enhanced survival rate in 2CA and 2CA plus B\textsubscript{12}CN treated progressive EAE mice .................................................................................. 81

Fig. 5-5. Mean clinical scores of PBS control and treated EAE mice during disease progression ................................................................. 82

Fig. 5-6. PAD inhibition by 2CA and 2CA plus B\textsubscript{12}CN treatment in EAE mouse organs · 85

Fig. 6-1. Mean EAE scores of 2-month-old PTX-injected ND4, 5-month-old PTX-injected ND4, and 5-month-old PTX-injected, 2CA-treated ND4 .................................................. 89

Fig. 6-2. PAD enzyme activity in normal, untreated, PTX-injected, and PTX-injected, 2CA treated ND4 mouse brain at 5 mo of age with standard deviation ........................................ 90

Fig. 6-3. Hematoxylin & Eosin histological stain of cerebellum from untreated ND4 (2 M ND4 No Treatment), PTX-injected ND4 (2M ND4 PTX-treated), Normal (2M normal) at 2 months of age ........................................................................................................................................ 92

Fig. 6-4. Hematoxylin & Eosin histological stain of cerebellum from Normal (5M normal) untreated ND4 (5M ND4 No Treatment), PTX-injected ND4 (5M ND4 PTX-treated) at 5 months of age ........................................................................................................................................ 93

Fig. 6-5. Luxol fast blue histological stain of optic nerve from normal (Normal), non-treated ND4 (ND4 no treatment), PTX-injected ND4 (ND4 PTX treatment) at 5 months of age ........................................................................................................................................ 94

Fig. 6-6. Immunohistochemistry of brain sections from normal (Normal), MOG-EAE - (MOG-EAE), non-treated ND4 (ND4 no treatment), PTX-injected ND4 (ND4 PTX treatment) at 5 months of age ........................................................................................................................................ 96

Fig. 6-7 Immunohistochemistry of brain sections from normal (Normal), non-treated ND4 (ND4 no treatment), PTX-injected ND4 (ND4 PTX treatment) at 5 months of age .......... 98
## Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>2CA</td>
<td>2-Chloroacetamidine</td>
</tr>
<tr>
<td>Arg</td>
<td>Arginine</td>
</tr>
<tr>
<td>BAEE</td>
<td>(\alpha)-N-Benzoyl-L-arginine Ethyl Ester</td>
</tr>
<tr>
<td>BBB</td>
<td>Blood Brain Barrier</td>
</tr>
<tr>
<td>B(_{12})CN</td>
<td>Vitamin B12 Cobalamin</td>
</tr>
<tr>
<td>CFA</td>
<td>Complete Freund’s adjuvant</td>
</tr>
<tr>
<td>CNS</td>
<td>Central Nervous System</td>
</tr>
<tr>
<td>CREAЕ</td>
<td>Chronic Relapsing Experimental Autoimmune Encephalomyelitis</td>
</tr>
<tr>
<td>Cys</td>
<td>Cysteine</td>
</tr>
<tr>
<td>EAE</td>
<td>Experimental Autoimmune (Allergic) Encephalomyelitis</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>GFAP</td>
<td>Glial Fibrillary Acidic Protein</td>
</tr>
<tr>
<td>HRP</td>
<td>Horse Radish Peroxide</td>
</tr>
<tr>
<td>H &amp; E</td>
<td>Hematoxylin and Eosin</td>
</tr>
<tr>
<td>HEPES</td>
<td>N-2-Hydroxyethylpiperazine-N’-2-Ethanesulfonic Acid</td>
</tr>
<tr>
<td>IgG</td>
<td>Immunoglobulin-G</td>
</tr>
<tr>
<td>K1</td>
<td>Keratin K1</td>
</tr>
<tr>
<td>LFB</td>
<td>Luxol Fast Blue</td>
</tr>
<tr>
<td>MBP</td>
<td>Myelin Basic Protein</td>
</tr>
<tr>
<td>mg</td>
<td>Milligram</td>
</tr>
<tr>
<td>ml</td>
<td>Milliliter</td>
</tr>
<tr>
<td>mM</td>
<td>Millimolar</td>
</tr>
<tr>
<td>(\mu)g</td>
<td>Microgram</td>
</tr>
<tr>
<td>(\mu)mol</td>
<td>Micromole</td>
</tr>
<tr>
<td>MOG</td>
<td>Myelin Oligodendrocyte Glycoprotein</td>
</tr>
<tr>
<td>MRI</td>
<td>Magnetic Resonance Imaging</td>
</tr>
<tr>
<td>MS</td>
<td>Multiple Sclerosis</td>
</tr>
<tr>
<td>NAWM</td>
<td>Normal Appearing White Matter</td>
</tr>
<tr>
<td>ND4</td>
<td>DM20 transgenic line 4</td>
</tr>
<tr>
<td>ng</td>
<td>Nanogram</td>
</tr>
<tr>
<td>nmol</td>
<td>Nanomole</td>
</tr>
<tr>
<td>O.D.</td>
<td>Optical Density</td>
</tr>
<tr>
<td>PAD</td>
<td>Peptidyl Arginine Deiminase</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
</tr>
<tr>
<td>PLP</td>
<td>Proteolipid Protein</td>
</tr>
<tr>
<td>PPMS</td>
<td>Primary Progressive Multiple Sclerosis</td>
</tr>
<tr>
<td>PTX</td>
<td>Pertussis Toxin</td>
</tr>
<tr>
<td>PVDF</td>
<td>Polyvinylidene Fluoride</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>RRMS</td>
<td>Relapse Remitting Multiple Sclerosis</td>
</tr>
<tr>
<td>SPMS</td>
<td>Secondary Progressive Multiple Sclerosis</td>
</tr>
<tr>
<td>ST</td>
<td>Synovial Tissue</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor Necrosis Factor- alpha</td>
</tr>
<tr>
<td>WM</td>
<td>White Matter</td>
</tr>
</tbody>
</table>
Chapter 1
Introduction

A. Multiple Sclerosis (MS)

Multiple sclerosis (MS) is a chronic inflammatory demyelinating disease of the human central nervous system (CNS). Focal areas of myelin and axonal damage known as plaques with lymphocytic infiltration are common pathological features presented in patient’s brain and spinal cord (Compston and Coles, 2008). Although remyelination usually occurs during the early course of the disease, the later disease progression is dominated by a widespread astrocytosis associated with failure of remyelination and extensive neurodegeneration (Compston and Coles, 2008). Such chronic demyelination leads to a variety of clinical signs and disabilities including motor weakness in one or more limbs, fatigue, blindness, bladder dysfunction, cognitive impairment and eventually full paralysis (Bakshi et al., 2000).

MS is a complex disease of unknown etiology, as geographic, genetic, and immune factors are all believed to contribute to its pathogenesis. The exact mechanisms of MS disease progression and new lesion formation with failures of myelin regeneration remain currently unknown, but may be revealed in appropriate animal models.

I. Clinical features

MS is a clinically heterogeneous disease with each patient showing specific clinical signs and lesion patterns that can be remarkably different from one to another. In an attempt to better establish prognosis and therapeutic decisions, the United States National Multiple Sclerosis
Society has standardized four subtypes of MS to describe patterns of disease progression as shown in Fig. 1-1 (Lublin and Reingold, 1996).

**Fig. 1-1. Four clinical subtypes of MS.** (adapted from Lublin and Reingold, 1996.) (A). Relapse-remitting (RR) MS is characterized by clearly defined acute attacks with remission and residual deficit upon recovery. (B). Secondary progressive (SP) MS begins with an RR course followed by a continuous progression with occasional remissions. (C). Primary Progressive (PP) MS is characterized by a continuous progression of disability from onset with little improvement. (D). Progressive-relapsing (PR) MS is characterized by progression from onset but with clear acute relapses with or without full recovery.

To summarize, the majority of MS patients initially suffer from a relapsing and remitting form of disease characterized by alternating periods of symptom exacerbation and subsequent recovery (Thompson et al., 1997). To fulfill the diagnostic criteria for relapsing-remitting multiple sclerosis (RRMS), patients present with an acute episode (known as the clinically
isolated syndrome) and one or several sites of white matter abnormalities have to suffer from a subsequent attack of demyelination (Compston and Coles, 2006). The proportion of patients being diagnosed with RRMS increases from 50% at 2 years after first episode to 82% at 20 years (Compston and Coles, 2006).

Since recovery from each acute episode is incomplete, 65% of patients progress to the secondary progressive multiple sclerosis (SPMS) due to the accumulation of persistent symptoms (Thompson et al., 1997). SPMS, which commonly occurs in patients in their 40s or early 50s, is characterized by significant neurological deficits that accumulate over time with or without relapses (Compston and Coles, 2006).

Primary progressive multiple sclerosis (PPMS) is a less common subtype that is observed in around 10% of patients with a slow and steady progression of impairment from the onset without periods of remission (Compston and Coles, 2006). The least common subtype, progressive relapsing multiple sclerosis (PRMS), is observed in patients suffering from a steady neurological decline as well as clear superimposed attacks (Lublin and Reingold, 1996).

Numerous demyelinating diseases may be part of the MS spectrum in addition to the four pathologic variants due to similarity in clinical features. Marburg’s disease is a more malignant and fortunately rare form of fulminating MS. This variant is characterized by a rapid onset and almost continual demyelination resulting in death within months from onset (Capello and Mancardi, 2004). Balo concentric sclerosis is another aggressive borderline form of MS marked by large plaques of demyelination with concentric bands of preserved myelin (Capello and Mancardi, 2004). It was originally considered a distinct demyelinating disease, but more recent Magnetic Resonance Imaging (MRI) evidence suggested that some Balo-like lesions display
improvement overtime, and thus Balo concentric sclerosis may have a disease course typical of RRMS (Herndon., 2003).

As one can see, whether MS and its variants comprise a single disease with various clinical courses or a group of syndromes with diverse underlying causes remains controversial due to the highly variable disease progression among individual patients. To clarify the relationships among the MS variants, a complete understanding of the underlying pathophysiology and etiology of these conditions is necessary.

II. Pathophysiology

MS is complex not only in terms of clinical progressions but also in its pathophysiological features. Since most MS lesions were examined with standard histopathological techniques after autopsy, the lesions varied in types and location as would be expected from the heterogeneous clinical features. However, studies on the pathology of the MS lesions have identified some common neurodegenerative and inflammatory features.

Recent reports by Lucchinetti et al. (2000) and Lassman et al. (2001) have summarized four different patterns of demyelination in active MS lesions to group all subtypes of demyelinating diseases with similar clinical and pathologic features as a single entity. The first pattern involves active demyelination with T-lymphocyte and macrophage-dominated inflammation without significant activation of antibody. Pattern 2 describes active periventricular demyelination with T-lymphocyte and macrophage infiltration with extensive antibody deposition. Pattern 3 indicates demyelination with T-lymphocyte infiltration but no IgG antibody deposition. There is also evidence of possible preservation of a rim of myelin around
axon and partial oligodendrocyte apoptosis in this type of lesion. Pattern 4 lesion presents with sharp borders and oligodendrocyte apoptosis with a rim of normal appearing white matter (NAWM). The first three patterns are commonly seen in RRMS brain whereas the fourth has only been associated with PPMS. Lesion patterns in MS patients may evolve over time, but none of the individual MS brains has been observed to exhibit two different types of lesion simultaneously.

Central to the pathophysiology of MS is the degeneration of the myelin components. These lesions are scattered throughout the CNS with a predilection for optic nerves, brain stem, spinal cord, and periventricular white matter (Adams, 1977). The multilamellar myelin sheath responsible for insulating axons and facilitating saltatory conduction of nerve impulse is formed by membranous extensions of oligodendrocytes within the CNS (Peters, 1960). Myelin sheath is composed of 70% lipid and 30% protein by weight. Destruction of myelin proteins has been the focus of numerous studies on MS pathogenesis because myelin protein-lipid interactions are considered crucial in maintaining myelin membrane architecture (Moscarello et al., 2007), and also because cells secreting antibodies against myelin proteins (Sun, 1991; Van der Goes et al., 1999) have been recovered from MS patients.

Degeneration of the blood brain barrier (BBB) appears to be a necessary component of pathology leading to demyelination (Hochmeister et al., 2006). The obvious consequence to BBB disruption is the influx of serum proteins. Prineas (1975) observed lymphocytes and macrophages forming perivascular cuffs around early MS plaques as a result of BBB destruction. This was followed by diffuse parenchymal infiltration by inflammatory cells, macrophages, astrocytes, and edema. Eventually increased numbers of lipid-laden macrophages and demyelinated axons were observed.
Whether immune cells initiate demyelination by autoimmune attack on myelin proteins or the inflammatory cellular response is generated secondary to neurodegeneration by leakage of myelin components into the periphery remains currently unknown. Nonetheless, autoimmune attack by cytotoxic T lymphocytes has been one of the foci of MS research as activated CD8\(^+\) (T lymphocytes) and CD4\(^+\) (helper T cells) cells specific to myelin proteins have been identified surrounding MS plaques (Pender, 1995). A recent finding of identical T cell clones in anatomically distinct areas of MS brain beyond classical plaque area extending into adjacent NAWM further implicated the role of T lymphocytes in MS pathogenesis (Junker et al., 2007).

Although the inflammatory response in MS was once widely believed to be mediated exclusively by T-cells, more recent evidence has implicated B-cell humoral immune response in MS, as aggregates of B-cells together with macrophages have been detected in meninges in about 40% of SPMS patients (Magliozzi et al., 2007). Evidence also indicated that the CNS of MS patients provides a fostering environment for B-cells, as B-cell activating factor of the TNF family (BAFF) secreted by astrocytes was found to be upregulated in MS lesions. Flow cytometry analysis found that B-cells detected at MS lesions were able to secret autoantibodies (mainly IgG isotype) against various myelin components (Van der Goes et al., 1999).

III. Etiology

MS is a complex disease with multifactorial causes involving environmental and genetic components. The etiology of MS remains unknown. Currently, the most widely accepted hypothesis proposes MS as a T-cell mediated autoimmune disease mainly based on research with autoimmune animal models of MS. According to the autoimmune theory of MS, this disease is
induced by a virus or other infectious agents breaking down BBB (Herdon, 2005). Subsequently, molecular mimicry whereby viruses with epitopes similar to myelin proteins and their activation of cytotoxic T cells activates the immune system for myelin destruction (Jahnke et al., 1985).

In support of the autoimmune theory, at least 14 different viruses have been isolated from the brains of MS patients (Johnson, 1994). Herpes virus in particular has been observed in a significant portion of MS plaque and anti-herpes drug acyclovir has shown to reduce the number of MS attacks (Sanders et al., 1996). However, no single viral agent has been found in all types of active plaques (Herndon, 2005) and most of viruses isolated from the MS brains have not been shown to be etiologically related (Johnson, 1994). Moreover, since most viral studies have been conducted in postmortem brain tissue, whether infectious agents initiate autoimmune attack and play a causative role in MS remains undetermined.

MS has alternatively been proposed as a neurodegenerative disease caused by metabolic changes in the myelin constituents (Moscarello et al., 2007). According to the neurodegenerative theory, inflammatory cellular responses in MS are secondary to degradation of myelin components caused by biochemical changes in myelin proteins and release of myelin peptides into the periphery. Recent evidence showing extensive oligodendrocyte apoptosis and myelin macrophages in newly forming lesion of RRMS tissue is suggestive of a primary neurodegenerative mechanism followed by the autoimmune attack as a secondary event (Barnett et al., 2004). Since myelin proteins are primarily responsible for maintaining the myelin membrane architecture, the chemical changes in myelin proteins that precede membrane degradation have been under extensive investigation. In the present study, I have focused on the enzymatic changes in myelin basic protein (MBP), which is not only a major component of myelin protein but also a candidate autoantigen in MS.
IV. Current Treatments

There is no known cure for MS. Since MS is widely believed as an autoimmune disease, most currently approved disease-modifying drugs are immune modulators based on research with autoimmune animal models of MS. As of 2009, five disease-modifying treatments have been approved by U. S. Food and Drug Administration (FDA) for treating MS, including interferon β-1a (The Multiple Sclerosis Collaborative Research Group, 1996), interferon β-1b (The INFB Multiple Sclerosis Study Group, 1993), glatiramer acetate (Bornstein et al., 1987), mitoxantrone (Fox., 2006), and natalizumab (Polman et al., 2006). All the approved medications are immunomodulators except mitoxantrone which is an antineoplastic agent. These drugs reduce the progression rate of MS at various efficacy rates with serious side effects and undetermined long-term effect as summarized in Table 1-1. None of the drugs affects the basic pathology of MS.
Table 1-1. Summary of current disease-modifying treatments for relapse-remitting MS. (Adapted from Millefiorini et al., 1997; Johnson, 2007) The average annualized relapse rate in the placebo groups in clinical studies ranges from 0.8 to 1.2 attacks. (1), (2), (3), (4) refer to the number of year in the perspective MS treatment trials. NA: data on annualized relapse rate at 3 and 4 years from mitoxantrone and natalizumab treatment is not available.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Possible mechanism</th>
<th>Annualized relapse rate at 4 years from perspective MS treatment trials with optimal dosage</th>
<th>Side Effects</th>
</tr>
</thead>
<tbody>
<tr>
<td>Interferon β-1a</td>
<td>Upregulating immunosuppressive cytokine IL-10</td>
<td>0.92 (1) 0.82 (2) 0.57 (3) 0.44 (4)</td>
<td>Flu-like symptoms, injection site disorders, liver and blood cell abnormalities</td>
</tr>
<tr>
<td>Interferon β-1b</td>
<td>Downregulating receptors for inflammatory cytokine IL-2; blocking formation of gelatinase capable of degrading myelin</td>
<td>0.96 (1) 0.85 (2) 0.66 (3) 0.67 (4)</td>
<td></td>
</tr>
<tr>
<td>Glatiramer acetate</td>
<td>Random polymer composed of four amino acids found in MBP; possibly acting as a decoy shifting T cell population from pro-inflammatory Th1 cells to regulatory Th2 cells</td>
<td>0.75 (1) 0.45 (2) 0.39 (3) 0.28 (4)</td>
<td>Injection site disorders; flu-like symptoms; shortness in breath and rapid heartburn; potential serious side effects to cardiovascular system</td>
</tr>
<tr>
<td>Mitoxantrone</td>
<td>Anthracenedione breaking cell DNA and interfering with RNA; inhibiting T-cell, B-cell, and macrophage proliferation</td>
<td>0.52 (1) 0.37 (2) NA (3) NA (4)</td>
<td>Highly toxic similar to other antineoplastic agents; potential cardiotoxicity, myelosuppression and leukemia</td>
</tr>
<tr>
<td>Natalizumab</td>
<td>Monoclonal antibody against α4β1 integrin blocking T-cells from passing through cell layers lining BBB</td>
<td>0.28 (1) 0.20 (2) NA (3) NA (4)</td>
<td>Fatigue; liver injury; two cases of death caused by progressive multifocal leukoencephalopathy</td>
</tr>
</tbody>
</table>
As one can see, currently approved MS therapies with anti-inflammatory mechanisms targeting the peripheral immune system modestly delay disease progression and reduce the frequency of relapses by 33-66% without inhibiting disease progression (Steinman and Zamvil, 2006). Since the biological mechanism underlying progression in MS is complex possibly involving biochemical, electrophysiological and genetic factors, targeting immune system alone may produce limited beneficial effect. Therefore, a novel therapeutic strategy which directly and specifically targets a basic mechanism of MS is urgently required.

B. Myelin Basic Protein (MBP)

Myelin sheath is composed of a number of lipids and proteins (Table 1-2). MBP is a major component of myelin in human CNS and the second most abundant myelin protein. Since changes in MBP (see Citrullination of MBP, pg 23-25) are central to development of a targeted treatment of MS, some of its properties will be examined in this section.
Table 1-2. Composition of human CNS myelin. (adapted from Norton 1976; Pfeiffer et al., 1993; Amiguet et al., 1992; and Quarles et al., 1992)

<table>
<thead>
<tr>
<th>Substance</th>
<th>Percentage Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Total Proteins</strong></td>
<td>30.0</td>
</tr>
<tr>
<td>Proteolipid Protein</td>
<td>50</td>
</tr>
<tr>
<td><strong>Myelin Basic Protein</strong></td>
<td>30-40</td>
</tr>
<tr>
<td>2',3'- cyclic nucleotide 3'-phosphohydrolase</td>
<td>4</td>
</tr>
<tr>
<td>Myelin-associated glycoprotein</td>
<td>1</td>
</tr>
<tr>
<td>Myelin oligodendrocyte glycoprotein</td>
<td>0.01-0.05</td>
</tr>
<tr>
<td><strong>Total Lipid</strong></td>
<td>70.0</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>27.7</td>
</tr>
<tr>
<td>Total Galactolipid</td>
<td>27.5</td>
</tr>
<tr>
<td>Cerebroside</td>
<td>22.7</td>
</tr>
<tr>
<td>Sulfatide</td>
<td>43.1</td>
</tr>
<tr>
<td>Ethanolamine phosphatides</td>
<td>15.6</td>
</tr>
<tr>
<td>Cholin phosphatides</td>
<td>11.2</td>
</tr>
<tr>
<td>Serine phosphatides</td>
<td>4.8</td>
</tr>
<tr>
<td>Inositol phosphatides</td>
<td>0.6</td>
</tr>
<tr>
<td>Sphingomyelin</td>
<td>7.9</td>
</tr>
<tr>
<td>Plasmalogen$^2$</td>
<td>12.3</td>
</tr>
</tbody>
</table>

1. Total protein and lipid are expressed as percentage of dry weight, all others as percentage of total protein or lipid

2. Plasmalogens are mainly ethanolamine phosphatides.
The gene for MBP located on chromosome 18 is very large in size (179 kb) and contains three transcription start sites (Pribyl et al., 1993). As a result, the MBP family is composed of a variety of members arising from different transcription start sites, alternative splicing, and post-translational modifications. The classic MBPs are the products of transcription start sites 2 and 3 (Fulton et al., 2010). In the human CNS, there are four main isoforms of classic MBP generated by differential splicing of a single transcript: 21.5 kDa, 18.5 kDa, 17.2 kDa and 14 kDa isoforms (Kamholtz et al., 1986; 1988). The 18.5 kDa isoform (Fig. 1-2), which has the exon II spliced out, has been identified as the most prominent isoform in adult human myelin (Moscarello, 1997).

![Diagram of MBP isoforms](image)

**Fig. 1-2. Organization of (A) murine classic MBP gene and (B) human classic MBP gene.**
(adapted from Harauz et al., 2004) Note that genes for 18.5 kDa isoform in both murine and human have exon II spliced out.
The classic human 18.5 kDA isoform is a single chain of 170 amino acids, 19 of which are arginyl residues (Carnegie, 1971). Since MBP is expressed almost exclusively in myelin-producing oligodendrocytes, it was believed to play a crucial role in myelin maturation. Evidence has indicated MBP knockout mice suffered from decreased CNS myelination and a progressive neurodegenerative disorder characterized by tremors, seizures, and early death (Popko et al., 1987). As one can see in Fig. 1-3, there is a high degree of sequence conservation of 18.5 kDA MBP in 15 different species suggestive of its importance in maintaining myelin function (Harauz et al., 2004).

The primary role of the 18.5 kDA MBP has long been accepted as membrane-associated protein stabilizing the myelin sheath (Sedzik et al., 1984). As an “intrinsically unstructured” protein (Harauz et al., 2004), it is characterized by a high net positive charge coupled with a low mean hydrophobicity (Tompa, 2002). MBP’s open conformation and high basicity (pI > 10) create a large effective surface to facilitate its hydrophobic and electrostatic interaction with negatively charged phospholipids. Since myelin protein-lipid interactions play a vital role in maintaining myelin structural integrity, alterations to the electrostatic charge on MBP may contribute to destabilization and subsequently to the process of demyelination.
Fig. 1-3. A BLASTP/CLUSTALW alignment of sequences of 18.5 kDa MBP from various species. (from Harauz et al., 2004). Mouse (*Mus musculus*), rat (*Rattus norvegicus*), chimpanzee
(Pan troglodytes), human (Homo sapiens), bovine (Bos taurus), pig (Sus scrofa), horse (Equus caballus), rabbit (Oryctolagus cuniculus), guinea pig (Cavia porcellus), chicken (Gallus gallus), African clawed frog (Xenopus laevis), little skate (Raja erinacea), spiny dogfish (Squalus acanthias), horn shark (Heterodontus francisci). The amino acids are coloured as follows: red (small and/or hydrophobic), blue (acidic), magenta (basic), green (polar), and gray (others). (*) residues are identical among all species, (:) substitutions are conservative according to the colour scheme, and (.) substitutions are semi-conservative.

I. Citrullination of MBP

The 18.5 kDa MBP exists as a number of charge isomers (as opposed to mass isomers) which are results of a myriad of post-translational modifications including N-terminal acylation, phosphorylation, methylation, glycosylation, deamidation, and deimination (Moscarello et al., 1997). Based on the net positive charge, the 18.5 kDa MBP has been separated into 8 charge isoforms termed C1-C8 by the cation exchange chromatography on a CM52 column (Wood and Moscarello, 1989).

The C8 isomer, being the least cationic and most post-translationally modified among all the MBP isomers, is generated by the deimination of 6 arginyl residues at positions 25, 31, 122, 131, 159 and 170 of the amino acid sequence (Wood and Moscarello, 1989). Deimination of MBP converts positively charged arginyl residues into neutral citrulline as shown in Fig. 1-4. The deimination of arginyl residues within peptide bonds is specifically catalyzed by the family of peptidylarginine deiminase (PAD) enzymes.
Deimination of arginyl residues within peptide bonds (adapted from Vossenaar et al., 2003) Deimination of arginine (a positively charged amino acid) generates citrulline (a neutral amino acid) with the release of ammonia and the loss of one positive charge from the MBP for each arginyl residue deiminated. This process is facilitated by peptidylarginine deiminase, a calcium dependent enzyme.

The role of protein citrullination in the pathogenesis of MS was initially proposed since less cationic MBP isomer exhibited reduced ability to aggregate phospholipid vesicles (Cheifetz and Moscarello, 1985). Moreover, the most citrullinated C8 isomer exhibited reduced interactions with the myelin lipid bilayer as compared with other more cationic isomers (Wood and Moscarello, 1989), suggestive that the loss of electrostatic interactions may contribute to the destabilization of myelin in MS patients.

In their study of MBP from chronic MS patients, C8 isomer was found to be elevated 2-3 fold compared to C-8 form from normal brain (Moscarello et al., 1994), but accounted for 80-90% of the MBP from fulminating MS (Wood et al., 1996). This was similar to the pattern of myelin heterogeneity in infant white matter in which the C8 isomer accounted for 80-90% of the total MBP (Moscarello et al., 1994). Thus MBP in the brains of MS patients may resemble developmentally immature myelin and is susceptible to neurodegeneration. This readily
degradable MBP may provide the initial antigenic material to the immune system, as hypercitrullinated MBP has been shown to be more susceptible to proteolytic digestion by cathepsin D (Pritzker et al., 2000). The presence of citrulline in brains of early onset MS patients was detected by a proton magnetic resonance study (Oguz et al., 2009) suggesting that the enhanced MBP citrullination is a basic mechanism in the pathogenesis of demyelinating disease and represents an initial event.

Evidence of protein hypercitrullination was found in various mouse models of MS. In the ND4 mouse model (overexpressing DM20) of neurodegenerative demyelination, an increased abundance of highly citrullinated C8 isomer was observed in animal brain prior to spontaneous demyelination (Mastronardi et al., 1996). In an autoimmune mouse model of MS, citrullinated proteins were detected in the areas of spinal cord showing the highest degree of inflammation, and increased with disease progression (Raijmakers et al., 2005). Therefore, increased citrullination of proteins was implicated in the pathogenesis of demyelinating diseases.

Protein citrullination has also been implicated in the pathogenesis of other neurodegenerative and autoimmune diseases such as rheumatoid arthritis (Vossenaar et al., 2003), psoriasis (Ehrlich et al., 2004), Creutzfeldt-Jakob disease (Jang et al., 2010; Jang et al., 2008), and Alzheimer’s disease (Ishigami et al., 2005). Therefore, reversing protein hypercitrullination is not only an important therapeutic strategy for MS but also for these other diseases.

C. Peptidylarginine deiminase (PAD)

Peptidylarginine deiminase (PAD, EC 3.5.3.15) enzyme is responsible for catalyzing the
conversion of arginine residues to citrulline residues in the presence of calcium ion. PAD activity has found to be enhanced by dithiothreitol (DTT), and was optimal at pH 7.5 at 50°C (Fujisaki and Sugawara, 1981). PAD activity is originally discovered in guinea pig hair follicle (Rogers et al., 1977) and subsequently in other tissues including bovine and rat epidermis (Kubilus et al., 1980; Fujisaki and Sugawara, 1981), skeletal muscle (Sugawara et al., 1982) and bovine brain (Kubilus and Baden, 1983). At the present time, five isotypes of PAD have been described and cloned (PAD 1, 2, 3, 4, 6; Table 1-3; Mechin et al., 2007). The five human PAD isozymes are highly conserved at the amino acid level (Table 1-4) and the active site structures (Table 1-5).

Table 1-3. Comparison of five isozymes of peptidylarginine deiminases (PADs). (Table adapted from Mechin et al., 2007; Guerrin et al., 2003)

<table>
<thead>
<tr>
<th>Tissue Localization</th>
<th>Molecular mass (kDa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAD1 epidermis, uterus, stomach</td>
<td>74.6</td>
</tr>
<tr>
<td>PAD2 skeletal muscle, brain, uterus, pancreas, salivary gland</td>
<td>75.3</td>
</tr>
<tr>
<td>PAD3 hair follicles</td>
<td>74.6</td>
</tr>
<tr>
<td>PAD4 haematopoietic cells including eosinophils, neutrophils</td>
<td>74.0</td>
</tr>
<tr>
<td>PAD6 oocytes, early cleavage-stage embryos ovary, testis, peripheral leucocytes</td>
<td>77.7</td>
</tr>
</tbody>
</table>
Table 1-4. Percentage of homology between primary human peptidylarginine deiminases (PAD) amino-acid sequences, theoretical isoelectric point (pI), and molecular weight. (from Mechin et al., 2007).

<table>
<thead>
<tr>
<th>% Identity</th>
<th>PAD1</th>
<th>PAD2</th>
<th>PAD3</th>
<th>PAD4</th>
<th>PAD6</th>
<th>pl</th>
<th>kDa</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAD1</td>
<td>100</td>
<td>65</td>
<td>68</td>
<td>71</td>
<td>59</td>
<td>6.01</td>
<td>74.6</td>
</tr>
<tr>
<td>PAD2</td>
<td>100</td>
<td>67</td>
<td>65</td>
<td>59</td>
<td>5.40</td>
<td>75.3</td>
<td></td>
</tr>
<tr>
<td>PAD3</td>
<td>100</td>
<td>68</td>
<td>61</td>
<td></td>
<td>5.25</td>
<td>74.6</td>
<td></td>
</tr>
<tr>
<td>PAD4</td>
<td>100</td>
<td>61</td>
<td></td>
<td></td>
<td></td>
<td>6.15</td>
<td>74.0</td>
</tr>
<tr>
<td>PAD6</td>
<td>100</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>4.97</td>
<td>77.7</td>
</tr>
</tbody>
</table>

Table 1-5. Amino acids involved in peptidylarginine deiminase active site cleft are conserved between 5 PAD isozymes.(from Mechin et al., 2007)

<table>
<thead>
<tr>
<th>PAD/Pad</th>
<th>Asp-350</th>
<th>Arg-372</th>
<th>Arg-374</th>
<th>Hls-471</th>
<th>Asp-473</th>
<th>Arg-639</th>
<th>Cys-645</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>D</td>
<td>R</td>
<td>R</td>
<td>H</td>
<td>D</td>
<td>L</td>
<td>C</td>
</tr>
<tr>
<td>2</td>
<td>D</td>
<td>R</td>
<td>G</td>
<td>H</td>
<td>D</td>
<td>F</td>
<td>C</td>
</tr>
<tr>
<td>3</td>
<td>D</td>
<td>R</td>
<td>G</td>
<td>H</td>
<td>D</td>
<td>L</td>
<td>C</td>
</tr>
<tr>
<td>4</td>
<td>D</td>
<td>R</td>
<td>R</td>
<td>H</td>
<td>D</td>
<td>R/Y</td>
<td>C</td>
</tr>
<tr>
<td>6</td>
<td>D</td>
<td>Q/R</td>
<td>A/S</td>
<td>H</td>
<td>D</td>
<td>E/N</td>
<td>A*</td>
</tr>
</tbody>
</table>
PAD is unusual in that it requires its substrate arginine to be within peptide bonds and is incapable of converting free arginine to citrulline unlike other enzymes involved in arginine metabolism such as arginine deiminase and nitric oxide synthase. Based on research using benzoyl-L-arginine-amide as a substrate, one possible mechanism responsible for this specificity is that PAD recognizes two main chain oxygen atoms of its substrate by Arg372, Arg374, and Arg639 located at the molecular surface of the enzyme (Arita et al., 2004). Recent crystallographic data proposed a reaction mechanism of deimination by PAD4: Ca$^{2+}$ binding induced conformational changes generating active site cleft in the C-terminal domain of the enzyme (Arita et al., 2004), Cys645 at the active site of PAD4 subsequently initiated a nucleophilic attack on the carbon atom in the guanidine side chain of substrate arginine forming covalent S-alkylthiouronium intermediates (Fig. 1-5).
Fig. 1-5. Proposed reaction mechanism of protein citrullination by PAD4. (from Arita et al., 2004)
Previous studies indicated PAD enzymes may be subject to sex hormone regulation. In tissue culture studies with pituitary cell lines containing PAD2, estrogen has been shown to increase the amount of PAD mRNA and protein (Nataga and Senshu, 1991) while 17β-estradiol elicited a dose-dependent increase in PAD activity (Nagata et al., 1990). Estrogen has also been shown to stimulate PAD4 expression through binding of estrogen receptor (ER)-α to the upstream of the PADI4 gene in human breast cancer MCF-7 cells (Dong et al., 2007). A study by Senshu et al. (1989) has found PAD activity in vivo in female rat pituitary undergo changes related to the estrogen and progesterone: ovariectomized rats had minimal PAD activity but increased activity was restored with the administration of 17β-estradiol, and administration of progesterone decreased the PAD activity. Moreover, changes in PAD activity in female rat pituitaries due to pregnancy were detected: small amounts of PAD protein were present at the onset of gestation, began to increase at day 12 reaching the maxima at day 18 and declined afterwards (Akiyama et al., 1993). Thus increase in PAD protein in this study was parallel to the increase of serum estrogen after day 12.

PAD activity has been detected in all vertebrates and in most organs, tissues and cells, and thus PAD is implicated in biological functions as diverse as epidermal differentiation, gene expression, and development. Several structural proteins have been found in vivo as substrates of PAD including intermediate filament (IF) proteins, such as keratins K1 and K10, vimentin and glial fibrillary acidic protein (GFAP), and IF-associated proteins like filaggrin and trichohyalin (Senshu et al., 1995; Senshu et al., 1996; Senshu et al., 1999; Tarcsa et al., 1996; Tarcsa et al., 1997; Nicholas et al., 2004; Ishigami et al., 2005). PAD4, unlike other isozymes, can translocate into nucleus where it deiminates histone (Mastronardi et al., 2006). Evidence has indicated that human PAD4 regulated the expression of estrogen-responsive genes by modifying the methylated arginine sites in histones H3 and H4 (Wang et al., 2004) even though contradictory
evidence showed that methylation of arginine residues interfered with protein citrullination by PAD (Raijmakers et al., 2007). Moreover, PAD may play a role in brain development and myelination as the amount of PAD transcription and PAD protein increase in mouse brain early in development (Pritzker et al., 1999).

PAD is implicated in pathology of various inflammatory diseases including psoriasis, and rheumatoid arthritis. By immunohistochemical analysis, a decrease in deiminated proteins, in particular K1, has been observed in the hyperproliferative epidermis of psoriatic lesions compared to normal epidermis (Ishida-Yamamoto et al., 2000). Two studies on rheumatoid arthritis have reported the presence of PAD4 in all areas of the synovial tissue (ST), colocalizing with cells exhibiting positive staining for T-, B-, macrophage-, granulocyte-, fibroblast- or endothelial cell-specific markers (Chang et al., 2005). In addition, a study on animal models of arthritis demonstrated the absence of PAD2 and PAD4 in the ST of naïve mice and the presence of only PAD 4 in the inflamed ST of mice with collagen-induced arthritis or streptococcal cell wall-induced arthritis (Vossenaar et al., 2004).

PAD enzymes have been implicated in MBP citrullination and demyelinating diseases. Of five PAD isozymes, PAD2 and PAD4 have been located in the CNS, to the myelin sheath, the oligodendrocyte and axon (Mastronardi et al., 2006). In our mouse model of neurodegenerative demyelination (over-expressing DM20), PAD 2 up-regulation was found at 2 months of age, and citrullinated MBP and demyelination followed at 4 months of age, suggesting a precursor-product type relationship (Moscarello et al., 2002). A subsequent study has found that hypomethylation of the CpG island in the MS brain PAD2 promoter may lead to PAD2 overexpression and MBP hypercitrullination with subsequent myelin destabilization (Mastronardi et al., 2007). Hypomethylation of protein may also contribute to MBP hypercitrullination as Raijmakers et al. (2007) has shown that methylation of arginine residues
interfered with protein citrullination by PAD in vitro. Using synthetic peptides that contain either arginine or methylated arginine residues, Raijmaker et al. (2007) demonstrated that the human PAD2, PAD3 and PAD4 enzymes and PAD enzyme present in several mouse tissues in vitro could only convert non-methylated peptidylarginine into peptidylcitrulline. A Western-blot analysis has also found elevation of PAD 4 protein in NAWM of MS brain and in brains of animal models of demyelination, associated with an increase in citrullinated histone 3 (Mastronardi et al., 2006). Cell culture analysis indicated the translocation of cytosolic PAD4 into nucleus was mediated by TNF-α and subsequent histone citrullination may contribute to oligodendrocyte apoptosis (Mastronardi et al., 2006).

D. 2-Chloroacetamidine, a PAD inhibitor

Since protein hypercitrullination by PAD overexpression has played an important role in pathogenesis of MS, we propose the use of an active site inhibitor to PAD as a novel therapeutic strategy to treat MS. There have been few useful PAD inhibitors reported until Stone et al. (2005) described a small molecule PAD inhibitor, 2-chloroacetamidine (2CA, Fig. 1-6).

![Structure of 2-chloroacetamidine (2CA).](image)

**Fig. 1-6. Structure of 2-chloroacetamidine (2CA).** Note the structural similarity between 2CA and guanidine site chain of substrate arginine.
To characterize the effect of 2CA on recombinant PAD 4, Stone et al (2005) added an excess of substrate (10 mM N-benzoyl-L-arginine ethyl ester) to preincubation mixtures containing PAD4 and 2CA of various concentration. The result indicated that inactivation of PAD4 by 2CA occurred with $K_I$ and $k_{\text{inact}}$ values of $20 \pm 5$ mM and $0.7 \pm 0.1$ min respectively as shown in Fig. 1-7. To determine the effect of 2CA on PAD 2 and PAD 4, the activities of recombinant human PAD2 and PAD4 were examined by our lab in the presence of increasing amount of 2CA in vitro. The inhibition curve (Fig. 1-8) revealed 50% inhibition of PAD2 and PAD4 with 15 mM of 2CA.

**Fig. 1-7. Time and concentration dependent inactivation of PAD4 by 2CA** (from Stone et al., 2005). (A). Exponential fits to the observed inactivation of PAD4 at pH 7.6 and 37°C with different concentrations of 2CA: 0 (●), 5 (■), 8 (◆), 15 (▲), 25 (right triangle pointing left), and 30 mM (right triangle pointing right). The dashed line indicates inactivation by 2CA (15 mM) in the presence of 10 mM substrate, BAEE. (B) Concentration of dependence of pseudo-first-order $K_{\text{obs}}$ values for inactivation.
Fig. 1-8. Inhibition of recombinant PAD2 and PAD4 activities by 2CA. Inhibition curve showing citrulline levels (O.D. 464 nm) as a measure of residual recombinant PAD2 and PAD4 enzyme activities in the presence of increasing amount of 2CA. The PAD2 and PAD4 50% inhibition doses for 2CA is indicated by the arrow.

To determine the mechanism of how 2CA reacted with PAD in vitro, our lab set out to map the binding site of 2CA and recombinant human PAD2 by mass spectrometric analysis. PAD2, either reacted with 2CA or unreacted control, was digested by trypsin and subjected to ESI-TOF MS analysis. We identified a peptide with a mass of 1330.45 for unreacted PAD2 whereas the corresponding peptide from PAD2 reacted with 2CA had a mass of 1386.68. This mass difference of 56 Da corresponded to one molecule of acetamidine. The peptide reacting with 2CA was identified to be FLGEVHCGTNVR, located at the PAD2 C-terminal containing the active site cysteine (C). Fragmentation analysis (Fig. 1-9) of the reacted and unreacted peptide confirmed the identity of the cysteine as the active site amino acid in PAD2. Since this
active site is conserved among all human PAD isozymes (Fig. 1-9), we propose 2CA reacted with PAD enzymes by covalent binding to the active site of cysteine as shown in fig. 1-10.

**Fig. 1-9. Identification of the binding site of acetamidine by mass spectrometry sequencing.**
The covalently bound adduct (asterisk) was found at Cys656 in the PAD2 active site by fragmentation mass spectrometry.
Fig. 1-10. Mechanism of reaction of 2CA with PAD. A schematic of the nucleophilic reaction between the Cys656 in the active site of PAD 2 and 2CA. The reaction results in a covalent acetamidine adduct at the Cys656 position increasing the molecular mass of PAD 2 by 56 Da (the molecular mass of acetamidine).

Since MS is a disease of the CNS and PAD has been detected in MS brain white matter, any effective disease-modifying drug for MS has to be able to cross the BBB. We propose 2CA is advantageous in this respect due to its small molecular weight. To establish that 2CA was able to cross the BBB and interact with PAD in brain, our lab prepared a synthetic peptide containing the acetamidine adduct on the cysteine residue (\textsuperscript{650FLGEVHC*GTVNVR}), coupled to keyhole limpet hemocyanin to prepare a monoclonal antibody (MAb4E12) specific for the modified PAD peptide containing 2CA. A secondary anti-mouse IgG conjugated with colloidal gold particles was used to detect MAb4E12 antibody in brain sections of transgenic mouse overexpressing PAD2, which had been injected with 2CA for 2 weeks. By electron microscopy of these sections, the antibody was detected in both nucleus of the oligodendrocytes and in the myelin sheaths, confirming the presence of PAD2-acetamidine adduct in the brain of 2CA treated mice (Fig. 1-11).
Fig. 1-11. Immuno-electron microscopy of optic nerve cryo-sections from untreated and 2CA treated PAD2 transgenic mice. The two left side panels show axon (Ax) and nucleus (N) in untreated mouse myelin. The right side panel shows acetamidine-PAD clusters in myelin (arrows). Dark dots indicate gold particles within the oligodendrocyte nucleus and myelin. Gold particles were not found in the untreated myelin sections.
E. Animal models and their value in studying MS

MS is a uniquely human disease with heterogeneous symptoms and clinical signs, and runs at least four clinical courses which might result from environmental differences and genetic variation among the patient population. However, most current understanding of MS pathogenesis and drug development is based on research with various mouse models of MS. There is no single perfect model for studying MS, as each MS mouse model represents some but not all facets of this heterogeneous disease.

Since MS is widely viewed as a T-cell mediated inflammatory demyelinating disease, most of the current animal experiments utilized experimental autoimmune (allergic) encephalomyelitis (EAE) models, the autoimmune models of MS. The EAE model originated from vaccination with rabies-infected rabbit spinal cord by Louis Pasteur in 1885: about 1 in 1000 vaccinated rabbits developed “neuroparalytic incidents” that later proved to be due to contamination by spinal cord components in the inoculums (Fries et al., 2006). From then on, the EAE models have been developed in different animals immunized with various antigens including whole spinal cord, myelin proteins, and defined peptides leading to disease that shares some clinical features with MS (Steinman, 1999).

Currently, there are two most common types of EAE mouse models (Mix et al., 2008). The first type, active EAE, is induced by subcutaneous injection of an auto antigen, mostly myelin protein peptides including myelin basic protein (MBP) 84-104, myelin oligodendrocyte glycoprotein (MOG) 35-55 or proteolipid protein (PLP) 139-151. The encephalitogenic peptide is emulsified in complete Freund’s adjuvant (CFA) containing Mycobacterium tuberculosis strain H37RA that stimulates CD4+ Th1 by activating certain toll-like receptors. In some models, pertussis toxin (PTX) is injected intraperitoneally to disrupt BBB(Berard et al., 2010). The
second type, adoptive transfer EAE (AT EAE), is induced by intravenous injection of myelin-reactive CD4$^+$ Th1 cells into naïve mice. All EAE diseases directly involve activation of immune system as shown in Fig. 1-12.

Fig. 1-12. A schematic of the induction of active EAE and adoptive transfer on mouse. (From Mix et al., 2008). Immunization with myelin peptides leads to actively induced EAE. Transfer of myelin-reactive T-helper1 cells induces adoptive transfer EAE (AT-EAE). Most EAE models are studied on their systematic (PB,LN,SP) and local (spinal cord and brain) immune reactions. CFA: complete Freund’s adjuvant; EAE: experimental encephalomyelitis; i.p: intraperitoneal; i.v: intravenous; LN: lymph node; MOG: myelin oligodendrocyte glycoprotein; PB: peripheral blood; PLP: proteolipid protein; s.c: subcutaneous; SP: spleen.
From early EAE models constructed to understand acute disseminated encephalomyelitis, later versions of more chronic EAE have been developed with pathology including demyelination, axonal damage, and relapsing and remitting episodes of paralysis (Zamvil and Steinman, 1990). Research based on EAE models has led directly to the development of three therapies approved for use in MS: glatiramer acetate (Johnson et al., 1995), mitoxantrone (Hartung et al., 2000), and natalizumab (Stuve and Bennett, 2007).

EAE models may be vital for studying processes of autoimmunity underlying MS, however there are serious limitations of current EAE models. As one can see, EAE is not a spontaneous disease like MS since the disease almost always has to be induced in animals (Steinman et al, 2006). Both active EAE and AT EAE models involve induction of CD4+ response by CFA or myelin-specific CD4+ lymphocytes resulting in little variation in disease pathways. Moreover, the clinical courses are very different from most EAE models and MS patients. Most EAE develops over days in most models resulting in cytotoxic demyelination and fatal adverse effects (Steinman, 1999), in contrast to typical MS disease progression over years from early relapsing-remitting and later chronic forms. As a result, many drugs that were successful in pre-clinical EAE trials have failed in clinical trials with MS patients (Table 1-6, Mix et al., 2008).
Table 1-6. Failed translation of experimental therapies from EAE models to clinical practice (adapted from Mix et al., 2008).

<table>
<thead>
<tr>
<th>Therapy</th>
<th>Results in MS patients</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti TNF-α infliximab</td>
<td>Increased MRI activity</td>
<td>van Oosten et al. (1996)</td>
</tr>
<tr>
<td>Anti-CD3 and anti-CD4 antibodies</td>
<td>No significant clinical effect</td>
<td>Weishaupt et al. (2002)</td>
</tr>
<tr>
<td>Anti-CD28 mAb TGN1412</td>
<td>Cytokine storm causing multi organ failure</td>
<td>Hunig. (2007)</td>
</tr>
<tr>
<td>Altered peptide ligands</td>
<td>Anaphylactic reactions and exacerbation of MS</td>
<td>Kappos et al. (2000)</td>
</tr>
<tr>
<td>Oral tolerogens</td>
<td>No significant clinical effect</td>
<td>Faria et al. (2006)</td>
</tr>
<tr>
<td>Sulfasalazine</td>
<td>Only transient clinical effect</td>
<td>Noseworthy et al. (1998)</td>
</tr>
<tr>
<td>Linomide</td>
<td>Cardiopulmonary toxicity</td>
<td>Noseworthy et al. (2000)</td>
</tr>
</tbody>
</table>
Alternatively, our lab has developed transgenic mouse models of MS undergoing spontaneous neurodegeneration. The ND4 transgenic mice carrying 70 extra copies of cDNA for DM20 protein (a splice variant of PLP) was well documented for its degenerative demyelinating condition (Mastronardi et al., 1993). The function of the DM20 protein has not been defined, but ND4 transgenic mice overexpressing DM20 undergo biochemical changes in MBP resulting in higher proportion of the less cationic components (C8) reminiscent of the changes in MBP found in MS prior to the onset of spontaneous demyelination at 3 months of age (Mastronardi et al., 1996). Most animals become moribund by 8 -10 months of age thus making ND4 a good model to study for progressive MS. Morphologically, demyelination in ND4 mice was characterized by 1). Loss of myelin with axonal sparing by 10 months of age; 2). Extensive astrocytosis in brain; 3). Myelin disruption; 4). Ingestion of myelin debris by reactive astrocytes; 5). Some infiltration into brain parenchyma by lymphocytes in white matter regions (Mastronardi et al., 1993).

Since PAD2 up-regulation was found at 2 months of age in ND4 mice prior to MBP deimination and onset of demyelination at 4 months of age, our lab recently generated a transgenic mouse line overexpressing PAD2 by incorporating 30 copies of the cDNA for PAD2 (padi2) directed to the oligodendrocyte by the MBP promoter (Musse et al., 2008). PAD2 overexpressors develop normally until 6 months of age when they start to develop progressive demyelination. With increased PAD2 expression, PAD activity, and MBP deimination in white matter, areas of focal demyelination and thinner myelin with extensive astrocytosis were revealed in PAD2 mouse optic nerve and brains (Musse et al). Thus the PAD2 overexpressor is a neurodegenerative model for mild MS in which PAD2 overexpression is causal. This model ideal to study mechanism representing an early biochemical change in the pathogenesis of demyelinating disease.
Although the neurodegenerative MS model represents a spontaneous disease with changes in microheterogeneity of MBP, these models are limited by a lack of inflammatory responses. Compared with brains from MBP-induced EAE mice (SJL strain), none of CD3\(^+\) (T cells), CD8\(^+\) (cytotoxic T-cells), CD68\(^+\) (macrophages) were found to be prominent in the brains of PAD2 overexpressor at 9 months of age (Musse et al., 2008). The present study has found similar result i.e. lack of cellular infiltration, in the brains of ND4 mouse as described in Chapter 6.

As one can see, current understanding of MS pathogenesis and developing new MS therapeutics rely heavily on research with animal models, but current models reflect limited pathogenetic, clinical and therapeutic features of the different forms of MS. Therefore, a unified model with autoimmune and neurodegenerative features may be more appropriate to answer specific questions of MS therapy including the search for new therapeutic targets and their validation. This problem is addressed in this thesis.
F. Hypothesis

*Is protein citrullination by elevated PAD activity associated with MS disease in vitro (MS patient NAWM) and in vivo (4 MS mouse models)?*

Changes in microheterogeneity of MBP, i.e. higher proportion of less cationic and more deiminated components have been previously observed in MS NAWM as well as white matter in neurodegenerative ND4 brain. Citrullinated proteins were previously reported in the areas of spinal cord showing the highest degree of inflammation in EAE mice (Raijmakers et al., 2005). Since MBP hypercitrullination was specifically facilitated by PAD enzymes, we propose that the elevation of PAD enzyme activity is responsible for protein deimination and subsequent demyelination in MS victims.

*Is 2-chloroacetamidine (2CA), a PAD active site inhibitor, able to attenuate disease in both neurodegenerative and autoimmune models of MS by affecting PAD activity in CNS?*

2CA is a small molecule capable of crossing BBB and interact specifically with PAD enzyme as an active site inhibitor. 2CA is predicted to interact specifically with PAD enzyme and thereby producing minimal toxicity and maximal efficacy in reducing PAD activity. Since we previously discovered that PAD2 promoter in MS NAWM was hypomethylated comparing with that in normal WM, combination treatment with B_{12}CN (a universal methyl donor), may have an added efficacy by reversing promoter hypomethylation. Since protein hypercitrullination may be a basic mechanism in demyelinating diseases, inhibiting PAD activity may reverse disease and allow remyelination.

*Is it possible to superimpose EAE-like inflammatory responses in ND4 mice already undergone neurodegeneration without injecting autoantigen?*
The nature of the demyelination in the ND4 mouse is primarily neurodegenerative process involving destabilization of the myelin membranes, the result of an increase of the cationic charge isomers of MBP with little inflammatory responses (Mastronardi et al., 1993). During active demyelinating phase after, MBP deamination leads to subsequent myelin destalization and the release of immunogenic peptides. Injection of pertussis toxin which disrupts the blood brain barrier will allow inflammatory cells to migrate into the CNS. In the presence of myelin antigens, sensitized cells may be formed possibly in the brain. Alternatively, the release of immunogenic peptides may sensitize T-cells in the periphery which later migrate into the CNS resulting in an inflammatory response similar to that of EAE mice superimposed upon the neurodegeneration. We therefore propose inflammatory cellular responses in this MS model are secondary to degradation of myelin components caused by biochemical changes in myelin proteins.
Chapter 2  
Materials and Methods

A. Materials

Alpha - N-benzoyl-L-arginine ethyl ester (BAEE), L-citrulline, and Vitamin B_{12} (cyanocobalamin) (B_{12}CN) were purchased from Sigma Chemical Company (St. Louis, MO). The PAD inhibitor 2-chloroacetamidine (2CA) was purchased from Alfa Aesar (Ward Hill, MA). The primary mouse monoclonal antibody F95 which recognizes peptidylcitrulline is a generous gift from Dr. A. P. Nicholas (University of Alabama at Birmingham, AL). The secondary antibody goat anti-mouse IgG HRP conjugate was purchased from Bio-Rad Laboratories (Hercules, CA). Cortical white matter samples from brains of MS patients and individuals without neurological diseases were obtained from the Human Brain and Spinal Fluid Resource Center (HSB) at University of California at Los Angeles, CA.

B. Animal Models

I. Neurodegenerative models

ND4 transgenic mice (CD1 strain) developed in our institute (The Hospital for Sick Children, Toronto, ON) carrying 70 copies of cDNA for DM20 (a splice variant of the myelin proteolipid protein) is a model for progressive demyelination as described by Mastronardi et al. (1993). Briefly, heterozygous ND4 mice develop normally until 3 months of age when they demyelinate spontaneously and develop progressive signs of demyelinating diseases. Most
animals become moribund when they reach 8 to 10 months of age. Although the mechanism of
demyelination in this model is not fully understood, a persistent high level of DM20 (a major
proteolipid protein in the young but a minor one in the adult) may result in improperly folded
myelin prone to disruption.

PAD2 overexpressing mouse (CD1 strain) was generated by incorporating 30 copies of
the cDNA for PAD2 (padi2) directed to the oligodendrocyte by the MBP promoter. The
homozygous PAD2 transgenic mice used in our experiment was reported recently by Musse et
al. (2008). This transgenic line develops normally until 6 months of age when the animals start to
demyelinate.

II. Autoimmune models

Chronic relapsing experimental autoimmune encephalomyelitis (CREAE) was induced in 6
– 8 wk old female SJL/J mice (Jackson Laboratories, Bar Harbor, ME) with 300 μg proteolipid
protein (PLP) peptide 139-155 emulsified in complete Freund’s adjuvant by subcutaneous
injection in the right and left flanks as previously described (Tuohy et al., 1995). Animals
develop various EAE signs of inflammatory demyelination at Day 10 post immunization and
reach the peak of disease at Day 16 post immunization followed by a chronic relapsing-remitting
phase.

Acute experimental autoimmune encephalomyelitis (EAE) was induced in 2 cohorts of 6-
8 wk old female C57BL/6 mice (Jackson Laboratories, Bar Harbor, ME) with 100 μg myelin
oligodendrocyte glycoprotein (MOG) peptide 35-55 emulsified in complete Freund’s adjuvant.
Each C57BL/6 cohort received intraperitoneal injection with either 150 ng or 50 ng pertussis
toxin (PTX) simultaneously followed by a second injection 48 h later. Acute EAE mice develop inflammatory demyelination Day 8 post immunization and progress to a stage of complete paralysis around Day 20.

All animal-use protocols and studies were approved by the Hospital for Sick Children’s Animal Care Committee.

C. Experimental Autoimmune Encephalomyelitis (EAE) induction in ND4 mice

Heterozygous ND4 transgenic mice carrying 70 copies of cDNA for DM20 develop normally until 3 months of age when the animals demyelinate spontaneously (Mastronardi et al., 1993). A first group of heterozygous ND4 mice were injected with 200 ng pertussis toxin (PTX) intravenously at 2 months of age before onset of demyelination and a second group at 5 months of age during active phase of demyelination.

D. Therapeutic protocol and clinical scoring

I. Transgenic mice

The first cohort of heterozygous ND4 mice was treated with either 2CA or a combination of 2CA and B12CN beginning at 3.5 months of age after the appearance of clinical signs of demyelination, and the second cohort were treated at 2 months of age before the disease onset. The third cohort of animals received 2CA or combination treatment beginning at 2.5 months of
age, and 2CA was removed from both treatment groups with $B_{12}$CN continued when mice reached 3.5 months of age. Homozygous PAD2 overexpressing mice were treated with either 2CA or a combination of 2CA plus $B_{12}$CN at 6 months of age at which time clinical signs of disease were observed.

2CA and $B_{12}$CN were diluted in phosphate buffered saline (PBS), pH 7.4, and sterilized through 0.2 μm PVDF syringe filters. 2CA group was treated with 5mg/kg 2CA via intraperitoneal injection three times weekly, and combination group was injected with 5mg/kg 2CA plus 15 mg/kg $B_{12}$CN three times weekly.

Clinical scoring for all ND4 and PAD 2 overexpressors was conducted twice weekly as described previously (Moscarello et al., 2002a). Briefly, animals were observed for clinical signs of demyelinating diseases including general body shaking, hindlimb/tail tremor, head tremor, wobbly gait, limp tail, balance, weakness, unsteadiness, seizure, and activity level. Animals were scored on a scale of 0-4, when 0 represented no signs and 4 represented severe signs. The score for each cohort was accumulated and averaged at the end of each week.

II. EAE mice

Clinical scoring of SJL/J and C57BL/6 mice was carried out daily after EAE induction. The clinical signs were measured using a standard scoring system for EAE signs, i.e. 0 – no signs, 1 – limp tail, 2 – inability of righting, 3 – paralysis of either hind limb or forelimb, 4 – full paralysis of both hind and forelimbs, 5 – moribund or death.
CREAE-induced SJL/J mice were separated into four groups according to the time when 2CA treatment started: day 14 (the peak of the acute phase), day 20 (the end of the acute phase), day 25 (the start of chronic phase), and day 30 (during the chronic phase). Treatment for C57BL/6 mice started at Day 8 after acute EAE induction when initial signs appeared. The 2CA and B_{12}CN were dissolved in PBS, pH 7.4, filter-sterilized through 0.2 μm PVDF syringe filter. For CREAE mice, 2CA treatment was conducted by 5mg/kg intraperitoneal injection three times weekly. For acute EAE mice, 2CA group was treated with 5mg/kg 2CA, and combination group was injected with 5mg/kg 2CA plus 15 mg/kg B_{12}CN every other day.

III. EAE-induced ND4 mice

Clinical scoring of ND4 mice was carried out daily for 2 wk after pertussis toxin injection using a standard scoring system for EAE signs as described previously. A group of 5-month-old ND4 mice was treated with 2CA (5 mg/ kg, 3 times weekly) 1 week after PTX injection as described earlier.

E. Histopathology

Brains from normal, untreated ND4, 2CA treated, and combination ND4 mice (all 6.5 months of age) were fixed in 10% formalin for 48 h at 4°C, embedded sagitally in paraffin, and sectioned to 5 μm thickness. Sections from the cerebellum of each brain were stained with Luxol Fast Blue (LFB) and hematoxylin for myelinated white matter and grey matter. Brains from normal CD1, untreated ND4, and pertussis toxin-treated ND4 mice (at 2 months of age or 5
months of age) were fixed in 10% formalin, embedded sagitally, sectioned to 5 μm, and stained with hematoxylin and eosin. Stained sections were imaged with an Olympus BX60 light microscope equipped with a CCD camera.

F. Immunohistochemistry

Immunohistochemistry of formalin-fixed, paraffin-embedded brain sections of normal CD1, untreated ND4, and pertussis toxin-injected ND4 mice (all at 5 months of age) using anti-CD3 antibody for T-cells and anti-GFAP antibody for astrocytes was performed by the Hospital for Sick Children Immunopathology Service Laboratory according to their standard operating protocols.

G. Electron Microscopy

Optic nerves from ND4 mice in 2CA study (normal, untreated ND4, treated ND4 mice at 6.5 months of age, and 2 months post-treatment ND4) were fixed with Karnovsky’s solution (2% paraformaldehyde, 1.25% glutaradehyde, 0.15 M CaCl₂ in 0.1 M sodium cacodylate buffer) for 7 d at 4°C. The nerves were then dehydrated in an ascending series of acetone, and infiltrated and embedded in plastic Embed 812. Ultrathin cross-sections of the optic nerves from each animal were prepared and mounted on copper grids, stained with uranyl acetate and lead citrate, and
viewed in a JEM-1230 transmission electron microscope (TEM) operated at 80 kV (JEOL U.S.A. Inc., Peabody, MA).

Sectioning and staining of all fixed mouse tissue samples were processed in the Division of Pathology, Department of Physiology & Experimental Medicine, the Hospital for Sick Children, Toronto.

H. PAD activity assay

Normal white matter and normal appearing white matter (NAWM) from individuals without neurological diseases and MS patients were homogenized in a HEPES buffer (50 mM HEPES pH 7.6, 1.0 mM ethylenediaminetetraacetic acid, 0.5 mM dithiothreitol, 0.43 mM phenylmethanesulphonyl fluoride, and 0.05% triton) and centrifuged at 12,000 rpm, 4 °C for 30 min in a Beckman Ti70.1 rotor. Freshly obtained C57BL/6 mouse spinal cord and spleen were processed using the same method as human white matter. Freshly obtained mouse brains were homogenized in double-distilled water and centrifuged at 40,000 rpm, 4°C for 1 hr in a Beckman Ti70.1 rotor. The supernatant was obtained, and protein concentration was determined by the method of Peterson (Peterson, 1977).

PAD activity assay was carried out as previously described (Lamensa et al., 1993). Briefly, 100 μL of each brain homogenate sample was used as enzyme source and incubated in 50 mM HEPES, 5 mM CaCl₂, 2 mM dithiothreitol and 12.5 mM alpha- N-benzoyl-L-arginine ethyl ester (BAEE) at 52°C for 30 min. The reaction was stopped by adding 100 μL of 5.0M
HClO₄. The reactants were centrifuged at 10,000 rpm at 4°C for 3 min. 450 μL of this supernatant was obtained to measure citrulline concentration using 0.5 mL of Reagent A (0.5% diacetyl monoxime, 15% NaCl) followed by 1 mL of Reagent B (0.1% antipyrine, 0.25% ferric ammonium sulfate, 25% H₂SO₄, 25% H₃PO₄). The mixture was boiled for 15 min and cooled on ice before the absorbance at 464 nm was measured. The PAD activity level was determined from a standard curve generated with various amounts of L-citrulline (0-20 μg). The PAD activity level was expressed in nmol citrulline/ min/ mg protein.

I. Immuno Slot Blot

Freshly obtained normal SJL/J, untreated CREAE, and 2CA treated CREAE mouse brains were homogenized in double-distilled water and centrifuged at 40,000 rpm, 4°C for 1 hr in a Beckman Ti70.1 rotor. The supernatant was obtained, and protein concentration was determined by the method of Peterson (Peterson, 1977). To measure citrullinated proteins, 10 μg protein extracts were applied onto a nitrocellulose membrane. The membrane was probed overnight at 4°C with mouse monoclonal antibody F95 specific to peptidylcitrulline. The membrane was washed four times with Tris-buffered saline containing Tween-20 (TBST), labeled with secondary goat anti-mouse monoclonal antibody conjugated to HRP for 1 h at room temperature, developed with enhanced chemiluminescence reagent after four additional washes with TBST, and exposed to X-ray film. The amount of citrullinated peptides was quantified from the pixel density analysis of immuno slot blot images with the Image-SXM image analysis software.
J. DNA Methylation Analysis

Brains were harvested from normal CD1, untreated, 2CA- treated, and 2CA plus B12CN- treated ND4 mice at 6 months of age. Genomic DNA was extracted from cortical white matter using QIAamp DNA Mini Kit (QIAGEN Sciences, Maryland, USA) according to manufacturer’s instructions. The mouse PADI2 gene sequence was identified by homology search of Mouse Protein Arginine Deiminase Type-2 cDNA using Ensembl database (www.ensembl.org). The flanking regulatory region spanning 1,000 bp from the methionine start codon was used for CpG island prediction. The criteria for choosing mouse Padi2 flanking regulatory region was consistent with our previous prediction on human Padi2 CpG island (Mastronardi et al., 2007a). Based on this prediction, a set of forward primer (5’ GTGGTAGAGGGGTGTTAGATATGA 3’) and reverse primer (5’ TAAAAAATAAAAACACTCCAAAAACAA 3’) was chosen.

To convert non-methylated cytosine residues to uracil, 2.0 µg of genomic DNA was treated with bisulfite using Epitect Bisulfite Kit (QIAGEN Sciences, Maryland USA) according to manufacturer’s instruction. Treated DNA was PCR-amplified with primers designed for bisulfite-modified DNA as previously described using a hot-start protocol (Qiagen, Mississauga, ON). PCR products were purified, sequenced using BigDye 3.1 chemistry and analyzed with the 3730 XL DNA Analyzer by Applied Biosystems in Foster City, CA.
K. Statistics

Clinical disease courses in different animal groups were compared by 2-way ANOVA test. PAD activities and peptidylecitulline levels in normal and MS groups were compared by parametric $t$ tests of paired individuals. All statistics were performed using Prism and Instat Software (GraphPad Software, San Diego, CA). All tests were two-tailed. $P < 0.05$ was considered as statistically significant.
The interest in PAD activity within MS brain in our laboratory initiated with the identification of a common biochemical change i.e. hypercitrullination of MBP in NAWM of MS brain which correlated with disease severity (Moscarello et al., 1994; Wood et al., 1996). Since the conversion of peptidyl arginine to citrulline is specifically catalyzed by PAD enzymes, elevation of PAD activity may be responsible for MBP citrullination and subsequent demyelination. This process of protein citrullination may be reversed with a PAD inhibitor. Thus the first part of my project was to compare PAD activities in normal and MS brain tissue and to examine whether 2CA can inhibit PAD activity in vitro in MS NAWM.

A. PAD activity in normal and MS white matter

In order to determine if PAD activity was increased with MS, homogenates were prepared from human brain white matter without neurological disease and normal appearing white matter (NAWM) from MS brains (Table 3-1). The homogenate was prepared in a HEPES (50 mM) buffer maintaining a physiological pH level at 7.6 with calcium-chelating agent EDTA (1.0 mM) to inhibit endogenous PAD enzyme.

PAD activity and protein concentration were measured in homogenate samples. Each sample was assayed for PAD activity by the absorbance at 464 nm using the method developed
by Lamensa and Moscarello (1993). Protein concentration was measured by the absorbance at 750nm using a modified Lowry’s assay specific for MBP developed by Peterson (1977). The specific PAD activity expressed as nmol citrulline per mg protein in MS white matter (17.0 nmol cit/min/mg protein) is significantly higher than that of normal white matter (12.4 nmol cit/min/mg protein) (p < 0.04, Fig. 3-1). The variation in PAD activity values of MS samples is possibly accounted by the differences in age and disease progression as well as the nature of autopsy samples. Samples from patients with secondary progressive MS showed the highest mean level of PAD activity (22.73 nmol cit/min/mg protein) possibly due to their disease severity and less protein degradation compared with that in primary progressive MS samples.
### Table 3-1. PAD activity of white matter from normal and MS brain samples.

<table>
<thead>
<tr>
<th>HSB#</th>
<th>Sample and Pathology*</th>
<th>PAD activity (nmol cit/min/mg protein ± s.d.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2181</td>
<td>Normal, MI (Male)</td>
<td>8.9 ± 0.7</td>
</tr>
<tr>
<td>2357</td>
<td>Normal, CHD, Hy, AF (Female)</td>
<td>11.5 ± 0.75</td>
</tr>
<tr>
<td>3080</td>
<td>Normal, AA, Pey, Ob (Male)</td>
<td>13.7 ± 0.02</td>
</tr>
<tr>
<td>3175</td>
<td>Normal, Pcy, DM, Hy (Female)</td>
<td>12.8 ± 0.84</td>
</tr>
<tr>
<td>3276</td>
<td>Normal, CHD, PVD, CRD (Male)</td>
<td>15.1 ± 1.3</td>
</tr>
<tr>
<td>3093</td>
<td>NA MS (Male)</td>
<td>18.5 ± 0.9</td>
</tr>
<tr>
<td>2935</td>
<td>2-MS (Female)</td>
<td>24.2 ± 0.58</td>
</tr>
<tr>
<td>3025</td>
<td>2-MS (Male)</td>
<td>15.1 ± 1.6</td>
</tr>
<tr>
<td>3185</td>
<td>1-MS (Male)</td>
<td>17.5 ± 1.1</td>
</tr>
<tr>
<td>2800</td>
<td>1-MS (Female)</td>
<td>8.24 ± 2.1</td>
</tr>
<tr>
<td>3509</td>
<td>1-MS (Female)</td>
<td>14.6 ± 0.7</td>
</tr>
<tr>
<td>3502</td>
<td>2-MS (Male)</td>
<td>26.2 ± 3.6</td>
</tr>
<tr>
<td>3928</td>
<td>2-MS (Female)</td>
<td>25.4 ± 1.6</td>
</tr>
<tr>
<td>3852</td>
<td>CP MS (Male)</td>
<td>11.6 ± 1.1</td>
</tr>
<tr>
<td>3840</td>
<td>1-MS (Female)</td>
<td>9.0 ± 0.3</td>
</tr>
<tr>
<td>3413</td>
<td>RR MS (Female)</td>
<td>17.1 ± 2.5</td>
</tr>
</tbody>
</table>

Homogenates were prepared from both normal human brain white matter and normal appearing white matter from MS brains for measurement of PAD activity with substrate alpha- N-benzoyl-L-arginine ethyl ester in the presence of Ca\(^{2+}\). *NA MS- MS subtype unavailable; 1-MS – primary progressive MS; 2-MS – secondary progressive MS; CP-MS – chronic progressive MS; RR-MS – relapse remitting MS; MI- myocardial infarction; CHD- coronary heart disease; Hy- hypothyroidism; AF- atrial fibrillation; AA- aplastic anemia; Pcy- pancytopenia; Ob- obesity; DM- diabetes mellitus; CHF- Congestive heart disease; PVD- peripheral vascular disease; CRD- Coronary renal disease.
Fig. 3-1. PAD activity in normal appearing white matter from MS brain compared with normal. Total PAD enzyme activity in normal brain white matter (n = 5) and MS normal appearing white matter (n = 11). PAD activity was calculated as nmol citrulline/ min/ mg protein.
B. Inhibition of PAD activity by 2CA in MS brain homogenate

Since the PAD activity in NAWM of MS brain is significantly higher than that of normal white matter, MS NAWM homogenate was allowed to react with 2CA in order to determine whether 2CA inhibits PAD activity in brain homogenate \textit{in vitro}. MS NAWM homogenate (100 μL, typically at a protein concentration of ~2 mg/ml) was pre-incubated with increasing amounts of 2CA (0 to 1500 μg or 0 to 26.7 μg) in the presence of Ca$^{2+}$ \textit{before} a PAD activity assay was conducted. The result revealed a ~20% inhibition of PAD activity in NAWM to a level comparable to that of normal white matter when 15 μmol or greater amounts of 2CA was added to the homogenate (Fig. 3-2).
Fig 3-2. Inhibition of PAD activity by 2CA with standard deviation in normal appearing white matter from human brain tissue. Pre-incubation of MS normal appearing white matter (n = 11) with 10 mmol or greater amounts of 2CA resulted in approximately 25% inhibition in PAD activity.

In summary, specific PAD activity per mg total protein was found to be significantly elevated in MS NAWM compared to that in normal WM (~1.37 fold). This elevation in PAD activity is consistent with elevated PAD 2 protein and citrullinated protein levels in MS brain as shown in immuno-slot-blot analysis with antibodies for PAD 2 and peptidyl citrulline by our lab. The increase in PAD activity may be accounted for MBP hypercitrullination in MS brain previously identified in our lab (Moscarello et al., 1994). 2CA, an active site inhibitor of recombinant PAD 2 in vitro, has been demonstrated to inhibit PAD activity in NAWM. This provides the basis for my subsequent in vivo study with various mouse models of MS.
Chapter 4
Inhibition of PAD in neurodegenerative mouse models of MS

Since 2CA has been found to effectively inhibit recombinant PAD activity and PAD activity in MS brain homogenates in vitro, I therefore became interested in examining the therapeutic effect of this PAD inhibitor in vivo in independent mouse models of MS. The first two models are neurodegenerative mouse models (ND4 and PAD2 overexpressors) which display a disease etiology directly related to protein hypercitrullination.

A. ND4 mouse model

ND4 neurodegenerative mouse model was developed in our lab by incorporating 70 extra copies of cDNA for DM 20 protein, a splice variant of myelin PLP (Mastronardi et al., 1993). The function of the DM20 protein has not been defined, but ND4 transgenic mice overexpressing DM20 undergo biochemical changes in MBP resulting in higher proportion of the less cationic components (C8) reminiscent of the changes in MBP found in MS prior to the onset of a spontaneous, progressive demyelination at 3 months of age (Mastronardi et al., 1996). Most animals become moribund by 8 -10 months of age thus making ND4 a good model to study for progressive MS. Microheterogeneity in MBP of this transgenic line was changed resulting in a higher proportion of the citrullinated and less cationic components reminiscent of the changes in MBP found in MS brain (Mastronardi et al., 1997). This post-translational modification on MBP
at 3-4 months of age was preceded by PAD 2 up-regulation at 2 months of age (Mastronardi et al., 2002). Thus the inhibition of PAD activity may target the basic mechanism of disease in this particular model. My project focused on examining the effect of PAD inhibition after the onset of disease and at the onset of PAD overexpression before disease onset. Two reagents were employed in attempt to inhibit PAD activity: 2CA, a PAD active site inhibitor, and Vitamin B$_{12}$ (cyanocobalamin) (B$_{12}$CN), a universal methyl donor to suppress PAD 2 transcription by methylating PAD 2 promoter site (Mastronardi et al., 2007b).

I. Clinical score after treatment with 2CA

ND4 mice were treated with either 2CA or a combination of 2CA and B$_{12}$CN beginning at 3.5 months of age after the appearance of clinical signs of demyelination (Fig. 4-1a) and scored twice weekly thereafter. Treatment was stopped when animals reached 6.5 months of age. Non-treated ND4 mice and mice from each treatment group were euthanized upon completion of treatment. The remaining animals were observed for clinical progression without further treatment for additional 1.5 months until they reached 8 months of age. Scoring was done by 2 independent observers.

2CA-treated mice showed fewer clinical signs than untreated ND4 mice (p < 0.01) at 6.5 months of age, but the residual signs were clinically significant (clinical score > 10). Mice receiving combination treatment showed significantly reduced clinical signs in comparison with non-treated animals (p < 0.001) to a level below clinical significance (clinical score < 10, Fig. 4-1a shaded area) at 6.5 months of age. Combination treatment with B$_{12}$CN extended the therapeutic effect of 2CA alone. Upon attenuation of treatment, clinical score of 2CA-treated
group rapidly rebounded after only 1 to 2 weeks post treatment, and the clinical signs continued to worsen up to 8 months of age (Fig. 4-1a). Clinical signs of the combination-treated group worsened as well, but more slowly than the 2CA-treated group (p <0.001).

To determine whether PAD inhibition prior to the disease onset could prevent disease, I treated ND4 mice with 2CA or 2CA plus B₁₂CN at two months of age prior to the onset of demyelination (Fig. 4-1b). Both treatment groups had no clinical signs throughout the treatment period of 4.5 months. Thus PAD inhibition by 2CA alone before disease onset prevented the onset of disease.

To examine the role of B₁₂CN in the prevention of demyelination, ND4 mice were treated with 2CA or 2CA plus B₁₂CN at 2.5 months of age before disease onset. 2CA treatment was stopped but B₁₂CN continued from 3.5 to 6 months of age (Fig. 4-1c). The clinical score indicated that removal of 2CA resulted in a rapid rebound of disease though continuation of B₁₂CN treatment showed some attenuation of clinical signs. Thus treatment of B₁₂CN alone was not sufficient for prevention of demyelinating disease, and 2CA treatment was necessary to produce the full effect reducing signs to the level below clinical significance. The beneficial effect of B₁₂CN is possibly explained by the methylation of PAD2 promoter thereby suppressing enzyme transcription which will be examined further in this chapter.
Mean clinical scores of non-treated and treated ND4 mice

Effect of treatment before disease
Fig. 4-1. Mean clinical scores of non-treated and treated ND4 mice. (a) Treatment began after the onset of disease at 3.5 months of age. The shaded region represents normal phenotypic range of clinical signs (score < 10). At 6.5 months of age when treatment stopped, mean clinical score of treated mice (n = 7 for both 2CA and 2CA plus B_{12}CN groups) was significantly reduced (p < 0.001) from the untreated mice (n = 4). Clinical signs were monitored for further 1.5 months during which time disease rebounded. The p value < 0.001 indicated that 2CA post-treatment group exhibited significant more severe disease at 8 months of age than combination post-treatment group. (b) Treatments started at 2 months of age before disease onset as indicated by the vertical arrow. The shaded region represents normal phenotypic range of clinical signs (score < 10). The p value of <0.001 indicates a reduction of clinical signs at 6 months of age between both 2CA (n = 5) and 2CA plus B_{12}CN (n = 6) treatment groups from the untreated controls (n = 2). (c) Treatment with 2CA (n = 3) and 2CA + B_{12}CN (n = 3) began at 2.5 months of age. Treatment of 2CA stopped but B_{12}CN was continued at 3.5 months of age. Removal of 2CA resulted in disease rebound much quicker (p < 0.01) in the 2CA group than the 2CA + B_{12}CN group.
II. PAD activity in brain

To determine whether reduction of clinical signs was associated with decreased PAD activity, total brain homogenate of mice 6.5-month-old from normal and treatment groups was used to measure PAD activity with BAEE (Fig. 4-2). ND4 mice of 6.5 months of age at 3 months after onset of clinical signs displayed a significantly elevated PAD activity level in the brain than that of the CD1 littermate controls (p < 0.05). The results indicated that 2CA treatment decreased PAD activity level significantly (p < 0.05) at 6.5 months of age and 2CA plus B12CN further reduced PAD activity to a level about half of the untreated group. Both post-treatment groups at 8 months of age demonstrated an elevated PAD activity level above that of the untreated control (p < 0.05) suggesting that disease rebound was associated with increased PAD activity and thus 2CA was responsible for the beneficial effects.
Fig. 4-2. PAD enzyme activity in treated, untreated ND4 and normal mouse brain homogenate. The PAD activities in brain homogenate from 2CA (n = 2) and 2CA + B12CN (n = 2) treated groups were compared with activities in brains from normal (n = 5) and non-treated ND4 (n = 4). The level of PAD activity in normal or treated ND4 brains was significantly lower than that of non-treated ND4 (p < 0.05). The PAD activity in brain homogenate from 2CA post treated (n = 2) animals was dramatically elevated to a level above that of non-treated ND4 mice.
III. Morphological changes in the central nervous system

Histopathological evidence of demyelination in 6.5-month-old normal, untreated ND4 mice, 2CA- treated ND4 mice, and 2CA plus B12CN- treated ND4 mice was examined by Luxol fast blue (LFB) hematoxylin staining of cerebellar sections (Fig. 4-3). Less staining was observed in non-treated mice indicative of extensive demyelination. A moderate loss of staining was observed in 2CA treated mice suggesting less severe loss of myelin. Combination treatment restored the LFB hematoxylin stain to a level almost comparable to that observed in normal mice suggestive of remyelination.
Fig. 4-3. Luxol fast blue hematoxylin histological stain of cerebellum from normal (Normal), non-treated ND4 (ND4 no treatment), 2CA treated ND4 (ND4 2CA treatment), and 2CA + B12 treated ND4 (ND4 2CA+B12 treatment) at 6.5 months of age. Cerebellum was stained for myelinated white matter (WM, blue area) and grey matter (GM, purple area). Demyelination was evident in non-treated and 2CA treated ND4 mice by the loss of staining. Combination treatment restored the staining level comparable to that observed in normal mice. The optical magnification of all images was at 50 x.
Morphological evidence of remyelination in mouse optic nerve was also examined by transmission electron microscopy (TEM) (Fig. 4-4) in collaboration with C.A. Ackerley in the Division of Pathology, Department of Physiology & Experimental Medicine, the Hospital for Sick Children, Toronto. A section of untreated ND4 mouse optic nerve showed extensive myelin loss (arrow) and a large amount of demyelinated nude axons (asterisk) (Fig. 4-4A), while intact, myelinated axons (Ax) were observed in normal mouse optic nerve (Fig. 4-4B). A similar section from 2CA-treated mice revealed some myelinated axons (Fig. 4-4C), and combination treatment of 2CA plus B12CN restored level of myelinated axon (Ax) comparable to that of normal mice (Fig. 4-4D). However, demyelinated axons (asterisks) were evident in 2CA and combination treated mice 2 months post treatment suggesting that the removal the treatment resulted in renewed demyelination (Fig. 4-4E, Fig. 4-4F).
Fig. 4-4. Morphological evidence of improved myelination after 2CA and 2CA plus B$_{12}$CN treatments. (A.) Electron micrographs of optic nerve from non-treated, 6.5-month-old ND4. Axons are indicated as “Ax”. Demyelination (arrow) was extensive. Demyelinated axons are indicated by asterisks enclosed within the white boundary. (B.) Normal mouse optic nerve with intact, myelinated axons (Ax). (C). 2CA- treated ND4 mice revealed some myelinated axons (Ax). (D). Combination treatment of 2CA and B12 restored level of myelinated axon (Ax) comparable to that of normal mice. (E). 2CA-treated ND4 mice 1.5 months post-treatment. Demyelinated axons (asterisks enclosed within the white boundary) were evident. (F). 2CA + B12 post-treatment mice with visible demyelination (arrow). Demyelinated axons (asterisks) and myelinated axons (bracketed) were evident. Bar lines = 2 µm.
IV. DNA methylation in cortical white matter

Previous studies have demonstrated an enhanced efficacy of immune suppressive therapies on ND4 mice when we combined treatments with B_{12}CN (Mastronardi et al., 2007a). In a previous study we also found hypomethylation of promoter of PAD2 gene in MS NAWM (Mastronardi et al., 2007b). Thus we propose the enhanced efficacy of 2CA plus B_{12}CN treatment is possibly due to methylation of the promoter on CpG islands in the mouse PAD2 gene. To determine the effect of the inclusion of B_{12}CN on DNA methylation, ND4 mice were treated with either 2CA or a combination of 2CA and B_{12}CN beginning at 3.5 months of age. DNA was extracted from cortical white matter of normal, untreated ND4, treated ND4 mice at 6.5 months of age after the treatment period of 3 months, and the methylation level of 11 CpG sites in Padi2 gene promoter was analyzed. Significant difference was detected on methylation level of 5 CpG sites between combination treated, and 2CA mono-treated or untreated ND4 mouse brains (Table 4-1). Since Dr. Zhen Li in our lab has also found a significant decreased PAD2 transcription in 2CA plus B_{12}CN mouse brain compared with non-treated or 2CA mono-treated group by a Real-time PCR analysis (Fig. 4-5), preliminary results suggested partial methylation on PAD2 promoter may account for the decreased PAD2 transcription and lower clinical score of combination treated animals.
Table 4-1. Methylation analysis on selective CpG sites of mouse Padi2 gene.

<table>
<thead>
<tr>
<th></th>
<th>CpG #20</th>
<th>CpG#23</th>
<th>CpG#25</th>
<th>CpG#27</th>
<th>CpG#28</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>3.8</td>
<td>3.1</td>
<td>1.3</td>
<td>9.9</td>
<td>2.5</td>
</tr>
<tr>
<td>Untreated</td>
<td>3.1</td>
<td>3.6</td>
<td>2.1</td>
<td>7.2</td>
<td>2.7</td>
</tr>
<tr>
<td>2CA-treated</td>
<td>3.1</td>
<td>2.5</td>
<td>0.9</td>
<td>9.1</td>
<td>2.7</td>
</tr>
<tr>
<td>2CA+B₁₂CN-treated</td>
<td>5.4</td>
<td>5.9</td>
<td>5.6</td>
<td>19.0</td>
<td>5.5</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Ratio of Treated/untreated</th>
</tr>
</thead>
<tbody>
<tr>
<td>2CA-treated</td>
<td>1.0</td>
</tr>
<tr>
<td>2CA+B₁₂CN-treated</td>
<td>1.7</td>
</tr>
</tbody>
</table>

DNA Methylation level of mouse cortical white matter in normal CD1, non-treated, 2CA treated, and 2CA+B₁₂ treated ND4 mice. Treatment began after the onset of disease at 3.5 months of age. At 6.5 months of age when treatment stopped, methylation level of 5 CpG sites (out of 11 CpG sites) in mouse Padi2 gene promoter was found to be elevated in 2CA+B₁₂CN treated animals compared to that of non-treated or 2CA treated group. The number represents the average of 2 independent determinations.
Fig. 4-5. RT-PCR of PAD 2 mRNA from ND4 mouse brain white matter. Levels of mRNA from white matter of mice treated with 2CA, 2CA + B12CN, non-treated ND4 compared with non-transgenic littermates. The p values of non-treated ND4 compared with the combination treated group indicated a significant change in transcription level.
B. PAD 2 overexpressing mouse model

The PAD2 overexpressing mouse was recently generated by incorporating 30 copies of cDNA for PAD2 (padi2) directed to the oligodendrocyte by the MBP promotor in CD1 mouse genome generating a homozygous PAD2 transgene (Musse et al., 2008). Thus PAD2 overexpression was causal to the neurodegenerative demyelination in this particular animal model.

I. Clinical score and PAD activity in brain

To examine the therapeutic effect of PAD inhibition in this transgenic line overexpressing PAD2 enzyme, Dr. Zhen Li of our lab treated a group of PAD2 overexpressing mice with 2CA and a second group with 2CA plus B12CN at 6 months of age at which time they started to demyelinate. Attenuation of clinical signs was observed by Dr. Li in both groups during the treatment period of 3 months (Fig. 4-5a). In this model, the addition of B12CN to the treatment had no additional effect on the clinical signs probably because no change in the methylation status of the PAD2 promoter was expected in this model.

I measured PAD activities in these mice and demonstrated that PAD activities were significantly reduced in the 2CA treated group (5.35 nmol cit/min/mg protein) and combination treated group (4.18 nmol cit/min/mg protein) than the non-treated control (7.46 nmol cit/min/mg protein) consistent with clinical scores (Fig. 4-5b). Therefore, the 2CA effect was observed when PAD enzymes were elevated either by promoter hypomethylation or increased gene dosage.
Fig. 4-6. Attenuation of clinical signs and PAD activity in the PAD2 overexpressing transgenic mice by 2CA treatment. (a). Mean clinical scores of PAD2 transgenic mice, homozygous for the PAD2 transgene, untreated (n = 3), treated with 2CA (n = 4) and 2CA plus B12CN (n = 4). The shaded region represents normal phenotypic range of clinical signs (< 10). The p value of <0.001 indicates that mean clinical signs were significantly reduced in both treated groups than non-treated control at 9.5 months of age. (b). PAD activities in PAD2 (n = 2)
and non-transgenic CD1 (n = 2) mouse brain homogenates. The p values of <0.01 and <0.001 indicates a significant difference between 2CA/2CA plus B_{12}CN (n = 3 for both treatment groups) and non-treated controls at 9.5 months of age.

In summary, the therapeutic effect of PAD inhibition was examined in two neurodegenerative mouse models of MS which display a disease etiology directly related to protein hypercitrullination by PAD overexpression. Two different reagents were used in an attempt to decrease PAD activity: 2CA, an active site inhibitor to all PAD enzymes, and B_{12}CN, a methyl donor possibly re-methylating Padi 2 gene promoter.

In ND4 (DM20 overexpressing) transgenic line, the treatment of 2CA alone at around 2 months of age when PAD 2 activity just became elevated before the onset of demyelination was able to prevent the disease onset. Treatment of 2CA at the onset of demyelination was able to halt disease progression and improve myelin morphology in the CNS possibly by inhibition of PAD activity in the brain where most pathological features were presented. Combination treatment with 2CA and B_{12}CN further extended the beneficial effects possibly by partial methylation of the promoter on CpG islands in the mouse PAD2 gene as described in our gene methylation analysis on DNA isolated from ND4 mouse cortical white matter. However, the therapeutic effect of B_{12}CN alone was limited as our lab previously demonstrated that B_{12}CN mono treatment was not able to suppress disease in either neurodegenerative ND4 mice or MS autoimmune mouse model (Mastronardi et al., 2007b). Furthermore, the evidence that removal of 2CA resulted in a rapid rebound of disease despite the continuation of B_{12}CN treatment in ND4 cohort with combination treatment at 2.5-month-old mice indicated the 2CA has to be administrated to produce a full treatment effect in this animal model.
In PAD 2 transgenic line, both treatments with 2CA or 2CA plus B\textsubscript{12}CN at the onset of disease was able to attenuate disease throughout the treatment period with PAD activity normalized in mouse brains. Since PAD 2 transgenic mice overexpressing PAD mainly by increased copies of gene and driven by a “recombinant” MBP promoter, the added beneficial effect of B\textsubscript{12}CN was not as obvious due to that Padi 2 gene promoter was not expected to be hypomethylated and thus methylation on CpG islands was not a factor.
Chapter 5

Inhibition of PAD in autoimmune models of MS

The above experiments have shown that in these two neurodegenerative models of MS, 2CA decreased PAD activity, attenuated disease, and induced remyelination. To further examine the therapeutic effect of PAD enzyme inhibition, I employed the experimental autoimmune (allergic) encephalomyelitis (EAE) models of MS which represent an autoreactive T-cell disorder directed against central nervous autoantigens leading to inflammatory demyelination (Sriram and Steiner, 2005). In addition to the utilization of EAE to study MS pathogenesis, EAE models have also been harnessed for developing therapeutic strategies, as the majority of the current MS disease-modifying therapies being planned for phase II and III trials were initially conducted on EAE animals (Steinman et al., 2006).

A. Chronic relapsing experimental autoimmune encephalomyelitis (CREAE) mice

This chronic relapsing EAE mouse model involves the sensitization of T-cells with myelin proteolipid protein (PLP), which is the most abundant myelin protein. CREAE mice experience various phases of disease including an early acute phase when the disease reaches the peak and a later chronic relapsing phase with alternating periods of disease rebound and transient recovery. Thus, CREAE model is an ideal model to study RRMS, the most common subtype of MS.
I. PAD activity during disease progression

To determine whether the role of protein hypercitrullination is also involved in the pathogenesis of an autoimmune model of demyelination, CREAЕ was induced in SJL/J mice by the injection of myelin proteolipid protein (PLP) peptide 139-155 in collaboration with Dr. Shawn Winer in Dr. Dosch’s lab. The pathogenesis of CREAЕ model is traditionally studied by the inflammatory responses including its immune cell profile and cytokine/chemokine secretion in addition to its pathophysiology, but the role of protein hypercitrullination is rarely investigated (Raijmakers et al., 2005). Therefore, to determine whether PAD activity in the CNS is associated with the disease progression of CREAЕ mice, animals were scored daily using a standard score for measuring EAE signs (Fig. 5-1a) while PAD activity in mouse brain was measured at various stages of disease development with our standard method using the substrate BAEE (Fig. 5-1b).

The results indicated that clinical score did not increase until after day 10 initiating the acute phase of EAE. Clinical scores reached maximum at day 16 which was at the peak of the acute phase, decreased slightly from day 17 to day 20 suggestive of the end of acute phase, and leveled off after day 21 suggestive of the start of the chronic phase. Consistent with the clinical scores, PAD activities were not significantly elevated on day 9 compared with those on day 0, but peaked at day 16 in the acute phase of CREAЕ. PAD activity during the chronic relapsing phase (day 20 and day 35) was lower than that in the acute phase (day 16) but remained significantly higher than that before disease onset (day 9). Therefore, increased PAD activity during disease progression of CREAЕ mice was associated with severity of clinical signs.
Fig. 5-1. Mean clinical scores and PAD enzyme activities of non-treated mice during CREAE progression. (a). Clinical progression of CREAE in SJL/J mice (n = 14) over 45 days. Mean clinical scores of non-treated mice were plotted with standard error of means (SEM). (b). PAD activity in mouse brain during CREAE progression (n = 3 at each time point). Consistent with clinical scores, PAD activity peaked at Day 16 when the mean clinical score was maximal. The p value of < 0.001 indicates a significant difference between PAD activities at Day 16 and Day 0. PAD activity remained high during the chronic phase.
II. EAE scores during treatment with 2CA

Since the disease severity of this CREAE model of demyelination was correlated with PAD activity in brain, our lab became interested in examining the therapeutic effect of PAD inhibition in this model. To determine whether inhibition of PAD is beneficial for the autoimmune model of demyelinating disease, 2CA treatment was started on CREAE mice at different phases of disease as determined by the clinical score (Fig. 5-2a). Mice were separated into four groups according to the time when 2CA treatment started: day 14 (the peak of the acute phase), day 20 (end of the acute phase), day 25 (the start of chronic phase), and day 30 (during the chronic phase). Treatment continued until day 40 when mouse brains were removed, and homogenates were prepared to measure PAD enzyme activity (Fig. 5-2b). The results indicated that 2CA treatment reduced mean clinical scores and PAD activity at all stages of disease indicative that PAD activity was associated with disease progression of CREAE mice.
Fig. 5-2. Attenuation of disease in CREAE by PAD inhibition. (a). Attenuation of clinical signs in CREAE by 2CA treatment. Mean clinical scores were plotted for non-treated (n = 14) and 2C-treated mice (n = 19). Treatments started on day 14 (acute phase), day 20 (end of acute phase), day 25 (chronic phase), and day 28 (chronic phase) as indicated by the arrows. (b). PAD activity in 2CA-treated mouse brain. All treatment groups (Day 14, Day 20, Day 25, and Day 28, n = 5 in each group) exhibited a reduced PAD enzyme activity level compared to that of non-treated CREAE (n = 5) at Day 40.

III. PAD activity and peptidylcitrulline in brain after treatment

Since citrullinated proteins are generated by PAD activity, citrullinated proteins were quantified by immune-slot-blot analysis of mouse brain homogenates. These results shown in Fig. 5-3 indicate a significant reduction of citrullinated proteins in 2CA-treated mice at all stages of disease (n = 10), compared with citrullinated proteins in untreated CREAE (n = 5), consistent with the decreased PAD activity. Therefore, the attenuation of disease in this autoimmune model of demyelination was associated with the reversal of protein hypercitrullination by PAD inhibition.
Fig. 5-3. Normalization of citrullinated proteins in 2CA-treated chronic relapsing EAE mice. CREAE mice in all treatment groups (Day 14, Day 20, Day 25, and Day 28, n = 12) exhibited a reduced citrullinated peptide level compared to that of non-treated CREAE at Day 40 (n = 2)(p < 0.001).

2CA has also been shown effective in reducing inflammatory demyelination in this autoimmune model as demonstrated by decreasing the secretion of inflammatory cytokines interferon-γ and interleukin-17 in splenocyte cultures. Therefore PAD inhibition is a potential therapeutic strategy for MS since it targets both neurodegenerative and inflammatory pathways involved in myelin stability and immune regulation.
B. Acute experimental autoimmune encephalomyelitis (EAE) mouse model

The immune response of this type of acute EAE model is elicited by inoculating with CNS antigen myelin oligodendrocyte glycoprotein (MOG) peptide 35-55. MOG is a transmembrane myelin protein expressed on the surface of oligodendrocyte cell and on the outermost surface of the myelin sheath. The function of MOG was speculated to provide adhesion and maintain structural integrity to the myelin sheath, to regulate oligodendrocyte microtubule stability, and to mediate interaction between myelin and the immune system (Johns and Bernard, 1999). Unlike the CREAE model induced by PLP peptide described in the previous section, pertussis toxin (PTX) is also used as an ancillary adjuvant to elicit autoimmune responses. The mechanism whereby PTX potentiates EAE response is unknown. Recent evidence proposed PTX may increase permeability of blood-brain barrier (BBB) by altering endothelial plasticity and angiogenesis (Lu et al., 2008). Therefore, acute EAE develops over days in this type of model resulting in cytotoxic demyelination and often fatal adverse effects possibly as a result the possible side effect of PTX toxicity on other organ’s plasma membrane.

I. Clinical score and survival rate during the treatment

To confirm if this autoimmune model of demyelination could be attenuated by 2CA or 2CA plus B12CN combination treatment, progressive EAE was induced in C57BL/6 mice by the injection of myelin oligodendrocyte protein peptide 35-55 with 300 ng or 100 ng PTX in two separate cohorts in collaboration with Dr. Jason Yantha and Geoff Paltser in Dr. Dosch’s lab. Since this animal model typically experiences aggressive disease progression, 2CA or
combination treatment was started on day 8 when initial EAE signs appeared. The results indicated a significantly improved survival rate at 90% in both 2CA and combination treated groups (p <0 .05) at day 30 in the cohort induced with 100 ng PTX(Fig. 5-4a). In the cohort injected with 300 ng PTX, 2CA and combination treated groups exhibited 40% and 60% survival rate respectively at day 30, whereas none in the control group survived beyond day 19 (Fig. 15-4b). A significant attenuation of EAE signs (p < 0.05) was observed in both treatment groups in cohort induced with 100 ng PTX (Fig. 5-5a). The treated groups in cohort induced with 300 ng PTX also exhibited decreased EAE signs during the treatment period of 22 days (Fig. 5-5b).
Fig. 5-4. Enhanced survival rate in 2CA and 2CA plus B₁₂CN treated acute EAE mice. (a). EAE mice induced with 100 ng PTX in 2CA (n = 10) and combination treatment (n = 10) groups exhibited an improved survival rate since day 15 after EAE induction compared to that of PBS control (n=5)(p < 0.05). (b). 2CA (n= 10) and combination (n= 10) treated EAE mice induced with 300 ng PTX exhibited a survival rate of 90% at day 30 after EAE induction.
Fig. 5-5. Mean clinical scores of PBS control and treated acute EAE mice during disease progression. (a) Attenuation of disease in EAE mice induced with 100 ng PTX by 2CA (n = 10) and 2CA plus B12CN (n = 10) treatment (p < 0.05) at day 19. (b). A significant attenuation of disease in EAE mice induced with 300 ng PTX was achieved with 2CA plus B12CN (n = 10) treatment, whereas 2CA treatment (n = 5) moderately decreased clinical score. Mean EAE scores were plotted for non-treated, and combination treated mice with standard errors of mean. Arrows indicate the starting of treatment at day 8.
II. PAD activity in brain, spinal cord, and spleen.

To determine whether PAD activity in the CNS and immune system is associated with the beneficial effect of 2CA, brain, spinal cord and spleen were obtained from EAE–induced mice at the end of treatment period to measure PAD activity (Fig. 14). Although PAD activity was not found to be significantly elevated in non-treated animals compared to normal, PAD activity was reduced (~ 25% compared with non-treated animals) in brain and spleen of both 2CA and combination groups (Fig. 5-6a, b). PAD activity in EAE spinal cord (22.01 nmol cit/min/mg protein) was found to be significantly elevated from normal (6.91 nmol cit/min/mg protein) suggesting that disease in this model was mainly associated with elevated PAD activity in spinal cord (Fig. 5-7c). Treatment with 2CA and 2CA plus B₁₂CN both normalized PAD activity in spinal cord demonstrating that the beneficial effect was primarily due to 2CA on spinal cord.
a). PAD activity in acute EAE brain

b). PAD activity in acute EAE spleen

c). PAD activity in acute EAE spinal cord

[ < 0.05]
Fig. 5-6. PAD inhibition by 2CA and 2CA plus B12CN treatment in EAE mouse organs. PAD activity was not significantly elevated in non-treated progressive EAE (a) brain (n = 5) and (b) spleen (n = 5) compared to that in normal C57BJ/6 brain (n = 5) and spleen (n = 2). Both 2CA and combination treatments achieved a moderate inhibition in PAD activity in EAE brains and spleen. (c). PAD activity was significantly elevated in PBS-treated EAE spinal cord (n = 5) to a level three-fold that of normal (n = 4). Both 2CA (n = 4) and combination (n = 5) treatment significantly decreased PAD activity in EAE spinal cord (p < 0.05).

In summary, the therapeutic effect of PAD inhibition was examined in two autoimmune mouse models of MS in which the role of protein hypercitrullination remains unknown. Two different reagents were used in an attempt to decrease PAD activity: 2CA, an active site inhibitor to all PAD enzymes, and B12CN, a universal methyl donor which our lab have previously employed in combination with immunosuppressant to treat various mouse models of MS (Mastronardi et al., 2007b).

The result indicated that PAD activity in CREAE brain was correlated with EAE disease severity, i.e. PAD activity did not increase before the onset of EAE, reached maximum coinciding with the peak of the acute phase, and leveled off during the chronic relapsing phase of EAE. This finding on the association of PAD activity in brain and disease severity was consistent with the previous report of citrullinated proteins in spinal cord of CREAE animals which increased with disease progression (Raijmakers et al., 2005). Treatment of 2CA starting at different stages of disease produced an immediate beneficial effect on suppressing EAE signs regardless of the disease stage. This beneficial effect was associated with a decreased PAD activity as well as normalized citrullinated protein levels in brain of treated CREAE animals.

In my study with acute EAE animals, 2CA treatment was found to improve survival rate and suppress EAE signs mainly during the chronic phase of disease although 2CA was not found to alter the disease progression. The therapeutic effect of 2CA was mainly associated with a
significant, 3-fold reduction in PAD activity in spinal cord. Similar pattern of PAD inhibition was found in brain and spleen which is important in immune regulation although the decrease was not as large. This is consistent with previous observation that acute EAE displayed more severe demyelination and inflammation in spinal cord and optic nerves rather than in brain. The synergistic effect of B_{12}CN was observed in the cohort treated with a higher dosage of PTX (300 ng). Although the mechanism is uncertain, B_{12}CN possibly exerts its beneficial effect by facilitating remethylation since methylation of MBP is an essential requirement for myelination (Kim et al., 1997).
Chapter 6

Induction of EAE in a neurodegenerative mouse model of MS

The four models of MS I have used to investigate the therapeutic effect of 2CA are incomplete each in its own way. Although EAE has been the most studied model of MS, it is not a spontaneous disease since a mandatory induction by injection of myelin antigens is required (Mix et al., 2008). On the other hand, the spontaneous neurodegenerative model of ND4 mouse is limited by a lack of inflammatory response and T-cell activation in brain. In this chapter, I described an attempt to generate a unified model for MS based on our neurodegenerative ND4 model that has undergone protein hypercitrullination.

ND4 mice are designed to overexpress DM20, a splice variant of PLP protein which is a major known CNS antigen eliciting immune response and paralytic disease in EAE mice (Sriram et al., 2005). During active demyelinating phase after PAD overexpression, increased citrullination of MBP leads to subsequent myelin destabilization and the release of immunogenic peptides. Injection of pertussis toxin which opens the blood brain barrier will allow inflammatory cells to migrate into the CNS. In the presence of myelin antigens, sensitized cells may be formed possibly in the brain. Alternatively, the release of immunogenic peptides may sensitize T-cells in the periphery which later migrate into the CNS resulting in an inflammatory response similar to that of EAE mice superimposed upon the neurodegeneration. If successful, this study will suggest that a non-specific inflammatory response associated with biochemical change including protein hypercitrullination in brain results in an EAE-like disease without injecting a myelin antigen. This combined inflammatory and neurodegenerative disease may represent a better animal model than either alone.
A. Clinical scores after induction of EAE

To induce EAE in a neurodegenerative mouse model of MS, spontaneous demyelinating ND4 mice were injected intravenously with 200 ng pertussis toxin (PTX) at 2 months of age before onset of neurodegeneration (n = 2) or at 5 months of age during myelin degradation which releases myelin antigens (n = 5). Animals were observed for EAE signs using the same scale as the EAE scoring system described in Chapter 5. Animals treated with PTX at 2 months of age showed no clinical signs of EAE 2 weeks following PTX injection (clinical score = 0) as shown in Fig. 6-1. Mice at 5 months of age displayed obvious EAE signs 1 week after PTX injection and progressed to a moderate level of EAE disease (clinical score = 2) 2 weeks after induction.

To determine whether EAE induction is possibly associated with change in PAD activity, a group of ND4 mice were treated with 2CA 1 week after PTX injection (n = 5). Animals were scored daily for clinical signs of EAE (Fig. 6-1). Clinical signs of 2CA-treated, EAE-induced mice worsened as well, but more slowly than non-treated, EAE induced-ND4 mice (p < 0.05).
Fig. 6-1. Mean EAE scores of 2-month-old PTX-injected ND4, 5-month-old PTX-injected ND4, and 5-month-old PTX-injected, 2CA-treated ND4. EAE was induced in ND4 mice at 2 months or 5 months of age by intravenous injection of 200 ng PTX. ND4 mice at 2 months of age (n = 2) before onset of neurodegeneration displayed no EAE clinical signs in 2 week post PTX-injection. ND4 mice received PTX-injection at 5 months of age after onset of demyelination developed obvious EAE signs 1 week post injection when 2CA treatment started as indicated by the vertical arrow. The p value < 0.001 indicated that 2CA treated EAE-induced group (n = 3) exhibited significantly less severe EAE progression 2 week post PTX injection than the untreated, EAE-induced group (n = 5).

B. Treatment with 2CA and measurement of PAD activity

To determine whether induction of EAE signs and therapeutic effect of 2CA were associated with changes in PAD activity, total brain homogenate from normal, untreated ND4, PTX-injected ND4, and PTX-injected, 2CA-treated ND4 (all 5-month-old) was used to measure PAD activity (Fig. 6-2). The results indicated no significant difference in PAD activities between the untreated ND4 and the PTX-injected group. Both groups displayed an elevated PAD activity above that of normal (p < 0.05). This suggested that PAD activity was mainly elevated during the
neurodegenerative phase prior to PTX-induced inflammatory response. 2CA treatment significantly decreased PAD activity level (p < 0.05) in 2CA-treated, EAE-induced mice to a level comparable to that of normal mice, suggesting that the halt in EAE progression was associated with suppression of PAD activity.

Fig. 6-2. PAD enzyme activity in normal, untreated, PTX-injected, and PTX-injected, 2CA treated ND4 mouse brain at 5 months of age with standard deviation. The PAD activities in brain homogenate from PTX and PTX + 2CA treated groups were compared with activities in brains from normal and non-treated ND4. No significant differences were found between PAD activities in untreated (n = 5) and PTX-injected ND4 brains (n = 5). The level of PAD activity in 2CA treated PTX-injected group (n = 3) was significantly lower than that of PTX-injected ND4 (p < 0.05).

C. Morphological changes in the CNS

Cerebellar sections from 2-month-old normal, untreated, and PTX-injected ND4 mice were stained with hematoxylin and eosin (H & E) for histological examination of demyelination (Fig.
Fully myelinated white matter was observed in all three groups of animals suggestive of failure in EAE disease induction before the onset of neurodegeneration consistent with a clinical score of zero.

H & E stained cerebellar sections from 5-month-old normal, untreated, and PTX-injected ND4 mice were examined for evidence of demyelination. Extensive demyelination indicated by staining loss and vacuole formation was observed in white matter from untreated and PTX injected ND4 mice indicating that EAE induction on ND4 transgenic mice was achieved only after the onset of spontaneous neurodegeneration (Fig. 6-4).
Fig. 6-3. Hematoxylin & Eosin histological stain of cerebellum from untreated ND4 (2 M ND4 No Treatment), PTX-injected ND4 (2M ND4 PTX-treated), Normal (2M normal) at 2 months of age. Intact myelin structure was evident in all animal groups at 2 months of age before the onset of neurodegeneration. Bar line = 100 μm. Magnification = 100x.
Fig. 6-4. Hematoxylin & Eosin histological stain of cerebellum from Normal (5M normal) untreated ND4 (5M ND4 No Treatment), PTX-injected ND4 (5M ND4 PTX-treated) at 5 months of age. Cerebellum was stained for myelinated white matter (WM, pink area) and grey matter (GM, purple area). Demyelination was evident in non-treated and PTX–injected ND4 mice as indicated by loss of staining and formation of vacuole. Magnification = 100x.

Morphological evidence of myelin loss in 5-month-old normal, untreated, and EAE induced ND4 was examined by Luxol fast blue (LFB) staining of optic nerve (Fig. 6-5). Moderate staining loss and vacuole formation were observed in untreated ND4 mice indicative of demyelination. Demyelination was extensive in EAE-induced animals as indicated by severe loss of staining, and thereby suggesting optic nerve is a major site of impact from PTX treatment.
Demyelination in optic nerve after PTX Treatment

Fig. 6-5. Luxol fast blue histological stain of optic nerve from normal (Normal), non-treated ND4 (ND4 no treatment), PTX injected ND4 (ND4 PTX treatment) at 5 months of age. Optic nerves were stained with LFB for myelinated white matter. Myelin breakdown was evident in non-treated ND4 group as indicated by vacuole formation. Severe demyelination was evident in PTX-treated ND4 mice with a pale blue dye color and a large loss of staining. Magnification = 400x.
Evidence of cellular infiltration was further examined by anti-CD3 (Fig. 6-6) staining of cortical sections from 5-month-old normal, untreated, and EAE-induced ND4 mice. No sign of cellular infiltration was found in untreated ND4 brains even though demyelination was extensive. Moderate perivascular infiltration of lymphocytes was observed in EAE-induced ND4 brain (Fig. 6-6). The presence of CD3+ cells in PTX-injected ND4 brain only is suggestive of that destruction of blood brain barrier in the presence of sensitized antigen may generate an EAE-like inflammatory response.
Fig. 6-6. Immunohistochemistry of brain sections from normal (Normal), MOG-EAE (MOG-EAE), non-treated ND4 (ND4 no treatment), PTX injected ND4 (ND4 PTX treatment) at 5 months of age. Mouse cortical section was stained for T-cells with anti-CD3 antibody. T-lymphocyte infiltration was shown in acute MOG-EAE brain. Non-treated ND4 brain displayed extensive demyelination as indicated by vacuole formation but no signs of
cellular infiltration. Perivascular infiltration by T-lymphocytes as indicated by the area enclosed within the black boundary (area enlarged as shown by the picture below pointed by the double arrow) was observed in PTX-injected ND4 brain. Magnification = 400x.

Astrocytosis is an abnormal increase in the number of astrocytes due to the destruction of nearby neurons. To determine the effect of PTX injection on neuronal damage in mouse brain, cortical sections from 6-month-old normal, untreated, and EAE-induced ND4 mice were stained with anti-GFAP antibody to assess the extent of astrocytosis. Sections from untreated induced ND4 brains showed extensive astrocytosis that marked neuronal damage in the mouse brain undergoing neurodegeneration (Fig. 6-7). Extensive astrocytosis was also evident in PTX-treated ND4 brain (Fig.6-7) suggestive of an increase in reactive gliosis leading to a less permissive environment for remyelination.
Fig. 6-7. Immunohistochemistry of brain sections from normal (Normal), non-treated ND4 (ND4 no treatment), PTX injected ND4 (ND4 PTX treatment) at 5 months of age. Mouse cortical sections were stained for astrocytes with anti-GFAP antibody. Minimal signs of astrocytosis were observed in normal mouse brain. Both non-treated and PTX-injected groups displayed extensive astrocytosis. Bar = 200 μm. Magnification = 200x.
In summary, induction of EAE like clinical signs was only possible in 5-month-old ND4 mice experiencing neurodegenerative demyelination but not in 2-month-old ND4 animals prior to the onset of spontaneous demyelination. The lack of clinical symptoms was further confirmed in H & E stained cerebellum from 2-month-old animals where well-myelinated grey matter and white matter were observed. On the contrary, severe demyelination was observed in similar sections from 5-month-old ND4 brains suggestive of that imposing EAE like inflammatory signs was not possible before demyelination and possible release of immunogenic peptides.

Further histological examination suggested that optic nerve was a major site of impact from PTX-treatment as indicated by a severe loss of LFB staining. Anti-CD3 staining has confirmed the presence of some T-cells in PTX-treated brain perivascular area. Extensive astrocytosis as indicated by the presence of GFAP+ cells was observed in PTX-treated ND4 mice at 5 months of age suggestive of severe neurodegeneration.

Examination of PAD activity in the 5-month-old cohort has found an elevated PAD activity in both non-treated and PTX-treated ND4 animals above that of 5-month-old CD1 litter mates. This is suggestive that increased PAD activity and possible protein hypercitrullination occurred mainly during the neurodegenerative phase and may be required for later protein degradation, release of immunogenic peptide and induction of autoimmune responses.
Chapter 7
Discussion

The study of protein citrullination in the brain remains one in which very few questions have been answered. The presence of an increasing amount of citrullinated MBP within MS brain and its correlation with the disease severity (Wood and Moscarello, 1989) has suggested that protein citrullination is an important underlying mechanism in the pathogenesis of demyelinating diseases.

Citrullination of proteins has been shown to have a large effect on the structure and function of many proteins. Citrullinated MBP displays a more open conformation (Harauz et al., 2004), interacts with lipid less avidly than the more cationic MBP isoforms (Wood and Moscarello, 1989), and is enriched in the loose myelin peptide fraction isolated from human brain white matter (Cruz and Moscarello, 1985). Citrullination of Histone 3 by PAD 4 has also been demonstrated to induce oligodendrocyte apoptosis in vitro possibly contributing to failure of remyelination (Mastronardi et al., 2006). In addition to its contribution to the formation of a less stabilized myelin sheath, citrullinated MBP has been shown to affect antigen recognition and proliferation in MBP-responsive T-cell lines (Zhou et al., 1993; Martin et al, 1994). Evidence that citrullinated MBP showed an increased susceptibility to proteolytic digestion by cathepsin D (Cao et al., 1999; Pritzker et al., 2000a) further implicated the role of protein citrullination in autoimmune response in MS. Since the mechanism by which proteins are citrullinated in vitro is specifically facilitated by the family of PAD enzymes, the functional significance of PAD enzyme activity was addressed in this thesis.
Chapter 1 of this thesis compared PAD activities within MS brains and within brains without neurological diseases since past research has implicated protein hypercitrullination by PAD in the pathogenesis of MS. The result revealed a widespread PAD activity in MS brains whereas the average PAD activity were more consistent within brains free of neurological diseases. The nature of autopsy material and the limited sample size may partially contribute to this variation. Paradoxically, brains from PPMS patients exhibited a lower average PAD activity (12.33 nmol cit/ min/ mg protein, n = 4) than that of chronic relapsing and SPMS patients (20.50 nmol cit/min/ mg protein, n = 5). Brains from SPMS possibly experienced more protein degradation due to disease severity and more frequent attack on myelin components. Moreover, PAD enzyme might be self-regulating in the long run as evidence suggesting that PAD from mouse uterus deiminated itself at arginine 352 (Tsuchida et al., 1993). Therefore, I speculate self-deimination of PAD in vivo may result in modification of its function and results in a decreased PAD activity in PPMS brain even though evidence is limited.

The average PAD activity in MS brain white matter was found to be 25% elevated from that of normal white matter. This increase of PAD activity in MS brain was consistent but not striking. On the one hand, PAD enzyme was easily degraded as shown in previous enzyme purification studies. In a study by Ohsugi et al (1995), a highly active recombinant PAD enzyme (8,450 nmol /min/ mg protein) underwent a major loss of its activity (70 nmol/ min/ mg protein) after the purification process. Pritzker (1996) employed many additional purification techniques but all methods resulted in a complete loss of PAD activity. On the other hand, previous study has found that PAD was able to deiminate unmethylated arginyl residues of bovine MBP at a rapid rate of 0.081 mol of citrulline/min (Pritzker et al., 2000b). Moreover, the increase in deimination of arginyl residues has been observed to dramatically enhance the rate of MBP catalytic digestion by cathepsin D (Pritzker et al., 2000a). Therefore, a modest increase in PAD
activity level may be able to contribute to MS disease progression by altering the three-dimensional structure of MBP by citrullination and thus decrease both the charge and compact structure of myelin.

The PAD activities in brains from MS patients and from ND4 and PAD 2 transgenic mice could be inhibited with active site inhibitor 2-Chloroacetamidine (2CA) to a level comparable to that of normal (~20-30% inhibition, see Pg. 43). The inhibition of PAD activities by 2CA in brains of MS patients and mice underwent demyelination was not as complete as the inhibition in recombinant PAD enzymes. Previous study has found that the deiminated residues of human MBP were located at the N-terminal and C-terminal ends of the protein chain, whereas deiminated arginyl residues were rarely found in the middle of the protein (Wood and Moscarello, 1989). However, incubation of MBP with PAD in vitro resulted in a non-specific deimination of all arginyl residues. The specificity of PAD activity on individual residues was speculated to be affected by the lipid environment enabling protein-lipid interaction and protein conformational changes. Thus enrichment of the lipid environment in vivo in MS and transgenic mouse brain tissue compared to minimal lipid environment in vitro may result in the modification of PAD activity on substrate BAEE and the accessibility of inhibitor to the enzyme. Moreover, the PAD activity assay employed in the present thesis measured the amount of citrulline generated in the process of deimination. The presence of endogenous citrulline in MS brains accumulated from chronic PAD activity in vivo as a part of total PAD activity readout may result in an apparently incomplete inhibition of PAD activity in MS tissues.

Since MBP citrullination facilitated by PAD enzymes has been implicated in the pathogenesis of MS, our lab has previously employed various PAD inhibitors in attempt to reverse MBP citrullination and prevent demyelination. Since the activity of all PAD isozymes is
calcium dependent, Pritzker (1996) examined inhibitory effect of various divalent cations of which slight inhibition was obtained through the use of MgCl₂ (32%). Similarly, a slight inhibitory effect on recombinant PAD enzyme *in vitro* was achieved with substrate arginine analogs poly-L-ornithine (14%) and monomethyl-homoarginine (8%) but it was not enough to warrant possible use therapeutic agents. Our lab later investigated paclitaxel known for its ability to stabilize microtubules and its structural similarities to BAEE, an artificial PAD substrate. Paclitaxel was an effective PAD inhibitor that inhibited 80% PAD activity at 12.5 mM (Pritzker, 2000). However, paclitaxel is a highly toxic chemotherapeutic agent with many side effects and is not water soluble. 2-Chloroacetamidine (2CA) was discovered as the most effective PAD active site inhibitor so far as 80% inhibition of recombinant PAD 2 enzyme activity was achieved with 30 mM 2CA, and 50% inhibition of recombinant PAD 4 enzyme was achieved with 15 mM 2CA. 2CA is more suitable candidate for MS therapeutics than paclitaxel since it was a smaller and more soluble molecule able to penetrate the BBB.

To determine if we could reverse protein hypercitrullination, we employed 2CA in both neurodegenerative and autoimmune models of MS to inhibit PAD activity. The beneficial effect of PAD inhibition by 2CA was apparent in present study with transgenic mouse models of neurodegenerative demyelination. PAD enzyme overexpression was causal to demyelination in PAD 2 overexpressors (Musse et al., 2008). In ND4 mice, evidence also revealed that increase in PAD activity preceding the change in MBP microheterogeneity i.e. a decrease in more cationic isomers coupled with protein hypercitrullination was associated with disease progression (Mastronardi et al., 1996; Mastronardi et al., 2006; Pritzker et al., 1999). Our present study with PAD inhibitor therefore confirmed the role of protein hypercitrullination in basic disease mechanism of these two transgenic models. In our most extensively studied ND4 transgenic line,
remyelination was evident in brains and optic nerves of 2CA or 2CA+B\(_{12}\)CN treated animals as shown in fig. 4-3 and fig. 4-4. Since there is no direct evidence to implicate protein citrullination in the process of myelination, I propose that reversing protein citrullination attenuates further demyelination process and allows remyelination by the innate repair mechanism.

Protein hypercitrullination has been reported in various autoimmune diseases including rheumatoid arthritis (Vossenaar et al., 2003) and psoriasis (Ehrlich et al., 2004). Our present study with two autoimmune models of MS also confirmed the beneficial effect of PAD inhibition on inflammatory demyelination. A morphometric analysis by Dr. Mastronardi in our lab revealed a significant reduction of perivascular lymphocyte infiltration in 2CA treated CREAE mice. Thus PAD pathway may be involved in immune regulation. A splenocyte culture study on CREAE mice in collaboration with Dr. Dosch’s lab suggested 2CA reduced secretion of IL-17 and INF\(_\gamma\) without modifying lymphocyte response to PLP. Further investigation was required to determine how PAD affects the “effector” function of lymphocytes.

The observation that EAE like clinical signs and possible inflammatory responses were only induced in ND4 mice already experiencing neurodegeneration further implicated the possible role of PAD in the autoimmune aspects of MS. As one can see in Chapter 6, elevation of PAD activity in brains of unified mouse model mainly during the neurodegenerative phase prior to the onset of inflammatory signs. Therefore, protein citrullination was possibly required to cause fragmentation of myelin components and subsequently increase the susceptibility of the less cationic MBP to autolysis generating immunogenic peptides. The release of immunogenic myelin peptides might sensitize T-cells in the periphery which later migrated into the CNS resulting in an inflammatory response secondary to neurodegeneration in this unified model of MS.
Previous work by our lab has suggested one of the mechanisms by which PAD 2 was elevated was by the decreased methylation of the CpG island in the promoter of Padi 2 gene in MS NAWM (Mastronardi et al., 2007b). In the presence of B\textsubscript{12}CN, an increased methylation was detected on about 50% of surveyed CpG islands in the Padi 2 promoter of ND4 brain. The finding by Dr. Zhen Li in our lab that PAD 2 transcription was decreased in the combination treated ND4 model supported promoter methylation as possibly responsible for additional beneficial effect of B\textsubscript{12}CN. In addition, B\textsubscript{12}CN alone may contribute to the process of myelination in other ways as B\textsubscript{12}CN deficiency may lead to subacute combined degeneration of spinal cord characterized by demyelination, axonal damage and later fibric sclerosis of nervous tissues. B\textsubscript{12}CN may also interfere protein citrullination by methylating arginyl residues (Raijmakers et al., 2007) although conflicting data suggested PAD4 following hormonal induction was able to interfere with arginine methylation in histone H3 (Cuthbert et al., 2004; Wang et al., 2004). Since our lab previously demonstrated that B\textsubscript{12}CN mono treatment was not able to suppress disease in neurodegenerative ND4 mice or MBP/PLP induced EAE models (Mastronardi et al., 2007b), methylation may have played a role secondary to direct PAD inhibition in treating animal models of MS.

Current therapies for MS, most of which are immunosuppressive target the peripheral immune system, modestly delay disease progression and reduce the frequency of relapses by 33-66% (Steinman et al., 2006). Most drugs bind to many molecules producing non-specific effects on the immune system and possibly leading to toxic side effects. The most successful treatment, Glatiramer Acetate, targets diverse molecules of human leukocyte antigen system. Other therapies including Mitoxantrone, an antineoplastic agent against various tumors, is highly toxic. Natalizumab, an antibody to α4β1 integrin blocking T-cells from entering the brain, was
withdrawn after 3 cases of leukoencephalopathy caused death. Recently developed immunomodulators including Fingolimod (a sphingosine-1-phosphate-receptor modulator) and Cladribine (2-chlorodeoxyadenosine triphosphate), both targeting lymphocytes, have only shown to improve short-term relapsing rate with potential long-term complication such as lymphocytopenia and bradycardia (Kappos et al., 2010; Giovannoni et al., 2010). Therefore, a novel therapeutic strategy which directly and specifically targets a basic mechanism of disease is urgently required.

We have identified hypercitrullination as a basic mechanism of demyelinating disease which has led to a novel therapeutic strategy based on our research with four independent mouse models of MS. Protein hypercitrullination has also been implicated in the pathogenesis of many other serious diseases including Creutzfeldt-Jakob disease (Jang et al., 2010), rheumatoid arthritis (Suzuki et al., 2007), psoriasis (Ehrlich et al., 2004), and Alzheimer’s disease (Ishigami et al., 2005). Thus 2CA that specifically and directly targets PAD pathway responsible for protein citrullination in the CNS, a basic mechanism of MS disease, may add a new dimension to attenuate demyelination as well as treating other diseases.
References


