Inhibition of Peptidyl-Arginine Deiminases Reverses Protein-Hypercitrullination and Disease in Mouse Models of Multiple Sclerosis.

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Running Title: Inhibition of Citrullination Attenuates Demyelination
Abstract

Multiple sclerosis (MS) is the most common CNS-demyelinating disease of humans, showing clinical and pathological heterogeneity and a general resistance to therapy. We first discovered that abnormal myelin hypercitrullination, even in normal appearing white matter, by peptidylarginine deiminases (PADs) correlates strongly with disease severity and might have an important role in MS progression. Hypercitrullination is known to promote focal demyelination through reduced myelin compaction. Here we report that 2-chloroacetamidine (2CA) a small-molecule, PAD active-site inhibitor, dramatically attenuates disease at any stage in independent neurodegenerative as well as autoimmune MS mouse models. 2CA reduced PAD activity and protein citrullination to pre-disease status. In the autoimmune models, disease induction uniformly induced spontaneous hypercitrullination with citrulline+ epitopes targeted frequently. 2CA rapidly suppressed T cell autoreactivity, clearing brain and spinal cord infiltrates, through selective removal of newly activated T cells. 2CA essentially prevented disease when administered before disease onset or before autoimmune induction, making hypercitrullination and specifically PAD enzymes a therapeutic target in MS models and thus possibly MS.
Introduction

Multiple Sclerosis (MS) is the most common demyelinating disease of human adults. Its therapies have limited effectiveness to decrease relapse frequencies without affecting disease progression (Steinman and Zamvil 2006). Treatments are essentially immunosuppressive (Wingerchuk 2008; Comi and Martino 2006; Hemmer et al 2005) reflecting broad consensus of autoimmune effector mechanisms in MS. However, disease heterogeneity provides evidence for non-autoimmune, biochemical and epigenetic MS abnormalities, whose role in the complex hierarchy of pathogenesis remains unclear. In a study of 286 MS cases (mostly biopsies) (Lucchinetti et al 2000) MS has been categorized into four different patterns of pathogenesis, which we have recognized in our animal models, which combined, show most of the features of MS.

In early studies we showed that myelin basic protein (MBP) isolated from normal human brain contained about 20% of the citrullinated MBP (Moscarello et al 1994). In chronic MS white matter the citrullinated protein was 45% and in fulminating MS (Marburg’s Disease) it accounted for 90% of the MBP (Wood et al 1996). This less cationic MBP was unable to compact lipid bilayers in model systems as revealed with several techniques including X-ray diffraction (Brady et al 1981a; Brady et al 1981b) electron spin resonance (Boggs et al 1982) circular dichroism (Epand et al 1974) and NMR (Deber et al 1986). We postulated that the decreased protein-lipid interactions resulted in destabilization of the myelin which could be more readily degraded.

Citrulline in proteins is generated by a family of enzymes, the peptidylarginine deiminases
(PADs) of which five are known all localized to a single locus on chromosome 1 (lp36.1). Of the five isoforms, PAD 2 and PAD 4 are found in brain, localized in myelin and oligodendrocytes. PAD 4 is unique since it is the only PAD to carry a nuclear localization signal. We have shown that PAD 4 can be translocated to the nucleus in oligodendrocytes in culture in the presence of TNFα where it deiminated histone H3, suggesting a role in apoptosis (Mastronardi et al 2006). PAD 2 was found in myelin, the axons, and the periaxonal space at the contact between myelin and the oligodendrocyte (Wood et al 2008). Both processes, the decreased myelin compaction and apoptosis of the oligodendrocytes representing important pathways in the patho-mechanism of demyelination.

Further support for an important role of PAD enzymes and hypercitrullination of proteins in MS was obtained with two transgenic mouse lines which demyelinate spontaneously. In the ND4 line which contains 70 copies of the cDNA for DM20 (a myelin proteolipid) an increase in PAD enzymes was observed at 2 months of age, one month prior to the onset of both clinical signs of demyelination and increased protein citrullination at 3 months of age, suggesting a causative role of PAD enzymes (Moscarello et al 2002). In the other model generated by the addition of 30 copies of cDNA for PAD 2, signs of demyelination occurred at 6 months of age spontaneously, showing that increased PAD enzymes on their own induced demyelination (Musse et al 2008). These results, combined with the above mentioned data on citrullinated MBP provide a compelling case for a
prominent role for PAD enzymes and citrullinated proteins in the pathogenesis of MS. Here we report the use of a small molecule active site PAD inhibitor, 2-chloroacetamidine (2CA) (Stone et al 2005) in the treatment of 4 animal models of demyelination, two neurodegenerative and two autoimmune. In all four models, protein citrullination was decreased, PAD activities were decreased, disease was attenuated, and remyelination was observed. In addition, 2CA markedly decreased CD3⁺ T-cells in the autoimmune models. 2CA is an irreversible PAD inhibitor and we have exploited this property to localize the adduct (PAD -2CA) in brain. With these studies we have identified a novel therapeutic target readily available for intervention by small molecules.
Results

PAD expression and activity in white matter from MS patients

We compared protein citrullination, PAD protein expression and activity in extracts of normal brain and of normal-appearing white matter (NAWM) from MS patient brains using the slot blot method (Supplementary Tab.1). PAD protein expression was elevated in NAWM from MS brain (Fig. 1a) with a corresponding increase of PAD activity and protein citrullination (Fig. 1b, c). Following addition of 2CA to NAWM extracts of MS brain, PAD activity declined to normal levels demonstrating that 2CA was effective in human brain extracts (Fig. 1c).

Using recombinant PAD 2 and PAD 4 enzymes we then measured the residual activity with BAEE in the presence of increasing concentration of 2CA (Fig. 2a). A rapid decrease of activity was observed with both enzymes, more marked with PAD 2, yielding an IC₅₀ 14.4 mM.

Human PAD enzymes are well conserved in the C-terminus, containing the active-site region (Fig. 2a inset). To identify the 2CA binding site, recombinant human PAD 2 was reacted with 2CA. Trypsin digests of 2CA-reacted and unreacted enzyme were compared by LC-ESI-QToF-MS/MS analysis (Supplementary Fig. 1). We identified a tryptic peptide in 2CA-reacted PAD 2 with a mass of 1386.68Da, while the corresponding unreacted peptide had a mass of only 1330.45Da. The difference of 56Da corresponds to addition of one molecule of acetamidine (Fig. 2c). As shown in Fig. 2b, we propose the mechanism of the reaction is acetamidine binding to Cys 656 in a covalent bond between the cysteine sulphur and the carbon of the acetamidine in a covalent interaction as
originally described by Stone et al 2005. Fragmentation analysis (Fig. 2c) mapped the relevant peptide (FLGEVHC*GTNVR) to the C-terminal region containing the active site cysteine (C).

A similar analysis identified the same active-site cysteine in recombinant PAD 4 (unpublished).

Fragmentation analysis determined that the y-ions 688-326-17 (NH3) and in PAD 2 and PAD 4, respectively, contained the cysteine-adduct (Fig. 2c).

To determine if 2CA crosses into the brain, our therapeutic target, we generated monoclonal antibodies to FLGEVHC*GTNVR, carrying the acetamidine adduct on the cysteine residue. Antibody mAb4E12 recognized 2CA-modified, but not unmodified PAD 2 in Western blots. We injected 2CA i.p. twice/week into transgenic mice overexpressing PAD 2 in white matter (Musse et al 2008). Brain sections were stained with mAb4E12 and developed with gold-labeled anti-mouse IgG for immuno-electronmicroscopy (Fig. 3). Drug-PAD 2 adducts were scattered in myelin sheaths and nuclei of oligodendrocytes, indicating that the drug reached its target molecules in the brain. Clusters of gold particles were found in the periaxonal region (white arrows) while gold label was absent in similarly stained sections from untreated PAD 2 transgenics.

**Disease attenuation by 2CA in demyelinating mice**

We employed four independent mouse models of demyelination, two non-autoimmune transgenic (Musse et al 2008; Mastronardi et al 1993) and two induced autoimmune models of demyelinating disease (Iglesias et al 2001; Tuohy 1995) thus comparing 2CA responses across different genetic backgrounds, disease causes and progression programs.
The first of the neurodegenerative models was the ND4 transgenic mice (DM20 overexpressors) which are normal from birth until ~10-12wk of age, when they develop a non-autoimmune, primary progressive, ultimately fatal CNS demyelinating condition (Mastronardi 1993; Johnson et al 1995). Disease progression is associated with increased myelin PAD expression and hypercitrullination of myelin proteins (Moscarello et al 2002) and histone H3 proteins (Mastronardi et al 2006). ND4 mice received i.p. PBS, 2CA (5mg/kg) or 2CA + 10mg/kg B12 every other day, beginning well before disease onset (2mo, Fig. 4a) or early after disease onset (3.5mo, Fig. 4b). In another group with early treatment onset, 2CA injections were stopped after six weeks, while B12 injections continued (Fig. 4c).

Early and prolonged 2CA injections starting at 2 months of age essentially prevented disease, with or without B12 (Fig. 4a). Most untreated mice were sacrificed with severe disease by 6 months, none died in the treatment groups. Treatment of early disease at 3.5mo prevented progression, but mild disability continued, unless animals received 2CA plus B12. However, fully progressive clinical disease re-emerged promptly after therapy cessation at 6 months (Fig. 4b). The temporal link of disease protection by 2CA and relapse after therapy cessation suggests PAD-mediated citrullination and disease progression are a basic mechanism in the transgene-driven pathogenesis. Abbreviated 2CA treatment (2-3.5mo) was not very effective, but continued B12 injections generated milder disease and delayed progression (Fig. 4c). We previously reported that B12 by itself has little effect in the ND4 mouse (Mastronardi et al 2007; Mastronardi et al 2004).
which we confirm here. Overall, 2CA induced dramatic disease attenuation, which did, however, require continued injections due to obvious persistence of pathogenic transgene expression.

To determine how improved clinical scores were related to PAD activity, 6-month-old mice from the treatment groups in figure 4b were examined (Fig. 4d). 2CA normalized white matter PAD activity in brain homogenates, with considerable overshoot (and rapid disease progression) in such animals after therapy cessation. B12 further reduced PAD hyperactivity, preventing the post-therapy overshoot (Fig. 4d). PAD 2 gene expression (Fig. 4e) paralleled PAD activity, suggesting that disease induced elevations are regulated at the transcriptional level, where PAD promoter hypomethylation may explain PAD overexpression as well as the positive impact of B12 adjunct therapy (Mastronardi et al 2007).

Luxol-fast-blue staining of myelin (Fig. 4f) shows impressive myelin deficits and pronounced vacuolization in untreated ND4 mice, which were dramatically improved in treated mice. When treatment was removed, myelinolysis re-emerged in both treatment groups, albeit milder in those that received B12. Morphological changes in myelin structure were analyzed by transmission electron microscopy of optic nerve cross-sections from 6 months old mice described in figure 5 a-f. In non-transgenic (normal) CD1 littermates (Fig. 5a), axons (Ax) are well myelinated with myelin of uniform thickness. ND4 mice show wide areas of myelin loss, degradation, and nude axons are common (Fig. 5b). 2CA treatment (Fig. 5c) clearly improved this morphology, with few axons seriously affected,
but many still show thinning of the myelin thickness. Addition of B12 resulted in a morphological picture virtually indistinguishable from normal (Fig. 5d). When treatment stopped at 6 months with analysis at 8 months, showed reappearance of disease (Fig. 5 e-f) due to the continuing presence of the transgene.

To quantify the above myelin changes, we calculated G-ratios (axon diameter/fiber diameter) from ~500 non-contiguous semi-thin sections per treatment group (Table 1). Compared to healthy littermates (G-ratio 0.74 ± 0.13) ND4 mice showed a reduction in optic nerve myelin thickness: G-ratio 0.96 ± 0.3 (p < 0.0013). In 2CA-treated ND4, myelin thickness was slightly improved and showed less variation (G ratio: 0.9 ± 0.15) (p < 0.015) while 2CA+B12 treatment nearly normalized myelin thickness to a mean G-ratio of 0.81 ± 0.18 (p < 0.015). These treatment data are typical for remyelination, where the original myelin thickness is never re-achieved.

Transgenic mice overexpressing PAD 2 under control of the MBP promoter (Musse et al 2008) develop a demyelinating phenotype (Fig. 6). Treatment with 2CA or 2CA+B12 had little effects on early disease, but treatments prevented the second, more aggressive disease phase equally. PAD activity was high in transgenic white matter, and 2CA, with or without B12, normalized tissue enzyme activity levels (Fig. 6). B12 has little effect in this model.
**Survival from acute MOG EAE**

EAE was induced in C57BL/6 mice with 100µg of MOG35-55 peptide, emulsified in Freund’s complete adjuvant and 300ng of pertussis toxin. At the earliest sign of disease, usually 9d post-immunization, groups of mice received carrier (PBS) 2CA or 2CA+B12 as before (Fig. 7a). Untreated mice developed rapidly progressive disease and were sacrificed when moribund (~d19). 2CA and 2CA+B12 treatment did not affect the disease course observed in untreated controls until ~d14, when progression halted and recovery began, leaving ~50% survival by day 30 (Fig. 7b). When 2CA treatment was started before immunization, disease lethality was zero (unpublished observation).

Despite the severity of disease, there was relatively little histopathology in brain. However, vacuolar demyelination and lymphoid infiltration were prominent in the spinal cord (Fig. 7c left panel arrows). In contrast, surviving 2CA and 2CA + B12 recipients showed much improved, virtually normal spinal histology (Fig. 7c, center and right panels). More sensitive immunofluorescence still detected scattered CD3⁺ T cells in treated animals, but the heavy T cell clusters in PBS treated controls were absent (Fig. 8a) suggesting that one possible 2CA effect may be suppression of T cell expansion. White matter of PBS treated mice had elevated PAD activities, which were normalized after 2CA (Fig. 8b).
Spinal cord PAD activities of PBS-treated mice reached 3-fold elevations, but reductions to normal levels were once again observed following treatment (Fig. 8c).

To seek more mechanistic understanding of the 2CA targets in the disease process, we measured MOG-specific autoreactive T cell pools. All mice developed highly reactive, systemically distributed pools of pMOG35-55- reactive T cells, detected in *ex vivo* T cell recall assays (Fig. 8d). Drug treatments reduced immunity significantly and recall responses to MOG within the short disease course. There are two arginine residues in the immunodominant MOG peptide and we measured T cell recognition of replacement peptides, carrying citrulline instead of arginine in one or both sites (Fig. 8d, right panels). Single ARG to CIT replacement peptides were recognized by substantial T cell pools, each about half the size of those recognizing native pMOG35-55. T cell pools recognizing MOG35-55 with both ARG residues replaced by CIT, were much smaller; testimonial to the stringency of *in vivo* T cell repertoire selection. Cell responses to citrullinated MOG peptides were reduced in mice receiving 2CA or 2CA + B12, in keeping with 2CA’s ability to block citrullination and the reduced availability of citrullinated peptides to be attacked by the immune system. These data indicated that the disease-related T cell autoreactivity-repertoire prominently includes recognition of CIT-containing epitopes, an observation with precedence in the literature (Carillo-Vica et al 2010).
We concluded that the acute 2CA blockade of citrullination in the early phase of this model produced a major reduction of autoreactive T cell pools, providing a mechanistic explanation for the drug-induced failure to generate the massive T cell tissue invasion characteristic of the effector phase of this disease. The remaining infiltrates of scattered CD3+ T cells in treated survivors (Fig. 8a) may be either anergic or non-specific bystanders with little pathogenicity, since there were no relapses after therapy cessation in CD3+ T cell population. Our conclusion of immune expansion as major 2CA target may also explain the lack of B12 effects in acute MOG-EAE, where time is short for remyelination.

**Attenuation of chronic relapsing EAE (crEAE) by 2CA**

Acute MOG-EAE is a rapid and aggressive disease and we decided to include a crEAE model that better mimics the relapsing-remitting course common in MS. Disease was induced in SJL mice with a proteolipid protein peptide (pPLP139-155, 200µg/mouse) (Tuohy et al 1995; Mastronardi et al 2004). Treatments with 2CA and 2CA+B12 were started at disease onset (d10-11) with different treatment schedules indicated in the top bars of figure 9. Attenuation of disease was observed in all treated mice (Fig. 9a). In contrast to our previous findings, where B12 showed synergistic effectiveness in interferon-β-treated crEAE (Mastronardi et al 2004) there was little added benefit when combined with 2CA and it was omitted in some of the subsequent experiments.

After pilot experiments suggested steep excursions at disease onset, we followed PAD activity over the disease course in 20 mice (Fig. 9b). PAD activities indeed showed a sharp rise at disease onset, peaking at the height of the initial disease phase, and declining to still above-background
levels thereafter. Protein citrullination in white matter was consequently elevated, a process prevented by 2CA (Fig. 9c). Besides strain differences, the different EAE induction modes critically reflect choice of the inducing myelin peptide, thus involving a cognate recognition event in CD4+ and/or CD8+ T cells with potentially encephalitogenic T cell receptors (Zhu et al 2006). The extent of disease was closely reflected by the extent of cellular infiltration in white matter sections (Fig. 9d) confirmed by measuring CD3+ T cell invasion around blood vessels (Fig. 10a). As with the MOG EAE model, 2CA also prevented and/or reduced (see below) T cell tissue infiltrates in the chronic relapsing model of EAE (Fig. 9d, 10a).

We compared systemically distributed pPLP139-155-specific T cells, of treated and untreated mice, via their release of ex vivo, peptide-induced effector cytokines IFNγ and IL-17 (Fig. 10b, c). The dramatic depression of cytokine responses to near background values was interpreted to suggest that 2CA interfered with recruitment and/or expansion of the ‘inciting’ T cell repertoire.

The chronic phase of crEAE allowed us to examine 2CA effects in greater detail (Fig. 10d-f and Supplementary Fig. 2). PAD inhibition was effective in abrogating disease at all stages of disease progression (Fig. 10d, f). The prompt decline of clinical signs following 2CA application in mice with crEAE implies a very rapid local and systemic (Fig. 10b-c) loss of effector cells following inhibition of continued protein citrullination. Tissue half-life times of citrullinated proteins are unknown, as is the persistence of the relevant, processed peptides in local antigen presenting cells. The rapidity of 2CA effects therefore makes direct actions of the drug on T cells and/or APC likely: both cell lineages express PAD (Liu et al 2006).
Discussion

Multiple sclerosis (MS) the most common demyelinating disease of humans is a heterogeneous disease characterized into four types (Lucchinetti et al 2000). Pattern 1 showed inflammatory demyelination with macrophage infiltration. Pattern 2 showed demyelination with inflammatory infiltration with T-cells. Pattern 3 was characterized by loss of oligodendrocytes by apoptosis. Pattern 4 also shows loss of oligodendrocytes but is rare.

Understanding the pathogenesis of MS has relied heavily on animal models of which there are several. Although all models reproduce some of the features of MS, none reproduces all the features. In our studies described here we have used four animal models to try to bring together as many of the features of MS as possible. Two of the models were inflammatory autoimmune models (acute and chronic relapsing EAE) reflective of pattern 2 MS. The acute EAE was monophasic and rapid whereas the chronic EAE was more reflective of the relapsing-remitting course of MS. The ND4 transgenic mouse which contains 70 copies of the cDNA for the myelin proteolipid protein DM20 is representative of pattern 3 MS. Pathology was found in brain primarily with none in the spinal cord (Ludwin 2006). Demyelination was observed in the absence of cellular infiltration (in contrast to EAE where cellular infiltration is a prominent feature). The ND4 was characterized by apoptosis of oligodendrocytes showing early retraction of the inner tongue process (Ludwin 2006). In fact some have suggested that all MS begins in this way by apoptosis of oligodendrocytes (Barnett and Prineas 2004). The importance of apoptosis was demonstrated in a recent
publication, in which oligodendrocyte ablation produced extensive myelin loss with myelin fragments in the lymph nodes, but no CNS immunity was found, supporting a neurodegenerative hypothesis for MS (Locatelli et al 2012). Patterns 2 and 3 account for 80% of the MS cases. Therefore, our combination of animal models represents a relevant system to study MS.

Tissue-selective overexpression of PAD enzymes and consequent, local protein hypercitrullination has become hallmark of a growing and diverse group of pro-inflammatory diseases, which share challenging or unmet therapeutic needs (Vossenaar et al 2003; Ishigami et al 2008; Ehrlich et al 2004; Battacharya et al 2006; Jang et al 2010; Jang et al 2008). The fact that several animal models of such human disorders show comparable elevations of PAD activity and hypercitrullination (Nicholas 2005; Hill et al 2008; Kuhn et al 2008) beg the question of commonalities in the underlying pathomechanisms of hypercitrullination disorders: what causes local PAD overexpression and is hypercitrullination a marker or mediator of disease progression?

In an effort to understand the importance of PAD enzymes in the EAE model, a PAD2 knock out mouse was generated (Raijmakers et al 2005). These authors claimed that citrullinated MBP was not present in these KO mice as detected with an anti-modified citrulline antibody. Since EAE was still induced in these mice, they concluded that citrullination was not required for disease induction in the PAD2 knock out. Using mice from their colony we isolated MBP from PAD2 knockout mice; trypsin digested the MBP fraction and showed that several citrulline containing MBP peptides were identified by mass spectrometry (Wood et al 2008). We also showed that citrullinated CNPase, and MOG were detected. Although PAD2 was absent from the knockout, PAD4 was found at the same levels as found in wild type mice.
Furthermore we reported that PAD4 deiminated MBP, as well as, PAD2 in vitro (Wood et al 2008). In a recent report, (Coudane et al 2011) showed that the presence of citrullinated proteins in the PAD2-knock-out mouse was independent of the presence or absence of PAD2. Therefore, citrullination was active in the PAD2 knockout suggesting that ablation of citrullination requires knock out of all PAD activity.

Over the past number of years we documented an increased citrullinated MBP in MS tissue and an increase in PAD enzyme activities. In order to place protein hypercitrullination and elevated PAD activities in the important role of the pathogenesis in MS, we sought to establish that increased PAD activity preceded disease, which we demonstrated in the ND4 model of MS (Moscarello et al 2002).

Hypercitrullination in MS appears to work through several mechanisms involving biochemical change in CNS and immune system. First we have shown in our neurodegenerative mouse models that 2CA was able to attenuate clinical signs of demyelination accompanied by improved myelination and normalized PAD activity in CNS. It was also shown in our acute MOG EAE model that 2CA improves survival rate possibly by decreasing the amount of citrullinated MOG peptides. Since previous studies have reported that MOG may become citrullinated at arginines 41 and 46 of peptide 35-55 (both TCR contact residues) which in turn lead to immune response against the peptides and development of EAE (Carillo-Vico et al 2010) we propose that inhibition of PAD may prevent citrullination of MOG peptide resulting in less severe autoimmune responses.

The molecular mechanism of PAD up-regulation in the ND4 mice remains uncertain although the PAD 2 promoter hypomethylation reported in MS (Mastronardi et al 2007) may be responsible (Unpublished Data) in which decreased methylation has been found at several CpG sites in the
ND4 mouse. As the blood brain barrier is intact in this model, inflammatory infiltrates are absent and cannot contribute to increased PAD enzyme.

Our studies with 2CA argue cogently that hypercitrullination is a critical effector element in the pathogenesis of progressive demyelinating disease affecting both immune and non-immune elements. 2CA has excellent therapeutic efficacy in all phases of these quite diverse model diseases, able to prevent onset, suppress ongoing and allow considerable reversal of established demyelination, with no adverse toxicities observed at the dose and timeframes employed. As the active sites of PAD 2 and PAD 4 are similar, 2CA can bind to both thereby inhibiting citrullination totally (see PAD2 knock out above).

Of the growing number of hypercitrullination syndromes, one targets the eye (Battacharya et al 2006) and four the brain: Alzheimer’s disease and MS (Ishigama et al 2005) sporadic Creutzfeldt-Jakob Disease (Jang et al 2008) and scrapie (Wang et al 2009). It is tempting to speculate that the clustering of these disorders in neuronal tissue may indicate that PAD overexpression/hypercitrullination is a perhaps more predominant natural response factor to tissue stress in these neuronal organs recently supported by spinal cord injury studies where inhibition of PAD enzymes by a general PAD inhibitor decreased apoptosis of neural stem cells and improved regeneration of the cord (Lange et al 2011) suggesting an important role for PAD enzymes in spinal cord regeneration. Improved survival of neural stem cells following PAD inhibition can have a major impact on remyelination in MS.
Concluding Remarks

Multiple sclerosis, a heterogeneous disease of the CNS, arises from the combination of several pathogenic mechanisms. Two of these have been described earlier, i.e. bilayer instability and apoptosis of oligodendrocytes, to which we now add inhibition of T-cell expansion. The first results in myelin degradation while the second a failure to repair and the third to the inflammatory response. With the recent demonstration of the importance of PAD4 activity in the production of neutrophil extracellular trap (NET) formation by deiminating histone (Li, et al. 2010) and the subsequent demonstration that NETs can directly prime T-cells reducing their activation threshold (Tillack et al 2012) strong support is provided for an important role of citrullination in T-cell activation. In the present manuscript, we demonstrate the commonality between these diverse processes resides in protein hypercitrullination mediated by PAD enzymes. These processes are attenuated by PAD inhibition by a small molecule PAD active site inhibitor 2CA, which decreased hypercitrullination, PAD activity was reduced, CD3+ T-cells were reduced, disease was attenuated and remyelination was manifest in four relevant animal models of MS, validating PAD as a therapeutic target in MS by small molecule intervention. Because the 2CA is a weak general PAD inhibitor, we are now developing a library of highly active small molecule PAD inhibitors (nM range) which are reversible and therefore druggable.
Methods

Mice

SJL and C57BL/6 mice were purchased from Charles River (Montreal, QUE) and Jackson Laboratories (Bar Harbor, ME) respectively. ND4 (DM20) transgenic mice and homozygous PAD2 transgenic mice both on CD1 background developed in our institution have been described (Musse et al 2008; Johnson et al 1995; Mastronardi et al 1996). All animals were housed in a closed colony in the animal facility at The Hospital for Sick Children (HSC, Toronto, ON) in a controlled environment using a 12 hr light and dark cycle. Experiments were performed using sex- and age-matched mice under approved protocols and in agreement with animal ethics guidelines.

Therapy protocol and clinical scoring

2-Chloroacetamidine hydrochloride, 96% (2CA) (Alfa Aesar, Ward Hill, MA) and Vitamin B12 (Sigma-Aldrich, St. Louis, MO) were dissolved in phosphate buffered saline (PBS) pH 7.4, filter sterilized, and administered at 5 mg kg-1 (2CA) and 10 mg kg-1 (B12). I.p. injections of 50 µL were performed every other day for the periods indicated. ND4 mice were scored 3x/wk by two independent observers, with non-transgenic littermates serving as controls (Johnson et al 1995; Mastronardi et al 2004). 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, where 0 represented no signs and 4 represented severe signs. 6-8 week old SJL and C57BL/6 mice
were immunized subcutaneously with 200 µg of PLP139-155 (Alpha Diagnostics, San Antonio, TX) or 100 µg of MOG35-55 peptide (Alpha Diagnostics) respectively, emulsified in complete Freund’s adjuvant (Sigma-Aldrich). Pertussis toxin (Sigma-Aldrich) 100 or 300 ng (C57BL/6) was administered on the day of immunization and 48hr later. Mice were scored using a well-established scoring system for EAE signs (Tuohy et al 1995; Mastronardi et al 2004) 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.

**In vitro proliferation and cytokine secretion**

Splenocytes were isolated 45d after CREAE or 14d after acute EAE induction, and restimulated with the appropriate peptide. Cells were cultured for 72hr, with 1 µCi of [3H] thymidine added for the final 18hr prior to harvesting and liquid scintillation counting. To quantitate cytokine secretion, supernatants were collected after 72hr of culture and levels of IL-17 (R&D Systems, Minneapolis, MN) and IFN-γ. (BD Bioscience, San Jose, CA) were measured by ELISA, according to manufacturer’s instructions.

**Generation of mAb**

A synthetic peptide $^{650}$FLGEVHC*GTNVR containing an acetamidine adduct on the active site cysteine (C*) of the PAD2 enzyme was synthesized in our Biotechnology Center and conjugated to KLH and used to generate monoclonal antibodies by standard procedures in our monoclonal
antibody service. Two mAbs (mAb4B11 and mAb4E12) detected only the adduct-tagged peptide and the mAb4E12 clone was used for production.

**Reverse Transcriptase PCR (RT-PCR)**

cDNA from mouse white matter or spinal cord was prepared with a reverse transcriptase kit (Invitrogen, Burlington, ON) and amplicons of primer pairs for GAPDH and PAD2 were separated on agarose gel, stained and analyzed using Image SXM software (http://www.ImageSXM.org.uk).

**Immunoslot blotting and PAD enzyme assays**

Both PAD2 and citrullinated proteins were quantified from white matter homogenates by immunoslot blots. Membranes were probed with either anti-panPAD antibody (Nishijo et al 1991; Rus’D et al 1999; Takahara et al 1989; Terakada et al 1989), or anti-peptidylcitrulline antibody (F95) (Nicholas et al 2005) overnight/4°C, developed with secondary antibodies/1hr/20°C. Images were analyzed as above. PAD activity in aqueous white matter extracts from human and mouse whole brain was determined as previously described (Lamensa and Moscarello 1993; Watanabe et al 1988). In summary, 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 3min. 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.

**Electron microscopy**

Optic nerves from ND4 mice and non-transgenic littermates were removed at 6mo, immersion-fixed in Karnovsky’s solution (Fierabend et al 1994) as described (Moscarello et al 2002). For immunogold electron microscopy, optic nerves from untreated and 2CA-treated ND4 mice were fixed in 4% paraformaldehyde/0.1% glutaraldehyde in 0.1 M phosphate buffer pH 7.4 for 4-6hr (13).

**Mass spectrometry, peptide sequencing, and modification determination**

5 µg of recombinant PAD2 was reacted with 2CA in the presence of Ca²⁺ in Heps buffer pH 7.6 (BioShop Canada Inc., Burlington, ON) at 52°C for one hour, after which it was dialyzed and lyophilized. 2CA-bound and unbound recombinant PAD2 enzyme was digested with 0.05 µg trypsin (Roche Diagnostics, Laval, QUE) in 25 mM ammonium bicarbonate pH 8.6. After overnight incubation at 37°C, 10 µL of sample in 0.1% trifluoroacetic acid were used for
LCMS/MS analysis on a QSTAR XL electrospray ionization QTOF mass spectrometer (Applied Biosystems/MDS Sciex, Concord, ON), coupled with an Agilent LC system. The acquired data set was converted into a peak list file using the Mascot script, a component of the Analyst QS 1.1 software (Applied Biosystems). A custom modification on cysteine called acetamidination was built into the modification database with composition C(2)H(4)N(2) having a delta mass of 56.0374 (monoisotopic). Protein N-terminal acetylation, methionine oxidation, and acetamidination were set as variable modifications in the search. Peptide mass tolerance and MS/MS tolerance were set to 0.2 Da.

Statistics

Statistical significance between means was assessed by unpaired t-tests using Welch’s correction where appropriate. Analysis of clinical scoring curves was performed using two-way ANOVA or life tables where appropriate. Statistical significance was two tailed and set at 5%, with error bars showing a single standard deviation.
Acknowledgements

The immunological input in the EAE studies and the cell proliferation studies by Dr. H-M Dosch and his stuff are acknowledged without whom these studies would not have been possible. In particular we mention Geoffrey Palster, Jason Yantha. We thank Dr. A. Nicholas for the F-95 antibody. This research was funded by grants from the MS Society of Canada (MAM and FGM) and CIHR (HMD). Technical assistance was provided by Mrs. Teresa Miani (Molecular Structure and Function, HSC) Ms. Aina Tilups (Division of Pathology, HSC) Mr. Howard Rosenberg (Division of Pathology, HSC) for preparation of samples for electron microscope and Dr. Sudha Arya (Monoclonal antibody Facility at HSC) generation and purification of monoclonal antibodies 4E12. We also thank Dr. Herman Yeger (HSC) and Dr. Harry Schachter (HSC) for valuable suggestions, Dr. Rashed Nagra and Dr. Wallace Tourtellotte and their team for providing normal and MS samples from the Human Brain and Spinal Fluid Resource Center (VA West Los Angeles Healthcare Center, Los Angeles, CA, jointly sponsored by NINDS/NIMH, National Multiple Sclerosis Society (USA) and the Department of Veterans Affairs.
**Author Contributions**

H.L. an MSc graduate student did most of the enzyme assays, and peptide citrulline determination. She injected mice with 2CA, followed, and scored the mice both the ND4 transgenics and the acute EAE. F.G.M. was conceptually involved from the beginning of the study and maintained an involvement in injecting, and scoring of ND4, and EAE mice. S.H. and H.T. both from the division of immunology provided valuable assistance with the EAE experiments. Z.L. developed the PAD 2 transgenic mice. C.A. was responsible for the microscopy, in particular the electron microscopy of optic nerves and the immunogold studies. L.Z. carried out the mass spectrometry (Fig. 2). R.R. provided us with generous amounts of recombinant PAD 2 and PAD 4 for the in vitro studies. D.D.W. carried out inhibition assays, identified the PAD-2CA adduct and generated the monoclonal antibody.

**Competing Interest**

The authors declare no competing financial interest.
TRANSLATIONAL IMPACT

Clinical Issues

Multiple Sclerosis (MS) is the most common demyelinating disease of humans affecting about 2 million people worldwide. It is characterized by fatigue, muscle weakness, and cognitive impairment. These features are the result of degradation of the myelin sheath, which surrounds the nerves and must be intact for proper nerve conduction. The disease is generally progressive and may continue for many years. Although it is thought to be an autoimmune disease, a neurodegenerative etiology based dysregulation of one or more neurobiological process has become of considerable significance. The therapeutic needs of MS are largely unmet. Most therapies are immune modulatory directed at some aspect of the immune system. Although some improvement in relapse rates has been observed, the basic pathology continues.

Results

A number of years ago we reported the myelin basic protein (MBP), a major protein of myelin was hypercitrullinated in MS. We subsequently showed that the enzymes peptidylarginine deiminases (PAD) responsible for converting arginine in proteins to citrulline were also increased in white matter from MS brains. We proposed that inhibition of these enzymes should decrease the amount of citrulline formed and ameliorate disease. Using a small molecule PAD inhibitor, 2-chloroacetamidine (2CA), we showed that PAD enzymes were inhibited in in vitro assay and in vivo.
We identified the binding site of 2CA as cysteine 656 in the active site of the enzyme. In an vivo studies using 4 animal models of MS, two neurodegenerative and two autoimmune disease was attenuated in all models. PAD enzyme activities in brain and spinal cord were decreased, the amount of citrullinated proteins was decreased and remyelination was observed by electron microscopy. The four models included the ND4 (DM20 overexpressors), PAD overexpressors, acute EAE and chronic EAE. This combination reproduced most of the features of MS.

**Implications and Future Directions**

MS is a complex disease of the central nervous system. With our basic neurobiological approach, we found a commonality in models of disease reported here; i.e. protein hypercitrullination resulting from up regulation of PAD enzymes. PAD enzymes are a therapeutic target which can be regulated by small molecule PAD active site inhibitors. One such inhibitor is 2CA, which binds covalently to the active site of the enzyme. Since covalent inhibitors are generally not druggable our programme is to find, develop, synthesize new PAD inhibitors which are reversible and can be used in MS patients.
References


Human White Matter

Figure 1. PAD activity in NAWM of MS patients. (a) Quantification of PAD2 protein in white matter from normal and MS brain by immunoslot blot (n=5, p<0.0001). (b) Citrullinated protein in white matter from normal and MS brains by immunoslot blot as pixel density (n=4, p<0.01). (c) PAD enzyme activity in normal and MS tissue, with or without preincubation with 2CA (n=5, p<0.05). Each dot is one patient analysed 3 times. The means (bar) for all the MS patients were compared with the normal.
Figure 2. (a) PAD2&4 inhibition curves in the presence of increasing 2CA concentrations. Insert: PAD1-4 enzymes contain a common C-terminal active-site Cys residue (656Cys) bound by 2CA, confirmed by ESI mass spectrometry of tryptic digests of PAD2-acetamidine adducts. (b) A schematic of the nucleophilic reaction between 2CA and the 656Cys residue in the active site of PAD2, adapted from (Stone et al 2005). (c) Tabular summary of peptide fragment atomic masses for 2CA modified and native PAD2.

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Figure 3. mAb4E12 (anti-2CA adduct)-Immunogold labelled optic nerve cryosections from control and 2CA-treated PAD2 transgenic mice. Minimal labeling in untreated mice: arrows in left two panels. Numerous gold particles in nuclei (N) and cytoplasm of oligodendrocytes, myelin, and axonoplasm (Ax) of 2CA-treated mice (bar: 500 nm).
Figure 4. 2CA attenuates demyelinating disease in ND4 mice. (a) ND4 mice treated with PBS, 2CA (5 mg kg⁻¹), or 2CA+B12 (5 mg kg⁻¹ and 10 mg kg⁻¹) starting at 2mo, before disease onset (n=5, p<0.0001). (b) ND4 mice treated at disease onset (n=4, p<0.0001). (c) Stopping 2CA, but continuing B12 at 3.5mo in ND4 mice (n=5, p<0.0001) demonstrating that B12 alone does not attenuate disease. (d) PAD activity in brains from animals shown in Fig. 3b (n=5, p<0.05). The first 4 bars were from animals at 6 months of age, whereas, the post treatment animals were 8 months of age. (e) PAD2 RT-PCR in white matter extracts of normal, PBS, 2CA, and 2CA+B12 treated ND4 mice (n=9, p<0.05). (f) LFB and hematoxylin stain of cerebella from normal, PBS, 2CA, and 2CA + B12 treated ND4 mice (40X).
Figure 5. (a) Transmission EM of optic nerve sections from normal, PBS, 2CA, and 2CA+B12 treated ND4 mice at 6 mo (bar: 2 μm). (b) Transmission EM of optic nerve sections from 2CA and 2CA+B12 treated ND4 mice, 2mo after treatment cessation (bar: 2 μm). Asterisks show nude axons, arrows show thin myelin sheaths.
Figure 6. 2CA attenuates PAD2 Overexpressor. (a) Demyelinating disease in PAD2 transgenic mice treated with PBS, 2CA, or 2CA+B12 starting at 6 months of age (n=5, p<0.0001). (b) PAD activity in brain extracts of PAD2 transgenic mice treated with PBS, 2CA, or 2CA+B12, and non-transgenic littermates (n=4, p<0.05). PAD activity is reduced to normal levels by treatment.
Figure 7. 2CA attenuates acute EAE. (a) Clinical progression (n=5, p<0.0001) and (b) survival (n=5, p<0.01) during acute MOG-EAE in C57BL/6 mice treated with PBS, 2CA, or 2CA+B12. (c) H&E stain of spinal cord treated with PBS, 2CA, or 2CA+B12 (40X) showing decreased cellular infiltration after treatment (arrows).
Figure 8. (a) Spinal cord CD3+ lymphocytes in MOG EAE mice treated with PBS, 2CA, or 2CA+B12 (n=12, p<0.05). (b) PAD activity in brain (n=4, p=n.s.). (c) Spinal cord extracts (n=4, p<0.05) showing 3 fold increase in EAE reduced to normal levels by 2CA. (d) Ex vivo recall proliferative splenocytes responses of C57BL/6 mice with peak disease stimulated with MOG35-55 and MOG35-55 citrullinated at residue 41 (MOGcit41), residue 46 (MOGcit46), and residues 41 plus 46 (MOGcit1,46) (n=8, p<0.05 and p<0.01).
Figure 9. 2CA attenuates chronic relapsing EAE (crEAE). (a) Clinical crEAE progression in SJL mice treated with PBS, 2CA, or 2CA+B12 (n=5, p<0.0001). (b) PAD activity in SJL brain extracts during crEAE (n=4). (c) Protein citrullination in brains of SJL animals (n=5, p<0.0001) after termination of the experiment at 42 days. (d) H&E stain of SJL brain (40X) showing infiltration of cells (white arrows). Note the decrease after treatment.
Figure 10. (a) CD3+ lymphocytes in SJL spinal cord (n=12, p<0.01). (b) IFN-γ (n=3, p<0.05) and (c) IL-17 (n=3, p<0.05) secretion following in vitro stimulation of SJL splenocytes with PLP139-155. (d, e, f), Progression of crEAE following treatment with PBS or 2CA, beginning on day 20, 25, or 28 (n=5, p<0.0001).
Table 1 – G-ratios of untreated and treated mice.

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<th>Mouse phenotype</th>
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<td>ND4 + CA + B12 treatment terminated</td>
<td>0.87 ± 0.19</td>
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*N = 500 measures/group
*p > 0.015
Supplementary Figure 1. (a) Fragmentation of the peptide FLGEVHCNTGVR, with the peak at y6 corresponding to peptide RVNTGC containing an unmodified cysteine residue. (b) Fragmentation of the peptide FLGEVHCNTGVR following acetamidine-modification of cysteine, revealing a shift of 56 amu.
Supplementary Figure 2. PAD activity in SJL brain extracts after treatment with PBS or 2CA beginning on day 20, 25, or 28 (n=4, p<0.05).
## Supplementary Table I – PAD Activities of Normal and Diseased Human White Matter

<table>
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<th>HSB Identity*</th>
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**Supplementary Table 1.** * The human samples were provided by The Human Brain and Spinal Fluid Resource Center, VA West Los Angeles Healthcare Center, Los Angeles, who maintain sample identities using “HSB numbers” † 1° – Primary progressive; 2° – Secondary progressive; CP – Chronic progressive; RR – Relapsing remitting; 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 failure; PVD – Peripheral vascular disease; CRD – Coronary renal disease ‡ PAD assays were performed three times for each individual.