Peptidylarginine deiminase 2 (PAD2) overexpression in transgenic mice leads to myelin loss in the central nervous system

Abdiwahab A. Musse1, Zhen Li2, Cameron A. Ackerley3, Dorothee Bienzle4, Helena Lei2, Roberto Poma5, George Harauz1, Mario A. Moscarello2 and Fabrizio G. Mastronardi2,4

SUMMARY

Demyelination in the central nervous system is the hallmark feature in multiple sclerosis (MS). The mechanism resulting in destabilization of myelin is a complex multi-faceted process, part of which involves deimination of myelin basic protein (MBP). Deimination, the conversion of protein-bound arginine to citrulline, is mediated by the peptidylarginine deiminase (PAD) family of enzymes, of which the PAD2 and PAD4 isoforms are present in myelin. To test the hypothesis that PAD contributes to destabilization of myelin in MS, we developed a transgenic mouse line (PD2) containing multiple copies of the cDNA encoding PAD2, under the control of the MBP promoter. Using previously established criteria, clinical signs were more severe in PD2 mice than in their normal littermates. The increase in PAD2 expression and activity in white matter was demonstrated by immunohistochemistry, reverse transcriptase-PCR, enzyme activity assays, and increased deimination of MBP. Light and electron microscopy revealed more severe focal demyelination and thinner myelin in the PD2 homozygous mice compared with heterozygous PD2 mice. Quantification of the disease-associated molecules GFAP and CD68, as measured by immunoslot blots, were indicative of astrocytosis and macrophage activation. Concurrently, elevated levels of the pro-inflammatory cytokine TNF-α and nuclear histone deimination support initiation of demyelination by increased PAD activity. These data support the hypothesis that elevated PAD levels in white matter represents an early change that precedes demyelination.

INTRODUCTION

Multiple sclerosis (MS) is the most common demyelinating disease in young adults, and manifests itself in different ways (Lassmann et al., 2001). Although the cause of MS still remains elusive, the available evidence suggests a complex multi-factorial (genetic, immune and environmental) etiology (Lutton et al., 2004). The triggers that lead to the development of lesions in the ‘normal-appearing white matter’ (NAWM) have been hypothesized by us to provide some new pathogenic cues (Mastronardi and Moscarello, 2005; Moscarello et al., 2007). At the molecular level, we have shown that the severity of MS is associated with the degree of myelin basic protein (MBP) citrullination (conversion of arginine to citrulline) (Moscarello et al., 1994; Wood et al., 1996; Kim et al., 2003). In this context, the MBP cationicity found in MS patients was similar to that observed in children 4 years of age or younger, indicative of developmentally immature myelin (Moscarello et al., 1994). Various biochemical and biophysical studies have revealed the presence of increased amounts of citrullinated MBP. Hyperdeimination of MBP results in loss of myelin sheath integrity, which has been recently reviewed (Musse and Harauz, 2007).

The conversion of arginine to citrulline in proteins is carried out by the peptidylarginine deiminase (PAD) family of enzymes, of which five isoforms are known, with PAD2 (isoform 2) being the most abundant in the brain (Vossenaar et al., 2003b). The involvement of PAD in the pathogenesis of various autoimmune diseases such as rheumatoid arthritis has been established (Suzuki et al., 2003; Vossenaar et al., 2003a; Lundberg et al., 2005; Yamada et al., 2005). Given the increased citrullination of MBP and other proteins in MS (Kim et al., 2003) (reviewed in Harauz and Musse, 2007; Moscarello et al., 2007), we postulated that upregulation of PAD2 also represented an important early molecular change in demyelinating disease, and that increased citrullination of MBP results in reduced myelin stability in MS brain white matter (Moscarello et al., 1994). Evidence for PAD2 involvement has been obtained from the ND4 transgenic mouse model for demyelinating disease, in which increases in both PAD activity and mRNA levels were observed 1 month prior to an increase in citrullinated MBP and the concomitant appearance of any clinical or pathological signs (Moscarello et al., 2002a). We have also recently shown that the PAD2 promoter is hypomethylated in MS brain white matter (Mastronardi et al., 2007a), thereby explaining the increased synthesis of this enzyme. The sequence of events that we propose is as follows: decreased methylation of cytosines in promoter CG sequences by increased activity of a DNA demethylase results in increased transcription of the gene encoding PAD2 (Padi2) (Mastronardi et al., 2007a), an increase in PAD2, increased citrullination of MBP, and a decrease in the net positive charge on MBP and, subsequently, failure of myelin bilayers to compact leading to degradation of myelin and exposure of immunodominant epitopes (Moscarello et al., 2007; Musse and Harauz, 2007). A central player in this sequence is PAD2. Moreover, in a recently described Padi2-knockout mouse line, the amount of citrullination present in the central nervous system (CNS) was significantly diminished (Rajimakers et al., 2006), and demyelination was not observed. These observations were...
suggestive of PAD's association with disease, but were not indicative of a causal role.

In order to further define the involvement of PAD2 in the pathogenesis of demyelinating disease, we demonstrated that upregulation of PAD2 in oligodendrocytes leads to demyelination using a PAD2-overexpressing transgenic mouse line (PD2), in which the heterozygote contained 15 copies of the cDNA encoding PAD2 and the homozygote contained 30 copies. Using a set of previously established clinical criteria (Moscarello et al., 2002b), we compared the progression of the disease signs in transgenic mice with those of normal littermates. The increase in PAD2 expression was demonstrated by enzyme assays and reverse transcriptase (RT)-PCR. We used ultrastructural and histochemical analyses to demonstrate demyelination. The PD2 mice thus represent a new model for clinically mild variants of MS (Lassmann et al., 2001). Both clinical signs and morphological studies demonstrated that severity of signs correlated with gene dosage; the homozygote showed more severe signs and greater myelin destruction than the heterozygote. In addition, these data revealed that upregulation of PAD2, either by hypomethylation of the promoter (Mastronardi et al., 2007a) or by increased dosage of the PAD2 gene (in this study), can represent an important contribution to demyelination.

RESULTS
PAD2 transgenic mice and copy number
To determine whether increased expression of PAD2 in oligodendrocytes affected myelin structure, we generated transgenic mice expressing cDNA encoding the PAD2 protein under the regulation of the mouse MBP promoter. The construct used is shown in Fig. 1A. An EcoRI fragment of rat PAD2 cDNA consisting of 2.3 kb of DNA (Watanabe and Senshu, 1989) was inserted into the EcoRI cloning site of the pMG2 plasmid (Gow et al., 1992). The PAD2 minigene was restriction-digested with NcoI and injected into eggs from the CD1 mouse strain at the Hospital for Sick Children's transgenic mouse facility. Founder mice were obtained and assayed for the transgene by Southern analysis of tail clip DNA. The transgene-specific probe was a BglII fragment that straddled a portion of the MBP promoter extending into the 5'-untranslated region of the PAD2 cDNA (Fig. 1A). The results revealed a range in the number of transgene copies incorporated within the founder lines. A Southern blot of tail clip DNA from four mouse lines, numbers 9, 15, 25 and 28, is shown in Fig. 1B. Founder mouse line numbers 9 and 28 contained medium and high copy numbers of the transgene, respectively. To determine the relative abundance of the PAD2 transgene in the founder lines, we used the single copy proteolipid protein (Plp) gene as a loading control. The ratio of expression of the PAD2 transgene over the Plp gene revealed that line numbers 28, 9, 15 and 25 contained 15, 4, 1 and 0 copies of the transgene, respectively. Transgenic line number 28 was maintained by crossing homozygous mice with unrelated normal mice to generate 100% heterozygous mice. When homozygous mice were required, breeding pairs of unrelated heterozygous PD2 mice were used. In these crosses, inheritance of the transgene followed Mendelian ratios, with the progeny being, roughly: 25% homozygous PD2 mice, 50% heterozygous PD2 mice and 25% non-transgenic normal littermates, indicative of autosomal inheritance of the transgene.

Clinical phenotype and neurological examination
For this study, we maintained the high copy number PAD2 transgenic line, number 28 (containing 15 copies of the transgene), as heterozygous PD2 mice to assess the involvement of PAD2 in demyelinating disease. As a first qualitative approximation of the neurological deficits of the PD2 mice, we documented the clinical disease development, over a 6.0–6.5-month period from birth (longer in the homozygote), as mean clinical scores using previously established phenotypic behavior that included tail droop, tremors, unsteady gait, head shaking, convulsions, reduced physical activity and reduced righting ability (Moscarello et al., 2002b; Mastronardi et al., 2004). For the purpose of assessing the severity of the neurological deficits of the PD2 mice, these phenotypic behavioral displays were also compared with those observed for a previously characterized, spontaneously demyelinating transgenic mouse line, ND4 (DM20 over expressors) (Mastronardi et al., 1993), which had a more severe clinical course.

In our previous studies, we showed that the ND4 transgenic mouse, which contains supernumerary copies of a DM20 transgene, started to develop CNS demyelination 3 months after birth.
Disease Models & Mechanisms

Disease Models & Mechanisms

On the basis of these observed neurological signs, it was concluded that the lesions in the PD2 mice were localized to the CNS involving, bilaterally, the thalmo-cortex (leading to abnormal mentation, decreased interaction, and dullness). Furthermore, the neurological assessment of the abnormal gait suggested a cortical lesion or a concomitant cervical spinal cord lesion affecting the white matter between the C1-C5 spinal cord segments.

Expression of PAD2 protein in PD2 mice

To determine whether PAD2 was increased in the white matter tracts of heterozygous PD2 mice, we used a polyclonal antibody generated against whole muscle PAD2 (Takahara et al., 1983; Takahara et al., 1989) to stain mouse brain sections. The antibody labeled white matter tracts in the corpus callosum of both normal and PD2 mice, with a higher degree of labeling in the white matter tracts of the PD2 brain (Fig. 2A, arrows). Cerebellar white matter tracts also displayed higher qualitative labeling with anti-PAD antibody in PD2 mice (supplementary material Fig. S2). Similar increases were observed in spinal cord and cortical white matter (data not shown).

For an unambiguous quantitative analysis, the degree of PAD2 enzyme expression was determined from whole brain homogenates from 2-, 4- and 6-month-old heterozygous PD2 transgenic mice, and compared with non-transgenic littermates using anti-PAD2 antibodies by an immunoslot blot method. The loading control for each homogenate was the level of histone H3, which was determined by probing the immunoslot blot with anti-histone H3 antibody. The ratios of PAD2:H3 ± s.d. for each age group were

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**Fig. 2. PAD2 levels are elevated in PD2 white matter and in brain homogenates from PD2 mice.**

(A) Immunohistochemical analysis showing the extent of PAD2 expression in normal and heterozygous PD2 mice using anti-PAD2 antibody. Representative corpus callosum regions (arrows) of paraffin-embedded, formalin-fixed, sections of brain from 6-month-old normal control and heterozygous PD2 mice were probed with anti-pan-PAD2 antibody and counterstained with hematoxylin.

(B) Immunoslot blot showing quantitative expression levels of PAD2, normalized to histone H3, in normal and heterozygous PD2 mice, at various ages, using anti-PAD2 antibody and anti-histone H3 antibody (loading control). Significantly increased PAD2 levels are found at 2 (P < 0.0002), 4 (P < 0.002) and 6 (P < 0.002) months of age. (C) Specific PAD activity in isolated myelin from normal and heterozygous transgenic PD2 mice of different ages. All measurements represent the means ± s.d. of at least 10 independent determinations. Significant differences in specific PAD activity were observed in the myelin isolated from heterozygous PD2 transgenic mice compared with normal (N) mice at 1 (P < 0.01), and 2 (P < 0.0002) months of age. (D) Immunoslot blot analysis of the extent of MBP citrullination in isolated myelin from normal (N) and PD2 mice. The normalized relative ratios of deiminated and total MBP, determined using anti-modified citrulline antibody and anti-MBP antibody, respectively, are shown. The error bars indicate the s.d. from 9 determinations of each sample. Significant differences were observed at 2 (P < 0.002) and 3 (P < 0.0002) months of age, consistent with the elevated levels of PAD2 activity.
PAD2 activity and MBP citrullination in PD2 myelin

The involvement of PAD2 in myelin pathology was suggested previously by us (Moscarello et al., 2002a; Mastronardi and Moscarello, 2005; Moscarello et al., 2007). In order to determine whether PD2 mice possessed elevated levels of PAD2 in myelin, we isolated myelin from PD2 mice at different ages and compared PAD enzyme activities with those of non-transgenic littermates. When using the synthetic substrate BAEE (N-benzoyl-L-arginine ethyl ester), the results revealed that myelin isolated from PD2 animals at 1 and 2 months of age possessed significantly higher PAD2 activity (P<0.001 and P<0.0002, respectively) than their normal littermates (Fig. 2C).

To determine whether the observed in vitro increase in myelin PAD2 activity translated into higher activities or amounts of the enzyme, and thus elevated MBP citrullination, in vivo, we measured the amount of citrullinated MBP in myelin from PD2 mice by determining the ratio of citrullinated to total MBP (Fig. 2D). Special care was taken during preparation of the myelin to suppress the activity of the enzyme, and 5 mM EDTA was included in the isolation buffer to sequester Ca$^{2+}$, which is essential for enzyme activity. Both the monoclonal antibody F95 that recognizes peptidyl citrulline and a modified citrulline detection kit were used and yielded virtually identical results. Western blot analysis was used to confirm that citrullinated MBP isoforms were detected (data not shown). The ratio of citrullinated MBP to total MBP was slightly higher (~1.4-fold) in PD2 mice than in normal littermates (P<0.002) at 2 months of age; however, this ratio increased significantly by 3 months of age (P<0.0002), with a ~2.5-fold increase in citrullinated MBP in PD2 mice. The observed increase in MBP citrullination in younger PD2 mice correlated, in part, with the increased in vitro PAD enzyme activity (or quantity) in myelin isolated from these animals.

Morphological analysis of myelination by light and transmission electron microscopy (TEM)

In order to determine the degree of demyelination in PD2 mice, light and electron microscopic morphological analyses of appropriately fixed brain from both normal and transgenic mice were performed (Fig. 3). Images of optic nerves from normal mice, and heterozygous and homozygous PD2 mice, fixed with Karnovsky’s solution and processed for Toluidine Blue histology, are shown in Fig 3A-C, respectively. Light microscopy of Toluidine Blue cross-sections from normal mice (Fig. 3A) revealed well-myelinated fibers throughout the nerve. Optic nerve sections from heterozygous PD2 mice (Fig. 3B) revealed many normally myelinated fibers, but also apparent myelinolysis and thinly myelinated axons. Greater myelinolysis was observed in the homozygous PAD2 transgenics (Fig. 3C). Morphometric analysis of optic nerve fibers revealed a progressive reduction of myelinated fiber density from $8051\pm385$ fibers/mm$^2$ in the normal mice to $7820\pm296$ myelinated fibers/mm$^2$ in the PD2 heterozygous mice (P<0.001). Mice with the homozygous PD2 background showed a further reduction in myelinated fiber density to $7180\pm352$ fibers/mm$^2$ (P<0.001). These results suggested that PAD2 dosage had an effect on myelinated fiber density. We next assessed the G-ratio (axon diameter:fiber diameter) of myelinated fibers to determine whether myelin thickness was affected by PAD2 overexpression. The results revealed a progressive reduction in myelin thickness was apparent with increased PAD2 gene dosage. The heterozygous PD2 optic nerve had a mean G-ratio of 0.86±0.04 compared with 0.76±0.06 in normal mice, representing a significant reduction from the normal optic nerve myelin thickness (P<0.001). The myelin thickness in homozygous PD2 mice was further...
reduced, as evidenced by a G-ratio increase to 0.92±0.5, with more variation in myelin thickness. This represented a significant change compared with the G-ratios of non-transgenic mice (P<0.001).

In addition to the Toluidine Blue staining of myelin, we also assessed the degree of demyelination in PD2 mice using Luxol Fast Blue (LFB) staining of formalin-fixed, paraffin-embedded brain and spinal cord sections. For histopathology, sagittal sections of brain were stained with hematoxylin and eosin (H&E), and with LFB-Holmes – this histological stain is a combination of LFB (staining myelin) and a silver stain for axons, and is considered the most sensitive stain for demyelinating conditions. Representative LFB-Holmes stained sections of the cerebellar white matter of 6-month-old normal and homozygous PD2 mice is shown (Fig. 3D,E). H&E staining revealed no architectural changes in the brain structures of transgenic mice, whereas LFB staining revealed reduced myelin and irregular axonal staining in the homozygous mice (Fig. 3E).

Because the PAD2 transgene was under the control of the MBP promoter, we posited that peripheral nerve myelin might also be affected. Sciatic nerves from PD2 transgenic mice were examined for effects on peripheral nerve myelin pathology. Toluidine Blue-stained sections were examined by light microscopy. No differences in peripheral nerve myelin were observed between non-transgenic and PD2 mice (supplementary material Fig. S1). These results indicated that the effects of PAD activity were specific for CNS, and not peripheral nervous system, myelin.

Examination of optic nerves by electron microscopy revealed structural changes in the myelin of PAD2 transgenic mice. Micrographs of cross-sectioned optic nerves from non-transgenic and transgenic mice are shown in Figs 4 and 5. The micrograph of a normal optic nerve from a non-transgenic littermate (Fig. 4A) shows well-myelinated axons throughout the field. Well-myelinated axons were also present in the optic nerves from heterozygous PD2 transgenic mice (Fig. 4B); however, myelinolysis is also visible (Fig. 4C, arrowheads), representing subtle but definite changes in myelin structure. Changes in the optic nerves from homozygous PD2 transgenics were more pronounced. Fig. 5A shows a low-powered field, revealing an area of myelin degradation, nude axons and thinly myelinated axons in the presence of myelinolysis. In addition, astrocytic processes (asterisks) were prominent. Fig. 5B shows a high-powered field, containing thickened astrocytic processes (asterisks) and nude axons (arrowheads). Areas with variably myelinated axons also revealed nude axons (Fig. 5C,D, arrowheads) amidst astrocytic processes (asterisk). Therefore, increasing PAD2 gene dosage (homozygous) had a more severe effect on myelin structure than the lower heterozygous dosage.

**Cellular infiltration**

In order to ascertain whether infiltrating cells were prominent in the brains of PD2 mice, we used antibodies that recognize T-cells (CD3), cytotoxic T-cells (CD8) and activated macrophages/microglia (CD68). Neither CD3 nor CD8 were elevated in heterozygous PD2 brains (Fig. 6A). As a positive control for cellular infiltrates, we used brain extracts from SJL mice with MBP-induced experimental autoimmune encephalomyelitis (EAE) with each of these antibodies. Lymphocyte cuffing around blood vessels and subventricular white matter are common features of EAE (data not shown). The PD2 transgenic mouse was generated in a CD1 background – to date, this strain of mice has not been shown to be susceptible to EAE induction using myelin antigens. The extracts from the EAE brains had elevated levels of CD3, CD8 and CD68 markers relative to the levels found in PD2 mice brains. CD68 was the only marker that increased in PD2 mice from 2 to 4 months of age. The level of CD68 remained unchanged in 6-month-old PD2 brains and only increased slightly by 9 months of age. Overall, CD68 levels were less than half of those observed in the brain extract from mice with EAE. We found no histological evidence of lymphocytic infiltration in the PD2 mice, which was consistent with the very low levels of CD3 and CD8. Therefore, demyelination in homozygous PD2 mice was primarily neurodegenerative.

The amount of glial fibrillary acidic protein (GFAP) was shown to be elevated in PD2 mice by both immunohistochemical (supplementary material Fig. S2) and immunoslot blot methods. Representative anti-GFAP immunohistochemical images of the cerebellum and C1-C5 regions of the spinal cord from 6-month-old normal and PD2 mice revealed astrocytosis in the cerebellar white matter of PD2 mice (supplementary material Fig. S2B) compared with the cerebella of their normal littermates.
Because the neurological assessment suggested possible spinal cord lesions in and around C1-C5, we also examined spinal cords of PD2 mice in this same region. GFAP-labeling of a longitudinal-sectioned cervical region of the spinal cord from both normal (supplementary material Fig. S2C) and PD2 (supplementary material Fig. S2D) mice revealed that the level of astrocytosis, and the size of astrocytes, were greater in the PD2 spinal cord, indicating greater astrocytic involvement in the white matter of PD2 mice.

In order to determine the relative increase of astrocytosis, we quantified the amount of GFAP in whole brain homogenates from PD2 heterozygous mice at 1, 3, 6 and 8 months of age. Fig. 6B shows results from immunoslot blots for GFAP, in which the heterozygous PD2 mouse brain results have been normalized with respect to GFAP levels in non-transgenic littermates. The results in Fig. 6B reveal a slight, but non-significant, increase in GFAP in 1- and 3-month-old heterozygous PD2 brains. By 6 and 8 months of age, GFAP levels were moderately increased by up to ~1.7-fold of the non-transgenic littermates. These results were consistent with a moderate degree of astrocytosis in the PD2 brain sections (supplementary material Fig. S2A-D) and were also consistent with increased labeling of PAD2 in white matter regions of brain (supplementary material Fig. S2E,F).

Overexpression of PAD2 increases expression of PAD4 but not other PAD enzymes
All of the genes encoding the PAD enzymes are clustered on a single locus on human chromosome 1 at position 1p36.13. In order to determine whether the overexpression of PAD2 affected the expression of other PAD enzymes found in the brain, we conducted RT-PCR on RNA isolated from the white matter of 6-month-old normal and transgenic PD2 mice. The data shown in Fig. 7A revealed no PAD1 expression and very low levels of PAD3 expression in mouse white matter. PAD4 mRNA was expressed in skin (data not shown) and normal white matter, and was elevated in the white matter of heterozygous PD2 mice by ~4-fold relative to non-transgenic littermates. PAD4 mRNA levels were further
elevated in the PD2 homozygous line, by 5.3-fold. The primer set, specific for PD2 cDNA, revealed a band in the white matter cDNA from PD2 mice. As expected, the non-transgenic mice had no amplicons related to the PD2 transgene. As a control, we designed a mouse-specific primer set that was identical to both the endogenous mouse PAD2 cDNA and the rat PAD2 transgene (supplementary material Fig. S3A,B); therefore, the primers recognized both the natural PAD2 and the transgenic PAD2, both PAD2 cDNAs were detected (Fig. 7A). When compared with normal littermates, the total amount of PAD2 RNA in the heterozygous and homozygous PD2 transgenic mice was elevated by ~3.5-fold and ~7.5-fold, respectively.

Increased PAD4 and citrullinated histone correlate with increased TNF-α

In a recent study, we have shown that the cytokine TNF-α was elevated in the NAWM from MS individuals and in tissues from animal models of demyelination (Mastronardi et al., 2006). In the absence of other inflammatory features, such as microglial activation or lymphocytic infiltrates in the human NAWM, such observations suggested that the elevated TNF-α in these tissues might have been derived from astrocytes.

To determine whether TNF-α levels in PD2 animals were also affected, we measured the relative amounts of TNF-α in brain extracts prepared from 2-, 4-, 6- and 9-month-old heterozygous PD2 mice. The results revealed significant increases in TNF-α levels by 4 months of age (~1.7-fold of normal), and maximal levels by 6 months of age (~2-fold of normal) (Fig. 7B). To ascertain whether elevated TNF-α was accompanied by increased nuclear PAD4, we isolated white matter nuclear fractions from normal and PD2 mice, and assayed for PAD4 by using western blots. The results revealed elevated amounts of nuclear PAD4, peaking at 6 months of age, relative to the levels in non-transgenic brain nuclear fractions (Fig. 7C). To determine whether the elevated nuclear PAD4 was active, we measured the levels of citrullinated histone H3. Increasing amounts of citrullinated H3 were observed from 2-9 months of age, consistent with the elevated nuclear PAD4 levels (Fig. 7D).

DISCUSSION

MS is a complex human neurodegenerative disease of unknown origin, and is characterized by the active destruction of the insulating myelin sheath around the axons. The factors that contribute to lesion development are not fully understood. MS is unique to humans, and because of its complex etiology any animal model is only appropriate to represent a particular facet, or variant, of the disease. The most commonly used animal model for MS has been the EAE model, in which demyelination via an autoimmune response is induced in a susceptible background by injection of a myelin component. However, using this as a model for human MS has many limitations (Chaudhuri and Behan, 2005; Sriram and Steinier, 2005), partly because it does not faithfully replicate the altered post-translational modifications, such as deamination, of proteins such as MBP (Kim et al., 2003). We have previously proposed that myelin damage in MS white matter results from a failure to maintain compact adult myelin, reflecting abnormally altered post-translational modifications, such as deamination, of proteins such as MBP (Kim et al., 2003). We have previously proposed that myelin damage in MS white matter results from a failure to maintain compact adult myelin, reflecting abnormally enhanced citrullination of MBP. MBP citrullination results in fewer cationic MBP isomers and, therefore, less stable myelin multilayers, which are more susceptible to digestion by endogenous proteases (D’Souza et al., 2005; D’Souza and Moscarello, 2006; Musse et al., 2006). This irreversible post-translational modification of MBP is carried out by the PAD enzymes. In myelin this can be performed by PAD2 and PAD4 (Wood et al., 2008), both of which are found in human and mouse myelin.

In order to test the hypothesis that increased PAD activity can destabilize myelin structure, we generated a transgenic mouse containing supernumerary copies of PAD2 cDNA under the regulation of the mouse MBP promoter. A heterozygous line containing 15 copies of the transgene was used to prepare a homozygous line that developed more severe clinical and morphological features of demyelination. Toluidine Blue staining of optic nerve white matter revealed aberrant myelination and sporadic areas with myelin fragmentation and vacuolation. Light microscopic analysis of LFB-stained brain sections also revealed a reduction in myelin staining that was reminiscent of white matter pallor, and apparent vacuolation and the appearance of coarse fibers
in cerebellar white matter. All changes were more prominent in the homozygous line. The peripheral nervous system myelin appeared normal. Quantitative analysis of GFAP in total brain homogenates revealed an increase in the number of astrocytes that was most prominent in PD2 mice after 3 months of age.

The overall progression and magnitude of the disease signs appeared to be different between the new PD2 and established ND4 animal models for spontaneous demyelination. The PD2 mice displayed earlier clinical signs than ND4 mice, as early as 1.5 months compared with 3 months of age, respectively. Since myelogenesis begins at 2-4 weeks of age, the fact that both the heterozygous and homozygous PD2 transgenic mice began displaying clinical signs after the myelogenic period indicated that the disorder was a primary demyelinating one, as in the ND4 mouse, and not congenital as in the dysmyelinating leukodystrophies.

Recently, we reported that, in addition to MBP deimination and upregulation of PAD2, one of the hallmarks of demyelination in MS and in models of demyelinating diseases was the concomitant increase in PAD4, the nuclear isoform of this family of enzymes, with the resulting deimination of histones (Mastronardi et al., 2006). The production of TNF-α by astrocytes (Lieberman et al., 1989; Chung and Benveniste, 1990) and its involvement in the pathogenesis of CNS inflammation and demyelination is well documented (Probert et al., 2000). Direct involvement of TNF-α in non-immune-mediated primary demyelination has also been demonstrated using transgenic mice overexpressing murine or human TNF-α (Probert et al., 1995; Akassoglou et al., 1998; Akassoglou et al., 1999; Kassiotis et al., 1999), and the involvement of TNF-α induced PAD4 translocation into the nucleus (Mastronardi et al., 2006).

Taken together, the data presented in this report support our hypothesis that elevated PAD2 activity in brain white matter represents an early change preceding the onset of demyelination as summarized in the model presented in Fig. 8. Therefore, in addition to destabilization of myelin by PAD2-induced deimination of MBP, the deimination of histones with subsequent apoptotic events in oligodendrocytes results in a cascade of events causing primary demyelination. Myelin antigens are then released and can initiate an immunological cascade. Neurodegeneration may represent a primary event in demyelination, at least in some forms of MS in which cellular infiltration is not a prominent feature, as demonstrated in our models. Alternatively, some MS cases may also be initiated by immune-mediated processes. The process underlying lesion development appears to be heterogeneous, which in turn implies that therapeutic intervention may involve targeting more than one process, as we have suggested in earlier reports (Mastronardi et al., 2004; Mastronardi et al., 2007b). Thus, a unified model of MS recently proposed by Bruck (Bruck, 2007) represents a conceptual advance. In this report, we showed that an increased amount of PAD2 in a transgenic mouse led to myelin destabilization. The effects of this experimental increase of PAD2 were consistent with our earlier observations in which increased PAD2 in MS white matter was associated with both hypomethylation of the PAD2 gene and with increased citrullination of MBP. Therefore, the elevation of PAD2 by any mechanism represents an early change in the pathogenesis of demyelinating disease and PAD2 represents a therapeutic target.

**METHODS**

**Antibodies**

The primary antibodies used for this study were as follows: (1) mouse monoclonal anti-MBP MAb26 (a generous gift from Dr Nigel Groome, Oxford Brooks University, UK), which recognizes all isoforms of MBP; (2) mouse monoclonal antibody F95 (a...
generous gift from Dr A. P. Nicholas, University of Alabama at Birmingham, AL), which recognizes peptidylcitrulline; (3) modified citrulline detection kit (Upstate Biotech, Lake Placid, NY) containing rabbit anti-citrulline (modified) and goat anti-rabbit IgG HRP, which was also used for the detection of citrulline-containing proteins; (4) rabbit anti-pan PAD (a gift from Dr H. Takahara, Ibaraki University, Ibaraki, Japan), which recognizes all isoforms of PAD; (5) rabbit anti-histone H3 polyclonal antibody (Abcam, Cambridge, MA), which recognizes only PAD2; (5) rabbit anti-histone H3 polyclonal antibody Ab1791 (Abcam); and (6) rabbit anti-GFAP IgG (DAKO, Glostrup, Denmark). The secondary antibodies were goat anti-mouse and goat anti-rabbit IgG HRP conjugate (Bio-Rad Laboratories, Hercules, CA). Anti-TNF-α monoclonal Ab1793 (Abcam) was used to measure TNF-α in brain homogenates. Rabbit anti-CD3 (DAKO) was used to measure TNF-α in brain homogenates.

**Transgenic mouse lines and copy number**

The PAD2 transgene was constructed by ligating 2.3 kb of rat PAD2 (PD2) cDNA into the EcoRI cloning site of the pMG2 plasmid (Gow et al., 1992). This plasmid contains 1.8 kb of the mouse MBP promoter, and exon 2, intron 2 and exon 3 of the β-globin gene. The transgene construct was restriction-digested with NotI to remove bacterial pUC DNA from the MBP-PD2 minigene. The NotI fragment containing the minigene was purified from a 0.7% agarose gel using the Geneclean® kit (Qbiogene, Morgan Irvine, CA) according to the manufacturer’s instructions. The isolated DNA was further purified using Elutip-D columns (Schleicher and Schuell, Keene, NH). The minigene was microinjected into donor mouse CD1 eggs and transplanted into pseudo-pregnant surrogate female CD1 mice.

All animals were housed in a closed colony in the animal care facility at The Hospital for Sick Children (HSC, Toronto, Canada). Mouse colonies were established and maintained according to the guidelines and regulations of the HSC’s Animal Care Committee. The founder lines consisted of 15 mice with rat PAD2 transgenes incorporated into them. Animals carrying the transgene were initially determined by Southern blot analysis of DNA isolated from tail clips (~5 mm). A 32P-labeled BglII fragment derived from the transgene construct was used as a transgene-specific probe (Fig. 1). The transgene copy number was determined by Southern blot. The single copy myelin proteolipid protein (PLP) gene was used as a loading standard, and the semi-quantitative relative copy number of the transgene was determined through densitometric comparison with the PLP gene, using the open source Image-SXM image analysis software (version 1.75), which was based on the NIH image analysis program on a G4 Macintosh computer.

**Genotyping and tail clip DNA PCR assay**

The PD2 mice were heterozygous. For routine genotyping purposes, the transgenic mice were shown to carry the transgene by PCR analysis of isolated DNA. The DNA was isolated from mouse tail clips (~5 mm) following the ‘HotSHOT’ protocol (Truett et al., 2000). The presence of the transgene (rat PD2) was confirmed using the following primer sets: GAG CGG ACC GTA CGG CTG CAA and GAC CTI TTC ATC ACT GCT GCA GTCC, with an expected amplicon of 507 base pairs (bp) corresponding to the region between bases 70-576 of the cDNA, as described (Watanabe and Senshu, 1989). To determine whether the isolated DNA was a good substrate for PCR, the endogenous mouse β-globin gene was amplified with the following primer pairs: CCA ATC TGC TCA CAC AGG ATA GAG AGG GCA GG and CCT TGA GGC TGT CCA AGT GAT TCA GGC CAT CG, with an expected amplicon of 494 bp. Both PCR reactions were amplified for 34 cycles using the following profile: for the transgene, 1 min at 95°C, 1 min at 94°C, 1 min at 55°C and 1 min at 72°C; for β-globin, 1 min at 94°C, 1.5 min at 60°C and 2 min at 72°C.

**Reverse transcriptase PCR (RT-PCR)**

For mouse brain white matter, mouse-specific primers for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), PAD1, PAD2, PAD3 and PAD4 were used. To distinguish between endogenous PAD2 and PAD2 from transgenic mice, a rat-specific PAD2 primer set and a primer set matching sequences from both rat and mouse PAD2 cDNA were used. The specificity of the PAD2-specific primer set for rat PAD2 alone was confirmed by Blast 2 sequence alignment, using the NCBI Blast database (supplementary material Fig. S3). The forward and reverse primers, and PCR programs, were as follows:

- **G3Pdh (947 bp):** 5’-TG TGA GAT CTC GCC GTA ACG GGA A-3’ and 5’-CCA AAT TCA TGG TTA TAG CAG-3’; 3 min at 94°C, 30 cycles of (1 min, 94°C; 1 min, 50°C; 3 min, 72°C), 10 min at 72°C.
- **PAD1 (203 bp):** 5’-ATC CTG TTA GGA AAT TCA GAC T-3’ and 5’-GCA CAG AAG ACC TTC AG-3’; 3 min at 94°C, 40 cycles of (1 min, 94°C; 1 min, 55°C; 3 min, 72°C), 10 min at 72°C.
- **PAD2 (638 bp):** 5’-GCT TGA CCA GGG GCA GAC TCG AG-3’ and 5’-TCA GCA CCC AGC GAG CAG GAC TGG AG-3’; 3 min at 94°C, 35 cycles of (1 min, 94°C; 1 min, 60°C; 3 min, 72°C), 5 min at 72°C.

**Clinical scoring**

Animals were clinically scored three times weekly as described (Moscarello et al., 2002b; Mastronardi et al., 2004), and mean weekly scores (±s.d.) were plotted relative to age. The neurological signs measured included tail droop, tremors, unsteady gait, head shaking, convulsions, physical activity and righting ability. The scores for
each of these clinical signs ranged from 0 (no signs) to 4 (severe). Controls were non-transgenic littermates. For various comparative assays, the previously described ND4 transgenic mouse model was also used (Mastronardi et al., 1993).

Whole brain protein extracts and myelin preparation
Total brain protein extracts were prepared from non-transgenic and transgenic animals using a Teflon tissue homogenizer (Wheaton, Millville, NJ) in PBS, pH 7.6, 6 M urea. Membrane- and nuclear-containing fractions were prepared from transgenic and non-transgenic mouse brains using discontinuous sucrose gradients (Norton, 1974; Mastronardi et al., 2000).

For samples to be used for PAD activity assays, pellets were resuspended in 20 mM HEPES-KOH, pH 7.6, 1 mM EDTA, 0.5 mM DTT, with 0.43 mM PMSE. For samples to be used for western and/or immunoslot blots, pellets were resuspended in phosphate-buffered saline (PBS), pH 7.6, with 6 M urea. All samples were snap-frozen in liquid nitrogen and stored at −80°C. Protein concentrations were determined by a modified bicinichonic acid (BCA) method (Pierce, Rockford, IL).

EAE induction
Acute EAE was induced in 6–8-week-old female SJL mice using complete Freund’s adjuvant (CFA)-emulsified bovine MBP as the antigen. Mice were inoculated with two injections consisting of 200 ng of pertussis toxin (Sigma, St Louis, MO) in PBS, pH 7.4, via tail veins on day 0 and then again 48 hours later. The emulsified MBP-CFA was injected subcutaneously at the base of the tail. Mice were monitored for signs of EAE and scored according to the scale described by Mastronardi (Mastronardi et al., 2004). After the acute disease phase (day 20), brains were excised and quickly frozen in liquid nitrogen. Brain homogenates were prepared as above for immunoslot blot assays.

Protein analysis and immunoslot blot quantification
Both GFAP and PAD2 were quantified from total brain homogenate by immunoslot blots (Mastronardi et al., 2006). For quantification of citrullinated MBP, myelin was isolated as described above. For GFAP and PAD2 measurements, 5 μg protein extracts were applied onto a nitrocellulose membrane using a Bio-Dot SF Microfiltration Apparatus (BioRad, Hercules, CA) according to the manufacturer’s instructions. Membranes were probed with either anti-PAD2 antibody or anti-GFAP antibody overnight at 4°C. The membrane was washed four times with Tris-buffered saline containing Tween-20 (TBST), labeled with secondary goat anti-rabbit antibody or anti-GFAP antibody overnight at 4°C. For GFAP, mouse anti-actin monoclonal antibody (Chemicon International, Temecula, CA) was used as the loading standard. The relative amount of PAD2 and GFAP was calculated from the ratio of pixel densities in extracts of PAD2 versus histone H3, and GFAP versus actin, respectively. The amount of TNF-α, CD3, CD8 and CD68 was determined from whole brain homogenates as previously described (Mastronardi et al., 2006).

Transmission electron microscopy of optic nerves
Transmission electron microscopy of optic nerves
Histology of optic and sciatic nerves
Optic and sciatic nerves from non-transgenic and transgenic PD2 mice, at 6 months of age, were immersion-fixed with Karnofsky’s solution (Feirabend et al., 1994). Peripheral nerves were post-fixed in osmium tetroxide, sectioned, and stained with Toluidine Blue. Images of sciatic nerve cross-sections were captured using a Leica DM bright field microscope.

Immunohistochemistry
Immunohistochemistry of formalin-fixed, paraffin-embedded brain sections using anti-PAD2 polyclonal antibody was done as described previously (Liu et al., 2005; Nicholas et al., 2005). The slides were counterstained with hematoxylin, and viewed and
PAD2 overexpression causes CNS demyelination

TRANSLATIONAL IMPACT

Clinical issue
Multiple sclerosis (MS) is a complex brain-specific disease of unknown origin. Patients afflicted with MS exhibit a progressive decline in balance, coordination and movement, as well as fatigue, muscle weakness, and visual and sensory disturbances. These hallmark symptoms are caused by degeneration of myelin, which normally forms an insulating sheath around axons to enable rapid and efficient action-potential propagation. Myelin degeneration mechanisms in MS are not known, but an emerging theory implicates enzymatic conversion of amino acid charges in myelin basic protein (MBP). In this process known as deimination, peptidylarginine deiminase (PAD) converts positive arginine residues into the neutral amino acid citrulline. Prior studies demonstrate that MS severity is associated with increased citrulline in MBP, and that hyperdeimination leads to myelin sheath destabilization. The authors have previously identified increases in PAD2 and PAD4 away from lesions in MS brains, leading to the hypothesis that PAD upregulation is an early indicator of myelin degradation in MS.

Results
In this study, extra copies of the rat PAD2 gene were introduced into the mouse genome to create a transgenic model of human MS. PAD2-overexpressing mice exhibited abnormal gait, hind-limb weakness, and deficits in proprioception, balance, and coordination. CNS tissue analysis revealed white matter lesions and nude and hypomyelinated axons. Additionally, lesion sites had increased numbers of astrocytes, as seen in MS and MS-like diseases. The demyelination and neurological deficits were more severe in homozygous PAD2 mice than heterozygotes, indicating a dose-dependent effect of PAD2 overexpression. The authors also observed an increase in PAD4 expression in the transgenic mice, suggesting that PAD2 and PAD4 operate in the same pathway to produce the observed phenotypes.

Implications and future directions
This study provides in vivo evidence that PAD2 overexpression increases levels of citrulline in MBP and, consequently, triggers myelin destabilization. This confirms and extends previous data suggesting that the PAD-deimination pathway is important in the maintenance of myelin integrity. The PAD2 mouse described here is a novel model of demyelinating disease. Given the fact that PAD levels are increased in regions proximal to MS brain lesions, this animal model may be useful in studying the intervention of demyelination in MS. Furthermore, this work highlights the therapeutic potential of targeting PAD in demyelination disorders.

Quantification of TNF-α in PD2 brain homogenates
The amount of TNF-α in brain extracts was measured by immunoslot blots. Aliquots of brain extract consisting of 5 µg per slot were loaded in triplicate (in two separate experiments) onto a nitrocellulose membrane. Membranes were blocked with 5% blotto and then incubated with anti-TNF-α overnight at 4°C. The anti-TNF-α antibody was diluted 2000-fold. The secondary antibody was diluted 10,000-fold. Measurements of histone H3 were used as loading controls. The TNF-α probed membranes were stripped using Restore Western Blot stripping buffer (Pierce Biotechnology, Rockford, IL, as per manufacturer’s instructions) and then probed for histone H3 using anti-histone H3 antibody (Abcam Inc., Cambridgehire, UK) diluted 10,000-fold in 5% blotto.

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COMPETING INTERESTS
The authors declare no competing financial interests.

AUTHOR CONTRIBUTIONS
A.A.M. contributed Fig. 1C; Fig. 2; Fig. 6; Fig. 7B-D and supplementary material Fig. S2; A.A.M. also aided in the assessment of Toluidine Blue-stained optic nerve sections and contributed to the preparation of the manuscript; Z.L. aided in scoring the transgenic mice; generated the homozygous PD2 line and contributed the data related to the homozygous line; Z.L. also provided RT-PCR data presented in Fig. 6A and supplementary material Fig. S3; C.A.A. acquired electron and Toluidine Blue micrographs of normal and transgenic mice optic nerves, and calculated the g-ratios; D.B. assessed LFB brain histology of transgenic and non-transgenic mice; H.L. aided in the clinical scoring of the transgenic mice; R.P. assessed the clinical neurological signs of the mice; G.H. aided in the writing of the manuscript; M.A.M. aided in the conception and coordination of the study and contributed to the preparation of the manuscript; F.G.M. engineered the transgene, coordinated clinical scoring, organized the figures and contributed to the preparation of the manuscript.

SUPPLEMENTARY MATERIAL
Supplementary material for this article is available at http://dmm.biologists.org/content/1/4/5/229/suppl/DC1

REFERENCES

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Histopathology of brain tissue
Brains were excised following cervical dislocation. Care was taken not to damage the cervical region of the spinal cord during this procedure. Brain and spinal cord were immediately removed and fixed in 10% buffered formalin for 48 hours. For the brain, the tissues were embedded sagitally in paraffin and sectioned at a thickness of 5 mm. The corpus callosum and the cerebellum were routinely examined. For the spinal cord, the tissue was divided into three separate segments: (A) cervical (C1-C5), (B) thoracic, and (C) lumbar-sacral regions. Each section was cut longitudinally and embedded as a spread. Immunohistochemistry using anti-GFAP (Dako, Carpintera, CA) antibody for detection of astrocytes was carried out with an automated Ventana Benchmark XT (Ventana, Tucson, AZ) immunostaining system, as performed by the Neuropathology Service Laboratory using their standard operating protocols for these reagents. The slides were counterstained with hematoxylin, and viewed and photographed with an Olympus BX60 light microscope equipped with a CCD camera.

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