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Localisation of citrullinated proteins in normal appearing white matter and lesions in the central nervous system in multiple sclerosis

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ABSTRACT

Multiple sclerosis (MS) is a chronic inflammatory neurodegenerative disease, considered to be autoimmune in origin. Post-translational modification of central nervous system proteins, including glial fibrillary acidic protein (GFAP) and myelin basic protein (MBP), through citrullination of arginine residues, may lead to exposure of neoepitopes, triggering autoimmunity. Here we investigated the expression of citrullinated proteins in active MS lesions, MS normal appearing white matter and control brain white matter. We demonstrate increased citrullinated GFAP and MBP by immunohistochemistry and western blotting in areas of ongoing demyelination, suggesting a pivotal role for deimination of GFAP and MBP in MS pathogenesis MS.

1. Introduction

Multiple sclerosis (MS) is a chronic immune mediated disease of the central nervous system (CNS) affecting approximately 0.1% of Caucasians of north and central European ancestry (Noseworthy et al., 2000), resulting in focal demyelinated lesions or plaques. In general, these lesions are classified as either active or inactive. Active MS lesions are defined by the presence of activated microglia and infiltrating macrophages, which contain remnants of myelin, phagocytosed during the demyelinating process, in addition to large reactive astrocytes (Jack et al., 2005). T cells, B cells and plasma cells are also found in active lesions (Frischer et al., 2009). In contrast, inactive lesions consist of demyelinated foci which are sharply defined and hypocellular with no evidence of active demyelination or axonal loss, but instead prominent fibrillary gliosis (Lucchinetti et al., 2005).

There is increasing evidence that citrullination may play an important role in MS pathogenesis (Nicholas et al., 2004; Harauz and Musse, 2007; Musse and Harauz, 2007). Citrullination is a process whereby an arginine residue is converted to the non-standard amino acid citrulline (Beniac et al., 2000), resulting in the loss of a positive charge and an altered secondary and tertiary structure of the protein (Musse et al., 2006; Harauz and Musse, 2007). This post translational modification (PTM) is carried out by a family of five citrullinating enzymes known as peptidylarginine deiminases (PADs), with PAD2 and PAD4 being the most common PADs found in the brain (Rogers et al., 1977; Vossenaar et al., 2003). Excess citrullination has been reported in the CNS in postmortem MS brain tissue (Nicholas et al., 2004; Mastronardi et al., 2006). Previously, using myelin basic protein (MBP) isolated from normal appearing matter (NAWM) from MS patients and controls, and fractionation of the samples by column chromatography, Moscarello et al. found that 18% of MBP was citrullinated in control tissue compared to 45% of MBP in patients with MS (Moscarello et al., 1994). Further studies by the same group found that in Marburg's disease, as much as 90% of MBP is citrullinated (Wood et al., 1996).

Since citrullination alters the charge of the protein, citrullinated MBP becomes partially unfolded and its interaction with phospholipids is weakened, resulting in myelin sheaths that are not as tightly packed as in normal myelin (Wood and Moscarello, 1989; Beniac et al., 2000). Studies have shown that deiminated MBP is more susceptible to proteolytic digestion by myelin associated proteases (Cao et al., 1999; Pritzker et al., 2000; D'Souza and Moscarello, 2006; Musse et al., 2006). This greater surface exposure and increased cleavage of citrullinated protein by proteases would lead to increased release of immunodominant epitopes, which could then sensitize peripheral blood T cells (Musse et al., 2006; Musse and Harauz, 2007). Furthermore, citrullination

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appears to be necessary in order to elicit $CD4^+$ T cell responses, suggesting that 'altered-self' epitopes are only presented to T cells when certain arginine residues have been converted to citrulline (Hill et al., 2003; James et al., 2010). Recently Acharya et al. (2012) have proposed a role for citrullination of neuronal proteins, localised in regions of neurodegeneration, in the generation of autoantibodies in Alzheimer's disease.

Using an antibody which recognises all deiminated proteins (F95) it was reported that citrullination of glial fibrillary acidic protein (GFAP) was substantially higher in the NAWM compared with equivalent control brain tissue (Nicholas and Whitaker, 2002; Nicholas et al., 2004, 2005). In addition, presumed chronic inactive lesions were found to be devoid of citrullinated proteins in this study, except within astrocytes surrounding blood vessels (Nicholas et al., 2004). Using F95 antibody these authors also demonstrated that citrullinated proteins were present in both the brain and spinal cord of mice with myelin oligodendrocyte glycoprotein (MOG)-induced experimental autoimmune encephalomyelitis (EAE), and that citrullination was shown to co-localise within MBP positive regions surrounded by GFAP immunoreactive astrocytes, that were also positive for deiminated proteins (Nicholas et al., 2005).

Here, we investigated the immunohistochemical localisation of citrullinated proteins in post mortem brain tissue from MS patients and normal control cases and confirmed the citrullinated proteins present by western blotting as well as expression of PAD2 mRNA in the CNS by quantitative real-time PCR. PAD2 mRNA expression was also examined in *in vitro* studies of primary human astrocytes, a human foetal microglial cell line and a human brain endothelial cell line. Using these techniques we demonstrated that increased citrullinated GFAP was found in areas of both ongoing demyelination and myelin loss in active and chronic active MS lesions. Interestingly, where there was complete myelin loss, citrullinated proteins were absent. Lower levels of citrullinated proteins were observed in the MS NAWM and control white matter. Western blot analysis of brain tissue from these patients confirmed that in addition to MBP, GFAP was the major citrullinated protein in the CNS, and the amount of citrullination was increased in active disease, suggestive of a role in the pathogenesis of MS.

2. Materials and methods

2.1. Human tissue

Nineteen blocks of snap-frozen autopsy tissue from 12 clinically and neuropathologically confirmed secondary progressive multiple sclerosis (SPMS) cases, together with nine blocks from 6 normal control cases, were obtained from the UK Multiple Sclerosis Society Tissue Bank, Imperial College, London (Table 1). The MS cases included 10 females, mean age 67.7 years (range 37–86) and 2 males, mean age 58.5 years (range 55–62) and contained lesions typical of active and chronic active disease. Brain tissue was received fresh from autopsy with <24 h death-autopsy times for 11 out of 12 MS cases. For each MS case, between 1 and 3 cerebral tissue blocks, including cortical and perivascular areas with white matter demyelination, were examined. All of the patients had a confirmed diagnosis of MS by both histological and clinical criteria. Control and MS blocks were matched for CNS location.

Serial cryostat sections (10 μm) were processed for haematoxylin and eosin (H&E), oil red-O (ORO), and anti-HLA-DR immunohistochemistry to evaluate the general histology and extent of cellular activation within each block. Perivascular inflammation was graded using a four-point scale (negative, +, ++, +++). Lesions with ORO-positive cells throughout and with perivascular cuffing were classified as active lesions. Blocks that contained ORO-negative regions with hypercellular borders, including ORO positive cells, were classified as chronic active lesions, whereas blocks derived from macroscopically normal white matter with the absence of ORO staining or perivascular inflammation and with resting microglia, identified with anti HLA-DR staining, were classified as normal appearing white matter (NAWM).

2.2. Immunohistochemistry

Serial cryostat sections of 19 MS and 9 control tissue blocks were cut and mounted onto poly-L-lysine coated glass slides (polysine™ slides, catalogue number 631-1349, VWR International Ltd., UK), fixed in ice-cold acetone for 10 min and then allowed to air-dry. Sections were

Table 1
Clinical data of controls and multiple sclerosis cases included in this study.

Case number	Age	Gender	Diagnosis	Total disease duration (years)	Lesion type
MS1	72	Female	SPMS	41	Active Chronic active
MS2	73	Female	SPMS	43	Active
MS3	77	Female	SPMS		Chronic inactive Chronic active Chronic active Active
MS4	51	Female	SPMS	21	Chronic active Active Chronic active Chronic inactive
MS5	55	Male	SPMS	43	NAWM Chronic active
MS6	86	Female	SPMS	36	NAWM
MS7	78	Female	SPMS	42	NAWM
MS8	71	Female	SPMS	35	Chronic active
MS9	62	Male	SPMS	39	Chronic active
MS10	37	Female	SPMS	17	Chronic active Chronic active Chronic active
MS11	77	Female	SPMS	21	NAWM NAWM
MS12	55	Female	SPMS	25	Chronic active
C1	64	Male	Normal	–	–
C2	69	Female	Normal	–	–
C3	35	Male	Normal	–	–
C4	78	Female	Normal	–	–
C5	60	Female	Normal	–	–
C6	75	Male	Normal	–	–

(–) not applicable.

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