Neuroscience Letters 486 (2010) 246–251

Contents lists available at ScienceDirect

Neuroscience Letters

journal homepage: www.elsevier.com/locate/neulet

Alterations of the blood–brain barrier in cerebral white matter lesions in the ageing brain

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ARTICLE INFO

Article history:
Received 20 July 2010
Received in revised form 7 September 2010
Accepted 22 September 2010

Keywords:
White matter lesions
Blood–brain barrier
Tight junction proteins
Claudin-5
Occludin
ZO-1

ABSTRACT

White matter lesions (WML) are associated with dementia and are common in brain ageing. In order to determine whether alteration of the blood–brain barrier (BBB) may contribute to the pathogenesis of WML we assessed albumin leakage and expression of the tight junction (TJ) proteins claudin-5 (Cln-5), zona occludin-1 (ZO-1) and occludin in cases derived from the Medical Research Council Cognitive Function and Ageing Study. Albumin extravasation was widespread in the ageing brain and enhanced in WML, suggesting dysfunction of the BBB may contribute to the pathogenesis of WML. This was not accompanied by significant changes in the endothelial expression of TJ proteins. However, ZO-1 and occludin were expressed by glial cells throughout the parenchyma of both control white matter and WML, suggesting these TJ proteins may have other functions in the brain.

White matter lesions (WML), regions of high signal intensity on T2-weighted magnetic resonance images (MRI), are prevalent in over 90% of the elderly population aged 64 and over [11], and correlate with progressive cognitive deterioration [14,23] and depression [1]. WML, classified by location as deep subcortical lesions (DSCL) or periventricular lesions (PVL), are common in normal ageing and neurodegenerative disorders including Alzheimer's disease (AD), vascular dementia and mild cognitive impairment [1,4,12,17]. The causes of WML are uncertain, but there is evidence for blood–brain barrier (BBB) dysfunction [30,33], cerebral hypoperfusion [9,13,42] and axonal damage [28].

Tight junction (TJ) complexes between brain capillary endothelial cells are an important structural component of the BBB, and consist of interconnected strands of transmembrane proteins and accessory proteins [22]. Transmembrane proteins, such as occludin, claudins (Cln) and junctional adhesion molecules, seal endothelial cells together restricting fluid and small molecule diffusion [15,16,24]. Accessory proteins, such as the zona occludins (ZO) family, have regulatory roles and link TJ complexes to the cytoskeleton [39].

BBB dysfunction, resulting in 'leaky' blood vessels, is a feature of a number of inflammatory and degenerative brain disorders including multiple sclerosis (MS) [21], cerebral malaria [5], HIV encephalitis [7] and Alzheimer's disease [6,37] and is associated with loss of TJ protein expression. Increased BBB permeability is also described in normal ageing (as reviewed in Refs. [10,29]). We have previously shown that WML in the ageing brain are associated with fibrinogen-immunoreactive clasmatodendritic astrocytes [33] inferring abnormal BBB permeability. The aim of this study was to examine the extent of BBB leakage, as determined by albumin protein leakage and characterise the expression of TJ proteins as markers of BBB integrity in WML in comparison to ageing brain white matter.

Human autopsy CNS tissue was obtained from the Medical Research Council Cognitive Function and Ageing Study (MRC CFAS), a large- prospective multi-centre, population-based UK study. Informed consent was obtained from all participants during life, and a multi-centre research ethics committee approved the study [3]. Brains were dissected following a standard protocol [20]. Anatomically defined formalin-fixed coronal slices underwent magnetic resonance imaging (MRI) analysis to assess WM pathology [12]. The commercially available antibodies used in this study

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doi:10.1016/j.neulet.2010.09.063

Table 1
Age, sex and post-mortem delay (PMD) of MRC CFAS brain donors.

	Median age (years) (min–max)	Sex (F/M)	PMD (h) (min–max)
Control			
(8 blocks from 8 non-lesional brains)	87.5 (84–89) (75–95)	2/6	22 (12–24) (1–35)
(7 blocks from 7 lesional brains)	87 (84–93) (74–94)	6/1	12.5 (8–22) (6–34)
PVL			
(10 blocks from 10 individuals)	88 (84–93) (77–96)	7/3	23 (10–30) (1–86)
DSCL			
(10 blocks from 10 individuals)	88 (86–92) (74–104)	8/2	18 (8–46) (6–60)

did not work consistently on the formalin-fixed material of the CFAS cohort despite antigen retrieval, therefore frozen tissue was used. MRI of the formalin fixed brain slices was used to aid selection of contralateral frozen slices likely to contain WML, and the presence or absence of WML confirmed using a previously published protocol [34]. Severe PVL (10 cases), DSCL (10 cases) and control WM both from brains containing lesions (WM[L]; 7 cases) and non-WML brain (WM[C]; 8 cases) were selected (Table 1).

Sections (7 μm) were fixed in ice-cold acetone for 10 min, blocked with 1.5% normal sera for 30 min, and incubated with primary antibody (Cln-5, ZO-1, occludin, Invitrogen, UK, diluted 1:100) for 60 min at room temperature. The horseradish peroxidase-conjugated avidin–biotin complex method was used (Vectastain Elite kit, Vector Laboratories, UK), with diaminobenzidine (DAB, Vector Laboratories, UK) as substrate. Specificity of staining was confirmed using isotype-specific antibody controls.

Dual immunostaining for albumin or TJ expression and cell phenotype markers was performed sequentially. Immunostaining for TJ or CD34 (Vector Laboratories, UK; diluted 1:100) was carried out as described above, then blocked with the avidin–biotin blocking kit (Vector Laboratories, UK). The sections were incubated with GFAP (DakoCytomation, UK; diluted 1:500), CD68 (DakoCytomation, UK; diluted 1:100) or albumin (DakoCytomation, UK; diluted 1:1000). The alkaline-phosphatase-conjugated avidin–biotin complex method was used (Vector Laboratories, UK) and visualised with alkaline phosphatase substrate 1 (Vector Laboratories, UK).

Selected WM regions of interest were marked on H&E sections and mapped onto consecutive immunostained slides. Quantification of specific immunoreactivity was performed by capturing bright-field microscopic images (Olympus Cell R, Olympus Biosystems, Watford, UK), using a 20× objective, in five fields selected at hazard within the marked area. Digital images were thresholded and the area of immunoreactivity was measured as a percentage of total field area, using Analysis D̂ software (Olympus Biosystems, Watford, UK). Staining for Cln-5 and ZO-1 was associated with endothelium, therefore the vascular immunoreactivity of these TJ proteins was also expressed as a ratio of total CD34 staining.

Albumin leakage was assessed in the areas analysed for TJ protein expression by 2 independent observers blinded to the lesional status of the tissue (SBW, JC) using a semi-quantitative scoring system described below, based on a modified protocol grading serum protein detection in WML in cerebrovascular disease and AD [41]. Statistical comparisons of quantitative data between control, PVL and DSCL were carried out using the Kruskal–Wallis test. In the case of a significant result, pair-wise comparisons were made using the Mann–Whitney *U*-test, and the *p*-value corrected for multiple testing using the Bonferroni method.

Albumin reactivity was graded as follows: (1) some blood vessels displayed evidence of perivascular albumin deposition (Fig. 1a), (2) weak glial reactivity in addition to the perivascular deposition (Fig. 1b), (3) many positive glial cells within the parenchyma (Fig. 1c), (4) and intense, confluent perivascular and parenchymal albumin reactivity with many positive glia (Fig. 1d). Grade 1 and 2 albumin staining were associated with 36% (5/14) of both WM[L] and WM[C] but were not present in any WML cases examined (0/7). Because of the small number of cases examined in this study, WM[C] and WM[L] cases were grouped together, although it should be noted that grade 1 staining was only detected in the WM[C] group. In contrast 100% of lesional cases (7/7) and 64% of WM[L] and WM[C] (9/14) contained confluent albumin reactivity and intense staining of cells morphologically resembling clasmatodendritic astrocytes (grade 3 or 4, Fig. 1c and d). Cohen's Kappa showed moderate agreement for the grading of albumin between 2 observers ($\kappa = 0.53$).

Cln-5 was exclusively associated with endothelial cells, as confirmed by dual staining with CD34 (Fig. 1e). Cln-5 showed no significant difference in total immunoreactive area between WM groups ($p = 0.418$), or when expressed as a ratio to CD34 immunoreactivity ($p = 0.956$) (Fig. 2a).

ZO-1 was associated with endothelium (Fig. 1f) and microglia (Fig. 1g) in both lesional and non-lesional WM in the ageing brain. Quantitative analysis of ZO-1 expression in blood vessels revealed a significant difference between WM groups ($p = 0.017$), but this disappeared when adjusted for blood vessel density ($p = 0.101$) (Fig. 2b). No significant difference in glial immunoreactivity was detected between WM groups ($p = 0.804$) (Fig. 2c).

Occludin expression was exclusively associated with glial cells within the WM parenchyma. Dual staining demonstrated an association with microglia (Fig. 1h) and showed no significant difference in levels of expression between WM groups ($p = 0.356$) (Fig. 2d). Not all occludin positive cells were $CD68^+$ suggesting other glial cells also express this protein.

Neither age, sex nor post-mortem delay were associated with claudin (age $p = 0.109$, sex $p = 0.626$, PMD $p = 3.63$), occludin (p-0.537, $p = 0.487$, $p = 0.59$), ZO-1 glial expression ($p = 0.887$, $p = 0.132$, $p = 0.511$) or ZO-1 blood vessel expression ($p = 0.736$, $p = 0.525$, $p = 0.531$).

Although the cause of WML is unknown, it has been suggested that BBB dysfunction resulting in serum plasma extravasation and glial cell activation may contribute to their pathogenesis [30,33]. In the current study we show that severe WML are characterised by intense serum protein staining throughout the parenchyma and by the presence of albumin-positive clasmatodendritic astrocytes. We demonstrate that these alterations in BBB function are not associated with changes in TJ protein expression, and show that ZO-1 and occludin are expressed by glial cells in all lesional and control brains.

The BBB is required to strictly regulate the brain microenvironment [36]. Enhanced influx of serum proteins has been used to assess disruption of the BBB in a variety of neuropathologic conditions [18,33,40]. In the present study approximately one third of control WM from the ageing brain showed minimal serum protein leakage, while all WML and two thirds of control WM samples were associated with high levels of albumin extravasation. The unexpected high levels of serum extravasation in control cases are not explained by our data, but may reflect age-associated changes in blood vessel morphology that were not addressed by the markers we used. All severe WML examined in this study showed high lev-