

Blood Vascular Changes Associated with Chronic Active Multiple Sclerosis Plaques: Detection by Anti-Human Brain Vascular Endothelia-Specific Monoclonal Antibodies

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Abstract

Recent studies suggest that brain angiogenesis is an important component of multiple sclerosis (MS) lesions and may be a potential target for investigating the pathogenesis and treatment of the disease. In this preliminary study, a panel of blood vessel-specific mouse monoclonal antibodies raised against normal human whole temporal lobe homogenates was used to study changes in vascular immunoreactivity and morphological features in cerebral tissue obtained from two clinically and neuropathologically confirmed cases of chronic active multiple sclerosis. Immunoperoxidase and immunofluorescence staining techniques revealed more frequent detection of intensely labelled blood vessels within active multiple sclerosis plaques compared to the adjacent white matter and normal control brain sections. Strongly reactive vascular endothelial sprouts as detected by epifluorescence immunohistochemistry were observed in whole vessel mounts isolated by partial homogenization and sieving of cerebral tissue containing active plaques, but not in vessels isolated from normal control cerebral tissue. These vascular changes suggest increased angiogenesis and endothelial proliferation within the active multiple sclerosis plaques and may be part of the inflammatory response by the central nervous system to this disease. This study also suggests that monoclonal antibodies raised specifically against whole homogenates of human brain could potentially be useful tools for investigations of antigenic heterogeneity and morphologic characterization of vascular endothelial changes associated with multiple sclerosis lesions in the human model system. Plans are under way to further investigate the exact biochemical nature of the specific antigens being recognized by the individual monoclonal antibodies.

Introduction

Multiple sclerosis (MS) is an autoimmune demyelinating disease of the central nervous system and a major cause of morbidity and mortality in young adults in Europe and America [50, 40, 59, 20]. The exact pathogenesis of MS is yet to be completely elucidated but studies have been published dating back more than a century ago indicating that MS brain lesions typically are localized to the per ventricular regions where they are situated around the subependymal venules and small veins [26, 6, 2, 22, 46, 4, 24, 69]. These findings stimulated several subsequent studies that led to the current concept that the brain vascular endothelial cells play a significant role in the pathogenesis of MS, an aspect that is currently receiving a greater attention among investigators. One suggested hypothesis was that early in the course of the disease in genetically susceptible patients, sensitized leukocytes, particularly CD4 + T lymphocytes and macrophages secrete proinflammatory cytokines including TNF- α , IFN- γ and IL-1 [53, 15, 7]. These cytokines are thought to act synergistically with chemokines mainly CCL19, CCL21, CXCL12 produced by macrophages and microglial cells to mediate the activation of brain endothelial cells [30, 13, 41, 5, 51]. When activated, brain endothelial cells exhibit increased expression for several different cell adhesion molecules, notably vascular endothelial growth factor (VEGF), endoglin (CD105), platelet endothelial cell adhesion molecule-1 (PECAM-1), intercellular adhesion molecule-1 (ICAM-1), and vascular cell adhesion molecule-1 (VCAM-1) [8, 15, 44, 68, 53, 64, 29, 25, 5, 51]. In addition to enhancing intercellular adhesion, brain microvascular activation is also accompanied by alterations in tight junction proteins, morphologic disorganization of the blood-brain barrier (BBB) structures and infiltration of the cerebral white matter by monocytes, lymphocytes and macrophages. These events culminate in oligodendrocyte loss, demyelination that could contribute to neurological deficit in MS [36]. Although, damage to blood-brain barrier structures is a consistent finding in MS pathogenesis, the possibility of a crucial role for central nervous system (CNS) angiogenesis (formation of new blood vessels) in this process is a relatively recent concept and is rapidly attracting interest among researchers [38, 17, 37, 31].

A critical review of the literature suggests that angiogenesis is an important facet in the pathogenesis of MS and its corresponding rodent model, experimental allergic encephalomyelitis (EAE) [17, 38, 31, 62]. Based on data from several independent investigations of EAE- and MS-associated neocortical angiogenesis, it was proposed that the molecular processes connected to microvascular alterations may serve as useful targets in the treatment of MS [35]. More recently, it was reported for the first time, that treatment of mice with angiogenesis inhibitors B20-4.1.1 and angiostatin (K1-3) after exposure to EAC mitigated the disease process as evidenced by suppressed spinal cord angiogenesis and reduced activation of T lymphocytes [47]. In a related study conducted earlier, it was reported that prostaglandin I₂ (PGI₂) derived from young capillary endothelial sprouts enhanced neural healing as evidenced by formation of new sprouts from damaged axons in the descending central nervous system fiber tracts within focal lesions in a rodent model system of multiple sclerosis [52]. Taken together, published data from several investigations including the documented recovery of motor functions shown by the experimental animals in the study by Muramatsu and others [52], further emphasize the significant role of the brain vascular endothelium in the pathogenesis of MS and support the view that changes within brain vasculature may provide a potentially important target in the therapy for the disease. Immunohistochemical investigations of the brain vascular changes in rodent EAE and MS lesions in humans utilizing a variety of vascular endothelial antibodies have previously been published. Some of the most widely employed CNS vasculature markers include laminin, collagen type IV, fibronectin, VEGF, endoglin (CD105), PECAM-1, ICAM-1, VCAM-1, CD34 and Factor VIII-related antigen (FVIII-RAG) and α -smooth muscle actin [27, 34, 28, 65, 31, 35, 61, 58].

To date, the application of monoclonal antibodies specifically raised against the human whole brain to study morphological changes in brain vasculature within MS plaques, have not been reported. The purpose of this preliminary investigation was to find out whether blood vessel-specific monoclonal antibodies raised against whole homogenates of the human brain could be used for studying the vascular changes associated with plaque lesions in well characterized cases of chronic active multiple sclerosis. Our observations indicate that there is an increase in the density of immunoreactive blood vessel-associated filopodia vessels within the MS plaques. This study suggests an aberrant vascularization mechanism that could lead to potential target for new therapeutic approaches for MS.

Materials and Methods

Monoclonal Antibodies

Production of monoclonal antibodies, isotype determination, human tissue specificity and inter-species cross-reactivity has been described in detail previously [34]. Briefly, we immunized female BALB/c mice (F1 strain) with homogenates of whole human brain fragments removed during routine surgical operations for temporal lobe epilepsy at the Walton Center for Neurology and Neurosurgery, Liverpool (UK). Isolated spleen cells from the BALB/c mice were fused with mouse myeloma (P3-X63-Ag8-653, NS-1) cells [Molecular Biology and Immunology Unit, Liverpool School of Tropical Medicine (UK)], using the standard lymphocyte hybridization procedure [42]. Hybridoma supernatants were screened by immunohistochemical methods on frozen sections of normal human brain and human peripheral organs. Additional frozen sections from pieces (3-5 mm³) of cerebral cortex from a healthy squirrel monkey (*Macaca sciureus*), CBA/CA mice, Wistar rats (Liverpool School of Tropical Medicine, UK) and a cow (obtained within 1 hour of slaughter from a local slaughter house), were cut and used for cross-species immunoreactivity studies. A total of 2000 hybrid colonies were screened in 9 separate cell fusions, out of which 16 clones which exclusively reacted against blood vessels were isolated. Supernatants from the hybridoma stabilates were used as the source of antibodies described in this report.

Patients and Specimens

Deep frozen, unfixed human autopsy cerebral tissue from two patients was provided for this study by the Buxton Laboratories of Neuropathology at the Walton Center for Neurology and Neurosurgery, Liverpool, United Kingdom. Both cases were diagnosed and confirmed histopathologically by the Walton's neuropathologists [Coordinator Dr. John Broome, MB, ChB, FRCP, FRCPath]. Case one was from postmortem brain from a 47-year old female who died from bronchopneumonia following a 4-year history of primary progressive multiple sclerosis. Case two was from temporal lobectomy specimen from a 46-year old female with a 20-year history of relapsing remitting multiple sclerosis and who also had a 15-year history of temporal lobe epilepsy. In addition, deep frozen cerebral tissue from temporal lobectomy specimens from six patients coming to surgery at the Walton Center, for temporal lobe epilepsy in which there was no evidence of demyelinating disease were used as controls. The above samples were flash-frozen in liquid nitrogen and stored at -80°C until required [11].

Immunohistological Studies

The monoclonal antibodies (Table 1) were used as neat (undiluted) culture supernatants. Frozen sections (7 µm) were cut from multiple sclerosis and control brain specimens, mounted on poly-L-lysine-coated glass slides [33] and stored frozen (-70°C) until use. Sections were stained by the indirect avidin-biotin- and peroxidase (ABC-HRP complex) technique [32] as described previously [34] and in some experiments, the indirect immunofluorescence (biotin and streptavidin-FITC) technique [49] was used to confirm the vascular morphological characteristics detected by the immunoperoxidase technique. The indirect immunofluorescence technique was used to compare the *in situ* labelling patterns by blood vessels and their branches isolated from the multiple sclerosis plaques and normal control brains. For the histopathological assessment, 7 µm to 8 µm frozen sections were stained with hematoxylin and eosin, Oil Red O [16] and Luxol Fast Blue [39]. Oil Red O staining allowed the detection of the presence of lipid-laden macrophages and Luxol Fast Blue was employed in order to delineate pale staining areas indicative of demyelination. Adjacent sections were formalin-fixed and labeled by the indirect immunoperoxidase technique with monoclonal antibody against the neurofilament protein. Formalin-fixed sections were also stained with hematoxylin and eosin for routine microscopic evaluation. Vascular endothelial cells in frozen sections and in isolated vessels were confirmed by positive immunoperoxidase staining for factor VIII-related antigen (FVIII-RAG) (*Dako* Ltd, High Wycombe, UK) and the histochemical demonstration of vascular alkaline phosphatase based on Gomori's technique (1952). Additional sections were immunostained for endothelial markers alpha smooth muscle actin, SMA (Novocastra, Newcastle, UK) and Q-bend 10 (CD34, Novocastra, Newcastle, UK). Negative controls were treated with the same immunochemical reagents under identical conditions but the primary antibodies were omitted. The above immunohistochemical techniques and appropriate antibody dilutions were carried out as previously described (Ibiwoye et al., 1998).

Intact Brain Microvessels

For the detection of antibody binding by brain capillaries and their collaterals *in situ*, intact blood vessels were isolated from multiple sclerosis plaques and normal brains using a modified method of Brendel et al. [12] as described previously [34]. Briefly, pieces of frozen cerebral were allowed to thaw overnight at 5°C.

After removal of the meninges and large blood vessels from the external surface, the samples were placed in a Petri dish containing 3 ml cold (5°C) fetal calf serum (FCS) 10% in Iscove's Dulbecco's modified Eagle's medium (DMEM) (Imperial, UK), supplemented with L-glutamine, and cut into small pieces with a surgical blade. The pieces were transferred into a clean glass homogenizer maintained at about 5°C by immersion in a beaker of wet ice, to reduce protein degradation during subsequent processing. With a loose fitting, Teflon-coated pestle, the brain was homogenized manually by 15-20 upward and down ward movements. The homogenate was passed through a 153- μ m nylon mesh. Blood vessels were retained by the nylon fabric and the filtrate, consisting mainly of neurons and glia was discarded. The nylon mesh was placed in a second Petri dish containing 10 ml cold FCS 10% in Iscove's medium and rinsed thoroughly to suspend the vessels in solution. Homogenization and filtration were repeated until a relatively pure suspension of blood vessels was obtained and confirmed as such by examination under an inverted microscope. Aliquots (20 μ l) of the suspension were placed on clean glass slides, air-dried at room temperature, fixed for 10 minutes in cold acetone (-20°C) and stored at -20°C until required.

Grading and Comparison of Immunoreactivity in Frozen Sections

For a valid comparison of results, immunostained sections from the plaques and normal controls were mounted and examined in pairs under the Nikon Microphot Fx microscope outfitted with a SPOT-RT CCD digital camera (Nikon, Japan). Immunoperoxidase-stained slides were coded and assessed twice, in random order without knowledge of the individual monoclonal antibody's identity. Five equally-spaced fields were examined per slide using the x40 objective lens. Vascular immunoreactivity in MS sections was graded from 0 to +4 and compared with normal controls, based on antibody binding intensity and blood vessel morphological features as follows: labeled blood vessels but no filopodia or punctate profiles (+); positive staining but no difference in staining intensity (0); mild intensity with a few filopodia but no punctate profiles observed (++); moderate intensity with few filopodia and some punctate profiles (+++) and marked increase in intensity with numerous filopodia and punctate profiles (++++) (see Table 1).

Results

All formalin-fixed and unfixed frozen plaques used in this study were chronic active plaques characterized by infiltration of perivascular spaces by lymphocytes and plasma cells and reactive gliosis (Fig. 1a). Specimens from both multiple sclerosis patients showed evidence of myelin breakdown indicated in Oil Red O-stained slides by the presence of lipid-laden macrophages at the plaque margins. Preservation of axons was demonstrated by the positive immunoperoxidase staining for neurofilament protein (NFP) in adjacent formalin-fixed tissue sections (Fig. 1b).

Immunoperoxidase and Immunofluorescence Staining with Monoclonal Antibodies

There were no differences in immunoreactivity patterns within MS plaques and adjacent white matter in brain sections from the two patients examined. The immunoreactivity patterns of the monoclonal antibodies in brain sections from both patients are summarized in Table 1. Microscopically, the striking features seen within the MS plaques but not in adjacent white matter or normal control specimens, were greater numbers of immunoreactive blood vessels and the presence of numerous, intensely labelled punctate profiles (Fig. 1c, d, e). Another striking feature was that we detected greater concentration of intensely labelled blood vessels and punctate profiles within active MS plaques compared to the adjacent white matter in all the specimens examined (Fig. 1f). The punctate profiles were dispersed throughout the plaque but were more concentrated around the immunoreactive blood vessels (Fig. 1c and d). The vascular basement membrane appeared to be duplicated and the lining endothelial cells were swollen and disorganized (Fig. 1d, e, g). The punctate structures were intermixed with large numbers of strongly labelled filopodia (Fig. 1c, d, j). The filopodia appeared as thin filamentous processes randomly oriented within the plaque (Fig. 1c, d). They varied in length, had no recognizable lumen and were frequently attached roughly at right angle to the immunoreactive vessels (Fig. 1d). The structural relationship of the filopodial processes to blood vessels and their branches was better demonstrated in whole vessel preparation (Fig. 1j). Blood vessels isolated from normal control brains showed varying degree of immunoreactivity with the monoclonal antibodies but no punctate profiles or filopodia were detected (Fig. 1h). The blood vessels in both the MS and normal control frozen tissues were positive for FVIII-RAG, SMA and CD34 but filopodial processes and punctate profiles were not seen in vessels positively labelled by these monoclonal antibodies.

Discussion

In this light microscopic immunohistochemical study, monoclonal antibodies raised against human whole brain homogenate have been used to study the brain vascular changes associated with chronic active multiple sclerosis. Blood vessels within the active plaques showed more intense antibody binding and increased density than that shown by vessels in normal control brains.

A striking feature associated with active MS plaques in this study was the widespread distribution of discrete profiles and numerous filopodial processes radiating from the labelled blood vessels into the neuropil (Figs. 1c, d, and i). Brain vascular changes associated with chronic active human MS plaques and in rat model system of EAE have been studied previously by immunohistochemical technique using antibodies against endothelial markers including collagen type IV, VEGF, coagulation factor XIIIa, laminin, alpha smooth muscle actin (HHF 35), CD105, major histocompatibility antigen class II, and fibrinogen [28, 67, 62, 31]. The widespread disorganization and apparent reduplication of the vessel wall (Figs. 1e, g and i) appear to correspond to the advanced stage of vascular damage described by Wakefield et al. [67] and Gay and Esiri [28]. However, the endothelial filopodia (Figs. 1c, d, g, and j) as described in our study were not reported by Wakefield et al., [67] or by Gay and Esiri [28].

Based on their morphological features and direct attachment to blood vessels both *in situ* (Fig. 1j) and in frozen sections, particularly concentrated within the active plaques (Figs. 1c, d, f, g, i and j), we believe that the immunoreactive filopodial processes described in this study correspond to vascular endothelial sprouts or nascent blood vessels associated with active MS plaques. Sprouting endothelial filopodia morphologically similar to those described in this study have been previously reported as indicative of active vascular proliferation in normal developing human [57], cat and dog [18] brains. The histogenesis and morphological characteristics of the endothelial filopodia have also been well documented in the developing golden hamster [48], mouse [9] and rat [54, 45] brains, where they were shown to originate from, and determine the direction of the sprouting terminal vascular endothelial cells within the neuropil. The study of central nervous system angiogenesis [10] following a traumatic spinal cord injury indicate that newly-regenerated vessels initially lack a basal lamina, have poorly developed interendothelial junctions and are deficient in barrier functions. Snyder and colleagues [63] reported that in the spinal cord of guinea pigs exposed to experimental allergic encephalomyelitis (EAE), neuropathological changes included parenchymal fibrosis, widespread endothelial attenuation and fenestration and the accumulation of leukocytes and collagen within the perivascular space of Virchow-Robin of the affected vessels, suggesting disease-linked morphological alterations in the blood vessels. Immunohistochemical investigations have also been published by other researchers recently, confirming the occurrence of increased vascular density and endothelial cell proliferation in the cerebral white matter of patients suffering from multiple sclerosis [31]. Our observations suggest increase in the density of immunoreactive blood vessels within the MS plaques, and agree with the study by Holley et al., [31]. However, the blood vessel-associated filopodia observed in our present study were not reported in the data published by Holley and colleagues [31].

Because angiogenesis is being increasingly reported as having an important role in the pathogenesis of multiple sclerosis [1, 3, 67, 69, 38, 35, 62] the increased numbers of immunoreactive blood vessel profiles and widespread endothelial filopodia in our study suggest plaque-associated angiogenesis. In comparison with the studies by Gay and Esiri [28] and by Wakefield et al., [67], the differences in labelling patterns by our monoclonal antibodies probably indicate recognition of a different set of epitopes within the lesions examined. This aspect warrants further detailed investigation and in a future study, the exact biochemical nature of vascular endothelial monoclonal antibodies specifically raised against the human brain tissue will be determined. This preliminary study suggests that brain vascular changes constitute an important component in the pathogenesis and therapy of MS. Further investigations are underway to elucidate the biochemical nature of the specific antigens being recognized by the different monoclonal antibodies described in this study. Such investigations may lead to better understanding of the pathogenesis and more effective therapy of the disease.

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Table 1: Labeling Characteristics of the Monoclonal Antibodies in Normal Brains and in Active MS Plaques

Monoclonal antibodies	Immunoreactivity grading in cryostat sections	
	Normal controls	Active MS plaques
2D7	+	++++
3C4	+	++++
2G4	+	++++
6F7	+	++++
3D12	+	+++
6G6	+	++
3H2	+	++
5B7	+	++
6C3	+	++
4A2	+	++
3A1	+	++
4H1	+	++
4H4	+	++
6F9	+	++
6F6	+	0
1H5	+	0

Graded subjectively from 0 to +4 according to antibody-binding intensity and blood vessel morphologic features: +: positive staining; no difference in staining intensity (0): equally labeled blood vessels, no filopodia or punctate profiles (+); mild increase in intensity (++) : few filopodia but no punctate profiles; moderate intensity (+++) : few filopodia and some punctate profiles; marked increase in intensity (++++): many filopodia and numerous punctate profiles readily detected.

Figure 1 A: Immunohistochemical detection of vascular changes in chronic active multiple sclerosis plaque. (a) Formalin-fixed temporal lobe section from case one. Perivascular cuff of inflammatory cells (thin arrows) and reactive astrocytes (arrowheads) around thin-walled blood vessels were seen within the active plaque. Hematoxylin & eosin x 200 magnification. (b) Positive immunoperoxidase staining for neurofilament protein (NFP) in formalin-fixed temporal lobe section from MS case two showing preserved axons within the plaque (arrows). Immunoperoxidase staining of frozen MS plaque sections: Large numbers of strongly positive blood vessels, punctate profiles (arrowheads) and filopodia processes (arrows) seen with monoclonal antibodies 2D7 (c), 3C4 (d) and 2G4 (e). There is endothelial swelling and damage to the apparently duplicated vascular wall (monoclonal antibody 2G4) (arrow in Fig. e). Positive blood vessels are more concentrated within the plaques (PLQ) than in adjacent white matter (NAWM) (f). Tissue sections were counterstained with Mayer's Hemalum: Fig. 1c, d, e at x 400 magnification; Fig. 1A f at x40 magnification.

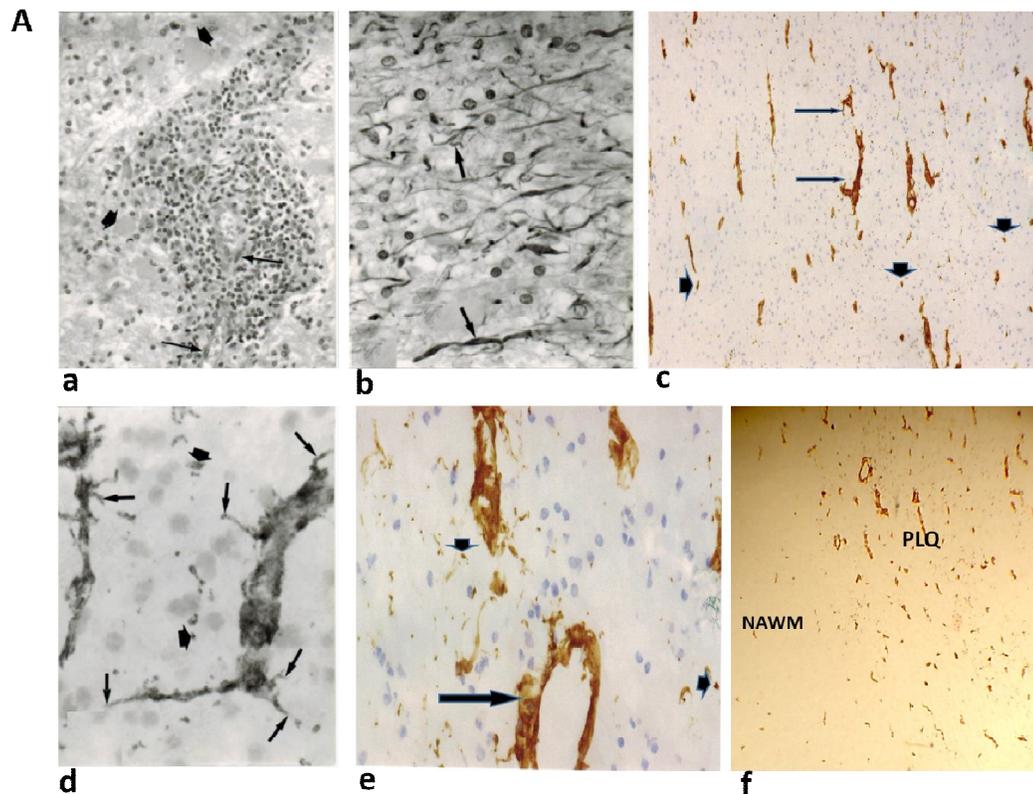


Figure 1 B: Streptavidin-FITC labeling (monoclonal antibody 2D7) of whole blood vessels isolated from MS case one, showing immunoreactive filopodia (arrows) attached to a uniformly-labeled blood vessel (j) but not in the vessel from vessel isolated from a normal control brain (h). Streptavidin-FITC labeling with monoclonal antibody 2D7 on frozen plaque sections from MS case two showing numerous strongly reactive blood vessels with duplicated walls (arrow, 1g); strongly labeled punctate profiles and filopodial processes (arrow) are seen (i). Streptavidin-FITC-labelled sections and vessels were counterstained with propidium iodide and viewed at x 400 magnification. No filopodial processes were seen in vessels isolated from normal control brains (compare h and j). Propidium Iodide counterstain. x 400 magnification.

