Involvement of Apolipoprotein E in Multiple Sclerosis: Absence of Remyelination Associated with Possession of the APOE ε2 Allele

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Abstract. Lipids are a major constituent of myelin and apolipoprotein E (apoE) plays a key role in lipid transport. We therefore hypothesized that apoE is involved in the processes of demyelination and remyelination. Furthermore as there is a biologically significant polymorphism in the APOE gene, the APOE genotype may influence the course of multiple sclerosis (MS). Specifically, as there is reduced affinity of the apoE E2 isoform for receptors on glial cells, we hypothesized that remyelination is impaired in individuals with the apoE ε2 allele. We determined the apoE genotypes of 71 archival cases of multiple sclerosis and 41 controls, reviewed the neurohistology, and performed apoE immunohistochemistry. ApoE immunostaining was markedly increased in areas of active demyelination, specifically in macrophages and astrocytes. The APOE allele frequencies of the cases of MS (ε2 = 0.06, ε3 = 0.8, ε4 = 0.13) resembled those of controls. Evidence of remyelination was identified in 25/71 MS cases (35%): in 25/64 patients (39%) without an ε2 allele and 0/7 (0%) patients with an ε2 allele (p < 0.05). In conclusion, we provide evidence that apoE is involved in the trafficking of lipid in MS and, although the number of cases with this allele was small, remyelination may be defective in patients with the APOE ε2 allele.

Key Words: Apolipoprotein E; Genetics; Lipid transport; Multiple sclerosis; Myelin; Remyelination.

INTRODUCTION

Although multiple sclerosis (MS) is the most common of the demyelinating diseases, its pathogenesis and etiology are as yet poorly understood. A striking aspect of MS is the variability in the features of the disease, for example, in symptomatology, severity, and disease progression. Pathologically, the variations manifest as differences in the number, size, and distribution of plaques, the degree of activity of demyelination, and the extent of remyelination (1, 2). These variables could potentially be influenced by as yet uncharacterized genetic factors.

Apolipoprotein E (apoE protein, APOE gene) is a lipid carrier that plays a role in regulating lipid metabolism. Systemically, it is synthesized predominantly in the liver and regulates transport of cholesterol and phospholipids around the body via interactions with a family of low-density lipoprotein (LDL) receptors (3). ApoE also plays a major role in lipid trafficking within the nervous system (3, 4). In the peripheral nervous system apoE is involved in the scavenging of degenerating myelin by macrophages in injured and demyelinated nerve and in the transport of lipids from macrophages to Schwann cells and neurons in regenerating nerve (5, 6). In the central nervous system apoE binds cholesterol and phospholipid to form lipoprotein complexes, the target for which is determined by receptors for apoE on neurons and glial cells (4). By this mechanism apoE is proposed to be involved in the transport of lipids between astrocytes and neurons in animal models of acute brain injury (4, 7, 8) and also putatively in humans (9). The receptors for apoE, their subtypes and cellular localization remain to be completely characterized. The LDLr-related protein (LRP) is expressed mainly in gray matter by neurons and reactive astrocytes. The very low-density lipoprotein receptor (VLDLr) is expressed by microglia and some neurons. The gp330/megalin receptor is expressed mainly by ependymal cells. The low-density lipoprotein receptor (LDLr) has a more widespread distribution in the CNS, including in the white matter, and appears to be the principal receptor expressed by oligodendrocytes (10). In view of its major role in lipid transport in the CNS, it is important to predict that apoE is intimately involved in the processes of demyelination and remyelination as these require the mobilization of myelin-associated lipids.

There is a polymorphism of the APOE gene that has profound biological significance in neurological disorders. The gene has 3 common alleles, designated ε2, ε3, and ε4, which encode corresponding isoforms of the protein (E2, E3, and E4) (4). Examples of the diverse influence of this polymorphism on CNS pathophysiology include APOE ε4 as a risk factor for Alzheimer disease (11–13), association of APOE ε4 with poor outcome following acute brain injury (14–17), and the association of both APOE ε2 and ε4 with cerebral amyloid angiopathy-related hemorrhage (18–20). Although the mechanisms underlying these associations are unclear at present, there are differing affinities of the 3 isoforms of apoE for the different receptors. In particular, apoE E2 isoform, compared with E3 and E4, has <2% affinity for the LDL
receptor (21–23). Relative binding affinities for the apoE isoforms have been determined rigorously only for the classic LDL receptor, and not for the other receptors in the LDL family. However, this finding is of specific interest to myelin pathophysiology because LDLr appears to be the predominant apoE receptor on glial cells, including oligodendrocytes (10). The very low affinity of E2 for the LDL receptor is of functional significance as it can manifest clinically as type III hyperlipoproteinemia in APOE ε2 homozygotes (3).

We hypothesized firstly that apoE, as a protein which mediates lipid transport in the CNS, is involved in the processes of demyelination and remyelination in MS, and secondly that the APOE gene polymorphism may influence the pathological features of MS. More specifically, as remyelination requires uptake of lipids by oligodendrocytes and apoE E2 has substantially lower affinity for the LDL receptor compared with E3 and E4, we hypothesized that MS patients possessing the ε2 allele may show evidence of reduced remyelination. We investigated these hypotheses, firstly, by assessing apoE immunoreactivity in plaques of demyelination compared with adjacent white matter and white matter in non-MS controls and, secondly, by correlating APOE genotype with a quantitative assessment of remyelination.

MATERIALS AND METHODS

Cases of Multiple Sclerosis and Controls

A search of the archive of the Neuropathology Department at the Institute of Neurological Sciences in Glasgow from 1981 onwards yielded 98 autopsy cases of MS. APOE genotypes could be determined from the archival formalin-fixed paraffin-embedded tissue (see below) for 71 of 98 cases, and these formed the basis for the study. The age of 2 of the MS patients...
had not been recorded. The average age at the time of death of the remaining 69 MS patients was 58.5 years (range 31–89 years), with a female to male ratio of 1.37:1. Controls comprised 41 archival cases without significant neuropathological abnormality. Their mean age was 50.8 years (range 18–82 years).

Review of Histology
The diagnosis of MS was confirmed by examination of sections stained with Luxol fast blue/cresyl violet (LFB/CV), hematoxylin and eosin (H&E), and Palmgren’s silver impregnation for axons. Individual plaques were categorized as chronic inactive plaques, chronic active plaques, and shadow plaques (containing areas of remyelination) according to previously described criteria (1, 2) defined in the Results section.

Assessment of Remyelination
Remyelination was identified in LFB/CV-stained sections as areas intermediate in LFB staining intensity between deeply stained normal white matter and completely unstained areas of

Fig. 1. (Continued)  E: Lipid laden macrophages in an area of active demyelination (CD68, ×40). F: Similar area of the same plaque illustrated in (E) showing apoE immunoreactivity of lipid laden macrophages (apoE, ×40). G: Central area of a chronic active plaque showing hypertrophic astrocytes (GFAP, ×40). H: Similar area of the same plaque as in (G) showing apoE immunoreactivity of astrocytes (apoE, ×40).

Fig. 1. (Continued) I: Remyelinated zone of a shadow plaque showing scanty apoE immunoreactivity, here probably confined to astrocytes (apoE, ×40).
demyelination (see illustrations). For each case in which remyelination was identified in any of the plaques, the total plaque area and total area of remyelination in that case were determined by image analysis (MCID). The percentage remyelination for each case was calculated as the total area of remyelination divided by the total plaque area (i.e. the sum of the areas of complete demyelination and remyelination).

Immunohistochemistry

Immunohistochemistry was performed on parallel paraffin sections of 6 chronic inactive plaques, 6 chronic active plaques, and 6 shadow plaques defined as described above. Sections of normal white matter from 6 of the controls were included. The primary antibodies used were goat polyclonal anti-apoE (1:5000 Chemicon International Ltd., Harrow, UK); mouse monoclonal anti-CD68 (1:200, Dako Ltd., High Wycombe, UK); and rabbit polyclonal anti-GFAP (1:1000, Dako). Positive controls for immunohistochemistry were Alzheimer disease cerebral cortex (apoE), tonsil (CD68), and astrocytoma (GFAP). Negative controls for immunohistochemistry were sections incubated with nonimmune sera and otherwise processed identically. Paraffin sections 8 µm in thickness for apoE and CD68 immunohistochemistry were pretreated in methanol hydrogen peroxide solution for 30 minutes, followed by heating in citrate buffer (pH 6) in a microwave for 30 minutes. Sections for GFAP immunostaining were pretreated with trypsin. Sections were washed in phosphate buffered saline and blocked using 2% horse serum for apoE staining and 2% bovine serum albumin for CD68 staining. Sections were incubated with the primary antibodies overnight at 4°C. Bound antibody was visualized using a Vectastain ABC kit (Vector Laboratories, Peterborough, UK) with 3,3′-diaminobenzidine as the chromogen.

Semiquantitative Assessment of Immunocytochemistry

Immunohistochemical preparations were compared with the adjacent LFB/CV and H&E-stained sections to confirm the localization of the plaques. Immunoreactivity was assessed semiquantitatively, blind to APOE genotype, on a 5 point scale as follows: none (0); equivocal (+); few immunoreactive cells (+++); moderate numbers of immunoreactive cells (+++); and many immunoreactive cells (++++). Astrocytes and macrophages, identified by morphology and by comparison with the corresponding sections immunostained for GFAP and CD68, were graded separately for apoE immunoreactivity.

APOE Genotyping

APOE genotypes were determined from the archival formalin-fixed paraffin-embedded tissue as previously described (19). Briefly, paraffin sections in 1.5 µl tubes were dewaxed using xylene, then washed with ethanol and dried on a heat block at 56°C for 1 h. The tissue was digested in 200 µl proteinase K (800 µg/ml) overnight at 56°C and then heated to 95°C to inactivate the proteinase. 1 µl of this solution was used as the target for hot start PCR using Amplitag Gold (Perkin Elmer, Applied Biosystems, Warrington, UK) and previously described primers (24) for 40 cycles: 94°C for 1 min; 65°C for 1 min; and 70°C for 2 minutes. PCR products were separated according to size by polyacrylamide gel electrophoresis and visualized by ethidium bromide staining and u.v. transillumination.

RESULTS

Review of Histology

The review of histology showed that 65/71 MS patients (92%) had exclusively chronic inactive plaques, comprising sharply demarcated zones of demyelination with relative preservation of axons containing reactive hyperplastic and hypertrophic astrocytes and no or few inflammatory cells (Fig. 1A). Chronic active plaques, in which macrophages that were identifiable on H&E-stained sections and myelin debris was visible within macrophages in sections stained with LFB/CV, were identified in 6/71 MS cases (8%). There were no acute plaques. Plaques with remyelination (shadow plaques), indicated by the presence of areas intermediate in LFB staining intensity between deeply stained normal white matter and completely unstained areas of demyelination were identified in 25/71 cases (35%) (Fig. 1B, C). For the cases with remyelination the total plaque area, assessed by image analysis, ranged from 25.3 to 523.1 mm² (median 177 mm²) and the total area of remyelination was 1.5 to 143.3 mm² (median 15 mm²). The area of remyelination as a proportion of the total plaque area sampled in each of the cases ranged from 1.4% to 81% (median 17.4%).

Immunohistochemistry

Immunohistochemistry was performed in order to assess alterations in apoE immunoreactivity in different plaque types: chronic active plaques from each of the 6 patients with this plaque type, 6 chronic inactive plaques, 6 plaques with evidence of remyelination, and white matter from 6 of the control cases. The immunohistochemistry is illustrated in Figure 1D–I and the semiquantitative assessments are shown in Figure 2. White matter in the MS cases adjacent to plaques resembled the white matter in control cases and showed faint apoE immunoreactivity in a small proportion of glial cells, judged to include both astrocytes and oligodendrocytes on morphological grounds (Fig. 1D). Areas of active demyelination in the chronic active lesions contained numerous lipid-laden macrophages that were strongly immunoreactive for CD68 and apoE (Fig. 1E, F). Reactive astrocytes, identified both morphologically and by immunoreactivity for GFAP, were also immunostained for apoE (Figs. 1G, H, 2). ApoE immunoreactivity was accentuated at the active rim of chronic active plaques, correlating with the localization of macrophages and reactive astrocytes. ApoE immunoreactivity in chronic inactive plaques and in areas of remyelination was confined to glial cells (Fig. 1I) and was less pronounced than in active lesions but more so than in control white matter (Fig. 2).

APOE Genotypes

The APOE allele frequencies of the MS cases were similar to those of neuropathologically normal controls
Fig. 2. Mean immunoreactivity scores for chronic active plaques, chronic inactive plaques, shadow plaques, and control white matter. Scored separately for each plaque type: macrophages (identified with anti-CD68); apoE immunoreactivity in macrophages; and apoE immunoreactivity in astrocytes. ApoE immunoreactivity was most pronounced in active lesions, in both astrocytes and macrophages. Astrocytes in chronic lesions (both chronic inactive plaques and shadow plaques) were also immunostained for apoE.

**DISCUSSION**

The increased apoE immunoreactivity of astrocytes and macrophages in areas of active demyelination supports the hypothesis that apoE is involved in lipid trafficking in MS. Co-localization of LDL and myelin basic protein within macrophages in actively demyelinating lesions provides further support (25). The lipid component of degraded myelin is potentially available for remyelination in a manner analogous to the proposed mechanism by which apoE mediates transport of lipid from degenerating myelin in the peripheral nerve for reutilization by Schwann cells. Rodent models have shown that cholesterol released from injured and demyelinated peripheral nerve becomes associated with apoE-containing lipoproteins (26) and that apoE can be synthesized by local macrophages (6). It is also proposed that apoE is synthesized by astrocytes and macrophages in response to CNS injury and that this facilitates transport of lipids to neurons for repair and regeneration (3, 4). Following acute brain injury apoE immunoreactivity is increased in astrocytes and subsequently in neurons (7–9), putatively as a reflection of upregulation of this lipid transport system. The increased apoE immunoreactivity seen in astrocytes and macrophages in active MS lesions in this study may also reflect upregulation of this mechanism for lipid transport and involvement of both astrocytes and macrophages in apoE-mediated trafficking of lipids. Although not clearly demonstrated in this study of MS, oligodendrocytes exhibit increased apoE immunoreactivity following injury of the rodent optic nerve (27), further emphasising the altered cellular distribution of apoE during redistribution.

(Table 1). Evidence of remyelination was identified in 25/64 cases of MS (39%) without an APOE e2 allele and in none of the 7 patients (0%) with an APOE e2 allele (p = 0.047, Fisher’s exact test; Table 2). Figure 3 shows for each MS patient the APOE genotype plotted against the percentage of remyelination in the plaques sampled. This graph shows that in patients with those genotypes associated with remyelination (3/3, 3/4, and 4/4), there was a wide variation in the area of the plaque that had been remyelinated, ranging from 0%–81%.
of myelin lipids. Additional evidence that apoE is involved in the MS comes from studies demonstrating alterations in apoE concentrations in cerebrospinal fluid (28, 29).

The APOE genotypes of the MS population were similar to those of controls, confirming the results of a previous study (30) and indicating that the APOE gene polymorphism does not influence the development of MS. However, we sought to determine if APOE genotype could explain some of the variation in the pathological features seen in MS. The very low affinity of the apoE E2 isoform for glial cell receptors, <2% compared with E3 and E4, prompted us to focus on remyelination as the pathological feature most likely to be influenced by the APOE gene polymorphism. Examination of MS pathology in autopsy tissue allows only a static picture of a dynamic process that usually extended over many years or decades. Particularly from studies of active lesions in progressive MS and biopsies of demyelinating lesions there is increasing recognition that remyelination occurs (31–35) and is associated with re-expression of a myelin basic protein gene normally expressed when myelination occurs during development (36). In such studies, optimally processed tissue examined by electron microscopy or by light microscopic examination of thin resin-embedded sections reveals the thin myelin sheaths and short internodes characteristic of remyelination. The presence of thin myelin sheaths, indicative that remyelination has occurred, is believed to be reflected in LFB-stained paraffin sections by the appearance of shadow plaques (Fig. 1A, B) (2, 3, 36). Examination of these areas of myelin staining intermediate in intensity between the deeply stained normal white matter and unstained completely demyelinated areas under a high power objective confirmed the presence of relatively thin myelin sheaths. Using this method none of the 7 MS patients with APOE e2 had any evidence of remyelination compared with 39% of the MS patients without APOE e2. This finding supports the hypothesis that remyelination may be impaired in patients who carry the APOE e2 allele.

There are a number of difficulties with this retrospective postmortem archival study. Firstly, despite the large number of MS cases in this study, the relative infrequency of the apoE e2 allele in the population resulted in few cases with this allele. Secondly, the variable degree of histological sampling among the study group precluded reliable assessment of other histological variables, such as the numbers and sizes of plaques, which might be influenced by APOE genotype.

From these observations it could be predicted that MS patients who carry the APOE e2 allele may follow a worse clinical course. More specifically, if remyelination influences the process of remission following acute exacerbation in MS then patients with APOE e2 may be less likely to have a relapsing-remitting course and more likely to have a chronic progressive course. Clinical studies are required to test this hypothesis. Of specific interest here is the very recent data to suggest that, by analogy with the response to other forms of brain injury, MS patients with APOE e4 may have a more aggressive type of disease (37). Possession of APOE e2 has not yet been assessed in such a study. If delivery of lipids to oligodendrocytes is a rate limiting process, particularly in patients with APOE e2, then this is a potential pharmacological target. Studies of experimental models in APOE transgenic animals may allow clarification of the respective roles of the isoforms of apoE in the processes of demyelination and remyelination. In addition, examination of polymorphisms in the genes encoding the receptors for APOE (38, 39) and of polymorphisms in the APOE gene promoter region (40) may be fruitful.

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REFERENCES


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