Fibronectin aggregation in multiple sclerosis lesions impairs remyelination

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Remyelination following central nervous system demyelination is essential to prevent axon degeneration. However, remyelination ultimately fails in demyelinating diseases such as multiple sclerosis. This failure of remyelination is likely mediated by many factors, including changes in the extracellular signalling environment. Here, we examined the expression of the extracellular matrix molecule fibronectin on demyelinating injury and how this affects remyelination by oligodendrocytes progenitors. In toxin-induced lesions undergoing efficient remyelination, fibronectin expression was transiently increased within demyelinated areas and declined as remyelination proceeded. Fibronectin levels increased both by leakage from the blood circulation and by production from central nervous system resident cells. In chronically demyelinated multiple sclerosis lesions, fibronectin expression persisted in the form of aggregates, which may render fibronectin resistant to degradation. Aggregation of fibronectin was similarly observed at the relapse phase of chronic experimental autoimmune encephalitis, but not on toxin-induced demyelination, suggesting that fibronectin aggregation is mediated by inflammation-induced demyelination. Indeed, the inflammatory mediator lipopolysaccharide induced fibronectin aggregation by astrocytes. Most intriguingly, injection of astrocyte-derived fibronectin aggregates in toxin-induced demyelinated lesions inhibited oligodendrocyte differentiation and remyelination, and fibronectin aggregates are barely expressed in remyelinated multiple sclerosis lesions. Therefore, these findings suggest that fibronectin aggregates within multiple sclerosis lesions contribute to remyelination failure. Hence, the inhibitory signals induced by fibronectin aggregates or factors that affect fibronectin aggregation could be potential therapeutic targets for promoting remyelination.

Keywords: fibronectin; multiple sclerosis; remyelination; astrocyte; oligodendrocyte
Introduction

Inflammation-mediated loss of myelin (demyelination) and incomplete remyelination are pathological hallmarks of multiple sclerosis. Remyelination is essential for both restoration of saltatory conduction and axonal protection (Franklin and ffrench-Constant, 2008). Although remyelination occurs in early stages of multiple sclerosis, it declines as the disease progresses, resulting in chronically demyelinated plaques and axonal loss (Goldschmidt et al., 2009). Oligodendrocyte progenitors, the cells responsible for CNS remyelination (Zawadzka et al., 2010), are present in most multiple sclerosis lesions, but ultimately fail to differentiate into mature myelinating oligodendrocytes, which results in remyelination failure (Franklin and ffrench-Constant, 2008; Kuhlmann et al., 2008).

Migration and proliferation of oligodendrocyte progenitor cells and their differentiation into myelinating oligodendrocytes are regulated by many factors, including the extracellular matrix (Baron et al., 2005). For example, laminin-2 provides oligodendrocytes with signals for both survival (Colognato et al., 2002; Baron et al., 2003) and myelination (Buttery and ffrench-Constant, 1999; Relvas et al., 2001; Siskova et al., 2006). In contrast, fibronectin promotes proliferation, but reduces myelin-like membrane formation (Buttery and ffrench-Constant, 1999; Baron et al., 2005; Maier et al., 2005; Siskova et al., 2006, 2009). Following CNS injury, the extracellular matrix is extensively remodelled, which is reflected in altered expression profiles of extracellular matrix molecules (Sobel and Mitchell, 1989; Gutowski et al., 1999; Back et al., 2005; van Horsen et al., 2005, 2006; Satoh et al., 2009). Therefore, aberrant extracellular matrix signals in the injury environment may inhibit oligodendrocyte maturation, contributing to remyelination failure in multiple sclerosis lesions. Indeed, while absent from healthy adult human tissue, expression of fibronectin is upregulated in multiple sclerosis lesions, particularly around blood vessels (Sobel and Mitchell, 1989; van Horsen et al., 2005), and also in the CNS parenchyma (van Horsen et al., 2006). Furthermore, astrocyte-derived high molecular weight hyaluronan, which inhibits oligodendrocyte maturation, appears in chronic demyelinating multiple sclerosis lesions (Back et al., 2005). Laminin expression is also increased in multiple sclerosis lesions (van Horsen et al., 2005). Therefore, the overall effects of lesion-induced changes in the extracellular matrix on oligodendrocyte progenitor cells remain to be established.

Here, we further characterized the nature of fibronectin signaling to oligodendrocyte progenitor cells in multiple sclerosis lesions, and how this fibronectin signalling might affect remyelination. We show that fibronectin specifically localizes in areas of demyelination, and identify cellular sources for its expression. In experimental demyelination, fibronectin is cleared on remyelination, whereas fibronectin aggregates and therefore persists in chronic multiple sclerosis lesions and chronic relapsing experimental autoimmune encephalomyelitis (EAE). Importantly, intralesion injection of fibronectin aggregates inhibits oligodendrocyte differentiation and remyelination in toxin-induced demyelinated lesions, which implies that aggregation of fibronectin contributes to remyelination failure in multiple sclerosis lesions.

Materials and methods

Multiple sclerosis lesions

Tissues were obtained from the Netherlands Brain Bank, Netherlands Institute for Neuroscience, Amsterdam. Studies were performed on brain material taken at autopsy from nine healthy subjects (without clinical or histological signs of neurological disease), eight subjects with (chronic) active multiple sclerosis lesions, nine with chronic inactive multiple sclerosis lesions and two with multiple sclerosis shadow plaques (Maier et al., 2007). Samples were selected according to their activity status as determined by MRI, split in half and either immediately frozen in liquid nitrogen or fixed in formaldehyde and paraffin embedded. Control white matter did not show any histological signs of inflammation and demyelination. Demyelinated lesions were identified by Luxol fast blue histochemistry and proteolipid protein staining. Active lesions were characterized by their indistinct margin, hypercellularity, intense perivascular T lymphocyte infiltration (CD3) and presence of hypertrophic astrocytes (glial fibrillary acidic protein, GFAP) and macrophages (CD68) in the centre of the lesions. Chronic active lesions were classified on the basis of a hypocellular lesion centre with fibrous astrocytes and some macrophages, sharp lesion border and a broad rim of macrophages. Chronic inactive lesions contained a hypocellular centre without macrophages and lymphocytes, and a sharp lesion border. Shadow plaques were characterized by a slightly reduced proteolipid protein expression compared with the surrounding normal-appearing white matter, without abundant expression of CD68. Notably, remyelinated areas, as determined by Luxol fast blue or proteolipid protein staining, were not visible in the other multiple sclerosis lesions analysed. For immunohistochemical analysis, sections were deparaffinized and subjected to antigen retrieval followed by fluorescent detection as described previously (Stanic et al., 2011). Control sections from which the primary antibody was omitted showed low non-specific binding. Sections were analysed using a conventional fluorescence microscope (Olympus AX70) equipped with analySIS software. For western blot and reverse transcription–PCR analysis, samples were homogenized and extracted for protein and total RNA as described (Maier et al., 2007). All material was collected from donors whose written informed consent for brain autopsy and the use of the material and clinical information for research purposes has been obtained by the Netherlands Brain Bank.

Toxin-induced demyelination

Lesions were induced in spinal cord white matter or in the caudal cerebral peduncle of 8–10-week-old female Sprague Dawley rats...
(Harlan) by injection of 1 μl of 1% lysolecithin (Sigma Aldrich) or 4 μl of 0.01% ethidium bromide (VWR), respectively (Fancy et al., 2004; Zhao et al., 2006). At the desired time points, animals were sacrificed and tissue processed as described previously (Woodruff and Franklin, 1999; Fancy et al., 2004; Zhao et al., 2006). The control spinal cord tissues consisted of similar fragments from unlesioned thoracic segments of spinal cord. The intralesion injection of fibronectin aggregates was performed at 7 days post lesion into lysolecithin-induced lesions in rat dorsal funiculus of spinal cord. A volume of 2 μl of fibronectin aggregates (0.3 μg/μl) was injected using a Hamilton syringe with a pulled glass tip attached. These animals were sacrificed 7 days after aggregate injection, i.e. 14 days post lesion. Experiments were performed in compliance with UK Home Office regulations.

**Chronic relapsing experimental autoimmune encephalomyelitis**

Chronic relapsing EAE was induced with recombinant rat myelin oligodendrocyte glycoprotein in adult male Dark Agouti rats (Harlan, weight 230–250 g) as described previously (Ledeboer, et al., 2003). Briefly, the rats were anaesthetized with isoflurane and immunized intradermally in the dorsal tail base with 75 μg of recombinant rat myelin oligodendrocyte glycoprotein (rrMOG1-125) emulsified in incomplete Freund’s adjuvant (Difco) together with 10 mM NaAc (pH 3.0). Control rats received incomplete Freund’s adjuvant and NaAc only. Rats were weighed and examined daily for neurological symptoms of EAE that were scored on the following scale: 0, no clinical symptoms; 1, only. Rats were weighed and examined daily for neurological symptoms of EAE that were scored on the following scale: 0, no clinical symptoms; 1, only. Rats were examined daily for neurological symptoms of EAE that were scored on the following scale: 0, no clinical symptoms; 1, only.

**Immunohistochemical analysis**

**Toxin-induced lesions**

Frozen sections (12 μm) of spinal cord and caudal cerebral peduncle were permeabilized and blocked with phosphate-buffered saline (PBS) containing 10 μM NaAc and then incubated with the appropriate primary antibodies (Table 1) diluted in PBS containing 3% normal donkey serum overnight at 4°C. For double labelling immunohistochemistry, primary antibodies were incubated sequentially. After washing in PBS, the sections were incubated with appropriate Alexa Fluor® (488 or 594)-conjugated secondary antibodies (Invitrogen 1:500) at room temperature for 2 h, followed by 4',6-diamidino-2-phenylindole (DAPI) to visualize cell nuclei. Following immunostaining, some slides were incubated in 0.1% Sudan Black solution (made in 70% ethanol) for 5 min at room temperature to stain myelin. Pale areas in the white matter indicate loss of myelin. Sudan Black staining has no detrimental effect on the cultures was routinely verified by staining for GFAP (astrocytes). Astrocyte cultures used were at least 97% pure. The cells were plated in 10-cm dishes (1.0 × 10⁶/dish) or 8-well chamber slides (10000/well). Deposited astroglial matrices were prepared by water-lysis of astrocytes for 2 h at 37°C.

**Table 1 Primary antibodies used during immunohistochemistry, immunocytochemistry and western blot**

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Manufacturer</th>
<th>Dilution western blot</th>
<th>Dilution IHC/ICC</th>
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<td>Anti-actin (mAb)</td>
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<td>1:500</td>
</tr>
<tr>
<td>Anti- GFAP (pAb)</td>
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<td>DAKO</td>
<td>n.a.</td>
<td>1:200</td>
</tr>
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</table>

n.a. = not applicable; mAb = monoclonal antibody; pAb = polyclonal antibody; IHC/ICC = immunohistochemistry/immunocytochemistry.

a Lee et al., 1999.
to immunofluorescence. Sections were analysed with a Zeiss Axio Observer A1 fluorescent microscope.

**Chronic relapsing experimental autoimmune encephalomyelitis**

Fresh frozen rat spinal cord sections (12 μm) were fixed with acetone for 20 min, washed in Tris-buffered saline, blocked in 5% milk in Tris-buffered saline with 0.5% Triton™ X-100 and 0.03% H₂O₂ and incubated with primary antibodies (Table 1) overnight at 4°C, followed by appropriate Alexa Fluor® (488 or 594)-conjugated secondary antibodies (1:400) for immunofluorescence. Sections were examined with a Leica confocal laser scanning microscope. For peroxidase-based analysis, sections were washed in Tris-buffered saline and incubated for 2 h at room temperature with secondary biotinylated IgGs (Jackson ImmunoResearch, 1:400), followed by washes in Tris-buffered saline and incubation for 1 h at room temperature with avidin-biotin-peroxidase complex (Vector Laboratories, 1:400). After washes in Tris-buffered saline and 50 mM Tris–HCl (pH 7.6), immunoreactivity was visualized using 0.5 mg/ml of diaminobenzidine (Sigma) in 50 mM Tris–HCl (pH 7.6).

**Immunocytochemical analysis**

Oligodendrocytes were fixed with 4% paraformaldehyde for 20 min and permeabilized with ice-cold methanol for 10 min. After a 30-min block with 4% bovine serum albumin, cells were incubated for 60 min with primary antibodies (Table 1) in 4% bovine serum albumin. Cells were washed three times with ice-cold PBS and incubated for 25 min with appropriate tetramethyl rhodamine isothiocyanate-conjugated antibodies (Jackson ImmunoResearch). Nuclei were stained with DAPI (1 μg/ml) and 1,4-diazabicyclo[2.2.2]octane-containing mounting medium was added to prevent image fading. For staining of astroglial matrices, fixation and permeabilization steps were omitted. Oligodendrocytes were characterized by their morphology, and in each experiment at least 500 cells were scored as myelin basic protein (MBP)-positive or -negative. In addition, positive cells bearing MBP-positive membranous structures spread between the cellular processes were identified as membrane sheet forming, irrespective of the extent of sheet formation.

**Reverse transcription–polymerase chain reaction**

Total RNA was isolated from cells and tissue homogenates using the RNeasy® Mini kit (Qiagen). Total RNA from tissue (0.5 μg) or cells (1.0 μg) was reverse transcribed in the presence of oligo(dT)12–18 and dNTPs (Invitrogen) with SuperScript® II reverse transcriptase (Invitrogen) according to the manufacturer's instructions. The resulting complementary DNA was amplified using primers specific to the different proteins (Table 2). Cycling conditions were optimized and PCR products were resolved by agarose gel electrophoresis. Changes in gene expression were analysed by Scion Image Software.

**In situ hybridization**

To generate a complementary RNA probe for fibronectin, complementary DNA acquired using fibronectin primers (Table 2) was cloned by reverse transcription–PCR and inserted into the pCR®II-TOPO plasmid, using the TOPO® TA Cloning® Kit (Invitrogen) according to the manufacturer's instruction. The probe is expected to recognize all variants of fibronectin messenger RNA. The digoxigenin-labelled proteolipid protein probe was generated as described (Chari et al., 2006). The details for probe labelling and staining procedures have been described in previous studies (Sim et al., 2000; Zhao et al., 2008).

**Deoxycholate (in)solubility assays and preparation of fibronectin aggregates**

Tissue homogenates (multiple sclerosis and chronic relapsing EAE) were incubated with deoxycholate buffer (2% deoxycholate and Complete Mini protease inhibitor cocktail (Roche) in 20 mM Tris–HCl, pH 8.3), for 30 min on ice. Proteins from lysolceithin-induced lesions were extracted from 30-μm thick slices through oscillation for at least 3 h in deoxycholate buffer. Deposited astroglial matrices were prepared by water-lysis of astrocytes for 2 h at 37°C. The efficiency of lysis was verified by DAPI staining, and only matrices without visible nuclei were used. Deposits were scraped in ice-cold deoxycholate buffer and further solubilized for 30 min on ice. Protein concentrations were determined by a Bio-Rad DC protein assay (Bio-Rad Laboratories) using bovine serum albumin as standard. For biochemical analysis, deoxycholate-soluble and -insoluble fractions from equal protein amounts of the deoxycholate extracts were separated by centrifugation at 13 000 rpm for 20 min at 4°C. The deoxycholate-insoluble pellets were dissolved in 2% SDS in 20 mM Tris–HCl, pH 8.8, whereas the deoxycholate-soluble supernatant was concentrated by trichloroacetic acid precipitation. For intraleisional injections, the aggregates present in the deoxycholate-insoluble fraction were dialyzed against PBS for 24 h at 4°C. After dialysis, the protein content was determined and the presence of aggregates confirmed by western blot.

**Lactate dehydrogenase and MTT assay**

Oligodendrocyte progenitor cells were plated in 24-well plates (Nunc) at a density of 50 000 cells in 500 μl culture medium.
Oligodendrocytes were treated with fibronectin aggregates (5.0 μg/ml) of the deoxycholate-insoluble fraction of rat astrocyte-derived deposits dialyzed against PBS at the onset of differentiation (oligodendrocyte progenitor cells), 3 days (immature oligodendrocytes) or 7 days (mature oligodendrocytes) after initiating differentiation. After 3 days, the medium (lactate dehydrogenase assay) and cells (3- (4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; MTT) were analyzed. To determine the cytotoxicity of deoxycholate-insoluble fibronectin aggregates, the release of lactate dehydrogenase into the medium was measured using a commercial lactate dehydrogenase assay kit (Roche) according to manufacturer’s instructions. The effect on cell viability was determined with an MTT assay. Briefly, after medium was removed for the lactate dehydrogenase assay, cells were incubated with MTT diluted in culture medium (0.5 mg/ml, Sigma) for 3–4 h. MTT-formazan crystals were collected in dimethyl sulphoxide and absorption was measured at 560 nm. Cytotoxicity (lactate dehydrogenase) and cell viability (MTT) are expressed as the percentage of vehicle-treated (PBS) cells, which was set at 100%.

**Electron microscopy analysis**

The animals were perfused with 4% glutaraldehyde and lesion containing spinal cord was coronally sliced at ~1 mm thickness and fixed with osmium tetroxide overnight before being subjected to a standard protocol of epoxy resin embedding (Zhao et al., 2008). Ultrathin sections of the lesion site were produced at transverse orientation and examined with a Hitachi H-600 electron microscope. Myelination of axons in the lesion was analyzed for g-ratio, which is calculated as the diameter of axons divided by the diameter of axons with surrounding myelin sheaths.

**Sodium dodecyl sulphate–polyacrylamide gel electrophoresis and western blotting**

Equal amounts of proteins (tissue homogenates, cultured cells and reducing conditions) or volume [deoxycholate-(in)solubility assays, non-reducing conditions] were loaded onto 6 or 8% SDS-PAGE gels and subjected to western blot analysis as described previously (Bsibsi et al., 2012). The signals were detected using the Odyssey™ Infrared Imaging System (Li-Cor Biosciences) and analyzed using Odyssey V3.0 analysis software.

**Results**

**Cellular fibronectin expression is transiently increased in toxin-induced demyelination**

Fibronectin effects on regeneration have been studied in various tissues (Scanzello et al., 2008). To assess whether on demyelination, changes in the extracellular matrix environment include fibronectin, we first examined the profile of fibronectin expression following toxin-induced demyelination. Injection of lysolecithin into spinal cord white matter creates focally demyelinated lesions without significant axonal loss (Blakemore and Franklin, 2008). These lesions undergo spontaneous remyelination, involving the activation of oligodendrocyte progenitor cells and their recruitment into the demyelinated area (0–10 days post lesion), and their subsequent differentiation and myelin sheath formation (10–21 days post lesion) (Zhao et al., 2006). In this model, the lesion area is characterized by an increased cellularity, which reflects well the area of demyelination as visualized with a Sudan Black myelin stain (Fig. 1A). Therefore, the demyelinated areas were identified based on their hypercellularity using DAPI staining for nuclei. Immunohistochemical analysis showed that fibronectin expression was highly detectable in demyelinated areas at 3 and 5 days post lesion, but progressively reduced at 10 and 15 days post lesion (Fig. 1B). Western blot analysis confirmed a clear and significant increase in fibronectin protein expression at 5 days post lesion, followed by a significant decrease at 14 days post lesion (Fig. 1C). Demyelination and ongoing remyelination of the lesions were confirmed by reduced expression levels of MBP at 5 days post lesion compared with unlesioned control, and increased MBP expression at 14 days post lesion as compared with 5 days post lesion, respectively (Fig. 1C). Transient expression of fibronectin was also observed using a second model of toxin-induced demyelination, in which ethidium bromide is injected into the caudal cerebral peduncle of rats (Supplementary Fig. 1; Woodruff and Franklin, 1999).

Fibronectin has two major variants: (i) plasma fibronectin, a soluble dimer that is secreted into the circulation by hepatocytes; and (ii) cellular fibronectin, which is produced by resident cells. On CNS injury, and likely also on toxin-induced demyelination, plasma fibronectin enters the brain owing to blood–brain barrier disruption (Sobel and Mitchell, 1989; van Horsen et al., 2005). To examine whether cells also express cellular fibronectin following CNS demyelination, we analyzed fibronectin messenger RNA expression in lysolecithin-induced demyelination via reverse transcriptase–PCR. As shown in Fig. 1D, total fibronectin messenger RNA levels increased significantly at demyelination (5 days post lesion) compared with unlesioned spinal cord, and decreased again in early remyelination (14 days post lesion). To assess cellular fibronectin protein expression, we performed double immunohistochemistry with anti-fibronectin and anti-EIIIA (IST9) specific antibodies. EIIIA-fibronectin, i.e. cellular fibronectin, was expressed in acute lysolecithin-induced demyelination, where it was confined to the lesioned area and mostly located around blood vessels (Fig. 1E). Notably, fibronectin expression was more widespread throughout the demyelinated area than EIIIA-fibronectin. Importantly, EIIIA-fibronectin protein expression was increased as early as 1 day post lesion and downregulated at 14 days post lesion (Supplementary Fig. 2), concomitant to the decrease of total fibronectin expression (Fig. 1B and C). Hence, these findings reveal that fibronectin expression was transiently upregulated on toxin-induced demyelination, and the detection of fibronectin messenger RNA and EIIIA-fibronectin indicate that CNS resident cells synthesize fibronectin in response to demyelination, which prompted us to identify these cells.

**Multiple cell types express cellular fibronectin in toxin-induced demyelination**

The cellular distribution of fibronectin messenger RNA in toxin-induced demyelination sections was examined by in situ
Figure 1  Expression of cellular fibronectin in lysolecithin-induced demyelination. Lesioned and non-lesioned tissue were analysed for the expression of fibronectin protein and messenger RNA using fluorescent immunohistochemistry (B and E), western blot (C) and reverse transcriptase–PCR (D) analysis. Focal demyelination of the rat spinal cord was induced by lysolecithin injection. In A–E, representative images, blots and gels are shown of three to four animals per condition. (A) Demyelinated areas, as visualized with a Sudan Black (SB) myelin stain, were identified based on their hypercellularity (DAPI staining). Images were taken at 5 days post lesion. Scale bars are 1000 μm (black) and 100 μm (white). (B) Fibronectin expression (red) is abundant at demyelination (3 and 5 days post lesion), and cleared on remyelination (10 and 15 days post lesion). The baseline expression of fibronectin was low on immunostaining of unlesioned control spinal cord and normal-appearing tissue around lesions (CTRL). Scale bar = 20 μm. (C and D) Fibronectin protein (C) and messenger RNA (D) is upregulated in demyelination [5 days post lesion versus control (CTRL)], but downregulated on remyelination (14 days post lesion versus 5 days post lesion). Data were quantified by normalizing the optical densities of fibronectin protein against actin (C), and fibronectin messenger RNA against the housekeeping gene cyclophilin (D). Data are expressed as value of the mean + the standard deviation (SD) (*P < 0.05, ***P < 0.0001 at one-way ANOVA followed by Tukey’s honestly significant difference test, three to four animals per condition). Demyelination of the lesions was confirmed by lower expression levels of the myelin protein MBP. (E) Fibronectin (red) and EIIIA-fibronectin (IST9, green) expression in lysolecithin-induced demyelination (5 days post lesion). Scale bar = 20 μm. EIIIA-fibronectin and fibronectin particularly co-localize (yellow) around blood vessels. Fn = fibronectin; DPL = days post lesion.
hybridization. Fibronectin messenger RNA expression was confined to the demyelinated area (Fig. 2A), consistent with fibronectin protein expression (Fig. 1A). Macrophages/microglia were identified as a possible source by two-colour double labelling in situ hybridization for fibronectin and osteopontin messenger RNA, a marker for cells of the macrophage/microglia lineage particularly in toxin-induced lesions (Zhao et al., 2008). There was clear colocalization at 1 day post lesion (Fig. 2B; 68.2 ± 11.2%) that was decreased at 5 days post lesion (Fig. 2C; 23 ± 5.8%), which might reflect the transition from monocytes/microglia to phagocytotic macrophages (Zhao et al., 2006). This is supported by double labelling with anti-fibronectin and anti-CD11b (MAC1) antibodies, showing fibronectin expression in a substantial number of MAC1-positive microglia/macrophages (Fig. 2D, 5 days post lesion). Fibronectin messenger RNA (Fig. 2E) and protein (Fig. 2F) was further detected in GFAP-positive astrocytes, indicating that astrocytes are another source of cellular fibronectin. Co-labelling with anti-Olig2 antibodies (Fig. 2G; 2.9 ± 1.0% fibronectin messenger RNA/Olig2 double positive), as a marker for the oligodendrocyte lineage, Nkx2.2 (H, green) as a marker for oligodendrocyte progenitor cells, and Von Willebrand factor (vWF, I, red), as a marker for endothelial cells. Protein and messenger RNA were visualized by indirect fluorescent immunohistochemistry and in situ hybridization, respectively. Lesioned areas were identified by hypercellularity, i.e. DAPI (blue) staining. Representative images of three to four animals per condition are shown. Scale bar = 50 μm for A, E and H, and 20 μm for B–D, F, G and I. Fn = fibronectin; DPL = day(s) post lesion.
Fibronectin aggregates perturb CNS remyelination

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Willebrand factor, a marker for endothelial cells, showed that some fibronectin co-localized with oval-shaped von Willebrand factor-positive cells (Fig. 2I, inset). This indicated that endothelial cells may also produce cellular fibronectin, as suggested before by the localization of EIIIA-fibronectin (Fig. 1), and co-localization of IST9 with von Willebrand factor (data not shown). Hence, macrophages/microglia, astrocytes and endothelial cells, but not cells of the oligodendrocytes lineage, contribute to cellular fibronectin expression in demyelinated lesions.

Fibronectin assembles into aggregates in demyelinated multiple sclerosis lesions

Previous studies suggest the expression of fibronectin in chronically demyelinated multiple sclerosis lesions (Sobel and Mitchell, 1989; van Horsen et al., 2005, 2006; Satoh et al., 2009). However, these studies do not provide information on fibronectin levels, its origin or its form. We therefore biochemically characterized fibronectin expression in white matter brain homogenates of healthy subjects (control white matter, n = 9), (chronic) active multiple sclerosis lesions (n = 8) and chronic inactive multiple sclerosis lesions (n = 9). In chronic inactive lesions, fibronectin was expressed around blood vessels (Fig. 3D), whereas an increased number of smaller fibronectin deposits were present throughout (chronic) active multiple sclerosis lesions (Fig. 3B and C). However, in control white matter, obtained from subjects without clinical or pathological signs of neurological disease, fibronectin was scarcely detectable and limited to the vasculature (Fig. 3A). Western blot analysis under reducing conditions confirmed the increased fibronectin expression in multiple sclerosis lesions as compared with control white matter (Fig. 3E). Although control white matter showed a variation in fibronectin levels among individual subjects, the amount of fibronectin in white matter homogenates of active and inactive multiple sclerosis lesions was generally higher (Fig. 3F). Therefore, our data provide quantitative evidence of fibronectin accumulation in multiple sclerosis lesions. Analysis of fibronectin messenger RNA expression via reverse transcriptase-PCR revealed low levels (Supplementary Fig. 3) that do not seem to be in line with the increased fibronectin protein levels in multiple sclerosis lesions (Fig. 3B), but could well be explained by the chronic status of these lesions. Accordingly, fibronectin messenger RNA might have been initially upregulated at the onset of demyelination, but downregulated over time, whereas the protein has not been degraded and subjected to post-transcriptional and post-translational modification, which we examined next.

Fibronectin normally appears as a disulphide-bound dimer with subunits of 220–250 kDa, but can assemble into a complex network of fibrils of high molecular weight aggregates, which are insoluble in the detergent deoxycholate (Wierzbicka-Patynowski and Schwarzbauer, 2003; Mao and Schwarzbauer, 2005). Under non-reducing SDS-PAGE conditions, fibronectin aggregates remain either in the stacking gel or just penetrate the resolving gel in lower percentage SDS cells. To examine whether fibronectin aggregates in multiple sclerosis lesions, we performed detergent deoxycholate (in)solubility assays. High molecular weight fibronectin complexes were observed in the deoxycholate-insoluble fraction from homogenates of multiple sclerosis lesions, whereas fibronectin dimers were the main form in control white matter (Fig. 3G). This suggests that fibronectin aggregates are predominantly present in both active and inactive multiple sclerosis lesions, and hardly in control white matter. Given our previous findings that fibronectin perturbs myelin-like membrane formation (Buttery and ffrench-Constant, 1999; Maier et al., 2005; Siskova et al., 2006, 2009) and given that clearance of fibronectin in toxin-induced demyelination preceded remyelination (Fig. 1), the detection of fibronectin aggregates in chronically demyelinated multiple sclerosis lesions led us to hypothesize that they may contribute to remyelination failure. If this is the case, fibronectin would not aggregate and/or aggregation would not persist in toxin-induced demyelination.

Fibronectin aggregates predominantly in inflammation-induced demyelination

Deoxycholate (in)solubility assays followed by SDS-PAGE under non-reducing conditions revealed that fibronectin aggregation was minimal in toxin-lesioned tissues (Fig. 3H). Hence, fibronectin expression was transiently upregulated on toxin-induced demyelination, but does not extensively aggregate. A major difference between multiple sclerosis and toxin-induced lesions is the mechanism of demyelination. In multiple sclerosis, demyelination results mainly from activity of the adaptive immune system, whereas in toxin-induced models, demyelination is caused by the toxin and this initiates a secondary inflammatory response, mainly involving the innate immune system. To assess whether fibronectin aggregation is mediated by chronic inflammation, we next examined fibronectin expression and aggregation in chronic relapsing EAE, an animal model of immune-mediated demyelination that resembles many clinical and pathological features of relapsing-remitting multiple sclerosis (Furlan et al., 2009; Pachner, 2011). On immunization with a myelin oligodendrocyte glycoprotein peptide, the animals suffer from neurological symptoms, usually restricted to the hind body, that develop in a relapsing-remitting pattern (Supplementary Fig. 4A). During the relapse phase, Major Histocompatibility Complex II inflammatory infiltrates can be observed in the rat spinal cord (Supplementary Fig. 4B), whereas as demyelinated areas can be observed in the lumbar and sacral region of the spinal cord (Storch et al., 1998; Ledeboer et al., 2003). Total fibronectin expression was increased at the relapse phase in chronic relapsing EAE by ~9-fold (Fig. 3I) compared with incomplete Freund’s adjuvant control. Furthermore, deoxycholate-(in)solubility assays showed that fibronectin aggregated in chronic relapsing EAE (Fig. 3J), similar to multiple sclerosis lesions (Fig. 3B). EIIIA-fibronectin expression was also evident at the relapse in chronic relapsing EAE (Fig. 3K), suggesting that cellular fibronectin was increased as an immediate response on inflammation-mediated demyelination. Hence, whereas fibronectin was transiently expressed and remained predominantly soluble in toxin-induced demyelination undergoing remyelination, fibronectin aggregates were formed in lesions of inflammation-induced demyelination, where remyelination often fails. Therefore, we
Figure 3  Fibronectin protein expression and biochemical characterization in human control white matter, multiple sclerosis lesions, chronic relapsing EAE and toxin-induced lesions. (A–D) Fibronectin expression (green) is abundant within the parenchyma of demyelinated multiple sclerosis lesion areas in active multiple sclerosis (B, arrows) and chronic active multiple sclerosis (arrow), and nearly absent from control white matter (CWM). In chronic inactive multiple sclerosis, fibronectin is abundantly expressed in association with blood vessels (D). Nuclei were counterstained with DAPI (blue). Representative images of control white matter (A) and centres of active (aMS, B), chronic active (caMS, C) and chronic inactive (ciMS, D) multiple sclerosis lesions are shown. Scale bar = 20 μm.  

(E) Expression levels of fibronectin and the other indicated proteins in 50 μg of brain white matter homogenates of human control white matter, (chronic) active and chronic inactive multiple sclerosis lesions as determined by western blot under reducing conditions (monomers). Demyelination of the lesions was confirmed by lower expression levels of the myelin protein MBP compared with control white matter. Decreased CNP levels, mostly in (chronic) active multiple sclerosis lesions, and decreased TuJ1-levels, particularly in chronic inactive lesions, were likely indicative of oligodendrocyte and axonal loss, respectively. The constant levels of actin in the samples served as internal controls.  

(F) Variation of fibronectin expression in individual human white matter tissue.  

(G) Fibronectin aggregates are abundantly present in (chronic) active multiple sclerosis and chronic inactive multiple sclerosis lesion homogenates compared with control white matter homogenates. Homogenates (100 μg) were subjected to deoxycholate–(in)solubility assays, and deoxycholate-insoluble (Ι) and -soluble (S) fractions were analysed by western blotting under non-reducing conditions.  

(H) Fibronectin aggregates are virtually absent from lysolecithin-induced demyelinated lesions. Deoxycholate-insoluble (Ι) and -soluble (S) fractions from 50 μg tissue were analysed by western blotting under non-reducing conditions. Three animals per condition were analysed.  

(I) Fibronectin expression is upregulated in chronic relapsing EAE animals as compared with incomplete Freund’s adjuvant controls. Spinal cord homogenates (20 μg) of control (CTRL) and chronic relapsing EAE (cr-EAE, relapse phase) were analysed by western blotting under reducing conditions (monomers). Data were quantified by normalizing the optical densities of fibronectin protein against actin, and expressed as mean ± SD (**P < 0.01 at Student’s t-test, three animals per group).  

(J) Fibronectin aggregates at the relapse phase in chronic relapsing EAE. Deoxycholate-insoluble (Ι) and -soluble (S) fractions from 100 μg tissue were analysed by western blotting under non-reducing conditions.  

(K) Fibronectin (red) and EIIIA-fibronectin (IST9, green) expression at the relapse phase of chronic relapsing EAE. Scale bar = 20 μm. EIIIA-fibronectin and fibronectin particularly co-localize (yellow) around blood vessels. Fn = fibronectin; AGG = aggregate; DPL = days post lesion.
Fibronectin aggregation by cultured astrocytes is induced by the inflammatory mediator lipopolysaccharide

Having identified multiple cellular sources in vivo, we first examined which of these cells express fibronectin messenger RNA and protein in (primary) monoculture in vitro. Only cultured astrocytes produced fibronectin messenger RNA and fibronectin protein in considerable amounts (Fig. 4A), which corroborate previous findings that astrocytes produce fibronectin (Price and Hynes, 1985; Liesi et al., 1986; Oh and Yong, 1996). However, fibronectin expression by the other cell types may occur in other, particular activation states. Water-lysis of the cultured astrocytes showed that untreated astrocytes deposited fibronectin in an evenly distributed diffuse pattern, whereas astrocytes exposed to the inflammatory mediator lipopolysaccharide deposited a more localized fibrillar fibronectin matrix (Fig. 4B, rat). Similar results were obtained when astrocytes were removed by EDTA-mediated detachment (data not shown). Notably, an increase in fibronectin messenger RNA and a slight increase in protein expression were observed in astrocytes treated with the inflammatory mediator lipopolysaccharide (Fig. 4A). Fibronectin aggregation also markedly increased after treatment with lipopolysaccharide in rat...
Astrocyte-derived fibronectin aggregates inhibit remyelination

We first studied whether astrocyte-derived fibronectin aggregates altered progression within the oligodendrocyte lineage in vitro. To allow investigation of cell–matrix interactions as such, oligodendrocyte progenitor cells were plated onto astroglial matrices obtained from untreated (Fig. 4B) or lipopolysaccharide-treated rat astrocytes (Fig. 4B). The astrocytes were removed by water-lysis, so that the effect of live astrocytes and their soluble factors on oligodendrocyte progenitor cells was eliminated. When oligodendrocyte progenitor cells were cultured on the astrocyte-derived matrices for 7 days, a small increase in the number of MBP-positive cells, indicative for differentiation, was observed (Fig. 5A and B). Myelin-like membrane formation, as defined by cells bearing MBP-positive membranous structures spread between the cellular processes, however, was prevented on astroglial matrices. Staining for MBP-positive cells indicated that cultured astrocytes did not induce aggregation unless additional factors, such as those linked to chronic inflammation, are present. This could explain why fibronectin aggregates were found at the relapse phase in chronic relapsing EAE and multiple sclerosis lesions, but not in toxin-induced demyelination. Because fibronectin aggregates in multiple sclerosis lesions, where remyelination fails, and not in toxin-induced lesions, where remyelination is complete, we next examined whether fibronectin aggregates contribute directly to remyelination failure.

Fibronectin aggregates are present at low level in remyelinated multiple sclerosis lesions

To assess whether fibronectin aggregates are expressed at remyelinating conditions, homogenates of remyelinated multiple sclerosis shadow plaque containing brain tissue were subjected to deoxycholate-(in)solubility assays. The status of shadow plaques was confirmed by the Netherlands Brain Bank, showing a slightly reduced expression of proteolipid protein compared with the surrounding normal-appearing white matter, without abundant expression of CD68. Fibronectin aggregates were weakly expressed in the examined multiple sclerosis shadow plaques, whereas fibronectin is mainly present as a deoxycholate-soluble dimer (Fig. 6A). Furthermore, western blot analysis at reducing conditions revealed that fibronectin levels are slightly increased in remyelinated multiple sclerosis lesions compared with control white matter, but to a lesser extent than in (chronic) active multiple sclerosis lesions (Fig. 6B). Fibronectin staining around blood vessels can be discerned throughout the shadow plaques (Fig. 6C), and seemed higher than fibronectin staining in surrounding normal-appearing white matter (data not shown). However, fibronectin is barely detectable in the parenchyma of the remyelinated area. Thus, fibronectin aggregates are present at a low level in
Figure 5 Effects of fibronectin aggregates on oligodendrocytes in vitro and in vivo. (A and B) Oligodendrocyte progenitor cells differentiate to MBP-expressing oligodendrocytes on control (CTRL) and fibronectin aggregate (LPS) containing astroglial extracellular matrices, but myelin-like membrane formation was retarded on fibronectin aggregates. This is confirmed by quantification of MBP-positive cells (B, differentiation) and the amount of MBP-positive cells elaborating myelin-like membranes (B, myelination). Astroglial matrices were obtained by water-lysis of astrocytes. In each experiment, at least 500 cells/well were counted. To compare different independent experiments, the data are expressed as per cent of control, i.e. values obtained from CTRL were set to 100. Each bar represents the mean + SEM of three independent experiments. Statistical differences were assessed with a Student’s t-test, and are indicated by asterisks (*P < 0.05, **P < 0.01). Representative images of three independent experiments are shown (A). Scale bar = 20 μm. (C–L) Fibronectin
remyelinated multiple sclerosis lesions, which corroborate the findings that fibronectin aggregates impair remyelination.

**Discussion**

Remyelination is crucial for functional recovery in demyelinating diseases, such as multiple sclerosis (Duncan et al., 2009). Here, we show that the extracellular matrix component fibronectin, which is largely absent from healthy adult white matter, rapidly accumulates as an acute response to demyelination, but disappears on remyelination. However, in multiple sclerosis lesions where remyelination fails, a persistent accumulation of stable aggregated fibronectin occurs. These fibronectin aggregates are most likely generated by astrocytes on engagement with inflammatory mediators, and most relevantly, these aggregates impair remyelination.

Aggregation of fibronectin is likely associated with the process of inflammation. This was suggested by the absence of fibronectin aggregates from toxin-induced demyelination, where an innate immune response is secondary to demyelination, compared with the abundance of fibronectin aggregates in chronic relapsing EAE and multiple sclerosis lesions, where demyelination is caused by the adaptive immune response. Also, our in vitro experiments with primary rat astrocytes suggest a role for inflammatory mediators in fibronectin aggregation. Astrocytes exposed to the inflammatory mediator lipopolysaccharide deposit fibronectin in fibril-like structures, whereas untreated astrocytes deposit fibronectin in a more diffuse pattern. This structural difference in fibronectin appearance is monitored by conversion from a deoxycholate-soluble dimeric form to deoxycholate-insoluble fibronectin aggregates. Fibrillar fibronectin assembly is primarily initiated by binding of soluble fibronectin dimers to integrin α5β1, followed by assembly into high molecular weight fibronectin that is cross-linked by non-covalent bounds (Wu et al., 1993, 1995; Wierzbicka-Patynowski and Schwarzbauer, 2003; Ohashi and Erickson, 2009). Integrin α5β1 is expressed on astrocytes (King et al., 2001), neurons (King et al., 2001) and activated microglia (Milner et al., 2007), but not on oligodendrocytes (Milner and ffrench-Constant, 1994). In addition to fibril formation, fibronectin can be covalently cross-linked by enzymes. Tumor necrosis...
Fibronectin aggregates perturb CNS remyelination

Fibronectin expression in multiple sclerosis lesions was previously suggested to result from blood–brain barrier disruption (Sobel and Mitchell., 1989; van Horssen et al., 2005). Our data reveal that on CNS injury, CNS resident cells also contribute to the fibronectin pool. This conclusion is supported by at least three observations: (i) next to a typical vasculature localization, small fibronectin deposits are present in multiple sclerosis, chronic relapsing EAE and toxin-induced lesions, suggesting additional sources to plasma leakage; (ii) fibronectin messenger RNA is upregulated in toxin-induced demyelination; and (iii) EIIIA-fibronectin protein is detected at the lesion site. The lack of fibronectin messenger RNA, likely due to the chronic disease status, precluded elucidation of cellular sources in multiple sclerosis lesions. However, in situ hybridization co-localization studies in toxin-induced lesion revealed that astrocytes, microglia/macrophages and endothelial cells likely synthesize fibronectin. Whether these cells also secrete and actively deposit fibronectin in vivo is rather difficult to determine. In vitro, fibronectin aggregation is dependent on astrocytes, as only astrocytes actively synthesize and deposit fibronectin, and plasma fibronectin alone, i.e. in the absence of astrocytes, does not aggregate (our unpublished observations). However, plasma fibronectin likely incorporates into astrocyte-derived fibronectin aggregates (McKeown-Longo and Mosher, 1983; Peters et al., 1990).

Importantly, our data revealed that aggregated fibronectin impairs remyelination. Fibronectin aggregates are hardly present in remyelinated multiple sclerosis shadow plaques, and injection of astrocyte-derived fibronectin aggregates into lysolecithin-induced demyelinated lesions resulted in a significant decrease of differentiated oligodendrocytes and concomitant increase in g-ratio compared with saline-injected lesions. Myelin-like membrane formation in vitro is inhibited by astrocyte-derived matrix that contains fibronectin aggregates, but not by astrocyte-derived matrix that contains dimeric fibronectin. This might be explained by the presence of additional (extracellular matrix) molecules that reside in the total astroglial matrix, such as laminin (Liesi et al., 1983, 1984; Fig. 4D and E). These additional molecules could overcome the myelinization-inhibiting effect of dimeric fibronectin that has been observed previously (Buttery and ffrench-Constant, 1999; Maier et al., 2005; Siskova et al., 2006, 2009). Indeed, laminin signals are known to dominate over dimeric plasma fibronectin inhibitory signals (Buttery and ffrench-Constant, 1999). The presence of additional proteins in total astrocyte deposits may therefore also explain why fibronectin aggregates affect oligodendrocyte differentiation only in vivo, as the intralesionally injected aggregates were deoxycholate-insoluble, therefore lacking laminin and other glycoproteins (Fig. 4D and E). The inhibitory effect of fibronectin aggregates may occur through different mechanisms. First, fibronectin aggregates could directly affect oligodendrocyte progenitor cells through the classical fibronectin receptors, αv integrins, which they upregulate in toxin-induced lesions (Zhao et al., 2009). Alternatively, the interaction of aggregates with other cell types in vivo might indirectly affect oligodendrocyte differentiation and remyelination. Finally, fibronectin aggregation likely involves biochemical restructuring of fibronectin (Johnson et al., 1999; Baneyx et al., 2002), which could expose different conformation-dependent binding sites that provoke altered signalling properties (Morla et al., 1994; Pasqualini et al., 1996; Sottile et al., 1998).

In conclusion, myelin regeneration following demyelination is a dynamic process, and requires a spatial and timely balanced response of the extracellular microenvironment. Temporal dimeric fibronectin expression by astrocytes might be important in regulating remyelination at earlier stages. Pathological fibronectin aggregates as observed in multiple sclerosis lesions do, however, likely contribute to remyelination failure. Therefore, strategies to promote remyelination should not aim at preventing fibronectin deposition, but at interfering with fibronectin aggregation and clearance. In addition, the effects of persistent fibronectin aggregates on other CNS cells, including microglia and neurons, as well as the mechanisms of how aggregation is mediated, warrant further investigation, particularly because protein aggregation is likely central to the pathology of several other neurodegenerative diseases (Jucker and Walker, 2011).

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Supplementary material

Supplementary material is available at Brain online.

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factor-α, for example, increases fibronectin aggregation in endothelial cell layers by an enhanced transglutaminase activity on their surface (Chen et al., 2000), a process that might be active in multiple sclerosis lesions (van Strien et al., 2011). Hence, both persistent and recurring inflammation as well as concomitant astrogliosis could gradually result in conformation and accumulation of aggregated fibronectin in multiple sclerosis lesions.

In vitro, fibronectin aggregates were deoxycholate-insoluble, therefore lacking laminin signals are known to dominate over dimeric plasma fibro-

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