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Macrophage-depletion induced impairment of experimental CNS remyelination is associated with a reduced oligodendrocyte progenitor cell response and altered growth factor expression

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Although macrophages are mediators of CNS demyelination, they are also implicated in remyelination. To examine the role of macrophages in CNS remyelination, adult rats were depleted of monocytes using clodronate liposomes and demyelination induced in the spinal cord white matter using lysolecithin. In situ hybridization for scavenger receptor-B and myelin basic protein (*MBP*) revealed a transiently impaired macrophage response associated with delayed remyelination in liposome-treated animals. Macrophage reduction corresponded with delayed recruitment of *PDGFRα*+ oligodendrocyte progenitor cells (OPCs), which preceded changes in myelin phagocytosis, indicating a macrophage effect on OPCs independent of myelin debris clearance. Macrophage-depletion induced changes in the mRNA expression of insulin-like growth factor-1 and transforming growth factor β1, but not platelet-derived growth factor-A and fibroblast growth factor-2. These data suggest that the macrophage response to toxin-induced demyelination influences the growth factor environment, thereby affecting the behavior of OPCs and hence the efficiency of remyelination.

Keywords: Remyelination; Inflammation; Oligodendrocyte progenitor cells; Macrophages; Growth factors; Phagocytosis; Demyelination; Regeneration

Introduction

Multiple sclerosis (MS) is characterized by an immune-mediated breakdown of myelin sheaths and axonal loss. Apart from T-cells, macrophages are believed to be important effector cells involved in the destructive process contributing to antigen presentation and secretion of proinflammatory cytokines and toxic factors, and may even directly damage myelin sheaths (Bitsch et al., 2000; Hill et al., 2004; Huitinga et al., 1990; Lassmann et al., 2001; Rose et al., 2004). Once axons have been demyelinated, a spontaneous regenerative response can occur in which the denuded areas are reinvested with new myelin sheaths. This process is termed remyelination and has been observed in both MS patients (Lassmann et al., 1997; Raine and Wu, 1993) and in a range of experimental models of demyelination (Franklin and Goldman, 2004). However, the extent of remyelination can differ widely, and the reason why it often fails in chronic MS is an important issue currently being explored (Prineas et al., 1993, 2002; Franklin, 2002).

While there has been a general consent on the involvement of the immune system in demyelination, a number of observations point to an association between inflammation and remyelination. Postmortem evidence from MS tissue (Prineas and Connell, 1979; Raine, 1997; Wolswijk, 2002) as well as experimental findings (Graça and Blakemore, 1986; Hinks and Franklin, 2000; Ludwin, 1980; Morell et al., 1998) suggests that remyelination is often associated with areas of robust inflammation and a large macrophage presence. Recently, several functional studies using toxin-induced models, where the myelin damage is not mediated by the immune system making it possible to isolate and study the contribution of inflammation to the repair process, have provided evidence that inflammation may beneficially contribute to remyelination. First, using a well-characterized method to deplete peripheral blood monocytes (van Rooijen, 1989), we were able to show that early depletion of macrophages leads to an impairment of remyelination (Kotter et al., 2001). Second, remyelination is impaired in transgenic animals lacking inflammatory cytokines such as TNF-α and IL-1β, MHCII, or T-cells (Arnett et al., 2001, 2003; Bieber et al., 2003; Mason et al., 2001).

Macrophages might benefit remyelination in two ways. On the one hand, they are the main cell type responsible for

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phagocytic clearance of myelin debris (Ousman and David, 2000; Copelman et al., 2001), which in vitro has been shown to impair oligodendrocyte precursor differentiation (Robinson and Miller, 1999). On the other hand, macrophages are able to secrete a wide variety of different factors including cytokines and growth factors known to be involved in remyelination (Arnett et al., 2001; Hinks and Franklin, 1999; Mason et al., 2001; Nathan, 1987). The present study tests the hypothesis that macrophages are involved in the process of remyelination by the phagocytosis of myelin debris as well as by contributing to the signaling environment. To do this, we have examined the consequences of a reduced macrophages response for (1) the oligodendrocyte progenitor cell (OPC) response to demyelination, (2) the efficiency of myelin debris phagocytosis, and (3) the expression profiles of several growth factors expressed during remyelination and that contribute to the signaling environment.

Materials and methods

Macrophage depletion

Depletion of peripheral blood monocytes was achieved using a previously published protocol (van Rooijen, 1989; van Rooijen and Sanders, 1994). Mannosylated multilamellar liposomes containing dichloromethylene diphosphonate (Cl_2MDP, clodronate) were prepared in PBS ready for intravenous injection. Clodronate was a gift of Roche Diagnostics (Mannheim, Germany). Rats were anesthetized with halothane and oxygen, and liposomes were injected into the tail vein at a dose of 1 ml of clodronate liposomes per 100 g bodyweight. Control animals received corresponding volumes of phosphate-buffered saline (PBS). Repeat injections of 0.5 ml/100 g clodronate liposome suspension or PBS for control animals were made every 5 days.

Induction of focal demyelination

A total number of 46 young adult female Sprague-Dawley rats (200–230 g) were used. Focal areas of demyelination were created in spinal cord white matter. In brief, rats were anesthetized with halothane and oxygen, and a laminectomy was performed on the first lumbar vertebra. One microliter of 1% lysolecithin (Sigma, UK) was injected into the left ventral funiculus, and an additional 1 μl was injected into the dorsal funiculus by means of a glass micropipette attached to a 10-ml Hamilton syringe held in a micromanipulator. Double injections were performed to increase the numbers of lesions for analysis without increasing animal numbers. For all variables assessed, no differences were found between dorsal and ventral lesions.

Solochrome cyanine staining

Air-dried frozen 10-μm cryostat sections were rehydrated with graded alcohol and stained with solochrome cyanine (Fisher Scientific Ltd., UK) for 15 min at room temperature. Following several washes with water, they were differentiated with 5% iron alum (Fisher Scientific Ltd.) again rinsed with water and counterstained with van Gieson staining for 20 s. After several washes, sections were dehydrated with graded alcohol, cleared in xylene, and mounted with DPX mountant.

Radioactive in situ hybridization

Four to six animals were sacrificed for each group and time point by intracardiac injection of pentobarbitone under deep halothane-induced anesthesia on experimental days 5, 10, and 20. One-centimeter lengths of spinal cord containing the lesion were rapidly removed and snap-frozen at −30°C in *n*-pentane and stored at −80°C prior to sectioning. Transverse cryostat sections (10 μm) of spinal cord were cut at −25°C and freeze–thaw-mounted onto the polylysine-coated slides. Sections were sampled from the center of lesion-containing lengths of spinal cords, air-dried, and fixed using 4% paraformaldehyde in phosphate-buffered saline (PBS) for 5 min, rinsed twice in 1× PBS for 1 min, and dehydrated in 70% ethanol for 5 min. Sections were stored in 96% ethanol at 4°C until further use. The following antisense oligonucleotides sequences specific for SR-B, myelin basic protein (MBP) exon1, insulin-like growth factor (IGF)-1, transforming growth factor (TGF)-β1, platelet-derived growth factor (PDGF)-A, and fibroblast growth factor (FGF)-2, whose efficacy was demonstrated in previous publications, were used:

SR-B: 5′-CTA CAG CTT GGC TTC TTG CAG TAC CGT GCC CTT GGC AGC TGG TGA CAT CA (Genbank accession no. D89655) (Hinks and Franklin, 2000); MBP exon1: 5′-TGT GGC CAG GTA CTT GGA TCG CTG TGA GGG TCT CTT CTG TGA TGC CAT (Sim et al., 2000); IGF-1: 5′-AGT AAA AGC CCC TTG GTC CAC ACA CGA ACT GAA GAG (Hinks and Franklin, 2000; Shinar and McMorris, 1995); TGF-β1: 5′-ATG GTA GCC CTT GGG CTC GTG GAT CCA CTT (Hinks and Franklin, 2000); PDGF-A: 5′-TGG GCG AGG TAT CCG CAG CCG AGG AGC AGC AGG CAA GCC CAG GTC CTC AT (Ueda et al., 1996) and 5′-CAC CTC ACA TCC GTC TCC TCC TCC CGA TGG TCT GGG TTC AGG TTG (Hinks and Franklin, 2000; Pollock and Richardson, 1992); FGF-2: 5′-CGG TTC GCA CAC ACT CCC TTG ATG GAC ACA ACT CCT CTC TCT TCT GCT TG (Hinks and Franklin, 2000; Ji et al., 1995) and 5′-CGG CCG TCT GGA TGG ATG CGC AGG AAG AAG CCG CCG TT (Barraclough et al., 1990; Hinks and Franklin, 2000).

Oligonucleotide probes were labeled at the 3′ end using a commercial kit (NEP100) and ^{35}S-dATP (1000 Ci/mmol). The radiolabeled probe was purified using Sephadex columns (Microbiospin6, Biorad), and dithiothreitol was added to a final concentration of 50 mM prior to dilution in hybridization buffer. Lesion-containing areas from both control and treatment groups were hybridized overnight in hybridization buffer, and ^{35}S-dATP-labeled oligonucleotide probes were diluted to a concentration of 3000 cpm/ml hybridization buffer. Sections were covered with parafilm and hybridized overnight at 42°C in a humid atmosphere. Excess unbound probe was removed using the following stringency washes: 1× standard saline citrate buffer (SSC) at room temperature for 30 min and then 1× SSC at 55°C for 30 min. Slides were finally rinsed in 1× SSC, dehydrated in ethanol, and air-dried. Sections from control and experimental animals were apposed to the same sheet of Biomax MR film before the film was developed under standard conditions. Antisense oligonucleotide sequences specific for *PDGF-A*, *FGF-2*, *IGF-1*, *TGF-β1*, *MBP*, and *SR-B* were used as previously published (Hinks and Franklin, 1999). Analysis of in situ hybridization autoradiograms was carried out using an MCID image analyzer (model M4, MCID, Toronto, Canada) to measure relative optical density (ROD) values. Expression levels within lesion areas, identified by solochrome cyanine staining of serial sections mapping throughout the entire lesion (8–12 sections per

animal), were measured using a semiautomated target detection facility that defined the region of increased optical density and then provided an averaged optical density reading across the selected region including both ventral and dorsal lesions weighted according to the area of expression within each animal from which background film levels were deducted. Measurements were taken within the linear range of optical density levels, and the in situ hybridization results were expressed in arbitrary units (ROD values × 100; film background was subtracted from readings prior to multiplication); measurements for each animal were processed independently on the MCID.

Nonradioactive in situ hybridization

The platelet-derived growth factor-α receptor (*PDGFRα*) probe was transcribed from a 1637-bp *Eco*RI cDNA fragment, encoding most of the extracellular domain of mouse *PDGFRα* cloned into pBluescript KS+ (a gift from Dr N.P. Pringle and Professor W.D. Richardson, University College, UK). *PDGFRα*-positive (+) cells in seven to nine dorsal and ventral funiculus lesions per group and time point were analyzed in animals sacrificed on experimental days 5 and 10. As detailed in Fig. 2, comparisons were performed between *PDGFRα*+ cells in normal white matter and on experimental days 5 and 10. Animals were perfused with 4% paraformaldehyde in PBS (phosphate-buffered saline), and tissue was prepared for in situ hybridization performed as described previously (Fruttiger et al., 1999; Sim et al., 2000). After in situ hybridization, RNA hybrids were visualized in situ by a standard technique as described previously (Fruttiger et al., 1999). Lesions were identified on digital images of solochrome cyanine-stained sections, and the lesion area determined using a public domain program (Image J 1.29×; free download on http://rsb.info.nih.gov/ij/) which calculates the area following a manual outline of the lesion border. *PDGFRα*+ cells within the lesions and within unaffected white matter were manually counted on digitized adjacent sections. OPC density was calculated by dividing the manual cell count by the established lesion area.

Oil-red-O staining

Oil-red-O (Sigma) working solution was prepared by adding 20 ml H_2O to 30 ml 1% oil-red-O in isopropanol. Sections were stained for 10 min then washed for 4 min and counterstained in Carrazi's hematoxylin for 4 min. Following a 4-min wash in H_2O, the sections were differentiated in 0.5% aqueous hydrochloric acid for 7 s and again washed in water for 10 min. Finally, the slides were mounted using an aqueous mounting medium. Representative images of oil-red-O stained lesions were digitized and blindly ranked with the highest staining density receiving the highest rank value.

Statistical analysis

ROD data were statistically analyzed using ANOVA followed by Student *t* post hoc pairwise comparisons for data at individual time points (Graphpad Software). *P* values for correlations of mRNA products across experimental groups were approximated in a computer simulation with $>10^{12}$ random sequence runs. Ranking analysis for *PDGFRα*+ and oil-red-O stained sections was done using Mann-Whitney test comparing data at individual time points. For all tests, $P = 0.05$ was taken as the minimum level of statistical significance.

Results

Clodronate liposome administration leads to a transient impairment of the macrophage response following lysolecithin-induced demyelination

To assess the effects of monocyte depletion on the presence of macrophages within the lesions, we measured the expression of scavenger receptor B (SR-B) mRNA. SR-B is expressed on blood monocytes, highly expressed in monocytes-derived macrophages, in neonatal microglia but not in resting adult microglia (Husemann and Silverstein, 2001; Husemann et al., 2002). A number of studies have used SR-B to identify the presence and activity of macrophages (Han et al., 1997), including within remyelinating lesions (Hinks and Franklin, 2000). Although levels of SR-B mRNA do not allow a clear distinction to be made between cell numbers and levels of activity within individual cells, they nevertheless provide a quantifiable indication of the total levels of macrophage function. SR-B mRNA expression was rapidly increased after lesion induction within the control group, whereas in liposome-treated animals, the rise was delayed, confirming that macrophage depletion was successful (Fig. 1A). Consistent with our earlier study (Kotter et al., 2001), a significant difference between the groups was found at day 5 ($P = 0.0007$). However, at days 10 and 20, SR-B levels were similar (Figs. 1A, C), indicating that despite the initial decrease in macrophage numbers within the lesion, the clodronate liposome-treated animals were able to restore normal numbers of macrophages, presumably due to a compensatory increase in microglia-derived macrophages.

The reexpression of MBP mRNA within lesions is impaired in monocyte-depleted animals

Myelin basic protein (MBP) is an important structural protein of myelin sheaths expressed in mature oligodendrocytes and can be used as a marker of remyelination (Jordan et al., 1990; Ludwin and Sternberger, 1984; Sim et al., 2000; Woodruff and Franklin, 1999b). *MBP* expression dropped sharply after lysolecithin injection. However, whereas a steady increase in *MBP* mRNA levels was observed in the controls, reexpression of *MBP* in the depleted animals was impaired. A significant difference of *MBP* levels between the groups was observed after 10 days ($P = 0.0007$), and *MBP* levels remained different after 20 days when *MBP* expression was unchanged in depleted animals, although it had increased in the control group ($P = 0.0061$; Figs. 1B, D). The lesion area was assessed on solochrome cyanine-stained sections, and statistical analysis yielded a significant difference between the treatment groups at day 20 ($P = 0.0203$) with lesion areas remaining large in depleted animals (data not shown). These findings are consistent with our earlier observations based on histological assessment of remyelination (Kotter et al., 2001).

A delayed macrophage response delays OPC recruitment and impairs differentiation during the course of remyelination

Oligodendrocyte progenitor cells (OPCs) are the primary source of remyelinating cells in the CNS and can be detected by the use of probes for *PDGFRα* (Gensert and Goldman, 1997; Levine and Reynolds, 1999; Sim et al., 2002a; Watanabe et al.,

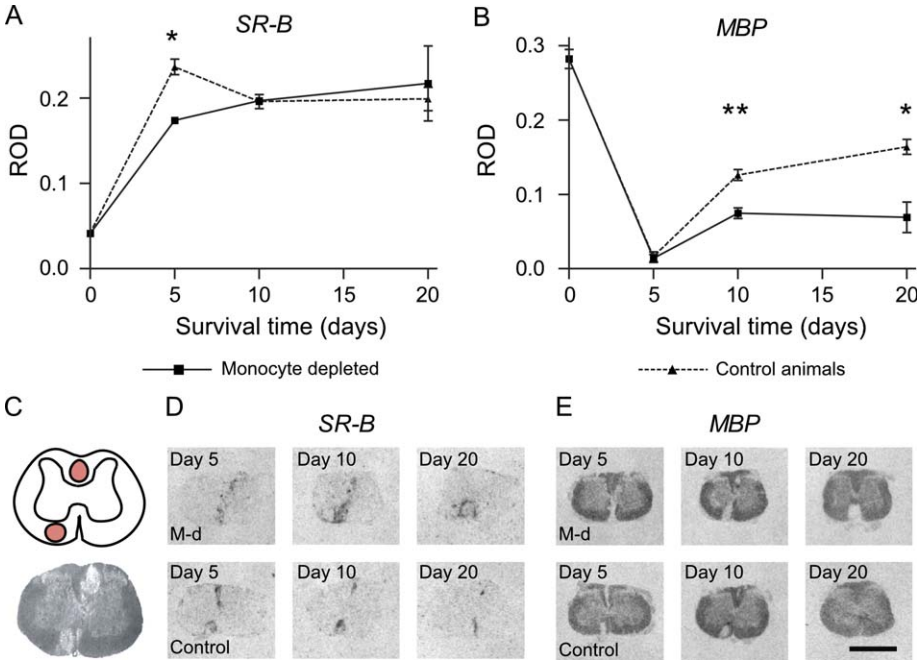

Fig. 1. Comparison of (A) *SR-B* and (B) *MBP* mRNA expression levels in monocyte-depleted and control animals following lysolecithin-induced demyelination. Although *SR-B* expression was only transiently decreased in liposome-treated animals, *MBP* reexpression was significantly impaired in these animals. (ROD: relative optical density, error bars: SEM; values of normal white matter were used as values for day 0). (C) Diagram and corresponding solochrome cyanine-stained transverse sections illustrating the location of lysolecithin-induced demyelination in the spinal cord. Focal areas of demyelination were induced in the dorsal and ventrolateral white matter of the spinal cord. (D, E) Autoradiograms showing in situ hybridization with ^{35}S-labeled oligonucleotides for *SR-B* and *MBP* mRNAs (scale bars: C = 2.65 mm; D, E = 5 mm).

2002). In theory, impaired remyelination could result from either impaired OPC recruitment or impaired OPC differentiation, or a combination of the two (Franklin, 2002). Assessment of OPC densities in the lesions revealed that they were significantly fewer in depleted animals compared to controls at day 5 (P = 0.0111). However, at day 10, similar densities of OPCs were found in both groups indicating a transient impairment in OPC recruitment (Fig. 2). Previous observations on lysolecithin-induced demyelination in rat spinal cord indicated that in young animals, oligodendrocyte differentiation starts around 10 days, when myelin protein gene mRNAs and new myelin sheaths can first be detected in the lesion (Woodruff and Franklin, 1999b). Thus, since the number of OPCs was the same in the depleted and control groups at this stage, it can be inferred that a key contribution to the impaired remyelination (Fig. 1B) was an impairment of OPC differentiation.

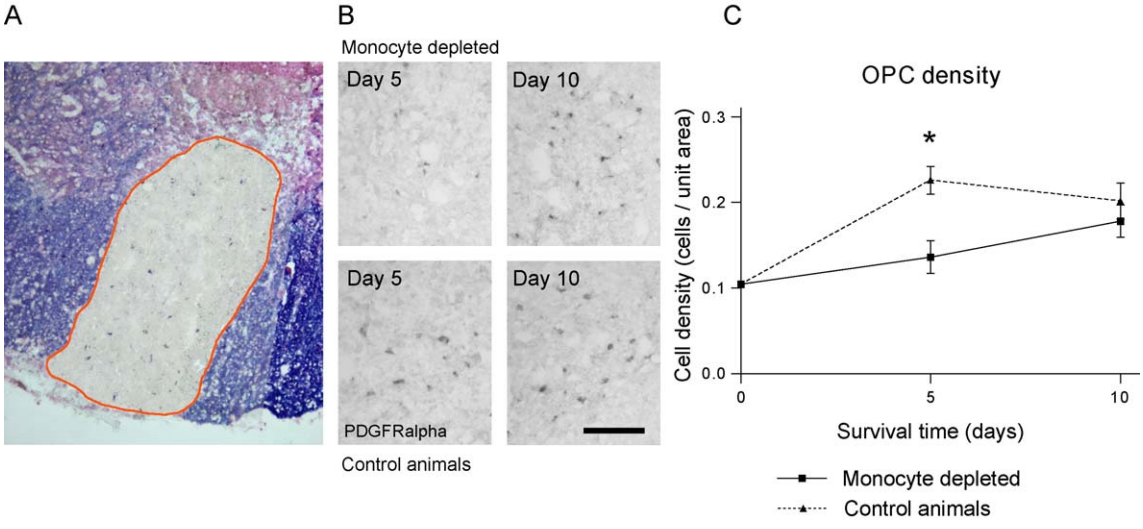

Fig. 2. (A) In situ hybridization for *PDGFRα* allows detection of oligodendrocyte progenitor cells (OPC) in the lesions. Digital images of solochrome cyanine-stained sections were overlaid with images of sections stained for *PDGFRα*. (B) Following detection of the lesion borders, *PDGFRα*-positive cells were manually counted, and the area of the lesion was detected using free-domain imaging software. (C) OPC recruitment was significantly delayed in liposome treated animals (scale bar: A = 0.4 mm, B = 0.1 mm).

Changes in the phagocytic activity appear during later stages of remyelination

Oil-red-O staining allows identification of neutral lipids that are formed once myelin debris has been ingested by macrophages and therefore can be used as an indicator of the efficiency of myelin debris phagocytosis (Eto et al., 2003). Blind ranking of oil-red-O-stained sections revealed no differences between the groups at experimental day 5 (Fig. 3), despite the differences in *SR-B* expression (Fig. 1A). However, at day 10, sections of liposome-treated animals exhibited significantly less staining than control animals (P = 0.0006), indicating that the depletion of macrophages is eventually reflected in differences in the efficacy of myelin debris clearance. Nevertheless, there was no direct temporal correlation between the OPC density and the efficiency of myelin debris clearance.

Impairment of the macrophage response alters IGF-1 expression

Macrophages are a major source of signaling molecules within areas of inflammation, and one would therefore predict that alterations in the macrophage response would be reflected in changes in the signaling environment. In order to test this prediction, we examined the expression profiles of several growth factors involved or implicated in remyelination, and whose expression patterns have previously been described in the rat lysolecithin lesion model (Hinks and Franklin, 1999, 2000) (Fig. 4).

Insulin like growth factor 1 (IGF-1) is associated with the differentiation of OPCs into mature phenotypes (Hsieh et al., 2004), and its expression patterns during remyelination suggest that it may act as a differentiation signal to recruited OPCs (Hinks and Franklin, 1999, 2000). Lesion induction led to a rapid increase in *IGF-1* mRNA expression in control animals that at day 5 contrasted with observed levels in depleted animals (P = 0.0363). The lower levels of *IGF-1* mRNA in the depleted animals showed a strong correlation with *SR-B* mRNA levels (P = 0.0041), suggesting that at this early time point, *IGF-1* expression is therefore related to the presence of macrophages. This observation supports the spatial expression correlations described previously (Hinks and Franklin, 1999) and is consistent with the observation of *IGF-1* production by macrophages as observed following cuprizone-induced demyelination and in other models of CNS injury (Mason et al., 2001; O'Donnell et al., 2002). The *IGF-1* mRNA levels were not significantly different at day 10 in PBS controls but somewhat surprisingly were significantly higher in the depleted group at day 20 (P = 0.0407, Fig. 4A). The high *IGF-1* mRNA in the depleted group was strongly correlated with low *MBP* expression (P = 0.00087) at day 20 and may have reflected an ongoing attempt to repair lesions that were more completely remyelinated in the control group.

TGF-β1 expression remains high in depleted animals at day 20

Transforming growth factor (TGF)-β1 has a multiplicity of biological effects including down-regulation of microglia functions and stimulation of astrocytes (Silberstein et al., 1996; Suzumura et al., 1993; Xiao et al., 1997). In the context of oligodendrocyte lineage cells, an in vitro association with proliferation arrest and differentiation of OPCs (McKinnon et al., 1993; Schuster et al., 2002) together with its expression profile following toxin-induced demyelination has suggested that it may serve a similar role to IGF-1 in stimulating OPC differentiation during remyelination. *TGF-β1* mRNA expression was rapidly up-regulated after lysolecithin-induced demyelination in both groups, and peak levels were reached at day 10 as previously reported. However, whereas a subsequent drop in *TGF-β1* mRNA levels was observed in the controls, *TGF-β1* mRNA levels remained high in depleted animals (P = 0.0282, Fig. 4), and there was a strong correlation between low *MBP* levels and high *TGF-β* levels (P = 0.00087), mirroring a similar effect of depletion on *IGF-1* mRNA levels (Fig. 4B).

Expression of the OPC mitogenic growth factors PDGF-A and FGF-2 remains unchanged in depleted animals

PDGF-A and *FGF-2* mRNAs are expressed following lysolecithin-induced demyelination (Hinks and Franklin, 1999), and both have been demonstrated to be OPC mitogens during remyelination (Frost et al., 2003; Woodruff et al., 2004). We therefore

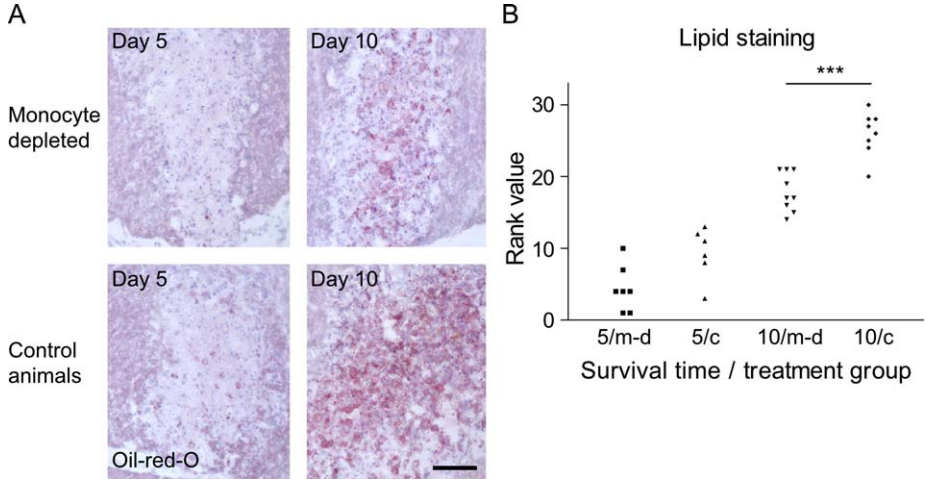

Fig. 3. (A) Transverse section of ventral funiculus lesions stained with oil-red-O to allow identification of neutral lipids. (B) Blind ranking analysis reveals an increase in the accumulation of intracellular lipids over time (rank value: highest density of oil-red-O staining received highest rank number). No differences were found between the treatment groups at day 5 (5/m-d: macrophage-depleted, 5/c: control). However, at day 10, monocyte-depleted animals exhibited significantly less staining than control animals, indicating a decreased phagocytic activity (10/m-d: macrophage-depleted, 10/c: control) (scale bar = 0.5 mm). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

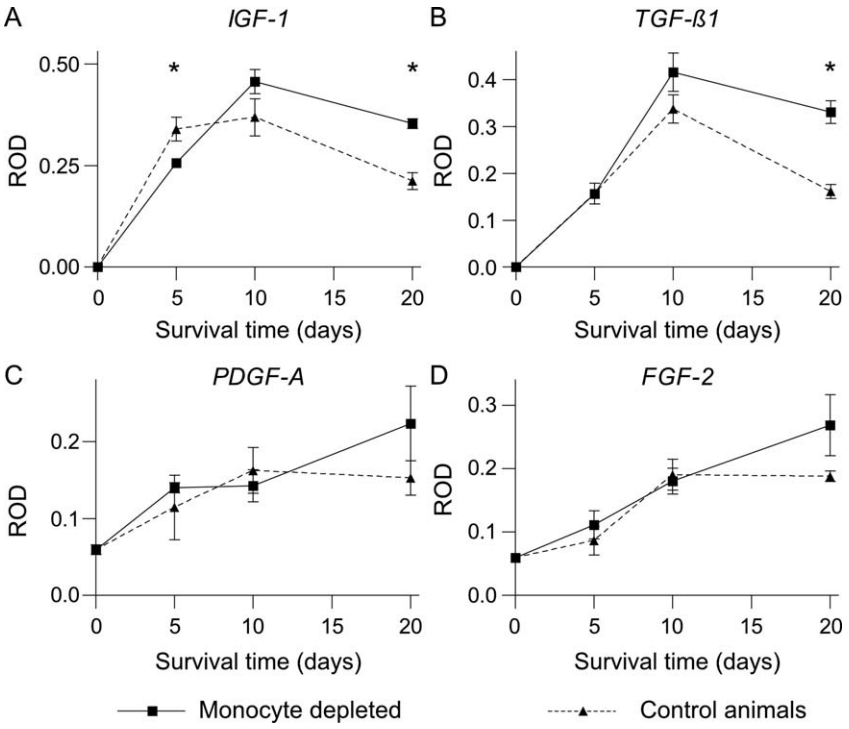

Fig. 4. (A–D) Comparison of *IGF-1*, *TGF-β1*, *PDGF-A*, and *FGF-2* mRNA expression in monocyte-depleted versus control animals. Monocyte depletion led to a delayed increase of *IGF-1* expression. In contrast, on experimental day 20, *IGF-1* expression remained high in liposome-treated animals. Similarly, *TGF-β1* levels remained high in liposome-treated animals. Liposome treatment did not affect the mitogenic growth factors *PDGF-A* and *FGF-2*. (ROD: relative optical density, error bars: SEM; values of normal white matter were used as values for day 0).

hypothesized that the decrease in OPC response in the depleted animals at 5 days (Fig. 2B) might be related to decreased levels of one or both of these factors. However, both factors exhibited similar expression profiles in control and depleted animals, including at day 5 after lesion induction when the OPC and macrophage abundance was lower in the depleted animals Fig. 4C and D.

Discussion

A number of in vivo studies provide evidence that remyelination is impaired when the inflammatory response is experimentally reduced (Arnett et al., 2001; Mason et al., 2001), and it is now evident that both lymphocytes and macrophages can contribute beneficially to remyelination (Bieber et al., 2003; Diemel et al., 2004; Kotter et al., 2001; Loughlin et al., 1997). Inflammatory cells may contribute to remyelination by a number of different ways. Their contribution could be either the removal of putative inhibitory factors or the supply of factors that are conducive to the regenerative process, specifically facilitating OPC recruitment or promoting the formation of new myelin sheaths.

To explore the role of macrophages in remyelination, we have removed the contribution that circulating monocytes make to the macrophage response to toxin-induced demyelination by injecting liposomes containing clodronate into the peripheral circulation. Once injected, clodronate liposomes are rapidly cleared by macrophages throughout the entire organism, decreasing monocyte populations of different maturation stages in the blood (Sunderkotter et al., 2004). Additionally, resident macrophages predominantly in the liver and the spleen are affected by the treatment (van Rooijen et al., 1990, 1997). Extensive use in vivo et vitro of the liposome technique has demonstrated its ability to specifically target cells of the macrophage lineage (Marin-Teva et al., 2004; van Rooijen et al., 1997).

Following liposome treatment, we find, somewhat surprisingly, that a transient delay in the macrophage response to demyelination is sufficient to impair remyelination. Similar to old animals (Sim et al., 2002b), monocyte depletion causes an impairment of OPC recruitment, as reflected by a difference in the OPC density within lesions of liposome-treated animals at day 5 (Fig. 2), and also OPC differentiation, inferred from the observation that OPC numbers were similar in both groups at day 10 when *MBP* mRNA and new myelin sheaths first appear in this lesion heralding the start of the differentiation phase of remyelination (Franklin and Hinks, 1999; Woodruff and Franklin, 1999a,b). The OPC recruitment impairment was not directly related to differences in the phagocytic activity, which instead bore a closer temporal correlation with the stage of OPC differentiation. Thus, the persistence of myelin debris in the lesion may inhibit OPC differentiation rather than recruitment, a conclusion consistent with in vitro studies on the effects of myelin on cultured OPCs (Robinson and Miller, 1999).

Differences in the density of OPCs in depleted animals at day 5 precede differences in the phagocytic response (detected by the degree of oil-red-O staining) and are therefore unlikely to be linked to the presence of myelin debris. It may thus be possible that the depletion of macrophages leads to a change in the signaling environment responsible for mediating OPC recruitment. Although many factors have been associated with OPC migration and proliferation in development, a smaller number have been shown to perform similar roles during remyelination. Of these, both PDGF-

A and FGF-2 emerge as key mediators of OPC recruitment and therefore as likely candidates for factors altered by monocyte depletion (Frost et al., 2003; Woodruff et al., 2004). However, the mRNA expression patterns of both factors remained unchanged in monocyte-depleted animals, and so the lack of OPC recruitment cannot be accounted for by these known OPC mitogens. Previous studies have indicated that reactive astrocytes are the primary source of both these factors following CNS demyelination (Albrecht et al., 2003; Hinks and Franklin, 1999; Redwine and Armstrong, 1998), and it would therefore seem that expression of these growth factor mRNAs is unaffected by macrophages. In contrast, parallel to the changes of OPC recruitment, *IGF-1* expression was lower in liposome-treated animals at day 5. Studies on *IGF-1r* knockout mice have implicated an important role of IGF-1 signaling for the survival of OPCs during early stages of remyelination (Mason et al., 2003). However, as *IGF-1* levels were only slightly reduced, it seems unlikely that the marked differences observed in respect to OPC density at early stages can be exclusively attributed to reduced *IGF-1* expression. There are a multiplicity of other factors potentially involved in OPC recruitment and either directly secreted by macrophages or be indirectly controlled by macrophage-derived factors. These include cytokines such as IL-1β (Vela et al., 2002) and chemokines such as CXCL1 (gro-α), (Robinson et al., 1998) or various extracellular matrix molecules influencing the migratory and proliferative properties of OPCs via their expression of αv integrins (Blaschuk et al., 2000; Milner et al., 1996). Given the numerous potential mediators of OPC recruitment and the interactions that occur between them, it is unlikely that its impairment results from changes in expression of single genes. Instead, it may arise from a broader disturbance of the signaling environment associated with this phase of remyelination, the identification of which will require the use of techniques for multiple gene analysis such as transcription profiling.

Consistent with the concept of a disrupted macrophage response altering the signaling environment following demyelination were the changes we found in the expression profiles of *IGF-1* and *TGF-β1* mRNAs. Two possible roles for IGF-1 as an OPC survival factor and differentiation factor during remyelination have been described. While there is some evidence that IGF-1 plays a role during the differentiation of OPCs into mature oligodendrocytes in vivo (Hsieh et al., 2004), a number of studies support the view that IGF-1 acts as a trophic factor on both OPCs and differentiated oligodendrocytes in vitro et vivo (Barres et al., 1992; Mason et al., 2000; Masters et al., 1991; McKinnon et al., 1993; Ye and D'Ercole, 1999). While the reduced levels of *IGF-1* at 5 days could be directly related to the reduced *SR-B* levels indicative of an impaired macrophage response, the high levels of *IGF-1* expression at day 20 were unexpected and suggest that early disruption of the macrophage response may disrupt a cascade of events which have unpredictable longer term consequences for the signaling environment. The functional significance of higher *IGF-1* mRNA levels in the slowly remyelinating depleted animals is unclear. One possibility is that it may help to counteract negative influences of factors such as TGF-β1 (McKinnon et al., 1993; Schuster et al., 2002, see below) or TNF-α which was up-regulated during unsuccessful attempts at remyelination in the *IGF-1r* knockout model (Mason et al., 2003). IGF-1 might also be involved in maintaining a robust macrophage response as a proliferative effect of IGF-1 on macrophages and microglia is well recognized (Li et al., 1996; Mueller et al., 1994; O'Donnell et al., 2002; Scheven and Hamilton, 1991).

Similar to *IGF-1*, the elevated levels of *TGF-β1* at 20 days suggested long-term effects on the signaling environment of an early disturbance in the inflammatory response to demyelination. The functional significance of this is difficult to assess since the biological effects of TGF-β1 are many. For example, TGF-β1 down-regulates a variety of macrophage functions such as cytotoxicity, the expression of lysosomal phosphatase, and surface expression of MHC-II (in rats; Hu et al., 1995; Merrill and Zimmerman, 1991; Panek and Benveniste, 1995; Suzumura et al., 1993). Thus, a potentially important role of TGF-β1 might be to modulate and prevent an exuberant and possibly damaging inflammatory response. Consistent with its role in limiting proliferation and inducing apoptosis during development, TGF-β1 has negative effects on the proliferation and survival of OPCs (McKinnon et al., 1993; Schuster et al., 2002). *TGF-β1* levels remained high in liposome-treated animals at day 20. Similar to *IGF-1*, there was a strong correlation between low *MBP* levels and high *TGF-β*. This might be regarded as efforts to limit ongoing processes involved in the attempt to repair. In this case, sustained high levels of IGF-1 might specifically counteract deleterious effects of TGF-β on OPCs ensuring their survival (Yu et al., 2000).

Our findings emphasize the importance of macrophages during remyelination and indicate that they are likely to contribute not only to the removal of myelin debris, but also to the creation of the growth factor signaling environment associated with the repair process. A caveat to our findings is that the effects we observe may only reflect the function of monocyte-derived macrophages, and it is possible that those derived from microglia perform different functions (Lassmann et al., 1993). Nevertheless, several conclusions regarding the role of macrophages can be drawn. Although some of the consequences of monocyte depletion, such as the early impairment of *IGF-1* mRNA expression, could be directly related to the reduced macrophage response, the later changes in *IGF-1* and *TGF-β1* appeared to be longer term consequences of an early disturbance of the normal inflammatory response. These results suggest a model whereby the creation of a pro-remyelination signaling environment is generated by a cascade of events triggered by the initial inflammatory response. Such a model would therefore explain why an early and even transient change in the inflammatory response causes long-term alterations in the environment that are not necessarily predictable but that occur as a result of any early change in the cascade becoming amplified with time. The implications of this model are that the long-term effects of early immunosuppressive therapies currently employed during acute relapses of multiple sclerosis may be unpredictable and deleterious to the regenerative process of remyelination. In the context of developing novel treatment strategies for de- and remyelination, inflammation should not be considered as being uniformly destructive, but instead, attempts should be made to differentiate regenerative inflammation as observed in the context of repair from destructive inflammation as seen in autoimmune disease or host defense.

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