Promoting oligodendrogenesis and myelin repair using the multiple sclerosis medication glatiramer acetate

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The formation of oligodendrocytes (oligodendrogenesis) and myelin is regulated by several neurotrophic factors. Strategies to increase the level of these trophic molecules may facilitate repair in demyelinating conditions, such as multiple sclerosis (MS). Because leukocytes are a source of neurotrophic factors, and as glatiramer acetate (GA) generates T helper 2 (Th2) lymphocytes that are not known to be harmful, we tested the hypothesis that GA regulates oligodendrogenesis and myelin formation. First, we generated GA-reactive Th2 cells and determined that they produced transcripts for neurotrophic factors, including insulin-like growth factor-1 (IGF-1). The conditioned medium from GA-reactive T cells elevated IGF-1 protein and promoted the formation of oligodendrocyte precursor cells (OPCs) from embryonic brain-derived forebrain cells in culture. We next subjected mice to lyssolecithin-induced demyelination of the spinal cord. At 7 days after the insult, the number of OPCs in the demyelinated dorsal column was higher than that in uninjured controls, and was further increased by the daily s.c. injection with GA. Increased OPC generation by GA was associated temporally with the elevation of IGF-1 and brain-derived neurotrophic factor (BDNF) in the spinal cord. Finally, the resultant remyelination at 28 days was higher in mice treated with GA during the first 7 days of injury compared with vehicle controls. These results indicate that GA promotes oligodendrogenesis and remyelination through mechanisms that involve the elevation of growth factors conducive for repair.

beneficial inflammation | neurotrophic factors | oligodendrocyte | regeneration | remyelination

Multiple sclerosis (MS) is characterized by inflammation and demyelination in the CNS. The demyelinated lesions can be repaired and, indeed, the extent of remyelination is substantial in some patients (1). There is increasing interest in facilitating remyelination as its benefits extend beyond restoration of nerve impulse conduction to preventing axonal degeneration, since axon and myelin units have dynamic interactions involving survival signaling.

Much has been learned about the process of remyelination (2) and key steps include the proliferation and maturation of oligodendrocyte precursor cells (OPCs) and the appropriate interactions of oligodendrocyte processes with axons to form myelin. A large number of molecules are critically involved in the proliferation, maturation and survival of OPCs, and among these are neurotrophic factors such as platelet-derived growth factor (PDGF), insulin-like growth factor-1 (IGF-1) and brain-derived neurotrophic factor (BDNF) (3–6). Accordingly, strategies that have been used to induce remyelination in animals have included the provision of neurotrophic factors through infusion or gene therapy.

Another means to deliver growth factors to the nervous system takes advantage of the observation that leukocytes are rich sources of neurotrophic factors. Indeed, even the disease-promoting inflammatory cells of perivascular cuffs in MS lesions

are immunoreactive for neurotrophic factors such as BDNF (7). However, any approach to use leukocytes to deliver neurotrophic factors for repair must balance their potential detriments in exacerbating the pathology of MS.

Glatiramer acetate (GA), a medication used in relapsing-remitting MS (8), generates GA-reactive T lymphocytes that are of the T helper 2 (Th2) anti-inflammatory bias (9, 10). GA-reactive Th2 cells accumulate in the CNS, where they have been shown to produce not only anti-inflammatory cytokines but also neurotrophic factors (11). Importantly, Th2 cells have not been found to be neurotoxic, unlike the proinflammatory Th1 or Th17 lymphocytes that destroy neurons in tissue culture (12). More recently, GA has also been found to modulate monocytoic cells into those that express anti-inflammatory cytokines (13).

Here, we have tested the hypothesis that T cells exposed to GA elevate neurotrophic factors that are important for oligodendrogenesis in culture. Furthermore, we have used mice with lyssolecithin-induced demyelination to address whether treatment with GA would increase OPC numbers and remyelination within the lesioned spinal cord. These studies have relevance to the biology of harnessing the benefits of inflammation to evoke repair.

Results

T Cells Exposed to GA Elevate Their Production of Growth Factors in Vitro. Cells obtained from the lymph nodes of GA-pretreated mice (Fig. S1) were incubated with APCs and GA, resulting in a proliferative response (Fig. S2) that suggested the presence of T cells reactive to GA. Cell conditioned medium from these GA-reactive cells was then examined for the accumulation of IFN- γ , a Th1 cytokine, and for IL-5, a Th2 cytokine. Increasing culture periods with GA resulted in loss of detectable IFN- γ and steady accumulation of IL-5 (Fig. S2), indicating that the GA-reactive T cells were of the Th2 phenotype, as reported by others (9–11). With this confirmation, we examined the capacity of GA-reactive T cells to produce growth factors. From several mouse donors previously treated with GA, lymph node cells were restimulated with GA and RNA was harvested. Fig. 1 shows that increases in transcripts encoding IGF-1 and PDGF α were evident after 3 days of GA treatment. Transcripts for BDNF was variable, with three cultures elevating this trophic factor in response to GA whereas three did not.

The cell-conditioned medium collected from T cells over 3 days of culture was analyzed for IGF-1 protein content. IGF-1

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ning 1 mm and centered around the lesion epicenter. Fig. 5 demonstrates that the remaining demyelination at day 28 was more extensive in vehicle-treated mice compared with that in the GA-treated group, and this was evident not only around the lesion epicenter, but also rostrally and caudally.

Finally, we documented the area of demyelination per section and summed these across 10 sections spanning 1 mm per mice to obtain an index of the lesion volume. Fig. 5 reveals that the volume of remaining demyelination at day 28 was significantly less in the GA-treatment group (average of $0.26 \pm 0.03 \text{ mm}^3 \times 10^{-3}$) (mean $\pm$ SEM) compared with vehicle controls (average of $0.55 \pm 0.10 \text{ mm}^3 \times 10^{-3}$) ($P < 0.05$, Mann-Whitney unpaired T test). This was not the result of differing lesion volumes at day 7 after the lysolecithin insult, which was found to be similar in both groups (average of $0.98 \pm 0.21 \text{ mm}^3 \times 10^{-3}$ vs. 0.72 ± 0.20 , vehicle vs. GA, mean $\pm$ SEM, n of 7 each, $P > 0.05$). Comparing across time points, the lesion volume at day 28 in the vehicle group was 56% of that at day 7 whereas that in the GA group at day 28 was 36% of that at day 7.

Overall, studies of indices of remyelination suggest that GA treatment in the first 7 days after lysolecithin administration favors a more pronounced repair over the ensuing 28 days.

Discussion

Summary of Results. We have determined that in culture, GA-reactive T cells produced growth factors described for oligodendrogenesis, and that the conditioned medium from GA-reactive T cells increased the number of OPCs that were formed from embryonic brain cells. After demyelination of the spinal cord in mice, the daily treatment for 7 days with s.c. GA elevated the injury-induced rise of OPCs correspondent with an mRNA profile in the injured tissue that was anti-inflammatory and proreparative. Although the size of the demyelination at day 7 was comparable between the two groups, suggesting that GA did not modulate grossly the initial demyelinating events, determinations at day 28 show increased remyelination when mice were exposed to GA during the first 7 days of injury.

Growth Factors Regulate the Generation of OPCs in Culture, in Development, and in Remyelination. Recovery after demyelination requires the presence of OPCs and the ability of these cells to divide, migrate, mature, and enwrap axons in response to signals in demyelinated lesions. The generation and maturation of OPCs are dependent on several growth factors. In tissue culture studies, the proliferation of OPCs is stimulated by PDGF, among others. BDNF increases the proliferation of OPCs and their maturation into oligodendrocytes (3, 4). IGF-1 has strong capacity to facilitate both OPC proliferation and maturation (5), and is a survival factor for oligodendrocytes.

Developmental studies have also highlighted the importance of growth factors. Transgenic mice overexpressing PDGF presented with hyperproliferation of precursor cells whereas PDGF null mice had severely reduced number of OPCs (6). IGF-1 transgenic mice have larger brain and higher myelin content than controls (17). Conversely, IGF-1 null mice have lower number of OPCs at 1–3 weeks of age, and mice with deficient IGF receptor 1 signaling have retarded developmental myelination (18).

Growth factors are also critical during remyelination. The increase of several growth factors within the demyelinated CNS has been reported (19). IGF-1 transgenic mice remyelinated more readily compared with wildtype after demyelination of the corpus callosum; moreover, type 1 IGF receptor null mice did not remyelinate adequately after insult, corresponding with the failure of OPCs to accumulate, proliferate or survive in mutants (20). It must be noted that although we have focused discussions to PDGF, BDNF, and IGF-1, a myriad of molecules has been described to influence myelin formation, including the chemokine GRO- α , hormones, notch signaling and LINGO.

Specific growth factors have been infused or genetically expressed in animal models of demyelination in attempts to promote remyelination. IGF-1 treatment of rats subjected to EAE increased the numbers of proliferating oligodendrocyte precursors (21). For BDNF, a single dose in rabbits subjected to cervical spinal cord ventral root avulsion and replantation promoted remyelination of axons that regenerated from the replantation (22).

The provision of a particular growth factor may sometimes result in proliferation of cells but not their subsequent maturation. This is highlighted by Woodruff et al. (23) where the transgenic increase of PDGF α led to a rise in numbers of OPCs after lysolecithin demyelination; however, there was a trend toward lower remyelination in the PDGF transgenics, which the authors speculated to be due to the prolonged mitogenic stimulus.

These results indicate that although single growth factor treatment may be adequate to affect repair, this may be suboptimal or counterproductive in some cases. The concomitant provision of multiple growth factors, to include those with proliferative and/or differentiating capacity, would appear to be required to enhance repair.

Beneficial Aspects of Neuroinflammation. One means to deliver multiple growth factors into the CNS takes advantage of the observation that leukocytes have the capacity to generate a range of growth factors (7). For example, activated T cells, B cells, and macrophages express nerve growth factor and BDNF in vitro, and inflammatory infiltrates of T cells, NK cells, and macrophages in the CNS of animals afflicted with experimental autoimmune encephalomyelitis (EAE) were immunoreactive for neurotrophin-3 and BDNF (24). These reports highlight that there are beneficial aspects to neuroinflammation, which should not be unexpected since a main role for an inflammatory response in other tissues is to enhance repair. In accordance, Schwartz and colleagues (25) have noted that even autoreactive T cells could prevent neuronal death in neurotrauma.

There is increasing evidence that inflammation aids remyelination. The depletion of macrophages (26) or T cells (27) impairs remyelination after lysolecithin-induced demyelination. Conversely, the promotion of an inflammatory response by creating a stab injury to the spinal cord (28) or by injecting zymosan along with OPC transplants into the retina (29) increased remyelination.

The benefits of inflammatory cells are attributed in part to their production of neurotrophic factors described above but other aspects of an inflammatory response may also help. For instance, proteases released by inflammatory cells may help remove nonpermissive molecules, such as proteoglycans that hinder recovery (14). The challenge of harnessing beneficial neuroinflammation is how to avoid its potential detriments, given the extensive literature that persistent neuroinflammation can harm the CNS.

Harnessing the Benefits of Inflammation with GA. GA has been in clinical use to treat MS for over 15 years where it has been shown to be safe, to reduce relapse rate and to produce longterm stabilization of disease in a significant proportion of patients (8). The mechanisms of GA in MS are multiple, and include the generation of Th2 cells and Type 2 monocytes (9, 13). These cells are not known to harm the CNS and may elicit CNS outcomes differently from other inflammatory cell types. When Th2 cells were contrasted with proinflammatory Th1 cells, it was found that Th2 cells interacted with glia to elevate neurotrophins; in contrast, the interaction of Th1 cells with glia did not affect neurotrophins but enhanced the levels of proinflammatory molecules (30). In our study, we were unable to reproducibly detect T cells at sites of demyelination by immunohistochemistry

or flow cytometry (data not shown), even though the PCR data (Fig. 4) suggested a Th2:Th1 bias in GA-treated mice. Future studies will need to examine representation of Th2 versus Th1 T cells, or Type 2 monocytes, in detail in the demyelinated spinal cord after GA-treatment.

In accordance with potentially useful outcomes of GA for the CNS, GA has been found to confer neuroprotection in animal models of optic nerve injury, EAE, ALS, Parkinson's, and Alzheimer's disease (31). GA has also been studied in the context of neural repair. Immunoglobulins harvested from GA-primed mice and injected into mice with viral-induced demyelination of the spinal cord promoted remyelination (32); whether similar outcome could result from daily s.c. injections to mimic the mode of treatment of MS patients with GA was not addressed. Treatment of EAE mice using GA has been reported to increase the extent of neurogenesis (33) and the number of OPCs (34) in the CNS. Overall, these studies support the repair potential of GA that is suggested by our study.

Conclusion

We have determined that the MS medication, GA, promotes oligodendrogenesis and indices of remyelination in mice subjected to lyssolecithin-induced demyelination. This demonstration of the myelination capacity of GA injections in a noninflammatory animal model, suggests the direct effect on myelin formation is not solely due to the reduction of a harmful inflammatory milieu. The results herein indicate that it is possible to employ GA to harness the benefits of neuroinflammation for repair. The data also suggest that the long term benefits observed in patients on GA treatment (8) may in part be due to remyelination, and this deserves further evaluation.

Materials and Methods

Assessment of Neurotrophic Factors Produced by GA-reactive T Cells in Vitro. Responder and APC populations (see *SI Text*) freshly harvested from mice were cocultured (3 million each, in 3 mL medium) in 6-well plates in the absence or

presence of 10 μ g/mL GA. Three days after, total RNA was extracted from cells with 1 mL of TRIzol solution (Life Technologies BRL). The mRNA expression of IGF-1, BDNF, PDGF α , and the housekeeping gene, 18s rRNA, was analyzed by real-time PCR. All PCR primer sets were purchased from SA Biosciences (IGF-1, PPM03387A; BDNF, PPM03006A; and PDGF α , PPM03103A). For 18s rRNA the following forward (F) and reverse (R) primers were used, F: GTACCCGTTGAACCCATT, R: CCATCCAATCGGTAGTAGCG.

Value of growth factor expression from each PCR was normalized to 18s rRNA value from the same RNA extract. By comparing the ratio of normalized growth factor value for a given T-cell culture exposed to GA versus a sister culture that is not, the relative fold change in transcript was thus obtained for that culture.

To verify that protein for growth factors was also elevated in GA-reactive T cells, conditioned media collected for cytokine analyses described earlier were subjected to the measurement of a selected growth factor, IGF-1, using ELISA (Biosource). Furthermore, the cell conditioned medium was analyzed for their capacity to promote OPC in culture as described in *SI Text*.

Lyssolecithin-Induced Demyelination in the Spinal Cord of Mice and Treatment with GA. C57BL/6 adult 2–3-month-old male mice were anesthetized i.p. with a mixture of ketamine (85 mg/kg) and xylazine (15 mg/kg). A 1.5 μ L solution of 1% D-lysophosphatidylcholine (lyssolecithin; Sigma) was injected over 1.5 min into the dorsal column of the T3 spinal cord segment using a 32 G needle, attached to a 5 μ L Hamilton syringe (Hamilton). The needle was left in the spinal cord for an additional 2 min to prevent backflow of the lyssolecithin. Muscles, connective tissue and skin were then sutured above the injection site.

Animals received daily injections of 2 mg per mouse of GA dissolved in saline s.c. for 7 days starting from the day of lyssolecithin administration. This dose was used by Aharoni et al. in mice to increase neurogenesis (33) or document OPC numbers (34) in EAE. Control animals were treated with daily s.c. injections of vehicle (PBS) after the lyssolecithin insult.

Mice were killed for the evaluation of OPC numbers and cytokine/growth factor content in the spinal cord at day 7 after demyelination, and for the evaluation of myelin reformation at day 28, as described in detail in *SI Text*.

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- Patrikios P, et al. (2006) Remyelination is extensive in a subset of multiple sclerosis patients. *Brain* 129:3165–3172.
- Franklin RJ, Ffrench-Constant C (2008) Remyelination in the CNS: From biology to therapy. *Nat Rev Neurosci* 9:839–855.
- Van't Veer ADY, Fischer TZ, Boetig DR, Wood MR, Dreyfus CF (2009) Brain-derived neurotrophic factor effects on oligodendrocyte progenitors of the basal forebrain are mediated through trkB and the MAP kinase pathway. *J Neurosci Res* 87:69–78.
- Du Y, Fischer TZ, Lee LN, Lercher LD, Dreyfus CF (2003) Regionally specific effects of BDNF on oligodendrocytes. *Dev Neurosci* 25:116–126.
- Kuhl NM, De Keyser J, De Vries H, Hoekstra D (2002) Insulin-like growth factor binding proteins-1 and -2 differentially inhibit rat oligodendrocyte precursor cell survival and differentiation in vitro. *J Neurosci Res* 69:207–216.
- Fruttiger M, et al. (1999) Defective oligodendrocyte development and severe hypomyelination in PDGF-A knockout mice. *Development* 126:457–467.
- Kerschensteiner M, et al. (1999) Activated human T cells, B cells, and monocytes produce brain-derived neurotrophic factor in vitro and in inflammatory brain lesions: A neuroprotective role of inflammation? *J Exp Med* 189:865–870.
- Ford CC, et al. (2006) A prospective open-label study of glatiramer acetate: Over a decade of continuous use in multiple sclerosis patients. *Mult Scler* 12:309–320.
- Chen M et al. (2001) Glatiramer acetate induces a Th2-biased response and cross-reactivity with myelin basic protein in patients with MS. *Mult Scler* 7:209–219.
- Yong VW (2002) Differential mechanisms of action of interferon-beta and glatiramer acetate in MS. *Neurology* 59:802–808.
- Aharoni R, et al. (2005) The immunomodulator glatiramer acetate augments the expression of neurotrophic factors in brains of experimental autoimmune encephalomyelitis mice. *Proc Natl Acad Sci USA* 102:19045–19050.
- Kebir H, et al. (2007) Human TH17 lymphocytes promote blood-brain barrier disruption and central nervous system inflammation. *Nat Med* 13:1173–1175.
- Weber MS, et al. (2007) Type II monocytes modulate T cell-mediated central nervous system autoimmune disease. *Nat Med* 13:935–943.
- Larsen PH, Wells JE, Stallcup WB, Odenakker G, Yong VW (2003) Matrix metalloproteinase-9 facilitates remyelination in part by processing the inhibitory NG2 proteoglycan. *J Neurosci* 23:11127–11135.
- Gregg C, et al. (2007) White matter plasticity and enhanced remyelination in the maternal CNS. *J Neurosci* 27:1812–1823.
- Watanabe M, Toyama Y, Nishiyama A (2002) Differentiation of proliferated NG2-positive glial progenitor cells in a remyelinating lesion. *J Neurosci Res* 69:826–836.
- Carson MJ, Behringer RR, Brinster RL, McMorris FA (1993) Insulin-like growth factor I increases brain growth and central nervous system myelination in transgenic mice. *Neuron* 10:729–740.
- Zeger M, et al. (2007) Insulin-like growth factor type 1 receptor signaling in the cells of oligodendrocyte lineage is required for normal in vivo oligodendrocyte development and myelination. *Glia* 55:400–411.
- Hinks GL, Franklin RJ (1999) Distinctive patterns of PDGF- α , FGF-2, IGF-I, and TGF- β 1 gene expression during remyelination of experimentally-induced spinal cord demyelination. *Mol Cell Neurosci* 14:153–168.
- Mason JL, Xuan S, Dragatsis I, Efstratiadis A, Goldman JE (2003) Insulin-like growth factor (IGF) signaling through type 1 IGF receptor plays an important role in remyelination. *J Neurosci* 23:7710–7718.
- Yao DL, Liu X, Hudson LD, Webster HD (1995) Insulin-like growth factor I treatment reduces demyelination and upregulates gene expression of myelin-related proteins in experimental autoimmune encephalomyelitis. *Proc Natl Acad Sci USA* 92:6190–6194.
- Lang EM, et al. (2008) Single-dose application of CNTF and BDNF improves remyelination of regenerating nerve fibers after C7 ventral root avulsion and replantation. *J Neurotrauma* 25:384–400.
- Woodruff RH, Fruttiger M, Richardson WD, Franklin RJ (2004) Platelet-derived growth factor regulates oligodendrocyte progenitor numbers in adult CNS and their response following CNS demyelination. *Mol Cell Neurosci* 25:252–262.
- Hammarberg H, et al. (2000) Neuroprotection by encephalomyelitis: Rescue of mechanically injured neurons and neurotrophin production by CNS-infiltrating T and natural killer cells. *J Neurosci* 20:5283–5291.
- Moalem G, et al. (1999) Autoimmune T cells protect neurons from secondary degeneration after central nervous system axotomy. *Nat Med* 5:49–55.
- Kotter MR, Setzu A, Sim FJ, Van Rooijen N, Franklin RJ (2001) Macrophage depletion impairs oligodendrocyte remyelination following lyssolecithin-induced demyelination. *Glia* 35:204–212.
- Bieber AJ, Kerr S, Rodriguez M (2003) Efficient central nervous system remyelination requires T cells. *Ann Neurol* 53:680–684.
- Foot AK, Blakemore WF (2005) Inflammation stimulates remyelination in areas of chronic demyelination. *Brain* 128:528–539.

29. Setzu A, et al. (2006) Inflammation stimulates myelination by transplanted oligodendrocyte precursor cells. *Glia* 54:297–303.
30. Roy A, Liu X, Pahan K (2007) Myelin basic protein-primed T cells induce neurotrophins in glial cells via α 5 β 1 integrin. *J Biol Chem* 282:32222–32232.
31. Arnon R, Aharoni R (2007) Neurogenesis and neuroprotection in the CNS—fundamental elements in the effect of Glatiramer acetate on treatment of autoimmune neurological disorders. *Mol Neurobiol* 36:245–253.
32. Ure DR, Rodriguez M (2002) Polyreactive antibodies to glatiramer acetate promote myelin repair in murine model of demyelinating disease. *FASEB J* 16:1260–1262.
33. Aharoni R, Arnon R, Eilam R (2005) Neurogenesis and neuroprotection induced by peripheral immunomodulatory treatment of experimental autoimmune encephalomyelitis. *J Neurosci* 25:8217–8228.
34. Aharoni R, et al. (2008) Demyelination arrest and remyelination induced by glatiramer acetate treatment of experimental autoimmune encephalomyelitis. *Proc Natl Acad Sci USA* 105:11358–11363.