The Immunomodulator FTY720 Has a Direct Cytoprotective Effect in Oligodendrocyte Progenitors

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ABSTRACT

The immunomodulator 2-amino-2-[2-(4-octylphenyl)ethyl]-1,3-propanediol (FTY720) has promising therapeutic effects in multiple sclerosis (MS), a degenerative disease in which demyelination of the central nervous system is accompanied by death of oligodendrocytes (OLGs), the myelin-producing cells. In vivo phosphorylation of FTY720 generates an agonist for G protein-coupled receptors for sphingosine-1-phosphate, a lipid mediator that plays a crucial role in the stimulation of OLG survival by neurotrophin-3 (NT-3). The mechanisms underlying the action of FTY720 in MS are not clearly understood, although the effects of this drug in autoimmune diseases are thought to stem from its ability to reduce lymphocyte infiltration and inflammation. Interestingly, we now found that FTY720 also has a direct effect on OLG progenitors. Treatment of these cells with FTY720 causes activation of extracellular signal-regulated kinase 1/2 and Akt, accompanied by protection from apoptosis. However, FTY720 also arrested OLG differentiation. Importantly, this effect was counteracted by NT-3, which not only enhanced the survival of OLG progenitors induced by FTY720 but also stimulated their maturation. Altogether, these observations suggest that in addition to its immuno-suppressive functions, FTY720 could also have a beneficial effect in MS by direct action on OLG progenitors. However, the finding that FTY720 blocks the differentiation of these cells raises the question of whether MS therapies with FTY720 should include the use of differentiation-enhancing factors such as NT-3. This approach would ensure both protection of existing OLG progenitor pools against immune-mediated insults as well as stimulation of remyelination by enhancing the maturation of these cells.

Multiple sclerosis (MS) is a chronic degenerative and debilitating disease of the central nervous system (CNS) characterized by inflammation and demyelination (McQualter and Bernard, 2007). The pathological hallmarks of MS also include axonal degeneration (Trapp et al., 1999) and death of oligodendrocytes (OLGs) (Barnett and Prineas, 2004; Lucchetti et al., 2004), the cells that make the myelin membrane in the CNS. Although the cause of MS remains unknown, the presence of serum antibodies against different myelin constituents and the existence of multiple inflammatory foci in brain and spinal cord support the idea of a predominant autoimmune component. For this reason, most therapeutic approaches involve the use of anti-inflammatory drugs and immunosuppressants.

2-Amino-2-[2-(4-octylphenyl)ethyl]-1,3-propanediol (FTY720), also known as Fingolimod, is among the latest immunomodulatory agents under evaluation for the treatment of MS. This synthetic drug was developed by chemical modification of ISP-I (myriocin), a sphingosine-like metabolite produced by the fungus Isaria sinclairii. FTY720 has been shown to prolong allograft survival in different animal models of transplantation (Chiba et al., 1996) and exert a protective effect in animal models of autoimmune diseases (Maki et al., 2002; Mizushima et al., 2004). Importantly, several studies have evaluated the effect of FTY720 in MS using both preclinical and clinical models. In preclinical studies, FTY720 has been shown to reduce inflammation and demyelination in animal models of MS, including the EAE (experimental autoimmune encephalomyelitis) model (Barnett and Prineas, 2004; Lucchetti et al., 2004). In clinical trials, FTY720 has demonstrated promising results in reducing relapse rate and expanding the disability-free period in patients with relapsing-remitting MS (McQuillan et al., 2006). Importantly, FTY720 has also been shown to have a direct effect on OLG progenitors. Treatment of these cells with FTY720 causes activation of extracellular signal-regulated kinase 1/2 and Akt, accompanied by protection from apoptosis. However, FTY720 also arrested OLG differentiation. Importantly, this effect was counteracted by NT-3, which not only enhanced the survival of OLG progenitors induced by FTY720 but also stimulated their maturation. Altogether, these observations suggest that in addition to its immuno-suppressive functions, FTY720 could also have a beneficial effect in MS by direct action on OLG progenitors. However, the finding that FTY720 blocks the differentiation of these cells raises the question of whether MS therapies with FTY720 should include the use of differentiation-enhancing factors such as NT-3. This approach would ensure both protection of existing OLG progenitor pools against immune-mediated insults as well as stimulation of remyelination by enhancing the maturation of these cells.
effect of FTY720 on acute experimental autoimmune encephalomyelitis (EAE), an animal model for MS. Treatment with FTY720 was shown to ameliorate EAE symptoms in both rats and mice (Fujino et al., 2003; Webb et al., 2004; Kataoka et al., 2005) and to be accompanied by inhibition of T cell infiltration (Brinkmann and Lyngh, 2002; Fujino et al., 2003; Kataoka et al., 2005) and reduced expression of pro-inflammatory ( interleukin-6) and inflammatory interferon-γ (IFN-γ) cytokines (Fujino et al., 2003). Moreover, recent results from a randomized clinical trial showed a significant reduction of both demyelinating lesions and clinical disease activity in patients treated with FTY720, further supporting the promising use of this drug for the treatment of relapsing MS (Kappos et al., 2006). The positive action of FTY720 in EAE and MS has been attributed to its capacity to sequester circulating lymphocytes, especially T cells, into the secondary lymphoid tissues such as lymph nodes and Peyer’s patches (Brinkmann and Lyngh, 2002; Mandala et al., 2002). However, several lines of evidence suggest that FTY720 may also exert additional immunomodulatory effects by interfering with dendritic cell function (Jan et al., 2005; Müller et al., 2005) and by inhibiting cytosolic phospholipase A2 and the production of eicosanoid inflammatory mediators in mast cells (Payne et al., 2007). Results from different laboratories have shown that FTY720 is phosphorylated by sphingosine kinase type 2 (SphK2) (Zemann et al., 2006) generating (S)-FTY720-phosphate (FTY720-P). FTY720-P is structurally similar to sphingosine-1-phosphate (SIP), a lipid metabolite that has emerged in recent years as a potent mediator in numerous biological processes (Herr and Chun, 2007). Because of its similarity to SIP, FTY720-P is able to bind to all of the high-affinity SIP receptors except SIP3 (Brinkmann and Lyngh, 2002). We have previously found that SIP signaling plays a crucial role as a mediator in the stimulation of OLG progenitor survival by neurotrophin-3 (NT-3) (Saini et al., 2005). This observation led us to hypothesize that FTY720 could, like SIP, have a direct effect on the OLG progenitors. This possibility is particularly attractive as several lines of evidence indicate that these immature cells are crucial for the replenishment of lost OLG populations and remyelination in MS (Zhao et al., 2005). In the present study, we have found that treatment of OLG progenitors with FTY720 results in rapid phosphorylation of ERK1/2 and Akt, two enzymes involved in cell survival pathways. Moreover, FTY720 protects the cells from apoptotic cell death induced by growth factor deprivation, cytokines, and microglial activation. Interestingly, FTY720 appears to inhibit the differentiation of the OLG progenitors, a phenomenon that is counteracted by NT-3. Taken together, these observations suggest that the beneficial effect of FTY720 in EAE and MS may not only result from its immunomodulatory properties but may also involve a direct effect on the OLGs. These actions may be further enhanced by combined therapies with NT-3.

Materials and Methods

Materials. Percoll and lipopolysaccharide (LPS) were purchased from Sigma-Aldrich (St. Louis, MO). Reduced growth factor Matrigel was from BD (Franklin Lakes, NJ). FTY720 was obtained from Cayman Chemical (Ann Arbor, MI). The active enantiomer of FTY720-P, (S)-FTY720-P, was prepared as described previously (Lu and Bittman, 2006). NT-3 and tumor necrosis factor-α (TNF-α) were from PeproTech (Rocky Hill, NJ). All cell culture media components were from Sigma-Aldrich. The phosphoinositide-3 kinase (PI3K) inhibitor LY294002, mitogen extracellular signal-regulated kinase kinase (MEK) inhibitor PD98059, and IFN-γ were from Calbiochem (San Diego, CA). The SphK inhibitor N,N-dimethylphosphoglycerine (DMS) was obtained from BIO-MOL International (Plymouth Meeting, PA). Anti-phosphorylated ERK1/2 (P-ERK1/2) and anti-total ERK1/2 (T-ERK1/2) antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-phosphorylated Akt (P-Akt), anti-pan Akt (T-Akt), anti-phosphorylated p38 mitogen-activated protein kinase (p38 MAP kinase), and anti-phosphorylated c-Jun N-terminal kinase (JNK) antibodies were from Cell Signaling Technology (Danvers, MA). The O4 antibody was kindly provided by Dr. Rashmi Bansal (University of Connecticut, Storrs, CT). Anti-β-actin antibody was from Sigma. All appropriate secondary antibodies were obtained from Santa Cruz Biotechnology, Inc.

Isolation and Culture of OLG Progenitors. OLG progenitors were isolated from 2- to 3-day-old Sprague-Dawley rat brains using a Percoll gradient followed by differential adhesion to eliminate the microglial cells, as described previously (Sato-Bigbee et al., 1999). The floating OLGs were plated in 48-well plates (Falcon) or 8-well chamber slides (Nalge Nunc International, Naperville, IL) coated with 12.5 µg/well reduced growth factor Matrigel. The cells were maintained overnight in chemically defined medium (CDM) (Dubecco’s modified Eagle’s medium (DMEM)-Ham’s F-12 medium (1:1), Invi-gro, Grand Island, NY) supplemented with 1 mg/ml fatty acid-free bovine serum albumin, 50 µg/ml transferrin, 5 µg/ml insulin, 30 nM sodium selenite, 0.11 mg/ml sodium pyruvate, 10 nM biotin, 20 nM progesterone, 100 µM putrescine, and 15 nM triiodothyronine. Cultures prepared from these cells are composed of immature OLG progenitors that are either bipolar or possess several simple processes and are stained with the A2B5/O4 antibodies (Sato-Bigbee et al., 1999). Astrogial contamination of these cultures, as assessed by glial fibrillary acidic protein staining, was less than 5%. Animal use and isolation of OLGs were conducted in accordance with the Institute of Laboratory Animal Resources (1996) and approved by the Virginia Commonwealth University Animal Care and Use Committee.

Microglial Cultures and Preparation of Microglia-Conditioned Media. Microglial cultures were prepared from 7-day-old rat brains following the method described above for isolation of OLG progenitors. After the Percoll gradient, the cell suspension containing both OLGs and microglial cells was subjected to differential adhesion for 20 min. The microglial cells that were firmly attached to the bottom of the plate were cultured in DMEM containing 2% fetal bovine serum. The microglia-conditioned medium was prepared as reported previously by Pang et al. (2000). For this, cultures grown to confluence were washed 3 times with PBS and then incubated in CDM or CDM with 5 µg/ml LPS. After 48 h, the medium was collected, filtered through a 0.45-µm syringe filter, aliquoted, and stored at −20°C until use.

Treatment with FTY720 or FTY720-P. After 1 day in culture, OLG progenitors were incubated for various times in DMEM-Ham’s F-12 medium with or without FTY720 or (S)-FTY720-P. Cells were then processed for Western blot analysis or for terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay as described below. In experiments to evaluate the role of different protein kinases, the cells were preincubated for 10 min in the presence of the following specific kinase inhibitors: PD98059 (MEK inhibitor, 50 µM), LY294002 (PI3K inhibitor, 30 µM), or DMS (SphK inhibitor, 5 µM). Cultures were then incubated for 15 min in the presence of either 1 µM FTY720 or the kinase inhibitor or a combination of both. For TUNEL assays, the inhibitors were added 10 min before the addition of FTY720. In these experiments, PD98059 was used at a concentration of 10 µM. Control media contained the same volume of
vehicle in which the inhibitors were dissolved (dimethyl sulfoxide for LY294002 and PD98059 and 4 mg/ml bovine serum albumin for DMS). Inhibitor concentrations are in agreement with those previously used by us and others to specifically inhibit these kinases in OLGs (Sato-Bigbee et al., 1999; Cui et al., 2006).

**Western Blot Analysis.** OLG progenitor cultures containing equivalent numbers of cells per well were lysed in 75 μl of 60 mM Tris-HCl buffer (pH 6.8) containing 10% glycerol, 2% SDS, and 5% 2-mercaptoethanol. Samples (15 μl) were subjected to SDS-polyacrylamide gel electrophoresis in 12% acrylamide, and the proteins were electrotransferred to nitrocellulose. The membranes were then subjected to immunoblot analysis as previously reported (Saini et al., 2005), with minor modifications. Nonspecific antibody binding to the blots was blocked by incubation in 10 mM Na2HPO4, 2.7 mM KCl, and 137 mM NaCl, pH 7.4, (PBS) containing 3% nonfat dry milk and 0.05% Tween 20 (blocking solution) for 1 h at room temperature. Blots were then incubated overnight with anti-P-ERK1/2 (diluted 1:1000), an antibody that reacts with p42 and p44 ERKs when phosphorylated at Tyr204 or anti-P-Akt (diluted 1:1000), an antibody that recognizes Akt when phosphorylated at Ser473 in the C-terminal regulatory domain. Anti-T-ERK1/2 (diluted 1:1000) and anti-T-Akt (diluted 1:1000) antibodies were used to detect the levels of total ERK1/2 and total Akt. Anti-β-Actin antibody (diluted 1:2000) were used as loading controls. After extensive rinsing with PBS, blots were incubated for 30 min in blocking solution, followed by incubation with the appropriate hors eradish peroxidase-conjugated secondary antibody for 3 h. All antibodies were diluted in blocking buffer. After two 5-min rinses in PBS containing 0.05% Tween 20 and four 10-min rinses in PBS, the immunoreactive bands were detected by chemiluminescence with SuperSignal West Dura reagent (Pierce Chemical, Rockford, IL). The relative amount of immunoreactive protein in each band was determined by scanning densitometric analysis of the X-ray films using the National Institutes of Health Image J program. After quantification of the bands, values were divided by β-actin levels to correct for loading differences.

**Detection of Apoptotic Cells.** After fixation of the cells with 4% paraformaldehyde for 1 h at room temperature, the number of apoptotic OLG progenitors was determined by detecting DNA fragmentation using a TUNEL assay (In Situ Cell Death Detection Kit; Roche Diagnostics, Indianapolis, IN), as reported previously (Saini et al., 2005). Fluorescein-labeled nucleotides incorporated at 3′-hydroxyl ends were visualized by incubation with horseradish peroxidase-conjugated anti-fluorescein antibody. The cells were then treated with diaminobenzidine (metal-enhanced DAB substrate kit; Roche Diagnostics) to detect peroxidase activity, followed by light microscopic examination. For each condition, at least 20 visual fields containing approximately 200 cells each were analyzed.

**[3H]Thymidine Incorporation.** After isolation, the OLG progenitors were plated on 48-well plates previously coated with 12.5 μl/well reduced growth factor Matrigel and maintained overnight in CDM. The next day, the medium was replaced with CDM containing 1 μCi/ml [3H]thymidine (75 Ci/mmol; Amersham Biosciences, Piscataway, NJ), in the presence or absence of 1 μM FTY720. After 18 h, the cultures were washed three times with ice-cold PBS, followed by incubation with 20% trichloroacetic acid for 1 to 2 h at 4°C. After four washes with 5% trichloroacetic acid, the cells were solubilized by incubation with 70% formic acid at 37°C for 1 h. Aliquots were then used to determine the radioactivity by liquid scintillation counting.

**Immunocytochemistry.** OLG progenitors were plated in 8-well chamber slides (Nalge Nunc International) coated with 12.5 μl/well
reduced growth factor Matrigel and maintained overnight in CDM. The next day, the medium was replaced with CDM alone or supplemented with 1 μM FTY720, 50 ng/ml NT-3, or a combination of both FTY720 and NT-3. The medium was replaced daily. On day 5, the cells were fixed in 4% paraformaldehyde, and immunocytochemistry was performed as reported previously (Sato-Bigbee et al., 1999). Nonspecific antibody binding was blocked by incubation of the cells for 1 h in PBS containing 5% nonfat dry milk, 0.05% Tween 20, and 0.5% normal goat serum (blocking solution). The cells were then incubated overnight with the O4 antibody (diluted 1:3) in blocking solution. After several washes in PBS, the cells were incubated for 30 min in blocking solution and for 2 h with Alexa 488-conjugated anti-mouse IgM (diluted 1:250). The cultures were analyzed using a Nikon Eclipse 800M fluorescence microscope.
Statistical Analysis. Statistical analysis was performed by one-way analysis of variance and an ad hoc Tukey-Kramer test (GraphPad Prism; GraphPad Software Inc., San Diego, CA). Differences were considered statistically significant when $p < 0.05$.

Results

Treatment of OLG Progenitors with FTY720 Results in Increased Phosphorylation of ERK1/2 and Akt. Because we previously found that S1P signaling plays a crucial role in the stimulation of OLG progenitor survival by NT-3 (Saini et al., 2005) and given that most actions of FTY720 are mediated by S1P receptors (Brinkmann and Lynch, 2002), it was of interest to examine the effects of FTY720 on OLG progenitors. Similar to S1P (Hida et al., 1999; Saini et al., 2005), treatment with FTY720 activated ERK1/2 (Fig. 1, A and B). A significant effect was observed at a concentration of 1 μM (Fig. 1A) and was maximal within 15 min (Fig. 1B). FTY720 also induced phosphorylation of Akt in a similar dose- and time-dependent manner (Fig. 1, C and D). However, treatment with FTY720 did not produce any significant changes in the levels of either total ERK or total Akt. Moreover, FTY720 did not activate p38 MAP kinase or JNK, as determined with phospho-specific antibodies (data not shown).

The FTY720-Dependent Induction of ERK1/2 and Akt Phosphorylation Requires SphK Activity and Is Mimicked by Synthetic (S)-FTY720-P. Several studies have implicated FTY720-P, an S1P analog, as the bioactive form induced phosphorylation of Akt in a similar dose- and time-dependent manner (Fig. 1, C and D). However, treatment with FTY720 did not produce any significant changes in the levels of either total ERK or total Akt. Moreover, FTY720 did not activate p38 MAP kinase or JNK, as determined with phospho-specific antibodies (data not shown).

The stimulation of both ERK1/2 and Akt phosphorylation induced by FTY720 was significantly reduced by the MEK inhibitor PD98059 (Fig. 2, A and C) as well as by the PI3K inhibitor LY294002 (Fig. 2, B and D). Thus, FTY720 has a direct action on OLG progenitors activating MEK/ERK1/2 and PI3K/Akt pathways, an effect that appears to involve the interaction between both signaling cascades.
responsible for FTY720 effects (Chiba et al., 2006). However, recent reports indicated that FTY720 also has actions independent of its phosphorylation by SphKs and the need for S1P receptors (Payne et al., 2007). These observations raised the question of whether FTY720 action in the OLG progenitors requires the activity of a SphK isozyme. To address this problem, we first investigated the capacity of FTY720 to stimulate ERK1/2 and Akt phosphorylation in cells treated with DMS, an inhibitor of both SphK1 and SphK2. Figure 3, A and B, shows that coincubation with DMS abolished the FTY720-dependent stimulation of both ERK1/2 and Akt phosphorylation, suggesting the requirement of FTY720 phosphorylation by SphK. To support this notion, we next examined whether the effects of FTY720 in the OLG progenitors were mimicked by its phosphorylated form, FTY720-P. These experiments were performed using the active (S)-enantiomer of FTY720-P, which is the in vivo phosphorylated product of FTY720 (Albert et al., 2005). Incubation of the OLG progenitors with (S)-FTY720-P resulted in a robust increase in the phosphorylation of both ERK1/2 and Akt (Fig. 4). This stimulation occurs at a lower drug concentration than the one required for FTY720 (Fig. 4, A and C).

Moreover, the kinetics of FTY720 versus (S)-FTY720-P actions indicate that (S)-FTY720-P induces ERK1/2 and Akt activation faster than its nonphosphorylated form, with significant effects being observed within 5 min (Fig. 4, B and D). These findings suggest that FTY720 action in the OLG progenitors is preceded by its phosphorylation by SphK. In agreement, activation of ERK1/2 by (S)-FTY720-P was not affected by DMS (Fig. 4E), substantiating the notion that phosphorylation of FTY720 is required for its actions in the OLG progenitors.

**FTY720 Blocks Apoptosis of OLG Progenitors Caused by Growth Factor Deprivation.** ERK1/2 and Akt are both known to be involved in regulating OLG survival (Ebner et al., 2000; Fragoso et al., 2004; Cui et al., 2006). Thus, the finding that FTY720 induces ERK1/2 and Akt phosphorylation led us to investigate its possible effect on the survival of OLG progenitors. To this end, the cell cultures were subjected to growth factor deprivation, a condition that induces apoptosis of OLG progenitors (Saini et al., 2005) and could be particularly important in the adult tissue where levels of growth factors are significantly reduced compared with those in the developing CNS. As detected by TUNEL staining,
overnight incubation in DMEM-Ham's F-12 medium alone resulted in approximately 50% of the cells undergoing apoptotic cell death (Fig. 5A). However, the number of apoptotic cells was significantly reduced upon treatment with FTY720, indicating that this drug indeed has a direct survival effect on the OLG progenitors. This effect truly reflects a change in the percentage of surviving cells as [3H]thymidine incorporation experiments indicated that FTY720 did not affect the proliferation of the OLG progenitors (Fig. 5C).

The Antiapoptotic Effect of FTY720 on OLG Progenitors Is Blocked by Inhibition of PI3K and MEK Signaling. Because FTY720 stimulated ERK1/2 and Akt phosphorylation, we then examined the extent to which these two protein kinases were involved in mediating the FTY720 effect on OLG survival. For this, the capacity of FTY720 to block apoptosis was investigated in cultures in which ERK1/2 and Akt signaling were blocked by inhibition of their upstream activators MEK and PI3K. As observed before, FTY720 protected the cells from growth factor deprivation. However, this effect was eliminated by cotreatment of the cultures with PD98059 or LY294002 (Fig. 5B), suggesting that ERK1/2 and Akt signaling is required for FTY720 to stimulate the survival of the OLG progenitors.

FTY720 and NT-3 Cooperate to Stimulate OLG Survival and Differentiation. NT-3 is known to stimulate the survival of OLGs and may play an important role in myelin regeneration after CNS injury and demyelination (Barres et al., 1993; McGtigue et al., 1998; Jean et al., 2003; Saini et al., 2005). We have previously found that NT-3 stimulates the survival of OLG progenitors by a mechanism that involves the activation of an S1P-dependent pathway (Saini et al., 2005). Thus, we next examined whether NT-3 and FTY720 could cooperate to stimulate the survival of OLG progenitors. Cultures treated with both FTY720 and NT-3 show significantly reduced numbers of apoptotic cells (15%), which is lower than the percentage observed in cultures treated with either of these alone (Fig. 6). Interestingly, FTY720 appears to arrest the differentiation of OLG progenitors, an effect that is overcome by cotreatment with NT-3. As shown in Fig. 7, cultures treated with FTY720 alone are predominantly composed of cells with a simple bipolar morphology characteristic of immature OLGs. On the other hand, the majority of the cells that were simultaneously exposed to both FTY720 and NT-3 exhibit complex and branched processes, a morphology indicative of mature OLGs. Altogether, these observations suggest that the combination of FTY720 and NT-3 could effectively promote the survival as well as differentiation of OLGs.

FTY720 Protects OLG Progenitors from Cell Death Induced by Cytokines and Microglial Activation. Death of OLGs in demyelinating diseases such as MS is also thought to be significantly increased by the presence of inflammatory cytokines such as TNF-α and IFN-γ or by oxidative stress by free radical species that are produced by activated microglia (McQualter and Bernard, 2007). Indeed, TNF-α and IFN-γ markedly induced apoptosis of the OLG progenitors (Fig. 8A). Importantly, FTY720 effectively protected the cells from this detrimental action of the two cytokines. Moreover, in agreement with previous reports (Pang et al., 2000), treatment of OLG progenitors with conditioned media from LPS-activated microglial cells induced significant apoptotic cell death (Fig. 8B). FTY720 also protected the OLG progenitors from these deleterious actions of microglial activation (Fig. 8B). Altogether, these observations suggest that FTY720 can protect OLGs from many of the factors implicated in their depletion in MS.

Discussion

MS is a chronic degenerative disease in which demyelinating lesions of the CNS are thought to be directly associated with an inflammatory process that involves the activation and recruitment of macrophages, microglial cells, and T lymphocytes (Lucchinetti et al., 2005). For this reason, most current therapies include the use of anti-inflammatory drugs. FTY720 is among the latest immunomodulators that exhibit promising use for the treatment of MS (Kappos et al., 2006). Our findings suggest that independently of its immunosuppressive and anti-inflammatory functions, FTY720 could also have a beneficial effect in MS as a result of a direct action on OLGs. Importantly, these effects occur at FTY720 concentrations that, according to recent pharmacokinetic
studies on tissue distribution (Meno-Tetang et al., 2006), are well within the FTY720 levels attained in brain after oral and intravenous administration.

We found that FTY720 protects cultured OLG progenitors from apoptotic cell death, an action that is preceded by the activation of MEK/ERK1/2 and PI3K/Akt signaling pathways. The observation that the capacity of FTY720 to induce ERK1/2 and Akt phosphorylation was abrogated by the SphK inhibitor DMS suggests that FTY720 actions in the OLGs depend on its phosphorylation by a SphK isozyme, most likely SphK2 (Zemann et al., 2006).

The ERK1/2 and PI3K/Akt signaling pathways have been implicated in promoting OLG survival under different conditions (Ebner et al., 2000; Fragoso et al., 2004; Cui et al., 2006). We found that FTY720 stimulates these pathways in the OLG progenitors by a mechanism that appears to require cross-talk between both signaling cascades. The results indicated that the FTY720-dependent phosphorylation of Akt involved both its classic activator PI3K as well as a MEK-dependent pathway. At the same time, both PI3K- and MEK-dependent pathways were required for ERK1/2 phosphorylation induced by FTY720. A close interdependence between PI3K/Akt and MEK/ERK1/2 signaling pathways has also been reported in other cell types. A crucial role of ERK1/2 in Akt activation was demonstrated in response to UVB irradiation, a situation that involved ERK1/2-dependent phosphorylation of the mitogen and stress-activated protein kinase 1 (Nomura et al., 2001). Another such example was observed in Akt activation induced by oxidative stress, which was shown to require ERK1/2-dependent phosphorylation of the adaptor protein p66shc (Hu et al., 2005).

It is important to note that the effect of FTY720-P on ERK activation in OLGs appears to depend on the developmental stage of these cells, as a recent report failed to detect ERK activation in morphologically mature OLGs (Osinde et al., 2007). In addition, the signaling pathways triggered by FTY720 appear to also exhibit cell specificity because previous studies on T lymphocytes have shown activation of p38 MAP kinase and JNK without ERK stimulation (Matsuda et al., 1999). On the contrary, we did not detect activation of p38 MAP kinase and JNK in the OLG progenitors (data not shown).

The present study further shows that FTY720 protects the OLG progenitors against the deleterious actions of activated microglia as well as the inflammatory cytokines TNF-α and IFN-γ, all of which have been implicated in the pathogenesis of MS (McQualter and Bernard, 2007). Previous studies (Fragoso et al., 2004) have shown that ERK has a protective effect against apoptotic cell death of OLGs induced by reactive oxygen species, known to be produced by activated microglia (Block et al., 2007). Akt has been shown to play a crucial role in the insulin-like growth factor-1-mediated protection of OLG progenitors from apoptosis induced by TNF-α (Pang et al., 2007). Thus, it is tempting to speculate that both ERK and/or Akt pathways may be involved in the FTY720-mediated protection of OLG progenitors against activated microglia and inflammatory cytokines.

The possibility of a direct protective effect of FTY720 on OLGs is particularly important as different studies have found that, together with myelin loss and axonal degeneration (Trapp et al., 1999), OLG death is a feature of MS (Barnett and Prineas, 2004; Lucchinetti et al., 2004). Moreover, analysis of early MS lesions showed OLG death with few or no infiltrating lymphocytes or macrophages, raising the possibility that, at least in some patients, OLG death may be a primary cause of MS (Barnett and Prineas, 2004).

Thus, whether OLG death is a cause or a consequence of MS remains controversial, but it is now clear that the loss of these cells is a major component of the disease. The finding that FTY720 is able to stimulate the survival of OLG pro-

Fig. 7. NT-3 counteracts the inhibition of OLG progenitor maturation induced by FTY720. Cultures were incubated for 5 days in CDM (control), CDM with 1 μM FTY720 (FTY720), CDM with 50 ng/ml NT-3 (NT-3), or CDM with 1 μM FTY720 and 50 ng/ml NT-3 (FTY720+NT-3). After fixation, cells were immunostained with O4 antibody.
genitors is particularly significant as these cells play a crucial role in the remyelination process by replacing lost mature OLG pools (Keirstead and Blakemore, 1999; Zhao et al., 2005). It should also be considered that FTY720 could influence remyelination by effects on other cells of the CNS. In this regard, it was recently demonstrated that (S)-FTY720-P has a direct effect on cultured astrocytes, stimulating both their migration (Mullershausen et al., 2007) and ERK phosphorylation (Oside et al., 2007) via S1P receptors.

The present findings in OLG progenitors, together with the observation of FTY720-P action in astrocytes, suggest that the mechanisms underlying the beneficial effects of FTY720 in MS are more complex than previously speculated. On one side, the therapeutic effect of FTY720 may result from its capacity to induce T lymphocyte sequestration (Mandala et al., 2002), interference with dendritic cell function (Lan et al., 2005), and inhibition of cytosolic phospholipase A2 and eicosanoid production (Payne et al., 2007). On the other hand, in addition to these important immunomodulatory effects, a positive outcome in MS may also reflect a direct action of FTY720 on different cell populations of the CNS, including cells within the oligodendroglial and astrocytic lineages. Our results indicate that FTY720 exerts a direct antipoptotic protective effect on OLG progenitor pools, which may potentially culminate in the replenishment of lost mature OLGs and, thus, promote the process of remyelination in MS. However, a key finding of this study is that the positive effect of FTY720 on the survival of OLG progenitors contrasted with an apparent inhibition of their maturation, an effect that was counteracted by NT-3. This observation suggests that FTY720-treatment for MS should include the use of remyelinating agents such as NT-3. This therapeutic approach would ensure both protection of existing OLG progenitor pools against immune-mediated insults as well as stimulation of remyelination by enhancing the differentiation of these cells.

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