Neurobiology of Disease xxx (2015) xxx–xxx

Contents lists available at ScienceDirect

Neurobiology of Disease

journal homepage: www.elsevier.com/locate/ynbdi

Fingolimod treatment promotes proliferation and differentiation of oligodendrocyte progenitor cells in mice with experimental autoimmune encephalomyelitis

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ARTICLE INFO

Article history:
Received 15 July 2014
Revised 24 December 2014
Accepted 29 January 2015
Available online xxxx

Keywords:
experimental autoimmune encephalomyelitis
oligodendrocyte progenitor cell
oligodendrocytes
remyelination
oligodendrogenesis
sonic hedgehog
cyclopamine

ABSTRACT

Multiple sclerosis (MS) is a major demyelinating disease of the central nervous system (CNS) leading to functional deficits. The remyelination process is mediated by oligodendrocyte progenitor cells (OPCs). In this study, we tested the hypothesis that Fingolimod, a sphingosine 1-phosphate (S1P) receptor modulator, stimulates OPC differentiation into mature oligodendrocytes, in addition to its well-known anti-inflammatory effect. Using an animal model of MS, experimental autoimmune encephalomyelitis (EAE), we performed a dose–response study of Fingolimod (0.15 or 0.3 mg/kg bw), which was initiated on the day of EAE onset. The neurological function was tested to determine the optimal dose of Fingolimod. Immunofluorescent staining was performed to measure the profile of OPC proliferation and differentiation. The mechanistic premise underlying the therapeutic effect of Fingolimod, was that Fingolimod stimulates the sonic hedgehog (Shh) pathway, and this pathway promotes OPC differentiation. To test this hypothesis, a loss-of-function study using cyclopamine, an inhibitor of the sonic hedgehog (Shh) pathway, was employed in vivo. Protein levels of the Shh pathway were measured by Western blot analysis. We found that Fingolimod treatment (0.3 mg/kg bw) significantly decreased cumulative disease score compared to the EAE control group. Concurrently, OPCs and proliferation of OPCs were significantly increased in the white matter of the brain and spinal cord at day 7 and day 30 after EAE onset, and oligodendrocytes, myelination and differentiation of OPCs were significantly increased at day 30 compared with the EAE control group. EAE mice treated with Fingolimod exhibited substantially elevated levels of Shh, its receptor Smoothened and effector Gli1 in the white matter of the CNS. However, combination treatment of EAE mice with cyclopamine–Fingolimod decreased Fingolimod monotherapy elevated protein levels of Smoothened and Gli1, and abolished the effect of Fingolimod on OPC proliferation and differentiation, as well as on neurological function outcome. Together, these data demonstrate that Fingolimod is effective as a treatment of EAE by promoting OPC proliferation and differentiation, which facilitate remyelination. In addition, the Shh pathway likely contributes to the therapeutic effects of Fingolimod on OPCs.

Introduction

Multiple sclerosis (MS) is an autoimmune demyelination disease of the central nervous system (CNS) with neurodegenerative aspects (Ransohoff, 2012), and varied and unpredictable symptoms that lead to disability. More than 2.3 million people acknowledge having MS worldwide. Current available MS therapies target immune modulation with some efficacy, however, concomitantly with adverse side effects (Gasperini and Ruggieri, 2012; Mi et al., 2007). Oligodendrocytes are the only myelin-producing cells in the CNS (Franklin, 2002; Franklin and Ffrench-Constant, 2008). Damaged oligodendrocytes no longer generate myelin and remyelination requires generation

Abbreviations: MS, multiple sclerosis; CNS, central nervous system; S1P, sphingosine 1-phosphate; BBB, blood–brain barrier; OPC, oligodendrocyte progenitor cell; Shh, sonic hedgehog; Smo, smoothened; EAE, experimental autoimmune encephalomyelitis; PLP, myelin proteolipid protein; FTY720, Fingolimod; BrdU, bromodeoxyuridine; HBC, 2-hydroxypropyl-β-cyclodextrin; CNPase, 2′,3′ cyclic nucleotide 3′ phosphodiesterase; NG2, chondroitin sulfate proteoglycan; SVZ, subventricular zone; MCID, microcomputer imaging device; MBP, myelin basic protein

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Available online on ScienceDirect (www.sciencedirect.com).

http://dx.doi.org/10.1016/j.nbd.2015.01.006
0969-9961/

of new mature oligodendrocytes from the differentiation of oligodendrocyte progenitor cells (OPCs) (McTigue and Tripathi, 2008; Scolding et al., 1998; Matsumoto et al., 2011; Young et al., 2013). Endogenous OPCs are extensively present in the adult CNS and comprise the majority of proliferating cells in the adult CNS (McTigue and Tripathi, 2008; Scolding et al., 1998; Matsumoto et al., 2011; Young et al., 2013; Dawson et al., 2003; Wolswijk, 2002). Although proliferating OPCs are recruited to MS lesions, differentiation of these OPCs into myelinating oligodendrocytes is compromised and leads to failure of remyelination and functional deficits (Franklin and Ffrench-Constant, 2008; Deshmukh et al., 2013; Kremer et al., 2011; Miron et al., 2013; Sloane et al., 2010; Back et al., 2005; Hanafy and Sloane, 2011). There are no effective remyelination therapies for MS patients. Thus, OPCs are a viable treatment target for MS clinical therapy (Kremer et al., 2011; Kotter et al., 2011; Mi et al., 2009; Miron et al., 2011; Syed et al., 2011; Kipp and Amor, 2012; Chari and Blakemore, 2002).

Fingolimod, a sphingosine 1-phosphate (S1P) receptor modulator, was the first approved oral agent to treat MS, with significant and robust effects evident on clinical function and MRI measurement (Al-Izki et al., 2011; Scott, 2011; Pelletier and Hafler, 2012; Kappos et al., 2006, 2010; Cohen et al., 2010; Montalban et al., 2011). Fingolimod inhibits lymphocyte egress from the lymph nodes into the blood and the CNS after it is phosphorylated, binds to S1P receptors located on lymphocytes, and thereby alleviates the symptoms of MS (Al-Izki et al., 2011; Scott, 2011; Pelletier and Hafler, 2012). The effects and underlying mechanisms of Fingolimod on immunomodulation have been well studied; however, the effects of Fingolimod on the oligodendrocyte lineage cells in the CNS after demyelination damage require clarification. The lipophilic nature of Fingolimod allows it to cross the blood–brain barrier (BBB) (Kipp and Amor, 2012; Miron et al., 2010, 2008a), and exert direct effects on oligodendrocyte lineage cells, which have S1P receptors (Miron et al., 2010, 2008a, 2008b; Jung et al., 2007), and thereby lead to remyelination in the CNS after demyelination (Rossi et al., 2012). However, there is controversy in the literature regarding the contribution of Fingolimod to remyelination (Miron et al., 2010; Jackson et al., 2011; Hu et al., 2011; Groves et al., 2013). The hypothesis of the current study is that the promotion of OPC differentiation into mature oligodendrocytes contributes to functional recovery after MS by Fingolimod treatment, since myelination is necessary for axonal integrity and brain function.

The sonic hedgehog (Shh) is a member of the family of the hedgehog proteins, and plays a critical role in OPC induction, survival, proliferation, migration and differentiation (Ortega et al., 2012; Wu et al., 2012; Lai et al., 2003). Shh signal binds to the transmembrane receptor protein, Patched, to activate another transmembrane receptor, Smoothened (Smo), and induces intracellular reactions that target the Gli family of transcription factors (Ruiz i Altaba et al., 2002). Gli1 is the principal effector of the Shh signaling in neural progenitor cells (Ahn and Joyner, 2005; Wang et al., 2007). The Shh–Gli1 axis stimulates OPC differentiation and remyelination poststroke (Zhang et al., 2009) and experimental autoimmune encephalomyelitis (EAE), a mouse model of MS (Zhang et al., 2008). Proliferation of premature oligodendrocytes is promoted by the Shh–Gli1 axis in vitro, and negated by cyclopamine, a selective inhibitor of Smo (Zhang et al., 2008; Lauth et al., 2007; Taipale et al., 2000).

In the present study, using the EAE mouse model, we investigated the effects of Fingolimod on neurological functional recovery and proliferation and differentiation of OPCs after EAE, and whether the Shh signaling pathway contributes to Fingolimod enhanced oligodendrogenesis.

Materials and methods

All experimental procedures have been approved by the Institutional Animal Care and Use Committee of the Henry Ford Health System.

EAE animals

To induce EAE, the peptide of myelin proteolipid protein ($PLP_{139–151}$) was subcutaneously injected into female SJL/J mice (Jackson Laboratory, 6–8 weeks old), and pertussis toxin (List Biological Laboratories, Inc.) 200 ng was injected intravenously on the day of immunization and 48 h later (Zhang et al., 2005). EAE mice were randomly enrolled into the following treatment groups: 1) Fingolimod treatment groups: Fingolimod (Novartis) at doses of 0.15 or 0.3 mg/kg bw were gavaged once a day for 30 consecutive days starting on the day of EAE onset, referred to as day 1 postonset (p.o.). These doses were translated from human safety dose studies (Scott, 2011; Reagan-Shaw et al., 2008). 2) EAE control group: EAE mice were gavaged with the same volume of saline. 3) Normal mice. To select the optimal dose of Fingolimod, the neurological function was tested daily up to day 30 p.o. using the 0–5 score, as follows: 0, healthy; 1, loss of tail tone; 2, ataxia and/or paresis of hind limbs; 3, paralysis of hind limbs and/or paresis of forelimbs; 4, tetra-paralysis; 5, moribund or dead. The higher the score, the more severe the disease (Zhang et al., 2005). Bromodeoxyuridine (BrdU) is a thymidine analog that is incorporated into the DNA of dividing cells during S-phase. To label the proliferating cells, BrdU (100 mg/kg, Sigma) was administered intraperitoneally (ip) into mice daily for 7 days initiated on day 1 p.o. The dose of Fingolimod that evoked the best functional benefit was employed in the subsequent studies.

To investigate the mechanism underlying the Fingolimod treatment with a focus on the Shh pathway, we employed 2 additional groups of EAE mice treated with cyclopamine, an inhibitor of the Shh pathway: 4) Combination of Fingolimod–cyclopamine treatment group: Starting on day 1 p.o., the optimal dose of Fingolimod were gavaged daily, and cyclopamine (10 mg/kg bw, Sigma) dissolved in 45% 2-hydroxypropyl-β-cyclodextrin (HBC) was injected ip every other day until sacrifice. 5) Cyclopamine treatment alone group: cyclopamine was injected every other day starting on day 1 p.o. until sacrifice.

Since the functional scores are not normally distributed data, we used the nonparametric Wilcoxon analysis to test the effect of the Fingolimod treatment on functional recovery after EAE. Data were analyzed as cumulative score.

Histopathology and quantification

Five groups of mice, including normal mice, EAE control mice, EAE mice with the optimal dose of Fingolimod treatment alone, EAE mice with combined treatment of Fingolimod and cyclopamine, and EAE mice with cyclopamine treatment alone, were euthanized at day 7 p.o. or day 30 p.o., respectively. They were intracardiac perfused with saline followed by 4% paraformaldehyde. Brain and spinal cord were removed, and coronal brain segments (bregma +0.86 mm/bregma −0.10 mm) (Franklin and Paxino, 1997) and lumbar spinal cord were obtained. A series of 6-μm-thick coronal paraffin sections were cut from each block. Every fifth section was used for immunostaining.

The antibodies against 2′,3′ cyclic nucleotide 3′ phosphodiesterase (CNPase, Chemicon) (Zhang et al., 2010) and chondroitin sulfate proteoglycan (NG2, Chemicon) (Parras et al., 2007) were employed to identify mature oligodendrocytes and OPCs, respectively. Immunohistostaining was performed using antibody against Shh (Santa Cruz) to measure the expression of Shh in the CNS of the EAE mice. The measured areas selected included the total length of subventricular zone (SVZ), 10 fields of the corpus callosum and 20 fields of striatum of the brain, and 10 fields in the white matter of the spinal cord (Fig. 1). The number of $NG2^+$, $CNPase^+$, and Shh^+ cells were measured under a 40× microscope (Olympus BX40) interfaced with a microcomputer imaging device (MCID) image analysis system (Imaging Research Inc., Cambridge, England).

Luxol fast blue (LFB) and Bielschowsky staining were used to demonstrate myelin and axons, respectively. The analyses were selected from the white matter of the lumbar spinal cord (Fig. 1) to assess demyelination. The counting areas were quantified using ImageJ software

Please cite this article as: Zhang, J., et al., Fingolimod treatment promotes proliferation and differentiation of oligodendrocyte progenitor cells in mice with experimental autoimmune encephalomyelitis, Neurobiol. Dis. (2015), http://dx.doi.org/10.1016/j.nbd.2015.01.006