International Immunopharmacology 30 (2016) 171–178

Contents lists available at ScienceDirect

International Immunopharmacology

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

Fingolimod ameliorates the development of experimental autoimmune encephalomyelitis by inhibiting Akt–mTOR axis in mice

Huiqing Hou [a], Runjing Cao [a], Jun Miao [b], Yafei Sun [a], Xiaoqian Liu [a], Xiujuan Song [a], Li Guo [a,*]

[a] Department of Neurology, Key Laboratory of Hebei Neurology, The Second Hospital of Hebei Medical University, Shijiazhuang, 050000, Hebei, China
[b] Department of Neurosurgery, North China Petroleum Bureau General Hospital of Hebei Medical University, Renqiu, 062552, Hebei, China

ARTICLE INFO

Article history:
Received 7 September 2015
Received in revised form 19 November 2015
Accepted 19 November 2015
Available online 26 November 2015

Keywords:
Multiple sclerosis
Experimental autoimmune encephalomyelitis
Fingolimod
mTOR
Treg cell
Th1 cell

ABSTRACT

Fingolimod is a new immunosuppressive agent approved by Food and Drug Administration (FDA) for treating multiple sclerosis (MS). It acts as a functional antagonist to downregulate the S1P1 receptor, which is known to signal through the Akt–mTOR pathway. We investigated the mechanism of fingolimod action in the classical animal model of MS: experimental autoimmune encephalomyelitis (EAE). Fingolimod treatment significantly reduced clinical scores and histopathology in this model, even when treatment was begun after the onset of pathology. The Akt–mTOR signaling pathway was shown to be activated in the EAE model, by measuring the abundance of downstream activation markers, pAkt and ps6k. And this pathway was inhibited when EAE mice were treated with fingolimod. Mice with EAE exhibited an increased frequency of Th1 cells in the spleen, with concomitant increases in the mRNA levels of Tbet and Ifng and increased IFN-γ production by activated splenocytes; the frequency of Treg cells, as well as mRNA levels of Foxp3 and Tgfb, was reduced, as was TGF-β production by activated splenocytes. After treatment with fingolimod, these parameters were reversed, suggesting that fingolimod treatment inhibits the Akt–mTOR axis in EAE, which affects the differentiation and function of Th1 and Treg cells. These results provide an insight into the mechanism of action of fingolimod treatment and may provide new ideas for treating EAE and MS.

1. Introduction

Multiple sclerosis (MS) is a globally distributed inflammatory demyelinating disease. In September 2010, fingolimod was approved as the first oral drug for treating MS by Food and Drug Administration (FDA) [1,2]. Previous studies have confirmed that fingolimod prevents lymphocytes egress from peripheral lymphoid tissue and blocks lymphocytes in secondary lymphoid organs [3–5]. The number of lymphocytes in the blood is reduced, which inhibits pathogenic lymphocytes from attacking the central nervous system [6–8]. Recently, fingolimod was demonstrated to affect the differentiation of different T subsets [9], but the exact mechanism has remained unclear.

Fingolimod is a nonselective antagonist of sphingosine 1-phosphate (S1P) receptors, acting against the S1P1, S1P3, S1P4 and S1P5 receptors, but primarily targeting the S1P1 receptor [10]. It has been shown that S1P1 induces Akt–mTOR pathway activation to impede the function of regulatory T (Treg) cells while promoting T helper type 1 (Th1) cells, and that S1P1 deficiency can lead to the opposite changes [11,12]. In experimental autoimmune encephalomyelitis (EAE), the classical mouse model for studying MS, therapeutic effects of fingolimod treatment have been observed [13–15]. We speculated that fingolimod, as an S1P1 receptor modulator, could block the Akt–mTOR pathway to modulate the differentiation and function of Th1 and Treg cells. The aim of this study was to determine the relationship between the Akt–mTOR pathway and the differentiation and function of Th1 and Treg cells in fingolimod treatment of EAE.

2. Materials and methods

2.1. Animals

Female C57BL/6 mice at 8–10 weeks of age were purchased from Vital River (Beijing, China). Mice were kept on a 12 h dark/light cycle with free access to food and water, and a constant temperature of 22 ± 0.5 °C. All mouse experiments were approved by the Institutional Animal Care and Use Committee of Hebei Medical University.

2.2. Induction and evaluation of EAE

EAE was induced by immunization with 250-μg myelin oligodendrocyte glycoprotein (MOG)p35–55 (Lysine Bio-system, Xian, China) dissolved in complete Freund's adjuvant (Sigma, St. Louis, MO, USA), which contained 4-mg/ml heat-killed *Mycobacterium tuberculosis* H37Ra (Difco Laboratories, Detroit, MI, USA). At time 0 and at 48 h post-immunization, C57BL/6 mice were injected intraperitoneally

* Corresponding author.
E-mail address: guoli6105@163.com (L. Guo).

http://dx.doi.org/10.1016/j.intimp.2015.11.024
1567-5769/

with 500-ng pertussis toxin (Alexis, San Diego, CA, USA). The mice were then randomly divided into an EAE group (EAE) (n = 29) and fingolimod treatment group (EAE + fingolimod) (n = 29). A group of healthy control mice were treated with normal saline (Control) (n = 29). Mice were examined daily after induction for clinical signs of EAE and scored according to the following criteria: 0, no paralysis; 1, tail paralysis; 2, hindlimb weakness or partial paralysis; 3, hindlimb paralysis; 4, forelimb and hindlimb paralysis; and 5, moribund and death. Subsets of these groups were euthanized at 21 days post immunization for investigation using the techniques described in Sections 2.4–2.9 below. An additional cohort of mice was treated with fingolimod after the onset of overt disease to assess its usefulness as a treatment. In this group, fingolimod treatment was begun in mice that developed a clinical score of ≥1.0 (n = 10) with untreated mice with EAE (n = 10) as controls.

2.3. Fingolimod treatment

Fingolimod (Novartis, Switzerland) was freshly prepared at a concentration of 0.2 mg/ml in saline, and administered to mice once daily by oral gavage. EAE mice were treated with fingolimod (1.0 mg/kg) or normal saline at a volume of 5 ml/kg once per day for up to 21 or 30 days after immunization, or after onset of disease.

2.4. Histopathology

Spinal cords were dissected carefully from mice (n = 6), fixed in 10% formalin in phosphate buffered saline (PBS), and embedded in paraffin, using standard methodology. Lumbar spinal cord sections (8 mm) were stained with hematoxylin & eosin (H&E) for evaluating overall histology and inflammation, and were stained with Luxol Fast Blue (LFB) for evaluating demyelination. The degrees of inflammation and demyelination were assessed semi-quantitatively on three non-serial sections of each mouse in a blinded manner, as described in previous study [16]. The level of inflammation was quantified as the following standards [17]: 0, no inflammation; 1, cellular infiltrates only around blood vessel and meninges; 2, mild cellular infiltrates in parenchyma (1–10/section); 3, moderate cellular infiltrates in parenchyma (11–100/section); and 4, serious cellular infiltrates in parenchyma (>100/section). The level of demyelination was quantified as the following standards [18]: 0, normal white matter; 1, rare foci; 2, a few areas of demyelination; 3, confluent perivascular or subpial demyelination; 4, massive demyelination involving one half of the spinal cord; and 5, extensive demyelination involving the whole cord.

2.5. Electron microscopy

The white matter of lumbar spinal cords (n = 1) was dissected out rapidly and made into small blocks, pre-fixed with 4% (v/v) glutaraldehyde, and immersed in PBS. After washing in PBS, the blocks were post-fixed in 1% (w/v) buffered osmium tetroxide for 1 h. After dehydration with graded acetone, blocks were embedded in epoxy resin Epon 812. Sections were cut (70 nm) and stained with uranyl acetate and lead citrate. Demyelination was measured with a JEM-1230 electron microscope (JEOL, Japan).

Table 1
Specific primers for qRT-PCR.

Target gene	Forward	Reverse
T-bet	5′-CAGTTCAACCAGCACCAGAC AG-3′	5′-CCACCAAGACCACATCCACA AA-3′
Foxp3	5′-CTCTAGCAGTCCACTTCACC AA-3′	5′-CACCCACCCTCAATACCTCT CT-3′
IFN-γ	5′-CTGATCCTTTGGACCCTC TG-3′	5′-CAGCCATGAGGAAGAGCTG-3′
TGF-β	5′-GCCCTGGATACCAACTATTG CTTCA-3′	5′-TAGGGGCAGGGTCCCAGACA GAAGT-3′

2.6. Enzyme-linked immunosorbent assay (ELISA)

For analysis of cytokine production, splenocytes were harvested (n = 6) and cultured in vitro with 10 μg/ml of MOG35–55 in RPMI 1640 medium containing 10% FBS (v/v) for 72 h to measure IFN-γ and TGF-β concentrations by quantitative ELISA. Cell supernatants were collected and the test of ELISA was carried out in accordance with the manufacturer's recommendations (BD Biosciences, San Jose, CA, USA). All experiments were repeated four times and results are expressed as the mean ± SD of triplicate wells.

2.7. Flow cytometry

For analysis of T cell subsets by flow cytometry, splenocytes (n = 6) were harvested and washed with RPMI 1640 medium. Samples were stimulated in triplicate with MOG35-55 in 24-well plates for 24 h.

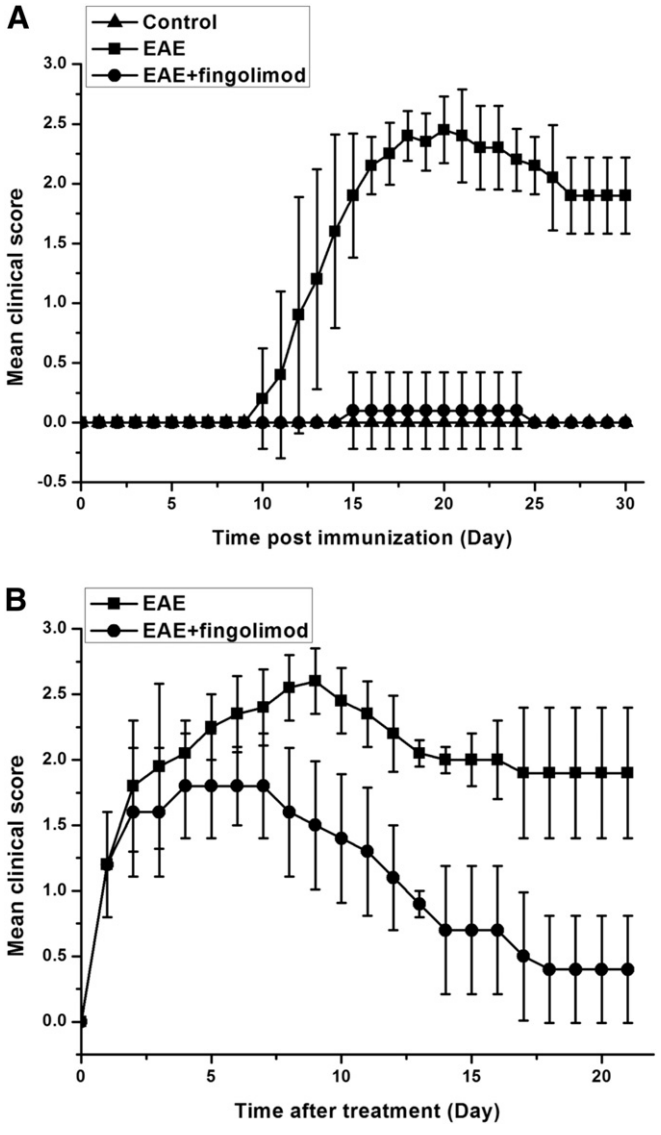

Fig. 1. Fingolimod treatment reduced the severity of EAE. (A) Fingolimod substantially prevents the development and progression of EAE (n = 10). (B) Fingolimod reverses the severity of EAE in mice (n = 10). Data are expressed as the mean ± SD of the clinical scores.