Original Article

Fingolimod (FTY720) ameliorates experimental autoimmune encephalomyelitis (EAE): I. Oral administration of FTY720 effectively inhibits relapse of EAE

Hirotoshi Kataoka\(^1\), Kyoko Shimano\(^1\), Noriyasu Seki\(^1\), Yasuhiro Maeda\(^1\), Mamoru Koyama\(^2\), Atsushi Fukunari\(^2\), Kunio Sugahara\(^1\), Takahisa Sugita\(^1\), and Kenji Chiba\(^1,\ast\)

\(^1\)Pharmacology Research Laboratories I and \(^2\)Advanced Medical Research Laboratories, Research Division, Mitsubishi Tanabe Pharma Corporation, Yokohama, Japan

Experimental autoimmune encephalomyelitis (EAE) is a CD4 T cell-mediated disease model for multiple sclerosis (MS). When SJL/J mice are immunized with myelin proteolipid protein (PLP), EAE symptoms were developed within 2 weeks, remitted thereafter, and then relapsed at 3 to 4 weeks after immunization, indicating that EAE induced by PLP in SJL/J mice shares a certain characteristic with relapsing remitting MS. In this study, we evaluated the preventing effect of fingolimod (FTY720), a sphingosine 1-phosphate receptor modulator, on relapse of EAE induced by PLP in SJL/J mice. When FTY720 at oral does of 0.1 and 0.3 mg/kg was administered daily after establishment of EAE, relapse of EAE was markedly inhibited during administration period. The relapse of EAE was significantly inhibited by subcutaneous administration of recombinant mouse interferon-$\gamma$ (rm-IFN-$\gamma$) at 10000 IU/mouse every other day at early period; however EAE was relapsed in the half number of mice in latter period. These results indicate that FTY720 shows a more marked preventing effect on relapse of EAE compared with rm-IFN-$\gamma$. By immunohistochemical staining, it is revealed that the area of demyelination and the infiltration of CD4 T cells are significantly reduced in the spinal cords of EAE mice by FTY720. Interestingly, FTY720 markedly decreased infiltration of PLP-specific, interleukin 17-expressing CD4 T cells (Th17 cells) into the spinal cords. Consequently, the preventing effect of FTY720 on relapse of EAE is likely due to reduction of infiltration of encephalitogenic CD4 T cells into the central nervous system.


\*Correspondence should be addressed to:
Kenji Chiba, Ph. D., Pharmacology Research Laboratories I, Research Division, Mitsubishi Tanabe Pharma Corporation, 1000, Kamoshida-cho, Aoba-ku, Yokohama, Kanagawa 223-0033, Japan. Phone: +81-045-963-4527, Fax: +81-045-963-3977, e-mail: Chiba.Kenji@mkt-pharma.co.jp

**Key words** experimental autoimmune encephalomyelitis, multiple sclerosis, FTY720, interferon-$\gamma$, Th17 cells
Introduction

Fingolimod (FTY720) is an orally active sphingosine 1-phosphate (S1P) receptor modulator with a structure closely related to sphingosine and is shown to be highly effective in various autoimmune disease models including experimental autoimmune encephalomyelitis (EAE)\(^{1,2}\). Phosphorylated FTY720 (FTY720-P) is converted from FTY720 by sphingosine kinases in vitro and it shows an agonistic activity at four types of S1P receptors (S1P1, 3, 4 and 5)\(^{2-5}\). Notably, FTY720-P strongly induces a long-lasting internalization of S1P1, expressed on cell surface and acts as a functional antagonist at S1P1 receptors on lymphocytes\(^{5,6}\). It is well documented that S1P1 play an essential role in lymphocytes egress from secondary lymphoid organs to lymph\(^{5,6}\). FTY720-P markedly reduces S1P responsiveness of lymphocytes and inhibits lymphocyte egress from secondary lymphoid organs thereby decreasing the number of circulating lymphocytes. Consequently, immunomodulating effects of FTY720 are likely due to sequestration of antigen-specific T cells into the secondary lymphoid organs.

There are several reports on ameliorating effects of FTY720 on EAE in mice and rats\(^{7-11}\). In myelin basic protein-induced EAE in LEW rats, prophylactic administration of FTY720 at 0.1 to 1 mg/kg almost completely prevents the development of EAE symptoms and EAE-associated histological change in the spinal cords\(^{7,9}\). In myelin oligodendrocyte glycoprotein (MOG)-induced EAE in DA rats, prophylactic therapy of FTY720 protects against the emergence of EAE symptoms, neuropathology, and disturbances to visual and somatosensory evoked potentials\(^{10,11}\). Consistent with rat EAE, development of EAE induced by myelin proteolipid protein (PLP) in SJL/J mice is almost completely prevented and infiltration of CD4 T cells into spinal cord is decreased by prophylactic treatment with FTY720 and FTY720-P\(^{8,9}\). When FTY720 or FTY720-P is given after establishment of EAE in SJL/J mice, marked therapeutic effects on EAE are observed accompanying with reduction of demyelination and infiltration of CD4 T cells in the spinal cords\(^{9}\). Similar therapeutic effects by FTY720 are obtained in MOG-induced EAE in C57BL/6 mice\(^{9}\).

Recently, it has been reported that oral administration of FTY720 at 1.25 mg and 5 mg is highly effective in relapsing-remitting multiple sclerosis (MS) patients\(^{12}\). When SJL/J mice are immunized with PLP, EAE symptoms were developed within 2 weeks, remitted thereafter, and then relapsed at 3 to 4 weeks after immunization, indicating that EAE induced by PLP in SJL/J mice shares a certain characteristic with relapsing remitting MS. Recombinant human interferon (rh-IFN)-\(\gamma\)-1a and -1b are widely used as standard therapeutic drugs for relapsing-remitting MS\(^{13}\). In the present study, we compared the preventing effects of FTY720 and recombinant mouse IFN-\(\gamma\) on the relapse of EAE induced by PLP in SJL/J mice.

Materials and Methods

1) Mice

Inbred strains of female SJL/J mice were purchased from Charles River Japan and were used at 8 to 12 weeks of age. All animal experiments were performed under an experimental protocol approved the ethics review committee for animal experimentation of Research Division, Mitsubishi Tanabe Pharma Corporation.

2) Agents and antibodies

FTY720 (2-amino-2-[2-(4-octylphenyl)ethyl]propane-1,3-diol hydrochloride) was synthesized according to the method described previously\(^{14}\) and was dissolved in distilled water for oral administration. Recombinant mouse IFN-\(\gamma\) (rm-IFN-\(\gamma\)) obtained from Calbiochem was diluted with saline for subcutaneous injection. PLP\(_{139-151}\) (HSLGKWLGHPDKF) was obtained from Peptide Institute. Fluorescein isothiocyanate (FITC)-conjugated hamster anti-mouse CD3\(\epsilon\) monoclonal antibody (mAb) (145-2C11), phycoerythrin (PE)-conjugated rat anti-mouse CD45R (B220) mAb (RA3-6B2), FITC- or Cy-Chrome-conjugated, or non-labeled rat anti-mouse CD4 mAb (GK1.5), rat anti-mouse CD8a mAb (53-6.7), FITC-conjugated rat anti-mouse IFN-\(\gamma\) mAb (XMG1.2), and PE-conjugated rat anti-mouse interleukin (IL)-17 mAb (TC11-18H10.1) were purchased from BD Bioscience. Rat anti-mouse CD16/32 mAb (93, FcR block) were obtained from eBioscience.

3) EAE induction

For EAE induction, SJL/J mice were immunized with 50 \(\mu\)g of PLP\(_{139-151}\) peptide and EAE symptoms were monitored using a clinical score ranging from 0 to 5 according to the method described previously\(^{9}\). Briefly, SJL/J mice received a single immunization of PLP\(_{139-151}\) in Freund's complete adjuvant containing killed Mycobacterium tuberculosis H37Ra subcutaneously on day 0. Individual mice were scored for clinical signs of EAE using the following criteria: 0, no paralysis; 0.5, stiff tail; 1, limp tail; 1.5, limp tail with inability to right; 2, paralysis of one limb; 2.5, paralysis of one limb and weakness of one other limb; 3, complete paralysis of both hind limbs; 4, moribund state; 5, death.
4) Flow cytometry

Peripheral blood was obtained from EAE mice on the next day of final administration (on day 42 after immunization). The numbers of lymphocytes, T cells, and B cells were determined by 2-color flow cytometry using FITC-conjugated anti-mouse CD3ε and PE-conjugated rat anti-mouse CD45R (B220) mAbs.

5) Immunohistochemical staining

The spinal cords of EAE mice were obtained on day 30 after immunization (on day 15 after primary administration). Six μm-thickness paraffin sections were prepared from the spinal cords and stained with hematoxylin and eosin after fixation in 4% formalin solution. For the immunohistochemical staining, 6 μm-thickness frozen sections were immediately fixed in cold-acetone and incubated with rat anti-mouse CD3 mAb (17A2), rat anti-mouse CD4 mAb (GK1.5), or rat anti-mouse CD8a mAb (53-6-7). The sections were then incubated with secondary mAbs conjugated with amino acid polymer and peroxidase (Histofine®), colorized with diaminobenzidine in the presence of hydrogen peroxide and counterstained with hematoxylin.

6) Intracellular cytokines staining

Single-cell suspensions were prepared from the spinal cords of EAE mice on day 30 after immunization. The cells were cultured in the presence of 50 μg/ml PLP139-151 in RPMI 1640 medium containing 10% fetal calf serum, 10 mM HEPES, 50 U/ml penicillin, 50 μg/ml streptomycin, 0.1 mM non-essential amino acids, 1 mM sodium pyruvate, and 50 μM2-mercaptoethanol for 66 h at 37°C in 5% CO2 and then were treated with 2 mM monensin for additional 6 h. After blocking by rat anti-mouse CD16/32 mAb, the cells were stained with Cy-Chrome-conjugated rat anti-mouse CD4 mAb and permeabilized with 0.5% Triton X-100. The intracellular cytokine staining was carried out using FITC-conjugated anti-mouse IFN-γ mAb and PE-conjugated anti-mouse IL-17 mAb. Flow cytometry analysis was conducted using LSR with CellQuest software (Becton Dickinson).

7) Statistical analyses

Differences of EAE clinical scores between groups were analyzed by Steel’s test or Mann-Whitney U test. Statistical differences of time to first confirmed relapse in each group were calculated by generalized Wilcoxon test with Holm’s multiple comparison test. The numbers of lymphocytes, T cells, and B cells in the peripheral blood were analyzed by Dunnett’s multiple comparison test or Student’s t-test. Differences between groups were considered significant when p<0.05.

Results

When SJL/J mice were immunized with myelin PLP139-151 in the presence of Freund’s complete adjuvant, EAE symptoms were developed on day 11 and EAE scores were rapidly elevated and reached a maximal level on day 15 after the immunization. To clarify the preventing effects of FTY720 on relapse of EAE induced by PLP139-151 in SJL/J mice, EAE-developed mice were pooled on day 15 after immunization with PLP139-151, divided into 3 groups, and administered FTY720 at oral doses of 0.1 and 0.3 mg/kg daily for 28 days (Fig.1). In vehicle-treated control group, the first phase of EAE was remitted with low EAE scores by day 23 after immunization; however EAE symptoms were relapsed on day 25 to 29 spontaneously, reached to maximal level on day 31, and maintained with high severity thereafter during administration period. On the other hand, the relapse of EAE was markedly inhibited in groups given FTY720 at 0.1 and 0.3 mg/kg orally. All 12 mice in the control group experienced the relapse of EAE from day 20 to 30, whereas only 3 mice in group given FTY720 at 0.1 mg/kg did the relapse during administration periods (Fig.2). Particularly, no EAE-associated clinical signs were observed from day 27 and thereafter in group given FTY720 at 0.3 mg/kg, indicating an almost complete prevention for the relapse of EAE.

Similarly, EAE-developed SJL/J mice were pooled on day 15 after immunization with PLP139-151, divided into 2 groups, and administered rm-iIFN-β at 10000 IU/mouse subcutaneously every other day for 28 days (Fig.3). The administration of rm-iIFN-β resulted in a significant reduction of EAE scores on day 27 to 35 suggesting a delay of the relapse in early period of administration; however rm-iIFN-β failed to prevent the relapse of EAE completely and EAE symptoms were relapsed in the half number of the mice in latter period (Fig.3). The numbers of lymphocytes, T cells, and B cells were markedly reduced in peripheral blood of mice given FTY720 whereas there was no clear effect on lymphocyte number in blood of rm-iIFN-β-treated mice (Fig.4).

It is well known that a striking feature of histopathology in EAE is demyelination and infiltration of encephalitogenic T cells in the central nervous system [14-16]. To elucidate the effects of FTY720 on demyelination and infiltration of CD4 T cells into the central nervous system in EAE induced by PLP139-151, the spinal cords were obtained from EAE mice given FTY720 or vehicle on day 30 after immunization. Demyelination and infiltration of inflammatory cells including lymphocytes were observed in the spinal cords of EAE mice in vehicle-treated control group (Fig.5A, B). Therapeutic oral administration of FTY720
Fig. 1 Effect of FTY720 on relapse of EAE in PLP\textsubscript{139-151}-immunized SJL/J mice

SJL/J mice were immunized with PLP\textsubscript{139-151} (50 µg/mouse) and Freund's complete adjuvant. EAE-developed mice were pooled, divided on day 15 after immunization, and administered FTY720 at oral dose of 0.1 and 0.3 mg/kg daily for 28 days. Results are expressed as the mean ± S.E.M. (n=12). Statistical differences were calculated by Steel's test (*p<0.05, **p<0.01).

Fig. 2 FTY720 significantly increases the number of mice remaining relapse-free in EAE induced by PLP\textsubscript{139-151}

SJL/J mice were immunized with PLP\textsubscript{139-151} (50 µg/mouse) and Freund's complete adjuvant. EAE-developed mice were pooled, divided on day 15 after immunization, and administered FTY720 at oral dose of 0.1 and 0.3 mg/kg daily for 28 days. Each group consisted of 12 mice. Statistical differences were calculated by generalized Wilcoxon test with Holm's multiple comparison test (***p<0.01).

Fig. 3 Effect of rm-IFN-\(\gamma\) on relapse of EAE in PLP\textsubscript{139-151}-immunized SJL/J mice

SJL/J mice were immunized with PLP\textsubscript{139-151} (50 µg/mouse) and Freund's complete adjuvant. EAE-developed mice were pooled, divided on day 15 after immunization, and administered rm-IFN-\(\gamma\) at 10000 IU/mouse subcutaneously every other day for 28 days. Results are expressed as the mean ± S.E.M. (n=10). Statistical differences were calculated by Mann-Whitney \(\bar{U}\) test (*p<0.05, **p<0.01).

Fig. 4 Effects of FTY720 and rm-IFN-\(\beta\) on the numbers of lymphocytes, T cells, and B cells in peripheral blood of EAE mice

SJL/J mice were immunized with PLP\textsubscript{139-151} (50 µg/mouse) and Freund's complete adjuvant. EAE-developed mice were pooled, divided at 15 days after the immunization, and administered FTY720 orally every day or rm-IFN-\(\beta\) subcutaneously every other day for 28 days. Results are expressed as the mean ± S.E.M. (n=6). Statistical differences were calculated by Dunnett's multiple comparison test for FTY720 groups or Student's \(t\)-test for rm-IFN-\(\beta\) group (**p<0.01).
Fig. 5 Therapeutic administration of FT720 decreases the area of demyelination and infiltration of inflammatory cells in the spinal cords of EAE mice
EAE-developed mice were administered FT720 at an oral dose of 0.1 mg/kg therapeutically for 14 days. On the next day of the final administration, the spinal cords were obtained and were stained with hematoxylin and eosin. Control: (A and B), FTY720 0.1 mg/kg: (C and D)

Fig. 6 Therapeutic administration of FTY720 decreases infiltration of CD4 T cells in the spinal cords of EAE mice
EAE-developed mice were administered FTY720 at an oral dose of 0.1 mg/kg therapeutically for 14 days. On the next day of final administration, the spinal cords were obtained and immunohistochemical staining was performed using anti-mouse CD4 mAb. Control: (A and B), FTY720 0.1 mg/kg: (C and D)

Fig. 7 Therapeutic administration of FTY720 decreases infiltration of Th1 and Th17 cells into the spinal cords of PLP139-151-induced EAE mice
EAE-developed mice were administered FTY720 at an oral dose of 0.1 mg/kg therapeutically for 28 days. On the next day of final administration, single-cell suspensions were prepared from the spinal cords of EAE mice and were cultured for 72 h in the presence of PLP139-151 (50 μg/ml). After the culturing, intracellular cytokine staining were performed by using anti-CD4, anti-IL-17, and anti-IFN-γ mAbs.

at 0.1 mg/kg resulted in a marked reduction of demyelination and infiltration of inflammatory cells in the spinal cords of EAE mice (Fig.5C,D). By immunohistochemical staining using mAbs against T cell subsets, it was revealed that CD4 T cells rather than CD8 T cells were infiltrated into the spinal cords, particularly the perivascular area and funiculus dorsalis in white matter under pia matter of EAE mice (Fig.6A,B). Therapeutic administration of FTY720 at 0.1 mg/kg orally resulted in a marked reduction of infiltration of CD4 T cells into the spinal cords (Fig.6C,D).

To clarify the involvement of PLP139-151-specific encephalitogenic CD4 T cells including Th17 cells,17-19, lymphocytes prepared from the spinal cords of EAE mice induced by immunization of PLP139-151 were re-stimulated with PLP139-151 for 72 h in vitro. Then, the numbers of IL-17-expressing Th17 cells and IFN-γ-expressing Th1 cells were determined by intracellular cytokine
staining using anti-mouse CD4, anti-IL-17, and anti-IFN-γ mAbs. As shown in Fig.7, significant numbers of Th17 and Th1 cells were found in the spinal cords from EAE mice, indicating the infiltration of myelin PLP19-151-specific Th17 and Th1 cells into the spinal cords in EAE induced by PLP19-151. Treatment with FTY720 markedly reduced the infiltration of PLP19-151-specific Th17 and Th1 cells into the spinal cords of EAE mice.

Discussion

In the present study, we demonstrate that oral administration of FTY720 effectively prevents the relapse of EAE symptoms induced by PLP after establishment of EAE. We also show that the preventing effects of FTY720 are more markedly as compared with rm-IFN-β in EAE induced by PLP. Several studies have demonstrated the ameliorating effects of FTY720 on EAE in rats and mice7-11. Prophylactic administration of FTY720 at 0.1 to 1 mg/kg almost completely prevents the development of EAE induced by myelin basic protein in LEW rats and by MOG in DA rats7-9,11. Consistent with the results in rat EAE, prophylactic administration of FTY720 markedly prevents the development of EAE induced by PLP in SJL/J mice8,9. Furthermore, FTY720 also shows marked therapeutic effects on EAE accompanying with reduction of demyelination and infiltration of CD4 T cells in the spinal cords when FTY720 is given after establishment of EAE induced by PLP in SJL/J mice or MOG in C57BL/6 mice9. Since EAE induced by immunization with appropriate myelin antigens is widely understood as a CD4 T cell-mediated model for MS, a common and often disabling disease of the central nervous system14-16; these results strongly suggest that oral administration of FTY720 provides a new therapeutic approach for MS.

Indeed, it has been reported that oral administration of FTY720 at 1.25 mg and 5 mg for 6 to 12 months is highly effective in relapsing-remitting MS patients22. FTY720 at an oral dose of 1.25 mg or 5.0 mg, or placebo was administered daily for 6 months to 281 patients with relapsing remitting MS and a total of 255 patients completed the clinical study. The median total number of gadolinium-enhanced lesions on magnetic resonance imaging (MRI) was significantly lower with 1.25 mg and 5.0 mg of FTY720 than with placebo. The annualized relapse rates in groups given 1.25 mg and 5.0 mg of FTY720 were 0.35 and 0.36, respectively and were significantly lower than that in the placebo group (0.77). By extension study for additional 6 months, the number of gadolinium-enhanced lesions and relapse rates remained low in groups given FTY720 and both measures decreased in patients who switched from placebo to FTY720. These results clearly demonstrate that oral administration of FTY720 reduces the number of lesion detected on MRI and clinical disease activity in relapsing remitting MS patients.

On the other hand, two types of rh-IFN-β (rh-IFN-β-1a and -1b) are widely used as a standard therapeutic drug for relapsing-remitting MS30. As we have described previously, FTY720 at 0.3 and 1 mg/kg orally shows a more marked therapeutic effect on PLP-induced EAE as compared with rm-IFN-β at 10000 IU/mouse 3 times a week intraperitoneally in SJL/J mice immunized with PLP30. Consistent with our previous data, we, in this study, demonstrate that daily oral administration of FTY720 at 0.1 and 0.3 mg/kg shows a superior therapeutic efficacy on relapse of EAE symptoms compared with rm-IFN-β at 10000 IU/mouse every other day subcutaneously in SJL/J mice immunized with PLP. Based on these results, it is presumed that that oral administration of FTY720 shows more marked therapeutic effects on the relapse of MS-associated symptoms compared with rh-IFN-β-1a or -1b in relapsing-remitting MS patients.

Early active MS lesions are characterized by the presence of infiltrated mononuclear cells around venules and small veins, followed by myelin breakdown and astroglialosis, resulting in irreversible disability34. The etiology of MS remains unknown, but is widely considered to involve the organ specific autoimmune destruction of myelin sheets mediated by myelin antigen-specific T cells16. Recently, it has been revealed that encephalitogenic IL-17-expressing Th17 cells play an important role in the development and progression of EAE and MS17-20. In the present study, we clearly demonstrate that oral administration of FTY720 shows a significant preventing effect on relapse of EAE symptoms induced by PLP accompanying with a marked reduction of infiltration of CD4 T cells including Th17 and Th1 cells into the spinal cords. Because oral administration of FTY720 leads to sequestration of circulating lymphocytes into secondary lymphoid organs by inhibiting S1P-dependent lymphocyte egress25; it is probable that the preventing effects of FTY720 on relapse of EAE is caused by sequestration of encephalitogenic Th17 cells into the secondary lymphoid organs such as draining lymph nodes. In our succeeding paper, we will describe that the ameliorating effects of FTY720 on EAE are likely due to reduction of the infiltration of encephalitogenic Th17 cells into the central nervous system by inhibiting S1P-dependent lymphocyte egress from secondary lymphoid organs.

Acknowledgements

We thank Sachiko Mochizuki and Mikako Murase for technical assistance.
References


