Original Article

FTY720 induces the sequestration of circulating lymphocytes into the bone marrow in alymphoplasia mice

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Fingolimod (FTY720), a sphingosine 1-phosphate (S1P) receptor modulator, is highly effective in various autoimmune disease models and decreases circulating mature lymphocytes by inhibiting S1P-dependent lymphocyte egress from secondary lymphoid organs (SLO). On the contrary, FTY720 at an oral dose of 1 mg/kg also induces a significant decrease in the number of peripheral blood lymphocytes (PBL), particularly T cells, in alymphoplasia (aly/aly) mice lacking SLO. We demonstrated that there was no contribution of thymus or spleen for the reduction of PBL by FTY720 because FTY720 could also decrease the number of PBL in thymectomized and/or splenectomized aly/aly mice. When mice were given FTY720 at 1 mg/kg orally, the blood concentration of FTY720 is approximately 0.2 μM or less. On the other hand, no apoptosis was seen in aly/aly lymphocytes when they were treated with FTY720 at a concentration of up to 1 μM. Moreover, we found that oral administration of FTY720 at 1 mg/kg to aly/aly mice induced the accumulation of mature lymphocytes, particularly T cells, into the bone marrow but not spleen or thymus. Consequently, it is highly probable that mature lymphocytes depleted from blood by FTY720 were sequestered into the bone marrow in aly/aly mice. The sequestration of T cells into the bone marrow was also induced by S1P lyase inhibitor that disrupts S1P gradients. These results demonstrate that the reduction of PBL in aly/aly mice by FTY720 is due to inhibition of S1P-dependent lymphocyte egress from the bone marrow, but not induction of lymphocyte apoptosis.


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Introduction

Lymphocyte recirculation through secondary lymphoid organs (SLO) is important for immune surveillance and the entry of lymphocytes into SLO is regulated by several chemokines such as CCL19, CCL21 and CXCL13. On the other hand, in the process of lymphocyte egress from SLO, an essential role of S1P and its receptor S1P1 on lymphocytes has been pointed out by several studies using fingolimod (FTY720), the S1P receptor modulator. FTY720 is immediately phosphorylated by sphingosine kinases in vivo, acts as an agonist on S1P1, and induces long-lasting internalization of S1P1 on lymphocytes. Consequently, FTY720-phosphate (FTY720-P) inhibits S1P1-dependent lymphocyte egress from the SLO, leading to the marked reduction of circulating lymphocytes in both blood and lymph.

Although it is strongly believed that SLO play a major role in the reduction of peripheral blood lymphocytes (PBL) by FTY720, it has been reported that FTY720 induces a significant decrease in the number of PBL in allografts (alan/aly) mice that lack SLO. Nagahara et al. hypothesized that FTY720 at 10 mg/kg decreases PBL in alan/aly by inducing apoptosis in lymphocytes. On the other hand, Luo et al. and Sugito et al. have reported that FTY720 even at lower dose (1 mg/kg) significantly decreases the number of PBL in alan/aly mice though they have not discussed about the fate of PBL in these mice after FTY720 administration.

To determine the fate of circulating lymphocytes in alan/aly mice after administration of FTY720 at 1 mg/kg, we investigated the effect of FTY720 on lymphocyte apoptosis in vitro and lymphocyte distribution in vivo. Our results imply that the reduction of PBL by FTY720 in alan/aly mice is predominantly due to sequestration of circulating lymphocytes into the bone marrow, but not induction of lymphocyte apoptosis.

Materials and Methods

1) Mice

Inbred strains of male alan/+ and alan/aly mice were obtained from CLEA Japan. All mice were used at 6 to 12 weeks of age. All animal experiments were performed under an experimental protocol approved the ethics review committee for animal experiment of Research Division, Mitsubishi Tanabe Pharma Corporation. In some experiments, 9 weeks old alan/aly mice were thymectomized and/or splenectomized and used at 2 weeks later.

2) Reagents and antibodies

FTY720 (2-amino-2-[2-(4-ocetylphenyl)ethyl]propane-1,3-diol hydrochloride) and an inhibitor of S1P lyase, 2-acetyl-4-tetrahydroxybutylimidazole (THI), were synthesized according to the respective methods as described previously. FTY720 and THI dissolved in distilled water were given orally. FITC-, PE-, or CyChrome-conjugated monoclonal antibodies recognizing the following mouse surface markers were obtained from BD Biosciences: CD3 (145-2C11), CD4 (GK1.5), and CD8 (53-6.7).

3) Flow cytometry

Peripheral blood (PB) and bone marrow (BM) were obtained from mice at various time points after a single oral administration of each compound. BM cells were flushed out from two femurs and tibias using RPMI 1640 medium and were passed through cotton to remove BM particles. After removing red blood cells by lysis with Tris-NaCl solution, the lymphocytes from PB or BM were stained with antibodies for CD3, CD4, and CD8. The numbers of each cell subset were determined using comparison to a known number of beads as an internal standard of Flow-Count fluorospheres (Beckman Coulter) by flow cytometry with a Cytomics™ FC500 (Beckman Coulter). Calculating formula to estimate total cell numbers in the PB and BM is following; the total volume of PB is almost 80 ml/kg in mice and the number of BM cells in whole body is 7.9 times a coefficient of two femurs combined.

4) Measurement for the blood concentration of FTY720

Blood samples were obtained from mice at 0.5-72 h after a single oral administration of FTY720 at 1 mg/kg. The concentrations of unchanged FTY720 in the blood were measured by gas chromatography/mass spectrometry according to the method as described previously.

5) Assays for cell viability and apoptosis

Spleens removed from mice were minced and passed through stainless mesh. After removing red blood cells by lysis with Tris-NaCl hypotonic buffer, the obtained spleen cells were cultured in 10% fetal calf serum (FCS)-containing RPMI 1640 medium (Sigma-Aldrich) in 10 cm-plastic dishes for 1 h to remove adherent cells and the recovered cells were used as splenic lymphocytes. For the detection of cell viability, splenic lymphocytes from control or alan/aly mice were seeded on 96-well plates in RPMI 1640 medium containing 10% FCS and were cultured in the presence or absence of FTY720 at concentrations of 0.03-30 μM for 24 h. The cells were then stained with TetraColor One kit (Seikagaku Kogyo) for 3 h and the absorbance at 490 nm (OD490) in each well was measured by a microplate reader.
Apoptosis was determined by flow cytometry using cellular Annexin V binding (Immunotech; apoptosis detection kit). In brief, splenic lymphocytes were cultured with or without FTY720 at concentrations of 1-10 μM for 5 h. The cells were then stained with Annexin V and propidium iodide (PI), and analyzed by flow cytometry. Apoptotic cells were identified as Annexin V+ PI.

6) Immunohistochemical staining

Samples of BM tissue were fixed in 10% neutral buffered formalin, EDTA-decalcified, and embedded in paraffin. Paraffin sections of the BM were deparaffinized, and incubated with goat anti-mouse CD3ε polyclonal antibody (Santa Cruz). The sections were then incubated with a secondary antibody which contains amino acid polymers highly labeled by both peroxidase and Fab’ fragment of anti-goat IgG (Nichirei Bioscience), and colored with diaminobenzidine in the presence of hydrogen peroxide. Digital images of the BM sections were obtained with Scan Scope XT (Aperio Technologies), and analyzed with Image-Pro Plus image analysis software (Media Cybernetics).

7) Statistical analysis

The results except for the blood concentrations were expressed as the mean ± S.E.M. Differences between groups were calculated by student’s t-test or Dunnett’s multiple comparison test and were considered to be statistically significant when p<0.05. The blood concentrations of FTY720 in mice were expressed as the mean ± S.D.

Results

We examined the effect of FTY720 on the time course changes in the number of PBL of homozygous aly/aly mice and heterozygous aly/+ mice. The number of total PBL in aly/+ mice was markedly decreased within 3 h after a single oral dose of FTY720 at 1 mg/kg and the reduction was maintained for more than 72 h (Fig.1). On the other hand, the number of PBL in aly/aly mice was significantly decreased at 12 h after FTY720 administration, reached its nadir at 24 h, and returned to the control value within 72 h (Fig.1). Although the number of PBL in aly/aly mice was significantly decreased to less than 50% of the control values at 24 h after the administration, the reduction was less marked in aly/aly mice compared with aly/+ mice (Fig.1).

To clarify the contribution of thymus and/or spleen for reduction of the number of PBL in aly/aly mice, thymectomized and/or splenectomized aly/aly mice were used for the experiments.

Fig.1  FTY720 induces reduction in peripheral blood lymphocytes in aly/+ and aly/aly mice

After oral administration of FTY720 at 1 mg/kg to aly/+ or aly/aly mice, the number of PBL was periodically determined by lymphocyte gating method of flow cytometry. The results are expressed as the mean ± S.E.M (n=3). The number of lymphocytes at pre-administration (0 h) was designated as control and statistical differences were calculated by Dunnett’s multiple comparison test (** p<0.01).

Fig.2  FTY720 decreases the number of peripheral blood T cells in thymectomized aly/aly mice

FTY720 at 1 mg/kg was administered orally to sham-operated (Sham) or thymectomized (Tx) aly/aly mice. At 24 h after the administration, the numbers of CD3 T cells, CD4 T cells, and CD8 T cells in the PB were determined by flow cytometry. The results are expressed as the mean ± S.E.M (n=3). Statistical differences were calculated by student’s t-test (* p<0.05, ** p<0.01).
As shown in Fig.2, FTY720 significantly decreased the number of PBL in thymectomized aly/aly mice with similar extent to that of sham control aly/aly mice. Similar results were obtained in aly/aly mice splenectomized or thymectomized/splenectomized (data not shown). These results clearly demonstrated that there was no contribution of the thymus or spleen to the reduction of PBL by FTY720 in aly/aly mice.

Since Nagahara et al. have been hypothesized that reduction of the number of PBL in aly/aly mice by FTY720 is due to lymphocyte apoptosis, we examined whether the blood concentrations of FTY720 after a single oral administration at 1 mg/kg are sufficient to induce lymphocyte apoptosis or not. As shown in Fig.3A, the blood concentrations of FTY720 are approximately 0.2 μM or less at 0.5 to 72 h after oral administration of FTY720 at 1 mg/kg to mice. When lymphocytes from control or aly/aly mice were cultured in the presence of FTY720 for 24 h in vitro, FTY720 at 0.3 μM or less showed no or only a slight reduction (about 80% of control at 0.3 μM) on the viability of lymphocytes (Fig.3B). On the other hand, FTY720 at 1 μM showed a significant reduction (about 60% of control) and 10 μM or higher did an extremely lower level of viability of lymphocytes from both control and aly/aly mice (Fig.3B). Moreover, we demonstrated by Annexin V staining that FTY720 at 1 μM induced no apoptosis in lymphocytes from control and aly/aly mice whereas higher concentration of FTY720 (3 to 10 μM) significantly induced apoptosis in lymphocytes (Fig.3C). Since the blood concentration of FTY720 in mice treated with 1 mg/kg is less than 0.2 μM and only high concentrations (>3 μM) of FTY720 can induce apoptosis in aly/aly lymphocytes, it would appear to be impossible for FTY720 to induce apoptosis of lymphocytes at a dose of 1 mg/kg in aly/aly mice.

Based on the results mentioned above, it is highly probable that FTY720 decreases the number of PBL in aly/aly mice by alteration of lymphocyte distribution between PBL and unrevealed sites excluding SLO, thymus, and spleen. To search for sequestration sites of circulating lymphocytes in FTY720-treated aly/aly mice, we focused on the BM because the BM is functioned as a pool of blood cells and thought to be one of SLO(15). As we expected, the numbers of lymphocytes, particularly mature (CD3+) T cells, CD4 T cells, and CD8 T cells were significantly increased in the BM to 140-190% of the control values at 24 h after FTY720 administration to aly/aly mice (Fig.4).

It has been known that in vivo administration of an imidazole compound, THI, inhibits the activity of S1P-degrading enzyme, S1P lyase and abrogates the endogenous S1P gradients between blood-lymph and SLO(16). The inhibition of S1P lyase raises the concentrations of S1P in SLO and then induces internalization of S1P1 on lymphocytes in SLO(18). Consequently, THI treatment inhibits S1P-dependent lymphocyte egress from SLO and causes the reduction in PBL(18). As shown in Fig.4, like FTY720, THI at an oral dose of 100 mg/kg also significantly increased the number of mature T cells in the BM in aly/aly mice, suggesting that
Fig. 4 FTY720 and THI increase the number of T cells in the BM of *aly/aly* mice

FTY720 at 1 mg/kg or THI at 100 mg/kg was administered orally to *aly/aly* mice. At 24 h after the administration, the numbers of CD3 T cells, CD4 T cells, and CD8 T cells in the BM were determined by flow cytometry. The results are expressed as the mean ± S.E.M. (n=3). Statistical differences were calculated by student’s *t*-test (* *p<0.01).

**Fig. 5 Immunohistochemical staining of T cells in the BM of *aly/aly* mice given FTY720**

At 16 h after oral administration of FTY720 to *aly/aly* mice, BM sections were prepared and used for immunohistochemical staining with anti-CD3ε polyclonal antibody ((A,B): vehicle control; (C,D): FTY720 1 mg/kg). (E): The number of CD3 T cells in the BM sections was counted under microscope. The results are expressed as the mean ± S.E.M. (n=8). Statistical differences were calculated by student’s *t*-test (* *p<0.05).

SIP gradients play an important role in lymphocyte recirculation through the BM.

Consistent with the results from flow cytometry analyses, immunohistochemical staining revealed that the frequency of mature T cells in the BM from FTY720-treated *aly/aly* mice was significantly higher than that from vehicle-treated *aly/aly* mice (Fig. 5; Control 1.701 ± 0.155 cells/1,000 μm², FTY720; 2.239 ± 0.197 cells/1,000 μm²). From these findings, we conclude that FTY720 decreases in the number of PBL in *aly/aly* mice by sequestration of circulating lymphocytes into the BM, but not by lymphocyte apoptosis.

**Discussion**

It is well documented that FTY720 decreases the number of circulating lymphocytes and sequesters them into SLO by inhibition of SIP-mediated lymphocyte egress from these organs. Nevertheless, it has also been reported that FTY720 decreases the number of PBL in *aly/aly* mice lacking SLO. In this study, oral administration of FTY720 (1 mg/kg) significantly decreased the number of PBL in *aly/aly* mice; however the reduction of PBL by FTY720 was lesser extent in *aly/aly* mice compared with *aly/+* mice. It is highly probable that this difference between *aly/+* and *aly/aly* mice is due to the presence or absence of
the SLO, because the sequestration of circulating lymphocytes into the SLO is a main mechanism of the reduction of PBL by FTY720. Nagahara et al. have hypothesized that the reduction of PBL by FTY720 in aly/aly mice was due to apoptosis of lymphocytes, because FTY720 (10 mg/kg) transiently increased the number of apoptotic cells in the PB in these mice. Indeed, our results also indicated that FTY720 at concentrations of 3 μM or higher induced the apoptosis of aly/aly lymphocytes in vitro. However, FTY720 at 1 μM induced no apoptosis and at 0.3 μM or less showed no or only a slight reduction of viability in aly/aly lymphocytes. Moreover, the blood concentration of FTY720 in mice given 1 mg/kg orally is less than 0.2 μM. From these results, it is unlikely that the reduction of PBL in aly/aly mice given FTY720 at 1 mg/kg orally is caused by lymphocyte apoptosis.

Since lymphocyte apoptosis is inadequate for explaining the reduction of PBL by FTY720 at 1 mg/kg in aly/aly mice, there is a possibility that circulating lymphocytes are sequestered into the thymus, spleen, or unrevealed sites in aly/aly mice after FTY720 administration. In this study, we found no contribution of thymus and spleen to the reduction of PBL by thymectomy and/or splenectomy of aly/aly mice. On the other hand, we demonstrated that the number of mature lymphocytes, particularly T cells, was significantly increased in the BM when FTY720 at 1 mg/kg was given to aly/aly mice, suggesting a sequestration of circulating lymphocytes into the BM. In this experiment, the total number of T cells decreased in the blood and spleen by FTY720 (1.72 ± 0.21 × 10⁷ cells) was almost identical to the increased number of BM T cells (1.67 ± 0.60 × 10⁷ cells). Immunohistochemical staining also revealed that the number of mature T cells was significantly increased in the BM of aly/aly mice given FTY720. Because FTY720 up to 0.3 μM shows no enhancing effect on the viability of aly/aly lymphocytes by culturing for 24 h, the increase of aly/aly lymphocytes in the BM by FTY720 administration is likely due to accumulation of lymphocytes rather than proliferation of them. Consistent with our results, it has been reported that FTY720 induces the accumulation of both normal CD4 T cells and colitogenic effector memory CD4 T cells in the BM of splenectomized, lymphotoxin-α-deficient mice lacking SLO. Based on these findings, we conclude that the reduction of PBL in aly/aly mice by FTY720 is caused by sequestration of circulating lymphocytes into the BM, but not by lymphocyte apoptosis. To confirm whether circulating lymphocytes in PB are sequestered into the BM by treatment with FTY720, we are currently performing lymphocyte transfer studies using fluorescein-labeled lymphocytes in aly/aly mice.

There is still a question whether the sequestration of circulating lymphocytes into the BM by FTY720 is caused by promotion of lymphocyte entry or inhibition of lymphocyte egress. In entry process, CXCR4, LFA-1 α, and integrin α4 are known to be important for T cell migration into the BM. There is no report of the promoting effect of FTY720 on CXCR4-dependent migration of PB T cells and we previously reported that FTY720 does not affect the expression levels of these adhesion molecules on these cells. The S1P-S1P1 axis has an established role in regulating T cell egress from SLO and there is a tight relationship between S1P1 surface abundance on T cells and local S1P concentrations. Most recent work by Jenne et al. has revealed that S1P1 on T cells in the BM was present higher amounts on parenchymal compared with sinusoidal cells, suggesting the existence of an increasing S1P gradient between parenchyma and sinusoid. In this study, we demonstrated that like FTY720, THI that disrupts S1P gradients in vivo also reduced PBL and conversely increased the number of BM T cells in aly/aly mice. Consequently, it is highly probable that the sequestration of circulating lymphocytes into the BM in aly/aly mice by FTY720 or THI is caused by inhibition of S1P-dependent lymphocyte egress from the BM. Since the BM is thought to function as one of SLO, it is presumed that the S1P-S1P1 axis regulates lymphocyte egress from the BM like SLO. To determine the contribution of S1P1 to lymphocyte egress from the BM, further studies using selective S1P1 agonist or S1P1-conditional knockout mice would be necessary.

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