Phosphorylation and Action of the Immunomodulator FTY720 Inhibits Vascular Endothelial Cell Growth Factor-induced Vascular Permeability*

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FTY720, a potent immunosuppressive agent, is phosphorylated in vivo into FTY720-P, a high affinity agonist for sphingosine 1-phosphate (SIP) receptors. The effects of FTY720 on vascular cells, a major target of SIP action, have not been addressed. We now report the metabolic activation of FTY720 by sphingosine kinase-2 and potent activation of vascular endothelial cell functions in vitro and in vivo by phosphorylated FTY720 (FTY720-P). Incubation of endothelial cells with FTY720 resulted in phosphorylation by sphingosine kinase activity and formation of FTY720-P. Sphingosine kinase-2 effectively phosphorylated FTY720 in the human embryonic kidney 293T heterologous expression system. FTY720-P treatment of endothelial cells stimulated extracellular signal-activated kinase and Akt phosphorylation and adherens junction assembly and promoted cell survival. The effects of FTY720-P were inhibited by pertussis toxin, suggesting the requirement for Gz-coupled SIP receptors. Indeed, transmonolayer permeability induced by vascular endothelial cell growth factor was potently reversed by FTY720-P. Furthermore, oral FTY720 administration in mice potently blocked VEGF-induced vascular permeability in vivo. These findings suggest that FTY720 or its analogs may find utility in the therapeutic regulation of vascular permeability, an important process in angiogenesis, inflammation, and pathological conditions such as sepsis, hypoxia, and solid tumor growth.

Sphingosine-1-phosphate (SIP), a multifunctional bioactive lipid mediator, mediates cellular responses by activating G protein-coupled receptors (originally termed as EDG receptors but that has been renamed as SIPn receptors). To date, five SIP receptors have been identified in different cell types, SIPn/EDG-1, SIPn/EDG-5, SIPn/EDG-3, SIPn/EDG-6, and SIPn/EDG-8 (1–7). Interaction of SIP with these receptors regulates a multitude of effects in the vascular system, including endothelial cell proliferation, survival, adherens junction assembly, migration, and morphogenesis (6, 7). In particular, activation of SIP1 and SIP3 receptors plays an important role in survival and morphogenesis in endothelial cells in vitro (8), and SIP1 is necessary for vascular maturation in vivo (9). However, SIP also exerts potent effects on other organ systems, such as the immune system and the reproductive system among others, indicating that it is a multifunctional lipid mediator (6, 7, 10).

SIP is generated by the metabolism of sphingomyelin, an abundant component of biological membranes. Specifically, sphingomyelinase degrades sphingomyelin to ceramide, which is degraded by a ceramidase to sphingosine, the substrate for sphingosine kinase (SK) (11), which adds the phosphoryl group to the 1-hydroxy group of the sphingosine molecule. SIP can be converted to sphingosine by the SIP-phospholysoleucinase (12, 13) or degraded irreversibly by SIP-lyase (14). Most cells exhibit a basal tone of SK activity that is further increased by different extracellular stimuli, such as growth factors, cytokines, and hormones. For example, platelet-derived growth factor (15), tumor necrosis factor (16, 17), vascular endothelial cell growth factor (VEGF) (18), epidermal growth factor (19), and estrogen (20) were all shown to induce the activation of SK activity. Such cellular activation brings about an increase in the intracellular levels of SIP. Similarly, cell activation has also been shown to activate the upstream enzymes of SIP synthesis, namely, ceramidase (21, 22) and sphingomyelinase (23, 24). These studies suggest that agonist-induced formation of SIP may serve a regulatory role in cellular signaling. The central enzyme in the pathway, SK, is encoded by two SK genes encoding SK1 and SK2 isoenzymes (25). However, the physiological role of each of these kinases remains to be elucidated.

Platelets, which exhibit high SK activity and lack the SIP lyase, are a rich source of SIP in vivo (26). Platelets contain stored SIP (27) and release it upon activation, thus contributing to serum SIP levels. Moreover, neutrophils, erythrocytes, endothelial cells, and mononuclear cells also release SIP (28, 29). However, the mechanisms whereby SIP is released are poorly understood. Studies from our laboratory recently showed that endothelial cells actively export the SK1 enzyme into the extracellular milieu, which could explain some of the biological actions of SIP (29). Thus, intracellular and extracellular SIP-generating systems exist and contribute to circulating SIP levels.

A novel pharmacologic modulator of SIP receptors has been described, namely, the immunomodulatory agent, FTY720 (30,
FTY720 elicits lymphocytes in blood and thoracic duct by sequestration of lymphocytes from circulation to secondary lymphoid organs, away from inflamed peripheral tissues and graft sites. FTY720 is phosphorylated in vivo, and the phosphorylated form (FTY720-P) is a potent agonist of S1P1, S1P3, S1P4, and S1P5 receptors (30, 31). Similarly, the analog (R)-AAL (but not the chiral analog (S)-AAL) was also phosphorylated (31). These findings provide a critical clue for the mechanism of action of this potent immunomodulatory agent. However, the cellular and molecular basis of FTY720 action remains to be elucidated. The vascular system contributes greatly to the innate as well as adaptive immunity and in fact plays a critical role in transplant-associated tissue rejection (32). In this report, we show that FTY720 possesses a heretofore unappreciated function as a profound regulator of the vasculature in vivo.

MATERIALS AND METHODS

Reagents—Fatty acid-free bovine serum albumin (BSA), 4,4-deoxyribo- doxine, β-glycerophosphate, and fluorescein isothiocyanate (FITC)-dextran were purchased from Sigma. Sphingosine, N,N-dimethylsphingosine (DMS), and S1P were purchased from Biomol Research Laboratories, Inc. (Plymouth Meeting, PA). 5′-3′-PdATP (specific activity 6,000 Ci/mmol) and [methyl-3H]thymidine (86 Ci/mmol) were from Amersham Biosciences. FTY720, phospho FTY720, (S)-AAL, (R)-AAL, and phospho(R)-AAL were kindly provided by Dr. V. Brinkmann, Novartis. Pertussis toxin was purchased from Calbiochem. Phospho-Akt, Akt, phospho-extracellular signal-regulated kinase 1/2 (ERK1/2), and ERK1/2 antibodies were from Cell Signaling Tech., and VE-cadherin and β-catenin antibodies were from Santa Cruz Biotechnology.

Cloning of SK1 and SK2—Mouse liver RNA (Ambion) was used to amplify the murine SK1 and SK2 cDNA by reverse transcriptase (RT) PCR with the forward primers 5′-AGG GCC ATG TGG TGT TGT TGT TGT-3′ and 5′-ATT ATG GCC CCA CCA CCA CTA CT-3′, respectively, and reverse primers 5′-TGC ACC ACC AAC TGC TTA GC-3′ and 5′-AGG TAC GCC TGG TGC CAT GAG-3′, respectively. The PCR product was cloned in pcDNA3.1 Topo vector (Invitrogen), and the DNA sequence was confirmed. SK1 and SK2 cDNAs were then cloned in pcDNA3.1 eukaryotic expression vector (Invitrogen). Full-length cDNA clones for human SK1 (IMAGE, ID 3851657; BC008040) and SK2 (IMAGE_ID; BC006161) were procured from Protein Tech Group, Chicago, IL. The SK1 and SK2 inserts were released by EcoRI and XhoI digestion, and the resulting 1.8- and 3.0-kbp fragments, corresponding to SK1 and SK2, were subcloned into pcDNA 3.0 mammalian expression vector at EcoRI and XhoI sites.

Reverse Transcriptase and Real Time PCR—Total RNA was isolated from cells using the RNA-Stat 60 reagent (Tel Test). 1 μg of total RNA was treated with RNase-free DNase (Promega, Madison, WI) and reverse-transcribed using random hexamer primers (Invitrogen) and Moloney murine leukemia virus reverse transcriptase (Invitrogen) according to the manufacturer’s instructions. Primers were designed using Primer ExpressTM 2.0 (Applied Biosystems) according to the software guidelines. All primer pairs were designed for a melting temperature of 60°C. Primer sequences were as follows: human glyceraldehyde-3-phosphate dehydrogenase forward (5′-TGC ACC ACC AAC TGC TTA GC-3′) and reverse (5′-AGG ATG TAC GCC TGG CAT GAG-3′), human SK1 forward (5′-CATCAGAACACGCCCCATGTTGTA-3′) and reverse (5′-GTC TTC ATG GGC ATC TCT ATC-3′), human SK2 forward (5′-CAA GCT CAC GAG GGA AGA AAC ACC ACG TCA-3′) and reverse (5′-GCT TCC TCC CAG TCA-3′), mouse SK1 forward (5′-GGA GGA GGA GAT AAC CTT TAA A-3′) and reverse (5′-GAC CCA CTT CTC CAC ACA-3′), mouse SK2 forward (5′-GGT GTG GTG ATG ATG TTA GAT G-3′) and reverse (5′-GCC CAC CCT CTG CAC ACA-3′). Real time PCR was performed using QuantiTect SYBR Green PCR kit (Qiagen) on a LightCycler 2.0 HT Sequencer (Roche Applied Biosystems, Foster City, CA). Each reaction was run in duplicate and contained 5 μl of cDNA, 900 nm primers and 12.5 μl of SYBR Green PCR master mix in a 25-μl final volume. The parameters for PCR were 95°C for 15 s and 60°C for 1 min for 40 cycles and a final dissociation stage of 95°C for 15 s. Melting curves were performed using the SDS2.1 software to confirm the phosphorylated state of the DNA. After optimization of the correct melting temperature was amplified. Quantification of RNA levels was calculated as previously described (33).

Cell Culture and Transfection—Human umbilical vein endothelial cells (HUVEC) (Clonetics, p4–11) were cultured in M199 medium sup-plemented with 10% fetal bovine serum and heparin-stabilized endothelial cell growth factor, as previously described (34). Human embryonic kidney 293T cells in 60-mm dishes were transfected with 4 μg of vector alone or with vectors containing SK constructs by the calcium phosphate method. 24 h after transfection, cells were harvested by scraping at 4°C with 400 μl of 25 mM HEPEPS, pH 7.5, 5 mM MgCl2, 1× protease inhibitor mixture (Calbiochem), and disrupted by brief sonication. Cellular homogenates were centrifuged for 10 min, 4°C at 20,000×g. SK activity was determined as described under “Materials and Methods.”

Cell Migration Assay—HUVEC migration was assayed using a 96-well chemotaxis microchamber (Neuro Probe, Inc.) as previously described (35). Briefly, polycarbonate filters with a pore size of 8 μm were coated with 5 μg/ml fibronectin. S1P, FTY720, AAL, FTY720-P, or BSA was dissolved in 0.1% fatty acid free BSA at a concentration of 25 μM. Triplicate 85 μl of the solutions or conditioned medium from HUVEC were added into the lower chamber. Approximately 5 × 104 cells suspended in 0.1% bovine serum albumin were placed in the upper compartment. The cells were allowed to migrate for 4 h at 37°C in a humidified chamber with 5% CO2. After the incubation period the filter was removed, and cells were stained in 0.1% crystal violet and eluted with 10% acetic acid in 96-well plates. Quantification was done based on absorbance at 575 nm by a Spectramax 340 (Molecular Devices) plate reader.

Activation of FTY720 and (R)-AAL by Endothelial Cells—Cells were washed 3 times with plain medium M199 and incubated with 0.1% fatty acid-free BSA for 10 min. Then they were washed and incubated in 0.1% fatty acid free BSA for the times indicated. After incubation, conditioned medium was removed, centrifuged at 1,000 × g for 10 min, and used for the kinase activity assay. Cells were washed at 4°C with 400 μl of 25 mM HEPEPS, pH 7.5, 5 mM MgCl2, 1× protease inhibitor mixture, and disrupted by brief sonication. Cellular homogenates were centrifuged for 10 min, 4°C at 20,000×g. SK activity was determined as described under “Materials and Methods.”

Analysis of Kinase Activity from Conditioned Media and Cells—Cells were washed 3 times with plain medium M199 and incubated with 0.1% fatty acid-free BSA for the times indicated. After incubation, conditioned medium was removed, centrifuged at 1,000 × g for 10 min, and used for the kinase activity assay. Cells were washed at 4°C with 400 μl of 25 mM HEPEPS, pH 7.5, 5 mM MgCl2, 15 mM NaF, 0.5 mM 4-deoxyribozidoxine, 40 mM β-glycerophosphate, and 300 μl of M199 and were incubated at 37°C for 30 min. Lipids were extracted as previously described (29), and samples were resuspended in 50 μl of chloroform. Lipids were resolved by TLC on silica gel G60 using 1-butanol/acetic acid/water (60/20/20 v/v) buffer and quantified with a PhosphorImager (Molecular Dynamics).

Western Blot Analysis—Confluent cultures of HUVEC cells were serum-starved for 2 h in 0.1% fatty acid-free BSA M199 before treatment. When they were washed and incubated with 5 mM sphingosine or 50 μM FTY720, or AAL, [γ-32P]ATP (10 μCi), 5 mM MgCl2, 15 mM NaF, 0.5 mM 4-deoxyribozidoxine, 40 mM β-glycerophosphate, and 1× protease inhibitor mixture. Samples were centrifuged, 10,000×g for 10 min, and protein concentrations of supernatants were determined by Bradford assay (Bio-Rad protein dye reagent). Equal amounts of protein were separated on a 10% polyacrylamide gel and blotted to a nitrocellulose membrane. Immunoblot analysis was performed using phospho-Akt, Akt, phosphoERK1/2, or ERK1/2 antibodies.

Apoptosis—HUVEC cells were labeled with [methyl-3H]thymidine (1 μCi/ml) for 18 h. Then they were washed, and 0.1% BSA M199 containing the different treatments was added. After 8 h, fragmented DNA was solubilized and quantified as described (36).

Immunofluorescence Analysis—2 × 105 cells were plated in 35-mm glass-bottom Petri dishes. Three days later, cells were washed and serum-starved for 3 h before the experiment. Then cells were washed with ice-cold phosphate buffered saline and fixed, and immunofluorescence analysis with VE-cadherin and β-catenin antibody (1 μg/ml; Santa Cruz) was conducted. Antibody staining was visualized with Alexa Fluor 488 donkey anti-goat (1:1000) IgG (Molecular Probes). Confocal microscopy was conducted on a Zeiss LSM 510 laser-scanning confocal microscope at the Center for Biomedical Imaging at the University of Connecticut Health Center. Fluorescence images were acquired using a 488-nm argon laser, and emitted fluorescence was detected with a 505-nm long-pass filter.

VEGF-induced Trans-cellular Permeability in Vivo—Mouse embryonic endothelial cells (5 × 104) were cultured for 2 days in Transwell
polycarbonate filters (6.5-mm diameter, 0.4-μm pores; Costar). Culture medium was replaced with serum/phenol red-free Dulbecco’s modified Eagle’s medium (0.1-ml upper chamber and 0.6-ml lower chamber). Cells were pretreated with FTY720, derivatives, or S1P for 1 h. FITC-dextran (average $M_w$ = 2000 kDa) was added to the upper compartment either in the absence or presence of murine recombinant VEGF (50 ng/ml). Media from lower wells were taken after the indicated time periods (5–45 min). Samples were placed in black solid-bottom 96-well plates (Costar), and fluorescence intensities were measured using a CytoFluor(R) fluorescence multi-well plate reader (Applied Biosystems) at 488-nm excitation.

**VEGF-induced Microvascular Permeability**—Normal FVB/N female or male mice were treated by gavage with FTY720 (50 μg), (S)-AAL enantiomer (50 μg), (R)-AAL enantiomer (50 μg) and water as the vehicle control. Five hours post-gavage animals were anesthetized with 2% Avertin® (0.5 cc/20 g) and infused through the tail vein with 100 μl of FITC-dextran (5 mg/ml, 165 KDa). Animals were placed on a warming table under a fluorescent dissecting microscope (Zeiss STV11, Zeiss, Inc.), and the central vessels in the ear were imaged. Control saline or mouse vascular endothelial growth factor (VEGF, 10 ng/ml) was injected subdermally into the middle ear using a 30-gauge needle (30 μl). Ear vasculature was then imaged with fixed exposure times in a stationary position from 5 to 120 min post-injection. Fluorescence images were then quantified using a pixel-based threshold in ImageProPlus (Media Cybernetics, Inc.) and quantified.

**Transmission Electron Microscopy of the Vasculature**—Animals pretreated by gavage with water, FTY, or (S)-AAL for 5 h were used to inoculate the ear subcutaneously with mouse VEGF (10 ng/ml, 50 μl) while under anesthesia. Five minutes post-injection, the ears were removed and fixed in 2% glutaraldehyde/sodium cacodylate buffer, trimmed to isolate the injection site, and processed for osmium tetroxide fixation. After postfixation, tissues were stained with uranyl acetate (0.5% in H2O), serially dehydrated with ethanol, and embedded in Polybed (Polysciences, Warning, PA). Analysis of the cross-section was performed by transmission electron microscopy on a Philips CM10. Sections (70–90-nm thick) were obtained, and vessels adjacent to the ear cartilage were photographed at various magnifications (8).

**Statistical Analysis**—All experiments were performed two to five times, and a representative experiment is shown. In migration and apoptosis experiments results represent mean values of triplicates. $p$ values were calculated by Student’s t test using Microsoft Excel software.

**RESULTS**

**Phosphorylation of FTY720 by Sphingosine Kinases in Vascular Endothelial Cells**—Because S1P is a potent inducer of
endothelial cell chemotaxis, we tested the effect of FTY720 and its analogues on endothelial cell migration. Neither FTY720 nor its structural analog (R)-AAL (Fig. 1a) induced endothelial cell migration in a wide concentration range (Fig. 1b). However, when FTY720 or (R)-AAL was incubated with HUVEC for 3 h, they were activated into potent chemoattractants for endothelial cells. This conditioned medium-induced migration was dose-dependent and pertussis toxin-sensitive, indicating the involvement of a Gt-coupled receptor. In contrast, the enantiomer (S)-AAL, which cannot be phosphorylated by SK (31), did not induce migration after incubation with endothelial cells. These data are consistent with the notion that endothelial cells phosphorylate and activate FTY720 and (R)-AAL into FTY720-P and (R)-APD, respectively, which are both potent chemoattractants for endothelial cells. Indeed we detected the presence of the SK1 and -2 transcripts in HUVEC cells by RT-PCR (Fig. 1c). We also tested the effect of DMS, which is a competitive inhibitor of the SK enzyme (25, 37, 38). DMS treatment (10 μM) potently inhibited the ability of endothelial cells to activate FTY720 and (R)-AAL (Fig. 1d). These results suggest that the endothelial cell-derived SK phosphorylates and activates both FTY720 and (R)-AAL in a stereospecific manner.

To confirm that FTY720 and (R)-AAL are indeed phosphorylated by endothelial cells, we performed an in vitro kinase assay with both total cell extract and conditioned medium from HUVEC. FTY720 and (R)-AAL were phosphorylated by the kinase activity present in HUVEC cytosol (Fig. 2a, top panel, specific activity 3.85 ± 0.75 and 15 ± 0.93 pmol/mg/min, respectively). As expected, sphingosine was an efficient substrate for this kinase activity (specific activity 75.7 ± 6 pmol/mg/min). In sharp contrast, (S)-AAL phosphorylation was undetectable, in agreement with the results obtained with the migration assay. Phosphorylation of both FTY720 and (R)-AAL was inhibited by DMS (50 μM). Similarly, we also detected this kinase activity in the conditioned medium from HUVEC, although this activity was much lower than the cytosolic extracts (Fig. 2a, bottom panel), suggesting that both intracellular and extracellular SK enzyme systems are capable of phosphorylating FTY720 and its analog, (R)-AAL. Interestingly, this phosphorylation was inhibited by Triton X-100 and increased by KCl (200 mM) (data not shown), which are conditions that favor the enzymatic activity of the SK2 isoenzyme (25, 37).

To definitively determine the role of SK isoenzymes in the metabolism of FTY720 and (R)-AAL, we utilized the transiently
transfected human embryonic kidney 293T cells expressing human and mouse SK1 and SK2 enzymes. Transcripts for the respective human and mouse SK isoenzymes were detected at high levels upon transfection (Fig. 2b). Endogenous sphingosine kinase activity was detected at modest levels in vector-transfected 293T cells. mSK1 transfection did not influence the phosphorylation of FTY720 or (R)-AAL kinase activity compared with vector-transfected 293T cells (Fig. 2c, left panel). However, sphingosine phosphorylation was significantly increased in mSK1–293T cells (5.8 ± 1.4-fold induction, specific activity 130.56 ± 44.3 pmol/min/mg). In sharp contrast, mSK2-transfected cells showed significant phosphorylation of FTY720 and (R)-AAL. The specific activity in mSK2–293T was 42.3 ± 15.9, 143.5 ± 45.2, and 193.7 ± 64.2 pmol/min/mg for FTY720, (R)-AAL, and sphingosine, respectively. (S)-AAL was not phosphorylated significantly by any of the transfected cells. Phosphorylation of FTY720, (R)-AAL, and sphingosine was markedly inhibited by DMS (50 μM). Similar to the mouse enzymes, transfection of human SK1 and SK2 also strongly stimulated sphingosine kinase activity (Fig. 2c, right panel) (3.1 ± 0.013 and 0.72 ± 0.064 nmol/mg/min, respectively); however, only the human SK2 was capable of phosphorylating PTY720 and (R)-AAL. Although hSK1 phosphorylated (R)-AAL, hSK2 did it more efficiently. These results indicate that the SK2 isoform is capable of phosphorylating FTY720 into FTY720-P, the S1P receptor agonist. Moreover, our results indicate that HUVEC contain the requisite enzyme systems for the phosphorylation of FTY720 and (R)-AAL.

**Effects of FTY720-P on Endothelial Cell Responses**—We next studied the effects of FTY720-P and its analog (R)-AFD (Fig. 1a) in HUVEC. Both FTY720-P and (R)-AFD were potent chemotaxtants for HUVEC (Fig. 3a). Both had their maximum effect at 10 nM (13- and 9-fold induction, respectively). FTY720-P and (R)-AFD-induced migration was blocked by per-
The time course of this phosphorylation was similar for FTY720-P and (R)-AFD at the indicated concentrations for 8 h. The percentage of fragmented DNA is shown. Data are the mean ± S.E. of triplicate values. *p < 0.01 versus non-treated. b, pertussis toxin (PTx) treatment abolishes the protective effect of S1P, FTY720-P, and (R)-AFD on the prevention of apoptosis.

Fig. 4. FTY720-P and (R)-AFD protect endothelial cells from apoptosis. a, S1P, FTY720-P, and (R)-AFD prevent serum deprivation-induced apoptosis in HUVEC in a dose-dependent manner. After 

\[ \text{meth-y}-\text{H} \] thymidine incorporation, cells were incubated with plain medium plus 0.1% BSA in the presence or absence of S1P, FTY720-P (FTY-P), or (R)-AFD or complete growth medium (CGM) at the indicated concentrations for 8 h. The percentage of fragmented DNA is shown. Data are the mean ± S.E. of triplicate values. *p < 0.01 versus non-treated.

FTY720-P modulates Adherens Junction Assembly and Vascular Permeability—We next evaluated the effect of FTY720-P and (R)-AFD on VE-cadherin assembly into adherens junctions, critical subcellular structures that are formed by homotypic adhesion of cadherin molecules from adjacent cells. It is well established that cadherins bind to their intracellular partners, the β- and α-catenins, which facilitate the anchoring of cadherin complexes to the actin cytoskeleton. Indeed, proper formation of adherens junctions is critical for endothelial cell morphogenesis into tubular structures and in established vessels for the proper regulation of vascular permeability (42, 43). We previously showed that S1P receptor signaling is critical in assembling adherens junctions (8).

Treatment of HUVEC with FTY720-P or (R)-AFD for 30 min resulted in a dramatic increase in VE-cadherin localization at the cell-cell contact sites (Fig. 5a). This effect was comparable with that observed for S1P. However, the non-phosphorylated precursors FTY and (R)-AAL did not induce VE-cadherin translocation to the adherens junctions. Similarly, the localization of the cytosolic partner, β-catenin, also paralleled the changes of VE-cadherin (Fig. 5b). These results suggest that FTY720-P and (R)-AFD act on vascular S1P receptors to induce adherens junction assembly in endothelial cells.

Several studies showed that VE-cadherin is an important determinant of vascular permeability (42–44). Indeed, VEGF-induced tyrosine phosphorylation of VE-cadherin resulted in increased vascular permeability in vitro (44). Blocking antibodies to VE-cadherin induced vascular leakage-like syndrome in mice (42). S1P is a unique factor in that it induces adherens junction assembly in endothelial cells and, thus, inhibits vascular permeability in monolayers of cultured endothelial cells in vitro (58).

We next used confluent monolayers of mouse embryonic endothelial cells to assess the effects of S1P, FTY720-P, and (R)-AFD on transmonolayer permeability in vitro. Confluent monolayers of mouse embryonic endothelial cells were cultured in Transwell filters and treated with VEGF (50 ng/ml) to induce transmonolayer permeability. The permeability of the monolayer to FITC-dextran (2000 kDa) was determined by measuring the fluorescence intensity of the medium in the lower chamber at different times. Without VEGF, the fluorescence in the lower compartment increased slightly over time (Fig. 6a). However, when the cells were treated with VEGF, a significant increase in the fluorescence in the lower compartment was observed, which was profoundly inhibited by S1P, FTY720-P, and (R)-AFD (Fig. 6b). These results indicate that VEGF-induced transcellular permeability is antagonized by the S1P receptor agonists FTY720-P and (R)-AFD.

Next we tested the role of plasma borne FTY720-P or (R)-AFD in the regulation of vascular permeability in vivo. Vascu-
lar permeability was assessed by injecting FITC-dextran in the tail vein followed by subcutaneous injection of VEGF in the mouse ear. Permeability-induced extravasation of the circulating FITC-dextran was evaluated over time by fluorescence imaging of the juxta-vessel structures at the injection site. FTY720 oral administration profoundly suppressed vascular permeability induced by VEGF (Fig. 7). (R)-AAL but not (S)-AAL also potently inhibited vascular permeability in vivo. These data suggest that phosphorylation of FTY-720 and (R)-AAL by sphingosine kinase in vivo and activation of S1P receptors on endothelial cells results in the inhibition of VEGF-induced vascular permeability. Transmission electron microscopy of vasculature in the ear tissue is shown in Fig. 8. VEGF treatment induced inter-endothelial cell spaces and reduced adherens junctions. In addition, the endothelial cell cytosol was swollen, and numerous cytoplasmic vesicular structures were observed throughout the cell. However, when FTY720 was administered, VEGF-induced inter-endothelial cell spaces and cell swelling were reduced, and increased quantities of adherens junctions were seen. Interestingly, VEGF-induced vesicular structures were still present; however, abundant caveolae-like, incompletely budded structures were observed. (S)-AAL administration, which is the inactive enantiomer in the phosphorylation reaction, was not able to inhibit VEGF-induced vascular changes at the ultrastructural level. These data support the concept that FTY-720-P action on endothelial S1P receptors induce adherens junctions to raise the threshold for VEGF to induce massive vascular permeability.

DISCUSSION

FTY720, originally derived from the fungal natural product ISP-1 by medicinal chemistry efforts, is a potent immunomodulator (45). Recent work shows that it sequesters T and B cells in peripheral lymphoid organs and Peyer’s patches (46). The efficacy of FTY720 has been demonstrated in animal models of organ transplantation and, therefore, is being tested in clinical trials for controlling kidney transplant rejection (46–49). Recent studies showed that FTY720 is phosphorylated into FTY720-P, a potent agonist for four of five S1P receptors (30, 31). The molecular basis of how FTY720 modulates various stages of lymphocyte trafficking, namely, homing, intravasation, and egress is not well understood. It is known that lymphocyte trafficking involves intimate interaction of blood-borne cells with vascular elements during the processes of intravasation and extravasation (50). Because S1P is a potent vasoactive mediator and a regulator of vascular development, we investigated the interaction of the FTY720 with the vascular cells.

A major finding of this work is that vascular endothelial cells contain enzyme systems to “activate” FTY720. Upon incubation...
with HUVEC-conditioned medium or cell extracts, FTY720 is phosphorylated and able to activate the endothelial cells to migrate in a pertussis toxin-sensitive manner, suggesting that it activates the G\textsubscript{i}-coupled S1P receptors (35). Our data also show that HUVEC express the mRNA for both SK1 and -2 isoenzymes. Moreover, in the ectopic expression system of human embryonic kidney 293T cells, only SK2 efficiently phosphorylated the FTY720 and the (R)-AAL analog. These data strongly suggest that SK2 is involved in the metabolism of FTY720 into FTY720-P. Although FTY720 is phosphorylated in a whole blood assay (30), the responsible SK enzyme isoform and its cellular source were not investigated. Although our data do not assess the cellular source of the SK2 enzyme in vivo, it suggests the possibility that FTY720 could be phosphorylated by the SK2 enzyme in specific vascular microenvironments, such as the sinus-lining endothelium. Although a previous study suggested that recombinant mouse SK1 can phosphorylate FTY720, albeit weakly (31), data in this report clearly show that SK2 is more efficient than SK1 in this reaction. However, it is important to point out that phosphorylation of FTY720 is less efficient than that of the natural substrate sphingosine. In addition, there may be additional lipid kinases that participate in the metabolism of FTY720. In the future, studies in SK1 and -2 knock-out mice will be needed to definitively determine the role of these isoenzymes in the metabolism of FTY720.

Another important conclusion that can be drawn from the present studies is that FTY720-P and its analog (R)-AFD activates the vascular S1P receptors and stimulates signaling pathways such as Akt and ERK. These events are important in the inhibition of endothelial cell apoptosis, which occurs in various pathological conditions when the vascular system is stressed. For example, in transplant vasculopathy, autoimmune-mediated vascular diseases, and systemic infectious diseases such as sepsis and Dengue hemorrhagic fever, endothelial cell apoptosis contributes to multisystem organ failure (51–53). Thus, the therapeutic potential of FTY720 as endothelial cell protective agents should be further investigated in the context of pathological conditions mentioned above.

Another physiologically relevant effect of S1P receptor agonists is the induction of adherens junction assembly in endothelial cells (8, 58). We have shown that S1P, FTY720-P, and (R)-AFD stimulates VE-cadherin and \(\beta\)-catenin translocation and assembly into cell-cell junctions, which may have important mechanistic implications. For example, tightening of cell-cell junctions at the sinus-lining endothelium induced by FTY720 administration could prevent egress of lymphocytes into the lymphatic sinus. In fact, FTY720-P inhibited chemo-kine-induced migration of T lymphocytes through an endothelial cell monolayer in vitro.\(^2\)

In addition, cell-cell junction molecules are an important target of mediators that regulate vascular permeability and angiogenesis (8, 43). We have also shown here that S1P,  

\(^2\) V. Brinkmann, unpublished observations.
FTY720-P, and (R)-AFD potently block VEGF-induced transcellular permeability in vitro and VEGF-induced vascular permeability in vivo. It is well accepted that VEGF is induced by tissue hypoxia, which occurs during solid tumor growth (54). In addition, VEGF treatment induces systemic vascular permeability (54). In fact, VEGF-induced vascular permeability may be critical for its angiogenic action, as it provides a provisional fibrin matrix essential for endothelial cell sprouting and migration. Our findings strongly suggest that FTY720 may have utility in pathologic conditions in which enhanced VEGF levels contribute to excessive vascular permeability. Further studies are needed to determine the role of S1P receptor signaling systems in various phases of angiogenesis.

The mechanistic findings described in this report warrant further investigations into the potential utility of FTY720 in a number of pathologic conditions. Thus, FTY720-P may inhibit vascular permeability in acute vasculopathic situations, such as in respiratory distress syndrome, which occurs as a complication of sepsis. Similarly, during acute infections, such as in Dengue hemorrhagic fever, uncontrolled vascular permeability leads to multiorgan failure (53, 55). In hematologic disorders such as thrombocytopenias, endothelial injury, increased vascular permeability, and tissue edema are prevalent (55, 56). In addition, chronic changes in dysregulated vascular permeability are important in peripheral vascular disorders associated with diabetes (57). Further work should investigate the role of S1P receptor agonism in the control of such conditions.

In conclusion, our data indicate that the potent immunomodulator FTY720 is phosphorylated by the SK2 isoenzyme and acts on S1P receptors to maintain the integrity and functionality of endothelial cells. These findings reveal a novel mechanism of action of FTY720 and its analogs in transplantation medicine. Equally important, potent vascular effects of S1P receptor agonists argue for their potential utility in vascular permeability disorders.

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