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The Immunosuppressant FTY720 Inhibits Tumor Angiogenesis Via the Sphingosine 1-Phosphate Receptor 1

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Abstract FTY720, a sphingosine 1-phosphate (S1P) analog, acts as an immunosuppressant through trapping of T cells in secondary lymphoid tissues. FTY720 was also shown to prevent tumor growth and to inhibit vascular permeability. The MTT proliferation assay illustrated that endothelial cells are more susceptible to the anti-proliferative effect of FTY720 than Lewis lung carcinoma (LLC1) cells. In a spheroid angiogenesis model, FTY720 potently inhibited the sprouting activity of VEGF-A-stimulated endothelial cells even at concentrations that apparently had no anti-proliferative effect. Mechanistically, the anti-angiogenic effect of the general S1P receptor agonist FTY720 was mimicked by the specific $S1P_1$ receptor agonist SEW2871. Moreover, the anti-angiogenic effect of FTY720 was abrogated in the presence of CXCR4-neutralizing antibodies. This indicates that the effect was at least in part mediated by the $S1P_1$ receptor and involved transactivation of the CXCR4 chemokine receptor. Additionally, we could illustrate in a coculture spheroid model, employing endothelial and smooth muscle cells (SMCs), that the latter confer a strong protective effect regarding the action of FTY720 upon the endothelial cells. In a subcutaneous LLC1 tumor model, the anti-angiogenic capacity translated into a reduced tumor size in syngeneic C57BL/6 mice. Consistently, in the Matrigel™ plug in vivo assay, 10 mg/kg/d FTY720 resulted in a strong inhibition of angiogenesis as demonstrated by a reduced capillary density. Thus, in organ transplant patients, FTY720 may prove efficacious in preventing graft rejection as well as tumor development. J. Cell. Biochem. 101: 259–270, 2007. © 2007 Wiley-Liss, Inc.

Key words: angiogenesis; FTY720; CXCR4; sphingosine 1-phosphate; immunosuppression; SEW2871; tumor growth

After decades of successful organ transplantation, de novo malignancies are now emerging as a significant cause of mortality, following organ transplantation. Specifically, the incidence of malignancies in kidney transplant recipients was significantly greater than that of age, sex, and race matched non-transplant patients [Sheil et al., 1993]. More recently, the United States Renal Data System (USRDS) reported that 35,765 primary kidney transplant recipients experienced a 3.3%, 5.5%, and 7.5% 1-, 2-, and 3-year non-skin malignancy rate, respectively [Kasiske et al., 2004]. The gravity of the post-transplantation malignancies is further underscored by data from 834 liver recipients, in whom 24% of deaths occurring after 1 year were caused by non-lymphoid de novo malignancies [Jain et al., 2000]. A similar report on cardiac transplant recipients revealed that 21% of deaths occurring after 2 years were due to neoplastic lesions [Gallo et al., 1997], indicating that the increased rate of de novo malignancies is not restricted to certain organs rather than constituting a general phenomenon.

In subsequent analyses, immunosuppressive drug therapy has been identified as one important etiological factor for the increased incidence of deaths from malignancies in transplant recipients. The higher incidence of post-transplant de novo malignancies was

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particularly prominent in renal transplant recipients, obtaining a standard dosage regimen of the calcineurin inhibitor cyclosporine A (CsA), as compared to patients on a low-dosage regimen [Dantal et al., 1998]. Consistent with experimental findings that CsA promotes tumor growth through its pro-angiogenic activity [Guba et al., 2002], the cumulative dosage of CsA was an independent factor driving the recurrence rate of hepatocellular carcinoma, following liver transplantation [Vivarelli et al., 2002]. Therefore, there is an emerging quest for developing immunosuppressive regimens that include drugs preferentially inhibiting tumorigenesis. In this respect, preliminary data indicate that the immunosuppressive agent FTY720, a synthetic myriocin (ISP-1) analog [Fujita et al., 1994], structurally also related to sphingosine 1-phosphate (S1P), may inhibit tumor growth [Tanaka et al., 2002; Lee et al., 2004a,b; Ho et al., 2005; Schmid et al., 2005]. After in vivo administration, FTY720 is readily phosphorylated [Brinkmann et al., 2002; Mandala et al., 2002], predominantly by sphingosine kinase-2 [Billich et al., 2003; Zemann et al., 2006]. Additionally, it was shown that ex vivo in murine and human blood [Mandala et al., 2002], in rodent lymphoid tissue [Brinkmann et al., 2002], and in human endothelial cells [Billich et al., 2003], FTY720 is converted to FTY720-P. This is highly important because FTY720 only represents a prodrug concerning its activity toward the S1P receptors and solely FTY720-P is able to bind the receptors [Brinkmann et al., 2002]. FTY720-P inactivates the $S1P_1$ receptor [Matloubian et al., 2004] and inhibits the immune response at low nanomolar concentrations, whereas it induces growth inhibition and apoptosis in several human cancer cells in vitro at micromolar levels. However, previous studies suggest that apoptosis induced by FTY720 may not be related to inactivation of the $S1P_1$ receptor because the two FTY720 enantiomers, NVP-AAL151 and NVP-AAL149, are both able to induce apoptosis in vitro, but only NVP-AAL151 inactivates the $S1P_1$ receptor [LaMontagne et al., 2006], and is active in vivo in transplant and autoimmune models. It is possible that FTY720, at high concentrations, acts as an intracellular second messenger and mimics sphingosine and ceramide, which both induce apoptosis independently of S1P receptors. To date, however, the exact molecular mechanisms of the anti-tumorigenic effects of FTY720 are undefined.

Since angiogenesis is a critical step in tumor progression and metastasis, we investigated FTY720 regarding its anti-angiogenic properties and the involved mechanisms in vitro and in vivo. Recently, a transactivation of the chemokine receptor CXCR4 (*fusin*) by S1P receptors has been described [Kimura et al., 2004]. Interestingly, CXCR4 by itself has been shown to be involved in the activation process of angiogenesis. Furthermore, experiments using $S1P_1$-deficient mice have shown that animals die in utero due to hemorrhage, as a result of insufficient vascular maturation, indicating that $S1P_1$ receptors are required for vascular development and regulation of angiogenesis [Liu et al., 2000]. Here, we hypothesize that FTY720-P has anti-tumorigenic properties that involve anti-angiogenic effects, which are mediated through the $S1P_1$-CXCR4 signaling pathway.

MATERIALS AND METHODS

Cells and Reagents

Human umbilical vein endothelial cells (HUVECs), smooth muscle cells (SMCs), and endothelial cell growth medium (ECGM) were purchased from Promocell (Heidelberg, Germany). In the assays, only HUVECs from passage 4 to 6 and SMCs from passage 4 to 8 were used to ensure an equal growth potential in all experiments. FTY720 was a kind gift from Novartis (Basel, Switzerland). A 10 mM stock solution was prepared in water and stored at 20°C. For in vitro experiments, the drug was diluted 1:10 in water and serial dilutions were made in the appropriate cell culture media. For in vivo experiments, serial dilutions were prepared in PBS. The specific $S1P_1$ agonist SEW2871 was obtained from Maybridge (Trevillet, Tintagel, Cornwall, UK) and was used in the spheroid angiogenesis assay. A stock solution was prepared in DMSO (3 mM) and subsequent dilutions were done in the appropriate cell culture media.

Spheroid In Vitro Angiogenesis Assay

To evaluate the anti-angiogenic properties of FTY720, we used an in vitro angiogenesis assay as described in detail, previously [Korff and Augustin, 1998]. Briefly, 10^3 HUVECs

(passages 4–6) per spheroid were plated into a non-adhesive, round bottom 96-well plate. In the case of coculture spheroids, 10^3 HUVECs (passages 4–6) together with 10^3 SMCs (passages 4–8) were plated into a round-bottom 96-well plate. After 24 h, the spheroids were harvested and half the spheroids of a 96-well plate (approximately 48 spheroids) were embedded in 1 ml of a collagen matrix and transferred into a 24-well plate. For the next 24 h, we incubated the embedded spheroids with the allocated treatment and thereafter, the spheroids were fixed in a 4% formalin solution to prepare them for the subsequent analysis. Spheroid sprouting was stimulated with human recombinant VEGF-A_{165} (20 ng/ml; R&D Systems, Wiesbaden, Germany) in the presence of FTY720 or SEW2871. Additionally, in the experiments including FTY720, a CXCR4 (*fusin*) neutralizing antibody (clone 12G5; R&D Systems) was used and as a control, we applied a mouse IgG_{2A} isotype antibody (R&D Systems). Coculture spheroids were stimulated with VEGF-A_{165} (20 ng/ml; R&D Systems) and with angiopoietin-2 (50 ng/ml; R&D Systems). We measured the cumulative sprout length per spheroid as the specific endothelial angiogenesis readout using an Axiovert 40 fluorescence microscope (Zeiss, Oberkochen, Germany), the AxioCam MRm digital camera (Zeiss), and the AxioVision 4.4 software (Zeiss). The pictures of the spheroids were taken under transillumination using an Achroplan objective (n.a. = 0.25), providing a 10-fold magnification (Zeiss). To calculate the mean cumulative sprout length, 10 spheroids per group were analyzed.

Cell Proliferation Assay

The TACS MTT (3-[4,5-dimethyl(thiazol-2-yl)]-3,5-diphenyltetrazolium bromide) cell proliferation and viability assay (R&D Systems) was used for the assessment of the effect of FTY720 on cell proliferation. The MTT assay was performed as outlined in the manufacturer's guidelines. A total of 4,000 HUVECs, SMCs, or LLC1 tumor cells were plated in each well of a 96-well microtiter plate, and after attachment to the surface they were incubated with increasing concentrations of FTY720 (ranging from 1 nM to 100 μM). After 72 h, absorbance at 570 nm was measured as a marker for metabolizing cells in an ELISA plate reader.

Murine Matrigel™ Plug Assay

To determine the effect of FTY720 on endothelial sprouting in vivo, a Matrigel™ plug assay was carried out as described in detail, previously [Kragh et al., 2003]. Mice were put under general anesthesia with an i.p. injection of a mixture of ketamine and xylazine at a final concentration of 100 and 10 mg/kg, respectively. An area around the left hip was shaved and 500 μl Matrigel™ (Becton Dickinson), supplied with VEGF-A (100 ng/ml; R&D Systems) and heparin (Braun, Melsungen, Germany) were injected subcutaneously in the left inguinal area. After 14 days, mice were euthanized for harvesting of the plug, followed by embedding in paraffin and preparation of 5 μm sections. Blood vessels were identified by immunohistochemistry using monoclonal, FITC-labeled antibodies against murine CD31 (Becton Dickinson). Four matrigel plugs per group (FTY720 and control) were used for analysis and five images per section were taken. The images covered areas from the whole section (center and border) and fluorescent blood vessels were quantified in a non-biased way. Sections were analyzed using confocal microscopy (LSM 510; Zeiss).

Subcutaneous LLC1 Tumor Model

C57BL/6 mice were ordered from the Charles River Laboratories (Sulzfeld, Germany). Animals were kept, fed, and treated according to the guidelines of the local animal care committee and according to federal laws. Lewis lung carcinoma (LLC1) cells were obtained from ATCC (Manassas, VA) and RPMI cell culture medium was acquired from GIBCO BRL (Eggenstein, Germany). LLC1 cells were cultivated in NUNClonCell T-75 or T-175 flasks (NUNC, Roskilde, Denmark) and generally used between passage 15 and 30. Fetal calf serum (FCS) was purchased from Biochrom (Berlin, Germany).

LLC1 cells (8×10^5 cells/mouse) were subcutaneously injected into the mid-dorsal region of syngeneic C57BL/6 mice. On day 6 following tumor cell injection, mice were randomized for daily i.p. injection of the drug (0, 1, 5, or 10 mg/kg), and the tumor volume was measured three times a week using a calliper. The tumor volume was calculated as the product of $\text{length}^2 \times \text{width} \times 0.25$. Twenty days after tumor cell injection, animals were sacrificed

for histological evaluation of the tumor. Tumor vessels were identified by immunohistochemistry using antibodies against Von Willebrand Factor (ab6994; Abcam, Cambridge, UK), a secondary goat anti-rabbit biotinylated antibody (Vector Laboratories, Burlingame, CA), and fluorescein-conjugated streptavidin (Jackson Laboratories, Lexington, Kentucky). Tumors from five mice per group were isolated for quantification and three random fields were analyzed per tumor. Since the central areas were necrotic in all tumor samples, the peripheral regions were used for this purpose. Sections were analyzed using an Axiovert 40 fluorescence microscope and a 20-fold magnification (n.a. = 0.45).

Statistical Analysis

Results for continuous variables are expressed as mean ± SD. Treatment groups were compared with the independent samples t test. In the case of non-normal distribution, the Mann–Whitney U test was used. Pairwise multiple comparisons were performed with the t test (two-sided) with LSD adjustment. P values <0.05 were considered statistically significant. All analyses were performed with SPSS 11.5 (SPSS, Inc., Chicago, IL).

RESULTS

FTY720 Inhibits Endothelial Cell Proliferation In Vitro

Tumor growth, in general, can be inhibited through various mechanisms. To elucidate the potential interactions through which FTY720 prevents tumor growth and reduces the number of metastases, we employed the MTT proliferation and viability assay to measure cell growth, in the presence of increasing amounts of FTY720 (Fig. 1). Repeated assays (n = 3) indicated that FTY720 had an IC_{50} between 1 and 2 μM, when used for the treatment of LLC1 cells. These data are consistent with results in which FTY720 has been used for the treatment of other tumor cell lines [Chua et al., 2005]. To further explore direct anti-angiogenic properties of FTY720, we performed the MTT assay using HUVECs and SMCs. HUVECs were used to examine direct effects on the endothelium, SMCs were used because in later experiments, SMCs revealed to have a protective effect on endothelial cells in the coculture experiments. In the MTT assay (n = 3) endothelial cells appeared to be more susceptible to the antiproliferative effects of FTY720, as compared to LLC1 tumor cells. However, SMCs were even less susceptible to FTY720 exposure (Fig. 1). Although the differences in the proliferation of

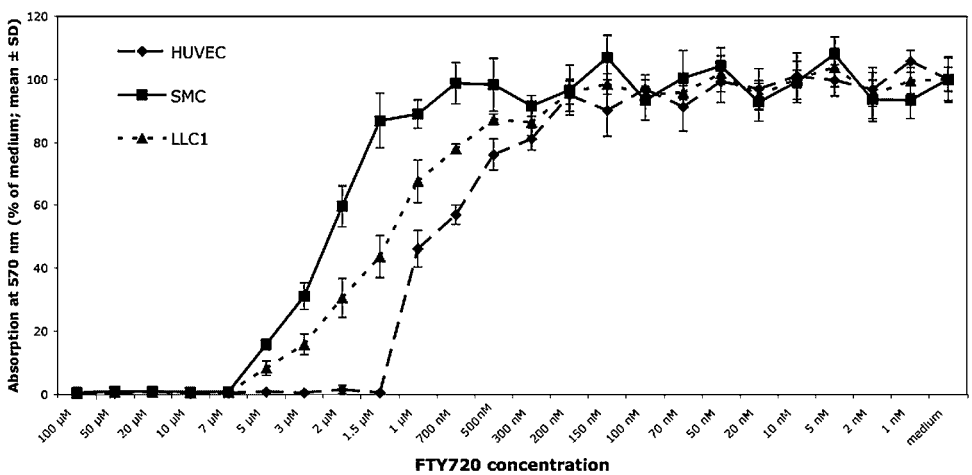

Fig. 1. MTT assay illustrating the proliferation of LLC1 tumor cells, HUVECs, and SMCs. The OD_{570} values are standardized to 100% medium-only controls. Cells were seeded into 96-well plates and after attachment of the cells to the surface, treatment with FTY720 started. After 72 h, the assay was stopped and the amount of viable cells was assessed by measuring the optical density at 570 nm in an ELISA reader. The figure shows the pooled results of three independent experiments with n = 4 per concentration and experiment. Treatment of cells with increasing concentrations of FTY720 showed that SMCs are less sensitive than LLC1 tumor cells, and endothelial cells are most sensitive to the drug.

the different cell types were not huge, these results suggest that the anti-angiogenic properties of FTY720 may also be mediated through an effect on the endothelial cells, in addition to the direct inhibition of tumor cells.

FTY720 Inhibits Endothelial Sprouting In Vitro

To investigate the complex process of angiogenesis in vitro, we have used a spheroid angiogenesis model. This model allows to assess the ability of FTY720 in modulating endothelial cells in their response to angiogenic stimuli. Interestingly, FTY720 inhibited sprouting of HUVEC at far lower concentrations as suggested by the MTT proliferation assay (inhibition in the sprouting assay 0.03 vs. 500 nM in the MTT assay). Even sub-nanomolar concentrations of FTY720 markedly inhibited the stimulatory effect of 20 ng/ml VEGF-A on endothelial sprouting (Fig. 2). At concentrations higher than the IC_{50}, sprouting of endothelial cell spheroids was completely prevented. Most importantly, the sprouting of coculture spheroids, consisting of a core of SMCs and a surface layer of HUVECs, showed an IC_{50} above 2 μM (Fig. 3), indicating that the SMCs are able to stabilize the endothelial cells and protect them from inhibitory effects of FTY720, confirming earlier findings on the stabilizing function of SMCs on angiogenesis in coculture spheroids [Korff et al., 2001]. Due to the stabilizing effect of the SMCs, it was necessary to induce the sprouting of coculture spheroids not only with VEGF-A, but with a combination of VEGF-A and angiopoietin-2 (Ang-2), as suggested earlier [Korff et al., 2001]. Ang-2 reverses the stabilizing effect of Ang-1 that is produced by SMCs and acts in a paracrine mode on HUVECs [Korff et al., 2001].

As extensively described, FTY720-P has agonistic properties towards four out of five S1P receptors (all but receptor 2). To prove our hypothesis that FTY720-P indeed mediates its anti-angiogenic effect through the $S1P_1$ receptor, we performed a set of experiments using the $S1P_1$-specific receptor agonist SEW2871 which is only capable of deploying its agonistic potential to the S1P receptor 1. Interestingly, SEW2871 inhibited vascular

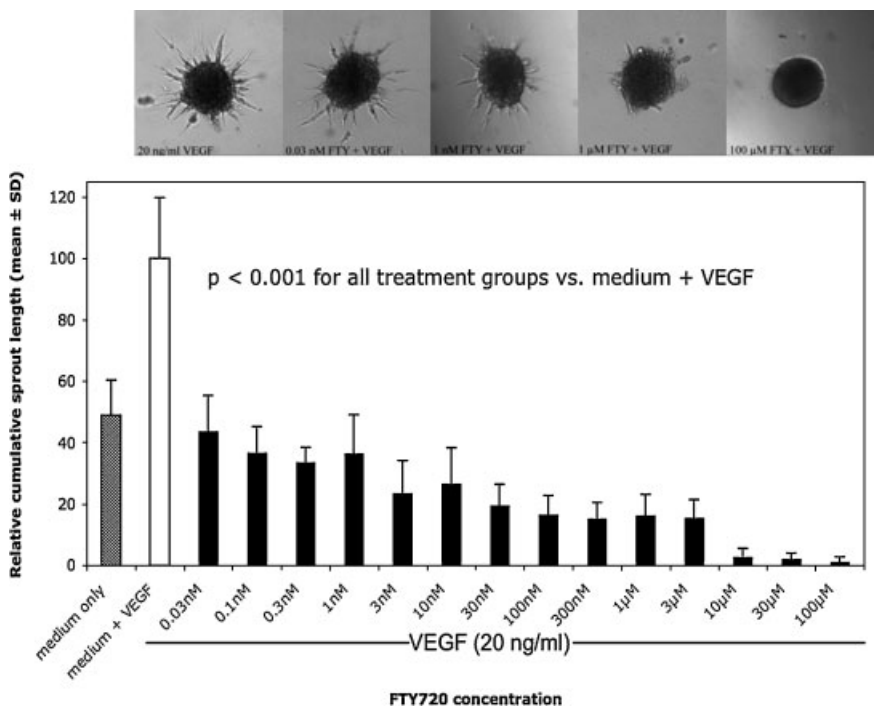

Fig. 2. The spheroid sprouting assay shows the ability of cells to form sprouts in response to angiogenic stimulation. As readout, the cumulative sprout length per spheroid was measured, and at least 10 spheroids were used per concentration to assess the mean cumulative sprout length. Mean values plus standard deviation are shown. Spheroids were stimulated with 20 ng/ml $VEGF\text{-}A_{165}$ and the inhibiting properties of FTY720 were examined. FTY720 was able to overcome the stimulating effect of VEGF-A even at sub-nanomolar concentrations. The **upper panel** shows representative pictures of spheroids from the spheroid assay (n = 10). In the **bottom panel**, a graph depicting the effect of FTY720 on the cumulative sprout length is shown.

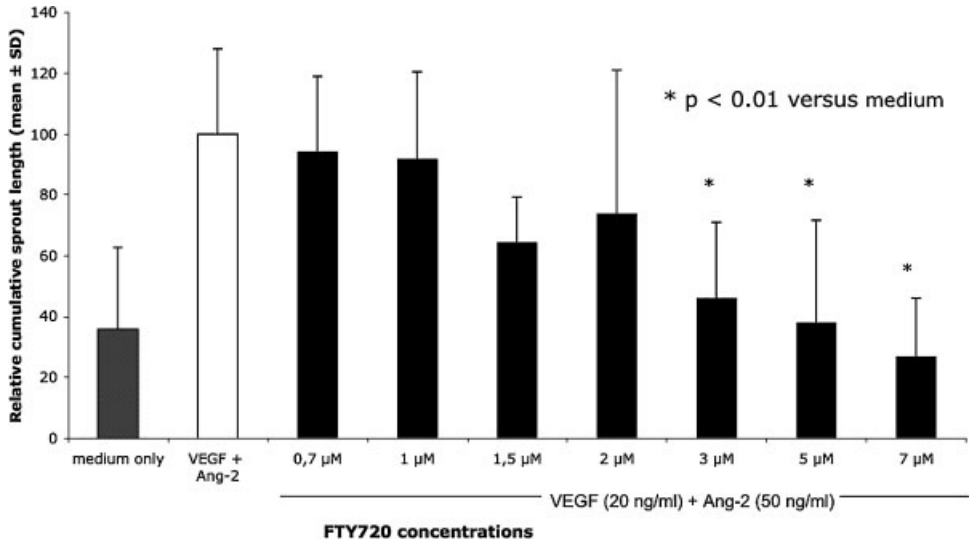

Fig. 3. A modified spheroid angiogenesis assay using two different cell types. Coculture spheroids of HUVECs and SMCs were stimulated with 20 ng/ml VEGF-A_{165} and 50 ng/ml angiopoietin-2 (Ang-2), and the inhibiting effect of FTY720 on the formation of sprouts was measured.

sprouting in our in vitro angiogenesis assay, almost as effective as FTY720 (Fig. 4a).

Since a transactivating effect of S1P receptors towards the CXCR4 receptor has been reported previously [Kimura et al., 2004], we examined whether pre-incubation of cells in the spheroid assay with a neutralizing antibody (clone 12G5) for the CXCL12 (formerly designated SDF-1 for stromal cell-derived factor-1) receptor CXCR4 may abrogate the anti-angiogenic activity of FTY720. Indeed, even for the relatively high FTY720 concentration of 10 nM, pre-incubation with the CXCR4-neutralizing antibody abolished the FTY720-mediated inhibition of endothelial sprouting (Fig. 4b). This finding was somehow surprising since migration, one of the basic requirements for sprouting, is activated by SDF-1/CXCR4 signaling [Murdoch et al., 1999; Koshiba et al., 2000]. However, the requirements for sprouting in our spheroid assay might be different from the situation in the migration assay. Additional experiments are certainly needed to clarify the mechanistic basis of our findings.

FTY720 Inhibits Tumor Growth Via Inhibition of Angiogenesis

To show that our in vitro results are also applicable in vivo, we implemented a subcutaneous tumor model. To examine the potential of FTY720 to inhibit tumor growth in vivo, we used C57BL/6 mice and LLC1 cells that originate from the same mouse strain. Following tumor cell injection, tumor volume was measured three times per week. Six days after tumor cell injection, when tumors had grown to a length of 5–8 mm, animals were randomized into four treatment groups (0, 1, 5, or 10 mg/kg bodyweight FTY720 as daily i.p. injection). These FTY720 concentrations are within the range commonly used in clinical trials. After 20 days following tumor cell injection, mice were sacrificed and tumors were resected for further histological analysis. Results revealed that treatment with FTY720 leads to a dose-dependent reduction in tumor size, with the maximum effect at a dose of 10 mg/kg (reduction to $51.7 \pm 10.9\%$ vs. control), a result that corresponded well with previously published data [Schmid et al., 2005]. Interestingly, we also found that the microvessel density (MVD) was significantly decreased in mice treated with 10 mg/kg/d FTY720 (Fig. 5b).

In organ transplantation, CsA is the standard immunosuppressant. However, only recently, the pro-neoplastic properties of CsA have been described [Guba et al., 2004; Kauffman et al., 2005], which may provide a rationale for the higher incidence of malignancies in patients on high-dose CsA [Dantal et al., 1998]. Based on our promising findings, we investigated the effects of FTY720, CsA, or a combination of both drugs, on tumor growth. We hypothesized that the combined use of both drugs in our animal

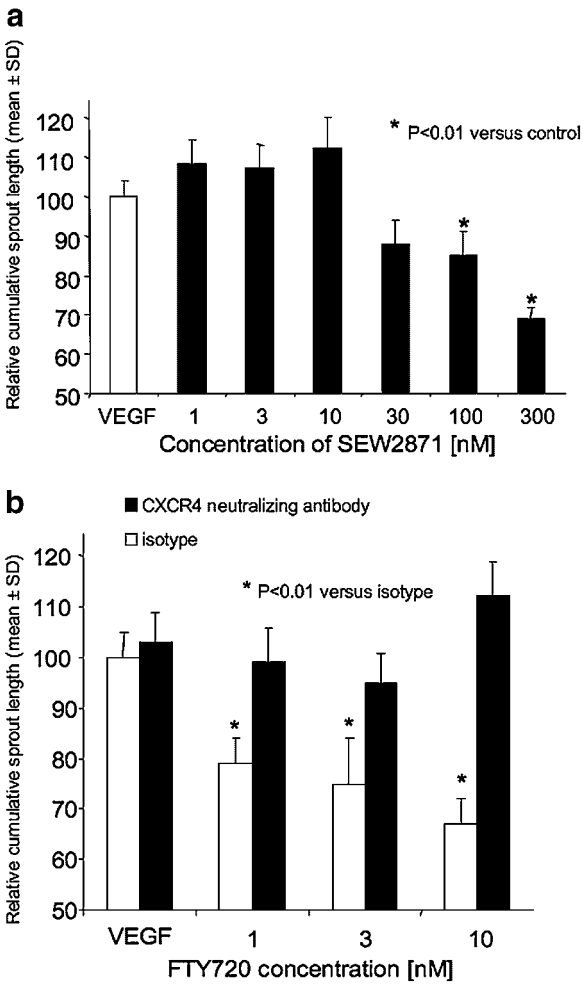

Fig. 4. **a**: A sprouting assay showing the effect of SEW2871 on spheroids. The specific $S1P_1$ agonist SEW2871 inhibits the sprouting of HUVEC spheroids that were stimulated with 20 ng/ml VEGF, in a dose-dependent manner. The inhibition reaches significance at a concentration of 100 nM of the drug. This is in concordance with the higher EC_{50}, as compared to FTY720. **b**: Pre-incubation of spheroids with a CXCR4-neutralizing antibody (murine IgG_{2A} isotype used as a control antibody) resulted in a complete abrogation of the inhibiting effect of FTY720 on the sprouting of HUVEC spheroids that were stimulated with 20 ng/ml VEGF. At a concentration of 1, 3, and 10 nM FTY720, the effect of the antibody resulted in a significant increase in sprout length.

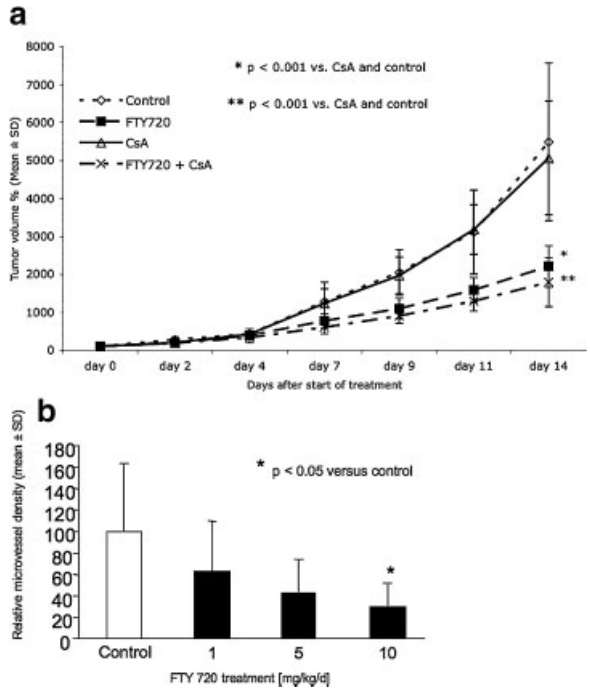

Fig. 5. In vivo experiment with C57BL/6 mice bearing a subcutanous LLC1 tumor. **a**: Comparison of tumor growth after treatment with 10 mg/kg FTY720, 20 mg/kg CsA, a combination therapy of FTY720 and CsA, or PBS (control). The lines show the mean size and the standard deviation from the tumors of one treatment group (n = 9 per group). FTY720 as well as the combination of FT720 and CsA treatment resulted in a highly significant reduction of tumor growth, as compared to CsA-only treated mice or control mice. **b**: The microvessel density is dose-dependently reduced in C57BL/6 mice after treatment with FTY720, reaching significance at a dose of 10 mg/kg/d. The vessels were stained using an antibody against Von Willebrand Factor. Results are shown as mean ± standard deviation.

model may abrogate the tumor-promoting activities of CsA. In our model, mice treated with 20 mg/kg bodyweight CsA exhibited a tumor growth, very similar to that of control mice treated with PBS (Fig. 5a). Most importantly, however, the anti-tumorigenic properties of FTY720 in the combination therapy translated into a tumor growth that was similar to the FTY720 treatment alone and significantly lower, as compared to CsA treatment alone or the control (PBS).

FTY720 Inhibits Vascular Sprouting In Vivo

To elucidate the biological basis for the retarded tumor growth in our subcutaneous in vivo model, we used a Matrigel™ plug angiogenesis assay [Kragh et al., 2003], to show that anti-angiogenic mechanisms are indeed operational. A total of 500 µl Matrigel™ was subcutaneously injected into the left inguinal area of C57BL/6 mice. The animals were then treated with daily i.p. injections of 10 mg/kg bodyweight FTY720 or with PBS (control). After 2 weeks, animals were sacrificed and 5 µm cryo-sections of the plugs were prepared for analysis of the MVD. Most importantly, FTY720-treated mice showed a significant

decrease in MVD of 87%, when compared to the MVD observed in PBS-treated animals (Fig. 6). It should be noted, however, that a daily treatment with 10 mg/kg FTY720 is a higher dosage than typically used in animal experiments.

DISCUSSION

The most evident findings presented in this study are that FTY720 potently inhibits angiogenesis in vitro as well as in vivo. Mechanistically, the effect was predominantly mediated by the $S1P_1$ receptor and involved transactivation of the chemokine receptor CXCR4. Consistently, in our animal model, the anti-angiogenic effect of FTY720 translated into a reduced tumor growth and, even more importantly, abrogated the pro-tumorigenic effect of the standard immunosuppressant CsA.

The synthetic myriocin analog FTY720 was recently identified as an agonist of S1P receptors [Brinkmann et al., 2002; Mandala et al., 2002], a novel class of five G protein-coupled seven-transmembrane receptors ($S1P_1$–$S1P_5$; formerly termed endothelial differentiation gene (EDG) receptors) [Lynch, 2002]. These receptors modulate cell migration and transduce intracellular signals involved in numerous other cellular processes [Spiegel and Milstien, 2003; Saba and Hla, 2004]. Their physiological ligand, S1P, is a potent lipid mediator. In vivo studies showed synergistic effects of S1P with angiogenic factors such as FGF-2 and VEGF-A to induce angiogenesis and vascular maturation in the murine Matrigel^{TM} plug model [Lee et al., 1999]. Moreover, $S1P_1$-deficient mice die in utero between embryonic days 12.5 and 14.5 due to hemorrhage as a result of insufficient vascular maturation, indicating that the $S1P_1$ receptor is required for vascular development [Liu et al., 2000]. A thorough analysis of the embryos showed that disruption of the $S1P_1$ gene changes the coverage of blood vessels with SMCs and other pericytes. These studies form the basis for the emerging concept that $S1P_1$ is a potent regulator of vascular growth and development. Preliminary studies suggest that FTY720 combines anti-proliferative and -angiogenic properties with cytotoxic effects on certain cell types, for example, hepatoma cells [Ho et al., 2005]. To more closely elucidate the function of the $S1P_1$ receptor in our in vitro experiments, we employed the specific $S1P_1$ receptor agonist SEW2871. Since SEW2871 and FTY720-P have different IC_{50} values for the $S1P_1$ receptor (20.7 nM for SEW2871 vs. 1.4 nM for FTY720-P) [Sanna et al., 2004], one would expect to see stronger inhibition using FTY720. Indeed, SEW2871 was able to recapitulate the

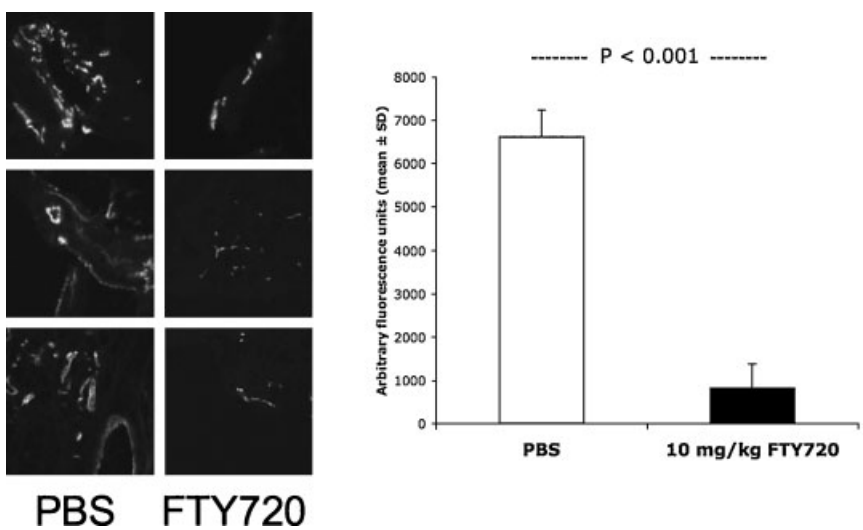

Fig. 6. The Matrigel^{TM} plug assay is an established assay to study angiogenesis in vivo. Blood vessels were assessed by immunostaining with FITC-labeled CD31 antibodies. The ingrowth of vessels was significantly reduced by a daily i.p. injection with 10 mg/kg bodyweight FTY720 for 2 weeks. Three representative pictures of CD31 antibody staining per group are shown on the **left**. The column graph is depicting the mean CD31 fluorescence staining including standard deviation (n = 4 animals per group and 5 sections per animal).

anti-angiogenic properties of FTY720 and as expected, the activity was correspondingly lower due to the different IC_{50} values.

Based on these data, it is not surprising that primary endothelial cells showed a lower IC_{50} than tumor cells in our in vitro MTT proliferation assay. Cancer cells are selected for disregarding growth arrest signals and, therefore, a hallmark of tumor cell development is acquisition of an unlimited growth potential. Additionally to the increased growth rate, tumor cells may not depend entirely on a single pathway that mediates growth-promoting signals. Surprisingly, SMCs showed a slightly higher IC_{50} in the MTT assay than tumor cells. To gain more relevant information in terms of angiogenic processes, we measured the ability of the endothelial cells to form sprouts in the spheroid assay as a result of angiogenic stimulation, a prerequisite for an engagement of these cells in the formation of blood vessels. Cells organized in a spheroid that is embedded in a collagen matrix, are much more sensitive to pro-angiogenic as well as anti-angiogenic stimuli, as compared to the same cells in a monolayer culture. This finding was also apparent in our experiments and is rationalized by the fact that proliferation is only one of the features of angiogenic processes. Additional processes such as cell plasticity and mobility are also pivotal features of angiogenesis. Therefore, our finding that FTY720 abrogated the stimulating effect of VEGF-A, even at sub-nanomolar concentrations demonstrates the high susceptibility of sprouting endothelial cells to FTY720, supporting the finding that FTY720 has a negative impact on stimulation by VEGF-A [Sanchez et al., 2003; Sanchez and Hla, 2004]. Still, clear inhibition of sprouting of HUVEC spheroids, even below the Kd values of FTY720 was surprising and might reflect the fine-tuned nature of pro- and anti-angiogenic pathways that can be influenced, even with a slight shift of the balance in favor of anti-angiogenesis. On the other hand, the inhibition of sprouting of HUVECs was weakened when the cells were cultured together with SMCs in coculture spheroids. Although the mechanism of protection of HUVECs against the action of FTY720 is not clear, it should be mentioned that a general stabilization of HUVECs by SMCs in coculture spheroids has been described [Korff et al., 2001] and SMCs also stabilize endothelial cells in vivo [Gerhardt and Betsholtz, 2003; Armulik et al., 2005]. This could also explain why FTY720 is not as potent in vivo as one might expect from the MTT assay and from the single cell-type spheroids. Nevertheless, FTY720 also manifested a clear effect in vivo, presumably also due to the fact that blood vessels in tumors are not well organized and are not completely covered with an intact layer of SMCs.

In vascular endothelial cells, S1P binds to three different S1P receptors, namely S1P receptors 1–3, inducing migration, proliferation, cell survival, and morphogenesis into capillary-like structures [Lee et al., 1999; Paik et al., 2001]. Importantly, such responses require the function of S1P receptor 1, which was originally isolated as an inducible gene from endothelial cells [Hla and Maciag, 1990; Lee et al., 1999], emphasizing the important role of the $S1P_1$ receptor in endothelial cells. To investigate, whether the inhibiting effect of the unspecific S1P receptor agonist FTY720-P in our experiments could also be achieved with the specific $S1P_1$ agonist SEW2871, we repeated the spheroid assay using this recently synthesized substance [Sanna et al., 2004]. Intriguingly, the inhibition of spheroid sprouting was mimicked by SEW2871, although higher concentrations were mandatory to achieve a similar inhibition as observed for FTY720. It is important to note, however, that SEW2871 has an EC_{50} approximately 15 times higher than that of FTY720-P [Sanna et al., 2004]. Therefore, these data strongly suggest that the anti-angiogenic effect of FTY720-P is, at least predominantly, mediated via the $S1P_1$ receptor. In principle, it cannot be ruled out that other S1P receptors may also contribute to the anti-angiogenic properties of FTY720-P. Since only S1P receptors 1–3 are expressed in significant amounts on endothelial cells [Lee et al., 1999; Kwon et al., 2001; Sanchez and Hla, 2004] and FTY720-P does not inhibit the S1P receptor 2 [Brinkmann et al., 2002; Sanna et al., 2004], it is clear that the effects of FTY720-P exerted towards endothelial cells must be mediated by S1P receptor 1 and/or receptor 3. Therefore, a specific $S1P_3$ receptor agonist would be crucial for addressing this issue. To our knowledge, however, no such $S1P_3$ receptor agonist is available to date. Nevertheless, our data are of paramount clinical importance, given that the immunosuppressive properties of FTY720-P are also mediated via the $S1P_1$ receptor, whereas the receptor responsible for bradycardia,

a frequently occurring undesired effect of FTY720 treatment, was found to be mediated by the $S1P_3$ receptor.

To further elucidate the downstream mechanism through which FTY720 exerts its anti-angiogenic effect, we used a neutralizing antibody directed against CXCR4. For this receptor, a pro-angiogenic function was described very recently [Guleng et al., 2005]. The physiological and specific ligand of this receptor is CXCL12 and, most importantly, a transactivating effect of S1P receptors on CXCR4 stimulation has been shown [Kimura et al., 2004]. Additionally, recent reports suggest an impact of the CXCL12/CXCR4 axis on homing of progenitor cells and metastasis [Kaplan et al., 2005] and on the recruitment of cells that support angiogenesis [Carmeliet, 2005]. In the spheroid assay, the inhibiting effect of FTY720 was completely abrogated by pre-incubation of spheroids with the CXCR4-neutralizing antibody. A very recent report [LaMontagne et al., 2006] showed that FTY720 does not change the VEGFR2 expression on HUVEC cells and that the specific VEGFR2 kinase inhibitor PTK787 exhibits synergistic effects with FTY720, suggesting that the VEGF-A/VEGFR2 pathway is not the main target of FTY720. Taken together, our mechanistic in vitro experiments demonstrate that FTY720 mediates its anti-angiogenic properties, primarily through the $S1P_1$ receptor and that this effect is, at least in part and under the circumstances used in our in vitro assay, dependent on transactivation of the CXCR4 receptor.

Based on these in vitro results, we performed dose-escalating in vivo studies using a murine tumor model. These experiments showed that FTY720 dose-dependently inhibits the growth of subcutaneous LLC1 tumors. Consistently, a similar dose-dependent reduction in the microvessel density was observed. These data further corroborate our finding that the reduction in tumor growth is due to the anti-angiogenic features of FTY720. However, effects seen in the in vivo experiments were not as strong as one would have expected, based on the in vitro experiments. It is likely that the stabilizing effect of perivascular SMCs and pericytes protects endothelial cells from the anti-angiogenic properties of FTY720. Indeed, stabilization of HUVECs by cocultured SMCs was also shown in the spheroid assay [Korff et al., 2001]. However, a strong anti-angiogenic effect could be shown in vivo in the matrigel plug assay. Presumably, this striking result is due to the fact that newly formed blood vessels are immature and therefore, are also not well covered with SMCs and constitute an easy target for the action of FTY720. It should be noted, however, that a dosage of 10 mg/kg/d is rather high and that unspecific effects (e.g., suppression of immune cells) could also contribute to the anti-angiogenic properties seen in this assay.

Since most routinely used immunosuppressants seem to have a pro-tumorigenic effect, we tried to abrogate the stimulation of tumor growth by combining CsA with FTY720. Despite the fact that CsA was reported to have a tumor-promoting effect [Guba et al., 2002, 2004], it did not significantly change the tumor growth in our in vivo model. However, combined treatment with FTY720 and CsA strongly retarded the growth of LLC1 tumors. This is of major clinical interest, since most immunosuppressive protocols cannot completely avoid so-called calcineurin inhibitors such as CsA. For future experiments, it will be highly interesting to combine FTY720 with other immunosuppressive drugs showing anti-tumorigenic properties [Guba et al., 2002], for example, mTOR inhibitors. The various side effects of CsA, FTY720, and mTOR inhibitors make it even more important to develop tailored immunosuppressive regimens for each patient, taking the individual situation into account. Especially, recent reports about side effects (e.g., bradycardia), after treatment with FTY720 suggest cautious consideration before its application. mTOR inhibitors also exert anti-angiogenic properties [Guba et al., 2002, 2004], but they show some adverse side effects, for example, a delay in wound healing [Valente et al., 2003; Dean et al., 2004] due to inhibition of fibroblast activity.

In conclusion, our data show that FTY720 has anti-angiogenic and subsequently, anti-tumorigenic properties in vitro and in vivo. The anti-angiogenic properties can be mimicked by the specific $S1P_1$ receptor agonist SEW2871, indicating that the anti-angiogenic properties of FTY720 are mediated by the same receptor that is responsible for the immunosuppressive effects of FTY720. Blocking of the S1P-mediated transactivation of the chemokine receptor CXCR4 abrogated the anti-angiogenic effect of

FTY720, indicating the crucial role of this receptor for downstream signaling. In experimental in vivo models, the combined use of FTY720 and CsA resulted in an antitumorigenic effect, suggesting that the combined use of these two immunosuppressive drugs may generate a clinical benefit for transplant patients, in terms of post-transplant tumor development under specific circumstances.

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REFERENCES

Armulik A, Abramsson A, Betsholtz C. 2005. Endothelial/pericyte interactions. Circ Res 97:512–523.

Billich A, Bornancin F, Devay P, Mechtcheriakova D, Urtz N, Baumruker T. 2003. Phosphorylation of the immunomodulatory drug FTY720 by sphingosine kinases. J Biol Chem 278:47408–47415.

Brinkmann V, Davis MD, Heise CE, Albert R, Cottens S, Hof R, Bruns C, Prieschl E, Baumruker T, Hiestand P, Foster CA, Zollinger M, Lynch KRœ. 2002. The immune modulator FTY720 targets sphingosine 1-phosphate receptors. J Biol Chem 277:21453–21457.

Carmeliet P. 2005. Angiogenesis in life, disease and medicine. Nature 438:932–936.

Chua CW, Lee DT, Ling MT, Zhou C, Man K, Ho J, Chan FL, Wang X, Wong YC. 2005. FTY720, a fungus metabolite, inhibits in vivo growth of androgen-independent prostate cancer. Int J Cancer 117:1039–1048.

Dantal J, Hourmant M, Cantarovich D, Giral M, Blancho G, Dreno B, Soulillou JP. 1998. Effect of long-term immunosuppression in kidney-graft recipients on cancer incidence: Randomised comparison of two cyclosporin regimens. Lancet 351:623–628.

Dean PG, Lund WJ, Larson TS, Prieto M, Nyberg SL, Ishitani MB, Kremers WK, Stegall MD. 2004. Wound-healing complications after kidney transplantation: A prospective, randomized comparison of sirolimus and tacrolimus. Transplantation 77:1555–1561.

Fujita T, Inoue K, Yamamoto S, Ikumoto T, Sasaki S, Toyama R, Chiba K, Hoshino Y, Okumoto T. 1994. Fungal metabolites. Part 11. A potent immunosuppressive activity found in Isaria sinclairii metabolite. J Antibiot (Tokyo) 47:208–215.

Gallo P, Agozzino L, Angelini A, Arbustini E, Bartoloni G, Bernucci P, Bonacina E, Bosman C, Catani G, di Gioia C, Giordana C, Leone O, Motta T, Pucci A, Rocco M. 1997. Causes of late failure after heart transplantation: A ten-year survey. J Heart Lung Transplant 16:1113–1121.

Gerhardt H, Betsholtz C. 2003. Endothelial-pericyte interactions in angiogenesis. Cell Tissue Res 314:15–23.

Guba M, von Breitenbuch P, Steinbauer M, Koehl G, Flegel S, Hornung M, Bruns CJ, Zuelke C, Farkas S, Anthuber M, Jauch KW, Geissler EK. 2002. Rapamycin inhibits primary and metastatic tumor growth by antiangiogenesis: Involvement of vascular endothelial growth factor. Nat Med 8:128–135.

Guba M, Graeb C, Jauch KW, Geissler EK. 2004. Pro- and anti-cancer effects of immunosuppressive agents used in organ transplantation. Transplantation 77:1777–1782.

Guleng B, Tateishi K, Ohta M, Kanai F, Jazag A, Ijichi H, Tanaka Y, Washida M, Morikane K, Fukushima Y, Yamori T, Tsuruo T, Kawabe T, Miyagishi M, Taira K, Sata M, Omata M. 2005. Blockade of the stromal cell-derived factor-1/CXCR4 axis attenuates in vivo tumor growth by inhibiting angiogenesis in a vascular endothelial growth factor-independent manner. Cancer Res 65: 5864–5871.

Hla T, Maciag T. 1990. An abundant transcript induced in differentiating human endothelial cells encodes a polypeptide with structural similarities to G-protein-coupled receptors. J Biol Chem 265:9308–9313.

Ho JW, Man K, Sun CK, Lee TK, Poon RT, Fan ST. 2005. Effects of a novel immunomodulating agent, FTY720, on tumor growth and angiogenesis in hepatocellular carcinoma. Mol Cancer Ther 4:1430–1438.

Jain A, DiMartini A, Kashyap R, Youk A, Rohal S, Fung J. 2000. Long-term follow-up after liver transplantation for alcoholic liver disease under tacrolimus. Transplantation 70:1335–1342.

Kaplan RN, Riba RD, Zacharoulis S, Bramley AH, Vincent L, Costa C, MacDonald DD, Jin DK, Shido K, Kerns SA, Zhu Z, Hicklin D, Wu Y, Port JL, Altorki N, Port ER, Ruggero D, Shmelkov SV, Jensen KK, Rafii S, Lyden D. 2005. VEGFR1-positive haematopoietic bone marrow progenitors initiate the pre-metastatic niche. Nature 438:820–827.

Kasiske BL, Snyder JJ, Gilbertson DT, Wang C. 2004. Cancer after kidney transplantation in the United States. Am J Transplant 4:905–913.

Kauffman HM, Cherikh WS, Cheng Y, Hanto DW, Kahan BD. 2005. Maintenance immunosuppression with target-of-rapamycin inhibitors is associated with a reduced incidence of de novo malignancies. Transplantation 80: 883–889.

Kimura T, Boehmler AM, Seitz G, Kuci S, Wiesner T, Brinkmann V, Kanz L, Mohle R. 2004. The sphingosine 1-phosphate receptor agonist FTY720 supports CXCR4-dependent migration and bone marrow homing of human CD34+ progenitor cells. Blood 103:4478–4486.

Korff T, Augustin HG. 1998. Integration of endothelial cells in multicellular spheroids prevents apoptosis and induces differentiation. J Cell Biol 143:1341–1352.

Korff T, Kimmina S, Martiny-Baron G, Augustin HG. 2001. Blood vessel maturation in a 3-dimensional spheroidal coculture model: Direct contact with smooth muscle cells regulates endothelial cell quiescence and abrogates VEGF responsiveness. FASEB J 15:447–457.

Koshiba T, Hosotani R, Miyamoto Y, Ida J, Tsuji S, Nakajima S, Kawaguchi M, Kobayashi H, Doi R, Hori T, Fujii N, Imamura M. 2000. Expression of stromal cell-derived factor 1 and CXCR4 ligand receptor system in pancreatic cancer: A possible role for tumor progression. Clin Cancer Res 6:3530–3535.

Kragh M, Hjarnaa PJ, Bramm E, Kristjansen PE, Rygaard J, Binderup L. 2003. In vivo chamber angiogenesis assay: An optimized Matrigel plug assay for fast assessment of anti-angiogenic activity. Int J Oncol 22:305–311.

Kwon YG, Min JK, Kim KM, Lee DJ, Billiar TR, Kim YM. 2001. Sphingosine 1-phosphate protects human umbilical vein endothelial cells from serum-deprived apoptosis by nitric oxide production. J Biol Chem 276:10627–10633.

LaMontagne K, Littlewood-Evans A, Schnell C, O'Reilly T, Wyder L, Sanchez T, Probst B, Butler J, Wood A, Liau G, Billy E, Theuer A, Hla T, Wood J. 2006. Antagonism of sphingosine-1-phosphate receptors by FTY720 inhibits angiogenesis and tumor vascularization. Cancer Res 66: 221–231.

Lee MJ, Thangada S, Claffey KP, Ancellin N, Liu CH, Kluk M, Volpi M, Sha'afi RI, Hla T. 1999. Vascular endothelial cell adherens junction assembly and morphogenesis induced by sphingosine-1-phosphate. Cell 99:301–312.

Lee TK, Man K, Ho JW, Sun CK, Ng KT, Wang XH, Wong YC, Ng IO, Xu R, Fan ST. 2004a. FTY720 induces apoptosis of human hepatoma cell lines through PI3-K-mediated Akt dephosphorylation. Carcinogenesis 25: 2397–2405.

Lee WJ, Yoo HS, Suh PG, Oh S, Lim JS, Lee YM. 2004b. Sphingosine mediates FTY720-induced apoptosis in LLC-PK1 cells. Exp Mol Med 36:420–427.

Liu Y, Wada R, Yamashita T, Mi Y, Deng CX, Hobson JP, Rosenfeldt HM, Nava VE, Chae SS, Lee MJ, Liu CH, Hla T, Spiegel S, Proia RL. 2000. Edg-1, the G protein-coupled receptor for sphingosine-1-phosphate, is essential for vascular maturation. J Clin Invest 106: 951–961.

Lynch KR. 2002. Lysophospholipid receptor nomenclature. Biochim Biophys Acta 1582:70–71.

Mandala S, Hajdu R, Bergstrom J, Quackenbush E, Xie J, Milligan J, Thornton R, Shei GJ, Card D, Keohane C, Rosenbach M, Hale J, Lynch CL, Rupprecht K, Parsons W, Rosen H. 2002. Alteration of lymphocyte trafficking by sphingosine-1-phosphate receptor agonists. Science 296: 346–349.

Matloubian M, Lo CG, Cinamon G, Lesneski MJ, Xu Y, Brinkmann V, Allende ML, Proia RL, Cyster JG. 2004. Lymphocyte egress from thymus and peripheral lymphoid organs is dependent on S1P receptor 1. Nature 427:355–360.

Murdoch C, Monk PN, Finn A. 1999. Cxc chemokine receptor expression on human endothelial cells. Cytokine 11:704–712.

Paik JH, Chae S, Lee MJ, Thangada S, Hla T. 2001. Sphingosine 1-phosphate-induced endothelial cell migration requires the expression of EDG-1 and EDG-3 receptors and Rho-dependent activation of alpha vbeta3- and beta1-containing integrins. J Biol Chem 276:11830–11837.

Saba JD, Hla T. 2004. Point-counterpoint of sphingosine 1-phosphate metabolism. Circ Res 94:724–734.

Sanchez T, Hla T. 2004. Structural and functional characteristics of S1P receptors. J Cell Biochem 92: 913–922.

Sanchez T, Estrada-Hernandez T, Paik JH, Wu MT, Venkataraman K, Brinkmann V, Claffey K, Hla T. 2003. Phosphorylation and action of the immunomodulator FTY720 inhibits vascular endothelial cell growth factor-induced vascular permeability. J Biol Chem 278: 47281–47290.

Sanna MG, Liao J, Jo E, Alfonso C, Ahn MY, Peterson MS, Webb B, Lefebvre S, Chun J, Gray N, Rosen H. 2004. Sphingosine 1-phosphate (S1P) receptor subtypes S1P1 and S1P3, respectively, regulate lymphocyte recirculation and heart rate. J Biol Chem 279:13839–13848.

Schmid G, Guba M, Papyan A, Ischenko I, Bruckel M, Bruns CJ, Jauch KW, Graeb C. 2005. FTY720 inhibits tumor growth and angiogenesis. Transplant Proc 37: 110–111.

Sheil AG, Disney AP, Mathew TH, Amiss N. 1993. De novo malignancy emerges as a major cause of morbidity and late failure in renal transplantation. Transplant Proc 25:1383–1384.

Spiegel S, Milstien S. 2003. Sphingosine-1-phosphate: An enigmatic signalling lipid. Nat Rev Mol Cell Biol 4:397–407.

Tanaka T, Takahara S, Hatori M, Suzuki K, Wang J, Ichimaru N, Suzuki S, Morozumi K, Okuyama A, Yamanaka H. 2002. A novel immunosuppressive drug, FTY720, prevents the cancer progression induced by cyclosporine. Cancer Lett 181:165–171.

Valente JF, Hricik D, Weigel K, Seaman D, Knauss T, Siegel CT, Bodziak K, Schulak JA. 2003. Comparison of sirolimus vs. mycophenolate mofetil on surgical complications and wound healing in adult kidney transplantation. Am J Transplant 3:1128–1134.

Vivarelli M, Bellusci R, Cucchetti A, Cavrini G, De Ruvo N, Aden AA, La Barba G, Brillanti S, Cavallari A. 2002. Low recurrence rate of hepatocellular carcinoma after liver transplantation: Better patient selection or lower immunosuppression? Transplantation 74:1746–1751.

Zemann B, Kinzel B, Muller M, Reuschel R, Mechtcheriakova D, Urtz N, Bornancin F, Baumruker T, Billich A. 2006. Sphingosine kinase type 2 is essential for lymphopenia induced by the immunomodulatory drug FTY720. Blood 107:1454–1458.