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G PROTEIN-COUPLED SPHINGOSINE-1-PHOSPHATE RECEPTORS: POTENTIAL MOLECULAR TARGETS FOR ANGIOGENIC AND ANTI-ANGIOGENIC THERAPIES

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Sphingosine-1-phosphate (S1P) is a plasma lipid mediator with pleiotropic activities; it is constitutively produced in red blood cells and vascular endothelial cells through phosphorylation of sphingosine by one of two S1P synthesizing enzymes, sphingosine kinase 1 and 2 (SphK 1, 2), and exported into plasma to bind to high density lipoprotein and albumin. Sphingosine-1-phosphate acts through five members of the G protein-coupled S1P receptors (S1PR1-S1PR5) to exert diverse actions, which include vascular maturation in embryonic stage and postnatal angiogenesis, maintenance of functional integrity of vascular endothelium, regulation of vascular tonus, and lymphocyte trafficking. Sphingosine-1-phosphate is unique in its ability to regulate cell migration either positively or negatively by acting through different receptor subtypes. S1PR1 and S1PR3 mediate chemotactic cell migration toward S1P via G_i/Rac pathway, whereas S1PR2 mediates S1P inhibition of chemotaxis via $G_{12/13}$/Rho-dependent inhibition of Rac. Sphingosine-1-phosphate positively or negatively regulates tumor cell migration, invasion in Matrigel, and hematogenous metastasis in manners strictly dependent on S1P receptor subtypes expressed in tumor cells. S1PR1 (and S1PR3) also mediates activation of G_i/phosphatidylinositol 3-kinase (PI3K)/Akt and stimulation of cell proliferation/survival, whereas S1PR2 could mediate suppression of cell proliferation/survival through $G_{12/13}$/Rho/Rho kinase/PTEN-dependent Akt inhibition. S1PR1 (and S1PR3) expressed in endothelial cells mediates angiogenic action of S1P by stimulating endothelial cell migration, proliferation and tube formation. In a mouse model of hindlimb ischemia after femoral artery resection, repeated local administration or sustained delivery of S1P, or transgenic overexpression of SphK1, accelerates post-ischemic angiogenesis, through the S1P actions on both endothelial cells and bone marrow-derived myeloid cells (BMDCs). In tumor cells, SphK1 is upregulated especially in advanced stages, through mechanisms involving both activating Ras mutation and hypoxia, which leads to increased S1P production and also decreased cellular content of pro-apoptotic sphingolipid ceramide, a metabolic precursor of S1P. Apoptotic tumor cells also produce S1P through SphK2 activation, thus implicated in tumor angiogenesis by acting on endothelial cells through S1PR1/S1PR3, as well as tumor-infiltrating macrophages and BMDCs. Inhibition of S1PR1 function by either an anti-

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S1P antibody or FTY720 inhibits tumor angiogenesis and tumor growth. Differently from S1PR1, S1PR2 expressed in host cells mediates inhibition of tumor angiogenesis and tumor growth, through mechanisms involving the suppression of endothelial cell migration, proliferation and tube formation, and inhibition of BMDC recruitment to tumor stroma with suppressed expression of pro-angiogenic factor and matrix metalloprotease 9. These findings provide the molecular basis for S1P receptor subtype-selective targeting strategies aiming at angiogenic therapy for occlusive peripheral arterial diseases, and anti-angiogenic and anti-tumor therapies against cancer. ***Biomed Rev*** **2011; 22: 15-29.**

Key words: sphingosine-1-phosphate, S1P receptors, peripheral arterial disease, tumor angiogenesis

INTRODUCTION

During the past two decades the existence of the novel intercellular signaling system has been elucidated, which comprises a plasma lysophospholipid mediator sphingosine-1-phosphate (S1P) (1), its synthesizing/degrading enzymes (2-6), membrane S1P transporters (7,8), S1P carrier proteins in the plasma (9), and five members of the G protein-coupled S1P-specific receptor subtypes, S1PR1~S1PR5 (10-22). The S1P signaling system plays crucial roles in mammalian embryonic development (24-26) and post-natal homeostasis in the cardiovascular (27-36), immune (37-47) and nervous systems (26,48). The S1P signaling system is also implicated as the target of therapeutic intervention in a variety of human diseases. Multiple sclerosis, a debilitating autoimmune disease, is now treated with the S1P receptor agonist prodrug FTY720 (49), whose phosphorylation product downregulates S1PR1 in lymphocytes to inhibit their recirculation, thus resulting in lymphopenia (38,50). In addition, accumulated evidence in experimental animal disease models indicates that targeting the S1P signaling system is a promising tactic for both angiogenic and anti-angiogenic therapies, for treating obliterative peripheral arterial diseases and malignant tumors, respectively (51-57). Before addressing this point, we will overview how blood vessels are generated during embryonic phase and after birth, and how the S1P signaling system is involved in blood vessel formation in physiological and pathological conditions.

VASCULOGENESIS, ANGIOGENESIS, AND VASCULAR MATURATION IN DEVELOPMENT

Mammalian blood vessels are generated by two different mechanisms, i.e. vasculogenesis and angiogenesis, in which endothelial cells derived from distinct sources are involved (58-61). In vasculogenesis, which is a process of *de novo* blood vessel formation in the early embryonic phase, endothelial progenitor cells (EPCs) or angioblasts differentiate from mesodermal cells, which proliferate, migrate and differentiate to become endothelial cells that are endowed with cell surface adhesion molecules including PECAM (platelet endothelial cell adhesion molecule) and vascular endothelial (VE)-cadherin. Endothelial cells then form aggregates through the homologous cell-cell adhesion, followed by dynamic morphogenesis and remodeling to form a primordial tubular network. The endothelium in the early embryo is then destined to either arterial or venous differentiation, leading to the formation of the dorsal aorta and cardinal vein. A part of arterial endothelial cells give rise to hematopoietic stem cells to initiate definitive hematopoiesis, whereas a part of venous endothelium differentiate to become lymphatic endothelial cells to form blind-ended lymphatic vessels (61).

Vasculogenesis is followed by angiogenesis, which takes place not only in embryonic period but also after birth. In angiogenesis, new vessels are created by sprouting of pre-existing capillaries (61,62). In response to pro-angiogenic chemoattractant signals in the microenvironment, capillary endothelial cells are destabilized, stimulated to migrate, invade through extracellular matrix (ECM) with its proteolytic degradation, and proliferate to form a cylindrical sprout, in which tip cells, a minor population of leading cells that sense and respond to pro-angiogenic cues, and stalk cells that trail the tip cells and form a cylinder structure, are distinguished. Sprouting new vessels get together to coalesce and undergo remodeling to build up microvessel networks in previously avascular areas (61,62).

Newly formed blood vessels then undergo the process of maturation, in which endothelial tubes are covered with mural cells (pericytes and vascular smooth muscle cells), which stabilize and strengthen the vessels, resulting in the formation

of a mature vascular bed (61-64). This process depends upon the recruitment of mural cells and heterologous endothelial-mural cell adhesion.

Angiogenic expansion, remodeling and vascular maturation leads to the formation of a fully functional embryonic vascular system. In addition, angiogenesis takes place after birth under both physiological and pathological conditions, which include wound healing, post-menstrual endometrial regeneration, inflammation-associated angiogenesis, post-ischemic angiogenesis and tumor angiogenesis (61,62,65). Importantly, bone marrow-derived myeloid cells and macrophages play a crucial role in preparing angiogenic microenvironment, through the production of pro-angiogenic growth factors, chemokines and cytokines, and proteases that degrade ECM and release ECM-bound growth factors, and by physically interacting with and thereby supporting vascular sprouting. In addition, under certain conditions circulating EPCs derived from bone marrow could be incorporated into angiogenic neovessels (65-67).

Vasculogenesis and angiogenesis are controlled by multiple arrays of pro-angiogenic growth factors, chemokines, cytokines, guidance molecules and their receptors, ECM components and their receptor/adhesion molecules, in a complex, spacio-temporally organized manner. Vascular endothelial growth factors (VEGFs) are the most critical driver of vasculogenesis and angiogenesis. Angiopoietins, ephrins and platelet-derived growth factors (PDGFs), and transforming growth factor-β (TGF-β) are required for vascular remodeling and maturation (58-67). In addition to these angiogenic factors, S1P is attracting increasing attention as a regulator of vascular formation.

TUMOR ANGIOGENESIS

Tumor angiogenesis plays a crucial role in tumor progression (68). As soon as a tumoral micronodule becomes hypoxic especially in the core region, the cellular level of hypoxia inducible factor1a (HIF1a) is upregulated, through the escape from von Hippel-Lindau protein-mediated proteasomal degradation (69). The formation of a HIF1a-containing transcription factor leads to stimulation in the expression of multiple genes that are involved in tumor progression, which include glucose transporters GLUT1/3, glycolytic enzymes, VEGF and other pro-angiogenic factors and SphK1 (69-72). These events switch on tumor angiogenesis, a critical event that allows abundant oxygen and nutrient supply to tumor cells to let the previously minute tumor proceed through the next stage, i.e. tumor progression with a rapid growth, invasion and eventual metastasis, processes dependent on newly constructed tumor vessels (68).

In tumor angiogenesis, endothelial cells in preexisting blood vessels located in the vicinity of tumors are induced to destabilize, migrate toward a tumor, proliferate and undergo morphogenesis to form networks of microvessels (65). These processes are reminiscent of developmental angiogenesis and regulated by multiple pro-angiogenic growth factors including VEGFs, angiopoietin-1, fibroblast growth factors (FGFs) and PDGFs, which are produced by both tumor cells and host stromal cells, among which are tumor-associated macrophages (TAMs) and other bone marrow-derived cells (BMDCs), which include $CD11b^+$ myeloid cells (58,59,65-69). TAMs are macrophages that have undergone phenotypic change in tumor microenvironment from a classically activated phenotype of proinflammatory anti-tumor immunocompetent M1 state to an alternatively activated, anti-inflammatory and angiogenic M2 state that favors tumor growth. Tumor-associated macrophages and BMDCs not only produce pro-angiogenic growth factors, tumor promoting growth factors and chemotactic factors that mobilizes BMDCs to tumor microenvironment, but also activate matrix metalloproteases (MMPs), which degrade ECM and liberate matrix-bound proangiogenic factors, thus establishing an angiogenic tumor microenvironment (69). The recruitment of pericytes and SMCs to the newly formed microvessels stabilizes them, establishing stable and abundant blood supply (60-64). Accumulated evidence now indicates that S1P is involved in tumor angiogenesis and tumor progression.

PRODUCTION AND DELIVERY OF S1P

S1P is present in the plasma at the order of 10^{-7} mol/L, which is for the most part bound to HDL and albumin (19,35,36,42,70). The major source of plasma S1P is red blood cells (43,71) and vascular endothelial cells (72), in which S1P is constitutively produced by SphK1 through phosphorylation of sphingosine, and exported by plasma membrane S1P transporters (7,8), followed by specific binding to HDL-associated apolipoprotein M (and non-specific binding to albumin) in the plasma (73). SphK1 and the other S1P synthesizing enzyme SphK2 share a conserved catalytic domain but are expressed in spacio-temporally distinct manners (74). Deletion of either SphK1 or SphK2 is functionally fully compensated by each other, whereas SphK1/SphK2 double knockout (KO) mice are embryonic lethal with an undetectable tissue level of S1P, indicating that S1P is produced exclusively by SphK1 and SphK2 in

vivo (26,75,76). In addition to erythrocytes and endothelial cells, activated platelets, mast cells and other types of cells release S1P (77). S1P thus released by blood cells and vascular endothelial cells activates endothelial S1PR1, the principal S1P receptor subtype expressed in this cell type, which mediates mitogenic, anti-apoptotic and chemotactic effects of S1P, as well as S1P-dependent suppression of vascular permeability, implicating critical role of S1P in maintenance of vascular integrity and wound healing (35,36). In addition, in various types of cells either of the SphKs could be stimulated to produce S1P in response to growth factors and cytokines (74).

It is of note that in tumor cells, especially in advanced stages, SphK1 is upregulated through multiple mechanisms, which include Ras activation, deletion of p53, and hypoxia (69-72, 78-89). SphK1 upregulation leads to not only increased production of S1P, but also a reduction in cellular levels of pro-apoptotic ceramide and sphingosine, which are metabolic precursors of S1P (90-94). Upregulation of SphK1 and a reduction in ceramide in tumor cells are closely associated with their acquisition of resistance against chemotherapeutic agents (78,80,81,84,95). On the other hand, SphK1 is implicated in upregulation of HIF1a (71,72). Thus, SphK1 and HIF1a may constitute a feed-forward amplification loop favoring tumor progression. In addition to S1P derived from hypoxic tumor cells through the action of SphK1, S1P is also released from apoptotic tumor cells, in which SphK2 is responsible for S1P production (96). S1P thus released from tumor cells is implicated in tumor angiogenesis, in which endothelial cells and tumor-infiltrating BMDCs and macrophages are targets of S1P actions (94,96-98). Indeed, S1P plays an essential role in inducing M2 phenotype in macrophages to render them to behave as TAMs, in which transcriptional upregulation of HIF1a under normoxia is implicated (98).

G_i-COUPLED CHEMOTACTIC S1PR1/S1PR3 MEDIATE A PRO-ANGIOGENIC ACTION OF S1P

Ubiquitously expressed S1P receptor subtypes, S1PR1, S1PR2 and S1PR3 have been studied extensively. They play crucial roles in mediating diverse actions of S1P, which reflect receptor subtype-dependent distinctive intracellular signaling and their cell type-specific expression patterns (Fig. 1) (12-19, 20-23,40,99-104). Vascular endothelial and smooth muscle cells, as well as leukocytes of both myelocytic and lymphocytic lineages, are the major targets of S1P action through either of the three major S1P receptor subtypes. The other members, S1PR4 and S1PR5, are relatively restricted in their expression to the immune and the nervous system, respectively (22).

S1PR1 couples exclusively to G_i and activates well known G_i-dependent pathways, including Ras-ERK and PI3K-Akt pathways, which lead to stimulation of mitogenesis and survival (Fig. 1). S1PR1- G_i-PI3K axis also activates Rho family small GTPase Rac, which is essential for regulating actin cytoskeleton and cell migration. In addition to Ras-ERK, PI3K-Akt and -Rac activation, S1PR1 mediates activation of phospholipase C (PLC) with consequent Ca^{2+} mobilization *via* G_i (13-15,19-23,40,99,102-104).

Rac activation leads to subcellular cortical actin assembly and focal contact to ECM, giving rise to lamellipodia or membrane ruffling (105) that features the leading edge of migrating cells toward chemotactic ligands and growth factors for G_i-coupled receptors and receptor tyrosine kinases. Rac is required for cell migration in vitro and in vivo for vascular formation in embryo (99,106).

S1PR1 is expressed in a variety of cell types including vascular endothelial cells, leukocytes of both lymphoid and myeloid origins, fibroblasts and tumor cells of epithelial and non-epithelial origins. S1PR1 expressed in these cell types, if not counteracted by an opposing function mediated by S1PR2, mediates chemotaxis toward S1P in pertussis toxin-sensitive, Rac-dependent manners (24,28-31,47).

S1PR1 expressed in lymphocytes is responsible for their egress from secondary lymphoid organs to lymph and then blood according to an increasing S1P concentration gradient imposed among these compartments, ensuring lymphocyte recirculation and immune surveillance. A Chinese medicine-derived sphingosine mimetic FTY720 (Fingolimod^R) is a potent S1P receptor modulator prodrug with multiple actions, which after phosphorylation by SphK2 *in vivo*, acts as an agonist for S1PR1, S1PR3, S1PR4 and S1PR5 but not S1PR2, and also as a functional antagonist for S1PR1 because of inducing its downregulation (38,39,44,45,107). S1PR1 downregulation in lymphocytes results in the failure of their egress from secondary lymphoid organs and, thereby, peripheral blood lymphopenia. FTY720 is now clinically employed as an immunosuppressive drug for the treatment of multiple sclerosis (49).

S1PR1 expressed in vascular endothelial cells, together with S1PR3, mediates angiogenic action of S1P, which include stimulation of endothelial cell migration, proliferation, survival and morphogenesis of tubular structures, which depends upon reinforcement of VE-cadherin mediated adhesion between endothelial cells (27,32-35,104,109). Endothelial

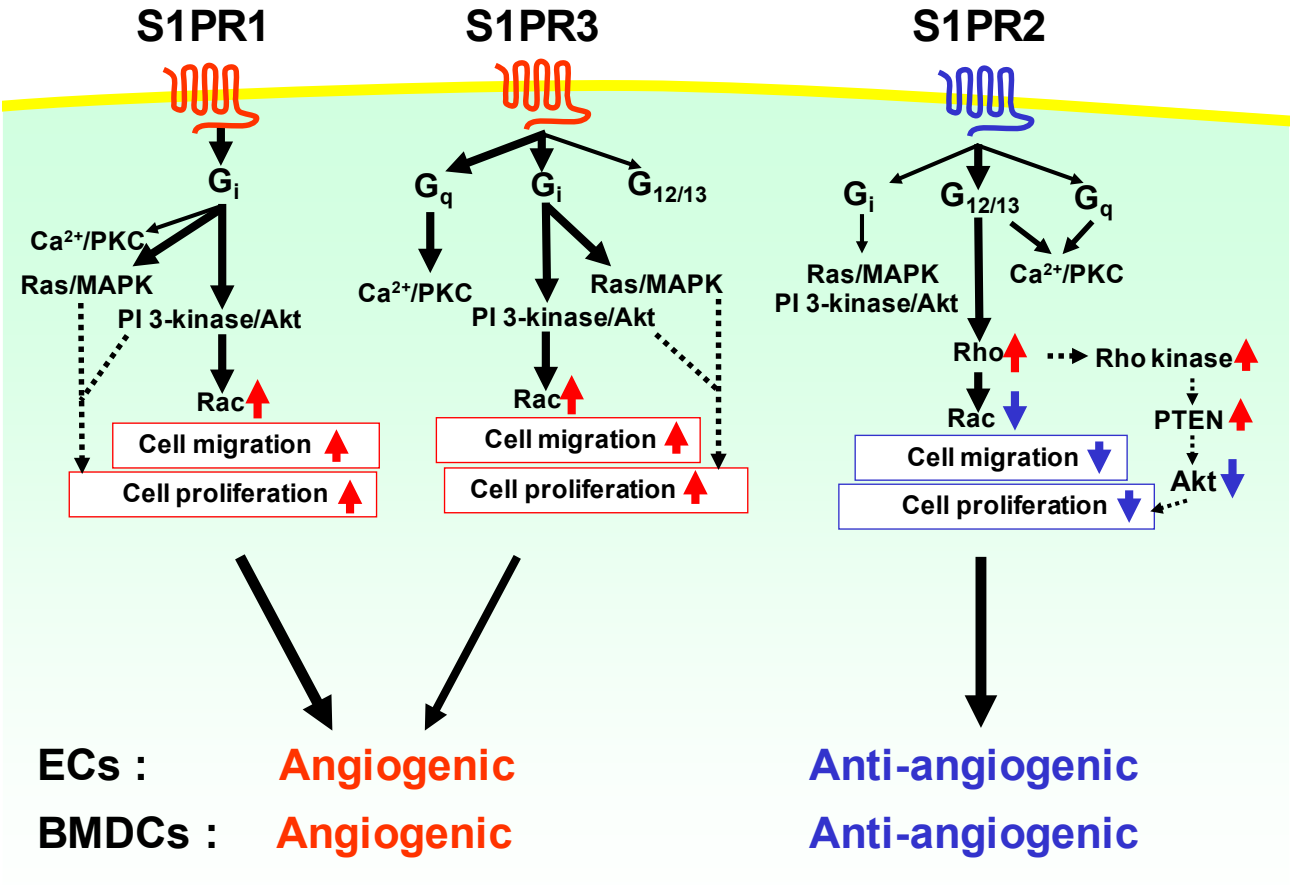

Figure 1. S1P receptor subtype-specific coupling to heterotrimeric G proteins and intracellular signaling pathways.
S1PR1 couples exclusively to G_i to activate Ras-ERK and PI 3-kinase (PI3K)-Akt/Rac pathways, leading to stimulation of chemotaxis and cell proliferation. S1PR3 activates G_q-PLC-Ca^{2+} pathway, and G_i-Ras-ERK and -PI 3-kinase-Akt/Rac pathways. S1PR3- $G_{12/13}$ –Rho pathway is masked and not evident unless G_i is inhibited by pertussis toxin. S1PR2 couples to $G_{12/13}$ to induce potent Rho activation, leading to inhibition of Rac and cell migration, and also inhibition of cell proliferation via inhibition of Akt. S1PR1/S1PR3 expressed in endothelial cells (ECs) and bone marrow-derived myeloid cells (BMDCs) mediate angiogenic effects of S1P. In sharp contrast, S1PR2 expressed in these cells mediates anti-angiogenic effects of S1P.

S1PR1 is also required for maturation and stabilization of nascent microvessels, which includes recruitment of mural cells and their tight coverage around naked endothelial tubes, in which heterologous endothelial-mural cell adhesion should take place. Indeed, in S1PR1-null mice vasculogenesis takes place normally but mural cell coverage is defective, leading to massive hemorrhage and embryonic death at mid-gestation (24). This phenotype is reproduced in endothelial cell-specific conditional S1PR1 knockout (KO) mice, and also in SphK1/2-double KO mice. PDGF-B null mice also show defective mural cell coverage and vascular maturation among other abnormalities. In endothelial-mural cell adhesion, endothelial S1PR1-G_i-PI3K-Rac signaling is required for phosphorylation of N-cadherin and N-cadherin/β-catenin/actin complex formation (110).

Endothelial S1PR1-dependent cell-cell adhesion is essential not only for angiogenesis and vascular maturation, but also for maintenance of vascular integrity through suppression of transmural permeability and leakiness. In this procss S1PR1-G_i-PI 3-kinase-Rac-dependent signalings are also operating, which include enhancement in formation of VE-cadherin-based formation of adherens junctions (111) and also that of tight junctions, in which junctional adhesion molecules such as zona occludens (ZO) proteins are involved (27,112).

It is reported recently that S1PR1/3 mediate macrophage phenotype switching from proinflammatory (classic) to anti-inflammatory (alternative) phenotype, leading to suppression of proinflammatory cytokine secretion. It is also reported that S1PR1/3 signaling contributes to induction of TAM phenotype (96).

S1PR3 resembles S1PR1 in that it activates Ras-ERK, PI 3-kinase-Akt and -Rac via G_i, mediating mitogenic/prosurvival and chemotactic effects of S1P (17-23,40,99-104,113). In addition, S1PR3 couples to Gq to potently activate PLC-Ca^{2+} signaling, and to $G_{12/13}$-Rho, the latter being a relatively minor pathway that becomes evident in pertussis toxin-treated, G_i-inactivated condition (99,113).

S1PR3 (and S1PR1) plays a pivotal role in S1P activation of endothelial nitric oxide synthase (eNOS), in which Ca^{2+}-dependent, Akt-dependent and ERK1/2-dependent mechanisms are operating (103,114-117). Thus, S1P induces NO-dependent vascular relaxation via S1PR3 (and S1PR1) activation in endothelial cells. In addition, NO plays crucial roles in protection of endothelial function and prevention of atherogenesis. S1P specifically binds to M apolipoprotein in HDL. HDL exerts its anti-atherogenic effects not only by subtracting excess cholesterol from vascular endothelium but also by providing endothelial S1PR1 and S1PR3 with S1P (114).

S1PR3 expressed in vascular smooth muscle cells, on the other hand, mediates vascular contraction via G_q-coupled Ca^{2+} mobilization and consequent activation of myosin light chain kinase and protein kinase C. Relative contributions of S1PR3 expressed in vascular endothelial vs. smooth muscle cells on vascular tone could depend upon vascular beds. In addition to mediating S1P actions on vasoconstriction or relaxation, S1PR3 expressed in the heart mediates negative chronotropic effect in a G_i-dependent manner, which is reminiscent of muscarinic acetylcholine receptor. S1PR3 KO mice are phenotypically normal (103) .

$G_{12/13}$-COUPLED CHEMOREPELLANT S1PR2 MEDIATES AN ANTI-ANGIOGENIC ACTION

S1PR2 is quite different from S1PR1 and S1PR3, and is the first G protein-coupled receptor to be identified that mediates inhibition of cell migration (99, 102,113,118-120). The repertoire of G protein coupling by S1PR2 overlaps with that of S1PR3, however, S1PR2 robustly couples to $G_{12/13}$ to induce potent activation of RhoA (16,19-23,99,101-104,113,118-120). At the site downstream of $G_{12/13}$-RhoA, S1PR2 potently inhibits its chemoattractant-induced Rac activation via stimulation of Rac GTPase-activating protein (Rac GAP) activity to inhibit cell migration toward these chemoattractants (19-23,99,102-104,118-120). Endogenous S1P2R expressed in B16 melanoma cells mediates inhibition of Rac, cell migration and invasion in Matrigel, which are abolished by S1PR2-selective antagonist, JTE013 (118).

S1PR2-mediated, $G_{12/13}$-coupled RhoA activation also leads to activation of Rho kinase or ROCK, and ROCK-dependent activation of PTEN, with consequent reductions in 3'-phosphorylated phosphoinositides, PIP_3 and PIP_2, which are required for Akt activation (121). Inhibition of Akt via S1PR2 leads to inhibition, rather than stimulation, of cell proliferation by S1P (121-124). S1PR2 mediates relatively weak activation of Ras/ERK and PI3K pathways via G_i (16,20-23,99,101-104,118), which may explain mitogenic actions of S1PR2 under certain conditions.

S1PR2-mediated, $G_{12/13}$-Rho-dependent inhibition of Rac and Akt underlies potent inhibitory action of S1PR2 on angiogenesis (123). Thus, S1PR2 expressed in murine lung microvascular endothelial cells (MLECs) mediates inhibition by S1P of their migration, proliferation, and tube formation in vitro, and angiogenesis in vivo in subcutaneous Matrigel plug assay and tumor angiogenesis. In S1PR2 KO mice tumor angiogenesis and tumor progression are significantly enhanced as compared to wild type (WT) littermates. Subcutaneous inoculation of tumor cells in WT mice together with MLECs obtained from S1PR2 KO mice markedly potentiated tumor angiogenesis and tumor growth as compared to tumor cell inoculation with WT MLECs, which implicates endothelial cell-autonomous function of S1PR2. In addition, the recruitment of BMDCs to tumor stroma, the expression of proangiogenic factors, and MMP9 activity were all enhanced in S1PR2KO mice as compared to WT littermates. Bone marrow transplantation experiments using $S1PR2^{LacZ/LacZ}$ mice demonstrated that S1PR2 expressed in tumor-infiltrating BMDCs, which include $CD11b^+$ cells implicated in tumor angiogenesis, play a crucial role in inhibiting tumor angiogenesis. Deletion of S1PR2 only in BMDCs significantly potentiated tumor angiogenesis and tumor growth in WT mice. These results indicate that S1PR2 expressed in endothelial cells and BMDCs in concert mediate inhibition of tumor angiogenesis and tumor growth (123).

Consistent with these observations, S1PR2 expressed in macrophages mediate S1P inhibition of their recruitment to inflammatory sites (123). However, S1PR2 in endothelial cells also mediates inflammatory effects of S1P, by upregulation of the proinflammatory enzyme cyclooxygenase 2 (COX2) and

downregulation of eNOS expression. In S1PR2 KO neonates that are subjected to ischemia-driven retinopathy, revascularization into avascular zones of the retina were augmented, whereas pathologic neovascularization in the vitreous chamber was rather suppressed, with reductions in endothelial gaps and inflammatory cell infiltration (117).

S1PR2 also mediates S1P stimulation of PLC and Ca^{2+} mobilization via Gq and $G_{12/13}$, (16,125), which in some vascular beds results in vascular contraction, contributing to normal hemodynamic regulation.

S1PR2 KO mice develop abnormalities in the inner ear, i.e., deafness and vestibular ataxia, in which hemodynamic derangement due to abnormally dilated capillaries in stria vascularis is involved (126). S1PR2KO mice also show occasional convulsion around weaning age with abnormal electroencephalogram (48), and spontaneous development of diffuse large B cell lymphoma in adulthood (127). Molecular mechanisms for these phenotypes are yet to be fully understood.

S1PR1/2 double null and S1PR1/2/3 triple null embryos show more severe defects in embryonic vascular formation and earlier death in utero, compared to S1PR1 single null embryos. S1PR2/3 double null embryos also show partial embryonic lethality and vascular abnormalities. These results indicate that coordinated functions of S1PR1, S1PR2 and S1PR3 are required for development of a fully functional vascular system during embryonic stage (126).

S1PR1, S1PR2 and S1PR3 are widely expressed in various types of cells. The overall outcome of S1P signaling in a given cell type largely depends upon relative expression levels of the S1P receptor subtypes. In addition, cross-talks between S1P receptor signalings and growth factor or cytokine receptor signalings have been reported. For example, S1PR1 and VEGF receptor activation in concert stimulate angiogenesis. Under certain conditions S1PR3 activation leads to activation of TGF-β signaling pathway and fibrosis (128,129). Update information regarding detailed cross-talk mechanisms is available in recently published excellent reviews (22,95,130,131).

STIMULATING S1P SIGNALING PATHWAY FOR TREATMENT OF POST-ISCHEMIC ANGIOGENESIS

Patients with occlusive peripheral arterial diseases suffer from limb ischemia, seeking for effective angiogenic therapy. For these patients, the clinical trials of either administration of proangiogenic growth factors such as VEGF, FGF-2 and HGF, or their expression vectors, or bone marrow mononuclear cell implantation which supplies myeloid lineage cells that produce proangiogenic factors and endothelial progenitor cells, have been conducted (132-136). These trials demonstrated that the angiogenic therapy conferred some beneficial effects such as reduced pain and decreased need for amputation, however, the effects are not satisfactory and obtained only in a portion of patients. Some drawback has been reported in the angiogenic therapy: administration of VEGF causes increases in vascular permeability and resultant edema as a serious side effect.

We demonstrated for the first time that S1P was effective in stimulating post-ischemic angiogenesis in a mouse model of hindlimb ischemia after unilateral femoral artery resection (51,52). Daily intramuscular administration of S1P dose-dependently stimulated blood flow recovery, resulting in up to twice as much blood flow at 10^{-7} M of S1P, which was accompanied by 1.7-fold increase in the capillary density in ischemic muscle compared with vehicle control. The optimal S1P effects were comparable with those obtained with FGF-2. Differently from VEGF, S1P injections did not increase vascular permeability, which was evaluated by Miles assay. We also analyzed post-ischemic angiogenesis in SphK1-overexpressing transgenic (TG) mice. SphK1 TG mice showed 40-fold higher sphingosine kinase activity and 1.8-fold higher S1P content in skeletal muscle compared with WT mice (51). In SphK1 TG mice the post-ischemic blood flow recovery and angiogenesis were both accelerated compared with WT mice, without an increase in the vascular permeability. These observations suggest that S1PR1/3-mediated angiogenic signals are dominant over anti-angiogenic signal mediated via S1PR2, and indicate potential therapeutic usefulness of S1P for tissue ischemia. We then explored the way to achieve sustained delivery of S1P, and found that poly(lactic-co-glycolic-acid) (PLGA)-based S1P-containing microparticles (PLGA-S1P) are biodegradable and continuously release S1P (52). We studied the effects of PLGA-S1P microparticles and found that intramuscular injections of PLGA-S1P dose-dependently stimulated blood flow and increased microvessel density in C57BL/6 mice with injections every 3 days conferring the optimal result. In Balb/c mice, which show retarded blood flow recovery compared with C57BL/6 mice and exhibit limb necrosis with apparent functional dysfunction, injections of PLGA-S1P stimulated blood flow with alleviation of limb necrosis and dysfunction. PLGA-S1P microparticles did not induce edema in ischemic limbs but rather suppressed VEGF-induced edema. Moreover, we observed that PLGA-S1P promoted the coverage of vessels by smooth muscle cells and pericytes, thus stabilizing vessels. PLGA-S1P microparticles stimulated Akt and ERK

with increased phosphorylation of eNOS in ischemic muscle. Experiments with a nitric oxide synthase (NOS) inhibitor showed that the stimulatory effect of PLGA-S1P on blood flow recovery was in part dependent on NO production. PLGA-S1P also stimulated the expression of pro-angiogenic growth factors in ischemic tissues, and enhanced the recruitment of $CD45^+$, $CD11b^+$ and $Gr\text{-}1^+$ myeloid cells, which are known to contribute to post-ischemic angiogenesis through production of pro-angiogenic growth factors (52).

These results indicate that PLGA-based, sustained local delivery of S1P is a promising therapeutic modality for stimulating post-ischemic angiogenesis. It is expected that S1P receptor subtype-selective agonists and antagonists could confer better effects in therapeutic angiogenesis.

TARGETING THE S1P SIGNALING PATHWAY FOR CONTROLLING TUMOR ANGIOGENESIS

Tumor angiogenesis has been the target of an anti-VEGF monoclonal antibody (mAb)(bevacizumab or Avastin^R) (132) and inhibitors of receptor tyrosine kinases or multi-kinase inhibitors. These modalities have limitations, because of side effects, including perforating peptic ulcer, hypertention and proteinuria associated with anti-VEGF therapy, and acquisition by tumor cells of resistance.

Accumulated evidence provides molecular basis for anti-angiogenic therapy through targeting the S1P signaling pathway. Anti-S1P mAb (Sphingomab) (53,54), has been developed and shown to act as a molecular sponge to efficiently bind S1P, preventing its binding to cell surface receptors. The anti-S1P mAb inhibits tumor progression in mouse xenograft and allograft models through inhibition of tumor angiogenesis. Thus, the anti-S1P mAb blocked endothelial cell migration and formation of tubular structures in vitro, and inhibited blood vessel formation induced by pro-angiogenic growth factors and arrested tumor-associated angiogenesis in vivo (53). The anti-S1P mAb also inhibited mitogenic and anti-apoptotic effects of S1P in tumor cells and their release of proangiogenic cytokine (53,54). Since the antibody is capable of triggering S1P release from erythrocytes (71), which constitute a large reservoir of plasma S1P, suitable antibody delivery system that bypass bloodstream to target primary and metastatic tumor tissues would improve cost performance status.

Since S1P stimulates and inhibits tumor angiogenesis through S1PR1/3 and S1PR2, respectively, either S1PR1/3-selective antagonists or S1PR2-selective agonists are expected to be effective in inhibiting tumor angiogenesis and tumor growth. At present the former strategy is under investigation in experimental models whereas the latter not yet available.

As described above, downregulation of S1PR1 by FTY720-phosphate in lymphocytes inhibits lymphocyte trafficking. FTY720-phosphate also induces downregulation of S1PR1 in endothelial cells and suppresses angiogenic activity of S1P (50). Pretreatment of HUVECs with nanomolar concentrations of FTY720 or FTY720-phosphate downregulated S1PR1 and inhibited endothelial cell migration in response to subsequent S1P. FTY720 potently inhibited angiogenesis in a variety of in vivo models, including corneal micropocket assay, subcutaneously implanted chamber assay, and tumor angiogenesis, tumor growth and metastasis (55-57). Moreover, FTY720 at micromolar concentrations directly induced apoptosis of a breast cancer cell line (55). It is also reported in ovarian cancer cells that FTY720 induced autophagy and necrosis (137). These observations may be related to recently reported novel actions of FTY720, which include inhibition of SphK1 (138) and autotaxin, the latter being phospholipase D in the plasma responsible for production of the lipid mediator lysophosphatidic acid (139). Because of the fact that FTY720 is a potent immunosuppressant, it is concerned that its clinical use for cancer patients is compromised by suppression in anti-tumor immunity and increased risk of opportunistic infection. In addition, endothelial dysfunction with reduced eNOS activity and increased permeability could occur. It is reported that the induction of vascular leak by FTY720 is associated with ubiquitin-dependent degradation of endothelial S1PR1 in mice (139).

FUTURE PERSPECTIVES

Recently novel pharmacological S1P receptor antagonists with anti-angiogenic potential have been reported. These include the S1PR1/3 antaonist, compound 5, {sodium 4-[(4-butoxyphenyl) thio]-2'-[{4-[(heptylthio)methyl]-2-hydroxyphenyl}(hydroxy) methyl]biphenyl-3-sulfonate} which inhibited endothelial cell migration, proliferation and tube formation and also inhibited hypotensive effect of S1P in rats (140), and an FTY720 derivative 1-(hydroxymethyl)-3-(3-octylphenyl)cyclobutane (VPC03090), which show inhibition of tumor growth in vivo (141). An S1P derivative [N-((2S,3R)-3-hydroxy-1-morpholino-4-(3-octylphenyl)butan-2-yl)tetradecanamide] (NHOBT) also shows inhibition of endothelial cell tube formation and endogenous neovascularization of the chick embryo chorioallantoic membrane (142). It is expected that S1PR1/3 antagonists with potent anti-angiogenic activity but relatively

weak inductive activities of vascular leak and eNOS inhibition would become promising therapeutics for controlling tumor angiogenesis. S1PR2 agonist, if developed, is also a promising candidate of anti-angiogenic medicine.

Investigation in-depth into molecular mechanisms for S1P-mediated regulation of angiogenesis, in combination with development of S1PR subtype-targeted, selective agonists and antagonists and their optimal drug delivery system, are expected to improve outcomes of both pro-angiogenic and anti-angiogenic therapies in the future. Their possible interference with biomolecules with angiogenic potentials, such as nerve growth factor (144-146) and leptin (147-149), might also be evaluated.

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