interactions between cancer cells and these stromal cells allow the cancer to progress by tumor growth, invasion, and metastasis. An excellent example is the contributions of blood endothelial cells in tumor-induced angiogenesis in cancer progression (2). It has become increasingly evident that tumor cells can release many factors that impact the tumor microenvironment, such as vascular endothelial growth factor (VEGF) for angiogenesis. In this review, we will discuss how cancer cells export one of these factors, sphingosine-1-phosphate (S1P), a pleiotropic bioactive sphingolipid metabolite that regulates many processes important for cancer progress in cancer cells themselves and in the tumor microenvironment.

S1P AND ITS ROLES IN CANCER

In the two decades since the discovery that S1P regulates cell growth (3, 4) and suppresses apoptosis (5), numerous reports have been published on S1P signaling and its functions as a bioactive lipid mediator. These clearly established that S1P regulates many important cellular processes involved in cancer, including growth, survival, migration, and invasion as well as angiogenesis and immune responses (6, 7). Growth factors, cytokines, hormones, and angiogenic factors important for cancer, including estradiol (E2) (8), epidermal growth factor (EGF) (9, 10), insulin-like growth factor-I (IGF-1) (11), and VEGF (12, 13) all stimulate sphingosine kinase (SphK), the enzyme that phosphorylates sphingosine to produce S1P. There are two isotypes of SphK, designated SphK1 and SphK2. SphK1 is located in the cytosol close to the cell membrane where its substrate sphingosine resides

Abstract Sphingosine-1-phosphate (S1P) is a bioactive lipid mediator that promotes cell survival, proliferation, migration, angiogenesis, lymphangiogenesis, and immune response; all are critical processes of cancer progression. Although some important roles of intracellular S1P have recently been uncovered, the majority of its biological effects are known to be mediated via activation of five specific G protein-coupled receptors [SIP receptor (S1PR)1–S1PR5] located on the cell surface. Secretion of S1P produced inside cells by sphingosine kinases can then signal through these receptors in autocrine, paracrine, and/or endocrine manners, coined “inside-out” signaling of S1P. Numerous studies suggest that secreted S1P plays important roles in cancer progression; thus, understanding the mechanism by which S1P is exported out of cells, particularly cancer cells, is both interesting and important. Here we will review the current understanding of the transport of S1P out of cancer cells and its potential roles in the tumor microenvironment.—Takabe, K., and S. Spiegel. Export of sphingosine-1-phosphate and cancer progression. J. Lipid Res. 2014. 55: 1839–1846.
SphK: A CRITICAL REGULATOR OF THE S1P RHEOSTAT AND INSIDE-OUT SIGNALING OF S1P IN CANCER

S1P levels are tightly regulated by the balance between synthesis by SphKs, reversible conversion to sphingosine by specific S1P phosphatases (SPP1 and SPP2), and degradation by S1P lyase (6, 22). In contrast to S1P, which is associated with growth and survival, its precursors, sphingosine and ceramide, are associated with cell growth arrest and apoptosis and the concept of a ceramide-sphingosine-S1P rheostat was proposed early in the beginning of the S1P research field (5). According to this model, the balance between these interconvertible sphingolipid metabolites, ceramide, sphingosine, and S1P, regulates cellular growth and survival in response to cellular and environmental stimuli (5, 22, 23). There are many examples in which this rheostat is functional in cancer cell survival, apoptosis, autophagy, and resistance to chemotherapy and radiation in vitro and in vivo (22, 24–28). SphK1 is a critical regulator of this rheostat, as it produces the pro-growth and anti-apoptotic S1P, and also reduces levels of pro-apoptotic ceramide and sphingosine (22, 26, 29). This concept has been borne out by many studies in cultured cells and preclinical cancer models, and the reader is referred to several excellent reviews on this topic (24–28).

Because S1P possesses a polar head group, S1P generated inside the cells is unable to freely pass through cell plasma membranes. Thus, S1P export requires a carrier or transporter. The release of S1P from cells was first described in blood cells. Blood cells, as a group, are generally able to release S1P efficiently, including erythrocytes, platelets, neutrophils, and mononuclear cells. Platelets store and release S1P following stimulation with thrombin (43) and shear stress (44), which is closely correlated to activation of protein kinase C (43). It has been shown that S1P is stored in the inner leaflet of the plasma membrane in platelets and released via transporters, and not by exocytosis in the presence of albumin (45). Erythrocytes which are able to release S1P even without stimulation (46) are now known to be a major reservoir of S1P (47, 48).

SIP EXPORT BY ABC TRANSPORTERS, THE MULTIDRUG RESISTANT PROTEINS

Because S1P possesses a polar head group, S1P generated inside the cells is unable to freely pass through cell plasma membranes. Thus, S1P export requires a carrier or transporter. The release of S1P from cells was first described in blood cells. Blood cells, as a group, are generally able to release S1P efficiently, including erythrocytes, platelets, neutrophils, and mononuclear cells. Platelets store and release S1P following stimulation with thrombin (43) and shear stress (44), which is closely correlated to activation of protein kinase C (43). It has been shown that S1P is stored in the inner leaflet of the plasma membrane in platelets and released via transporters, and not by exocytosis in the presence of albumin (45). Erythrocytes which are able to release S1P even without stimulation (46) are now known to be a major reservoir of S1P (47, 48).

Fig. 1. Inside-out signaling of S1P in the tumor microenvironment. S1P is generated from sphingosine (Sph), a ceramide (Cer) metabolite, by SphKs, especially SphK1. ABCG1, ABCG2, and possibly Spsn2 export S1P from cancer cells. S1P can then bind to S1PRs in autocrine, paracrine, and/or endocrine manners. S1P can promote cancer cell survival, proliferation, and migration, as well as angiogenesis and lymphangiogenesis, and possibly distant metastasis.
The ABC family of transporters was first implicated in release of S1P from cells. ABC transporters were originally identified as multidrug resistant proteins (MRPs) in cancer cells that were upregulated after treatment with cytotoxic chemotherapeutic agents. In addition to amphiphilic drugs, ABC transporters were later found to also enhance the transport of various lipids from the inner to the outer leaflet of the plasma membrane (49). ABC transporters contain two transmembrane domains with six membrane spanning α-helices that form a channel for substrate transport through membranes and two cytosolic ABCs. Based on phylogenetic analysis and amino acid sequence alignments, 49 human ABC transporters have been identified. Some members of this group have been implicated in export of S1P. Kobayashi et al. (45) suggested the existence of two independent S1P release systems in the platelet plasma membrane, an ATP-dependent system stimulated by thrombin and an ATP-independent system stimulated by calcium. In both platelets and erythrocytes, S1P secretion was mediated by an ABC transporter, as it was inhibited by glyburide. However, neither inhibitors of ABCB1 [previously called multidrug resistance-1 (MDR1) or P-glycoprotein (P-gp)] verapamil or cyclosporine A, nor the ABC1 inhibitor MK571, diminished S1P release, suggesting that an unidentified ABC transporter is involved in S1P export from platelets and erythrocytes (45, 50). Because the plasma S1P levels from Abca1-, Abca7-, and Abcc1/Mrp1-null mice were not significantly changed, it was suggested that either these transporters are not required in the maintenance of plasma S1P, or more likely, S1P release may be compensated by other transporters in vivo (51). Indeed, a more recent study demonstrated that scramblase-dependent transbilayer movement of S1P in erythrocyte membranes was important for S1P release rather than ATP-dependent ABC transporters. It was suggested that serum albumin or HDLs extract S1P from the cell membrane of erythrocytes. In addition, they observed transcellular transportation of S1P via contact between erythrocytes and endothelial cells (52).

Using pharmacological and molecular approaches, it was shown that ABC1 (also known as MRP1) is involved in transport of S1P out of rodent and human mast cells, especially after antigen stimulation independently of their degranulation (53). Discovery of an active transport system for mast cell secretion of S1P further supports the notion that S1P release may regulate migration of mast cells toward antigen and their arrival at sites of inflammation. We subsequently found that SphK1, but not SphK2, increased S1P export from MCF7 human breast cancer cells after E2 stimulation, quantitatively measured by LC-ESI-MS/MS (54). S1P export was suppressed either by pharmacological inhibition, as it was inhibited by glyburide. However, neither inhibitors of ABCB1 [previously called multidrug resistance-1 (MDR1) or P-glycoprotein (P-gp)] verapamil or cyclosporine A, nor the ABC1 inhibitor MK571, diminished S1P release, suggesting that an unidentified ABC transporter is involved in S1P export from platelets and erythrocytes (45, 50). Because the plasma S1P levels from Abca1-, Abca7-, and Abcc1/Mrp1-null mice were not significantly changed, it was suggested that either these transporters are not required in the maintenance of plasma S1P, or more likely, S1P release may be compensated by other transporters in vivo (51). Indeed, a more recent study demonstrated that scramblase-dependent transbilayer movement of S1P in erythrocyte membranes was important for S1P release rather than ATP-dependent ABC transporters. It was suggested that serum albumin or HDLs extract S1P from the cell membrane of erythrocytes. In addition, they observed transcellular transportation of S1P via contact between erythrocytes and endothelial cells (52).

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ABC1 has also been linked to transport of S1P in several other cell types. For example, transport of S1P from the cytosol of human fibroblasts to S1PR3 by ABC1 is involved in the cytoprotective effect of glucocorticoids (56). Moreover, ABC1 is also involved in S1P release from uterine leiomyoma cells and late pregnant rat myometrium (57). Based on measurements by immobilized metal affinity chromatography coupled with quantitative HPLC analysis, Lee et al. (51) suggested that ABCA1 and ABC1 are involved in the release of S1P from human umbilical vein vascular endothelial cells. In agreement, a recent study identified ABC1 as the transporter that mediates S1P efflux from brain and spinal cord endothelial cells (58).

Sato et al. (59) reported that the release of S1P from astrocytes is highly dependent on the ABC1 transporter, based on its inhibition by glyburide, a pharmacological inhibitor of ABCA1, or by ABC1-specific siRNA. Further, Polorget et al. (60) demonstrated that SphK1 and its product S1P stimulate ABCB1 expression and transport activity in endothelial cells from brain capillaries. Because SphK1 is overexpressed in endothelial cells from brain tumors, these data may suggest that SphK1 and S1P could contribute to the multidrug resistance phenotype in brain tumor-derived endothelial cells (60).

As mentioned above, ABC transporters were originally found as multidrug resistance genes, and have been shown to be overexpressed in a range of solid and hematological cancers and, in some cases, have correlated its expression with negative responses to treatment and poor disease outcome (61). Because several ABC transporters have been implicated in secretion of S1P from stromal, endothelial, and cancer cells, it is possible that these transporters are involved in pathological processes regulated by S1P, such as cancer progression and metastasis.

**SPINSTER HOMOLOG 2, A NEW S1P TRANSPORTER**

Because the ABC transporters, ABC1, ABCG2, and ABCA1, which have been implicated in export of S1P are promiscuous and also export other important lipids, the identification of spinner homolog (Spns)2 as a unique type of S1P transporter was a major breakthrough (62, 63). SPNS2 is a putative twelve transmembrane domain protein (504 amino acid residues) belonging to the Spinster family, which is part of the major facilitator superfamily (MFS) transporters based on amino acid sequence homology. Spinner was originally identified in *Drosophila* as the gene mutated in female flies that present strong rejection against male courtship. In zebrafish, a point mutation in the Spns2 gene causes the abnormal development of two hearts (cardia bifida), due to the failure in supplying S1P to the S1PRs that regulate the cardiac progenitor migration (62, 63). It was demonstrated that SPNS2, but not SPNS1, is able to export S1P and dihydro-S1P, but...
not sphingosine or dihydro sphingosine, from cells (63, 64). Dihydro-S1P is a saturated analog of S1P that does not contain the trans double bond at the position 4 in S1P and functions as an agonist for S1PRs, but its specific physiological role remains unclear. The S1P transport activity of SPNS2 increases in proportion to the amount of S1P inside the cells, which suggests that it acts as a passive transporter that does not require any energy source and is distinct from ABC transporters (65).

Although SPNS2 appears to be the predominant S1P exporter in vascular endothelial cells based on its downregulation both in vivo and in vitro (66), its effects on plasma S1P levels were small (64, 67–69) compared with erythrocyte-specific disruption of SphK1 that drastically decreased plasma S1P (47). These results suggest that SPNS2 may be not a major determinant of plasma S1P concentration (64, 67–69).

It is well established that mature single-positive T cells upregulate S1PR1, allowing T cells to exit the thymus in response to a S1P gradient (70). This gradient is produced by neural crest-derived perivascular cells that surround the thymus (71), and, subsequently, the T cells encounter the endothelium and respond to the high levels of S1P in the blood maintained by erythrocytes (47) and endothelial cells (67, 72). Several studies convincingly established that Sps2 knockout mice have decreased numbers of circulating T lymphocytes, particularly CD4 and CD8 single-positives, and accumulation of mature T cells in thymus. Mature recirculating B cells were also reduced in the bone marrow from these mice as well as in blood and secondary lymphoid organs (64, 66–69). However, the reduction in blood levels of S1P in Sps2 knockout mice cannot be the sole determining factor that influences this aberrant lymphocyte trafficking because Sphk1 knockout mice have an even greater reduction in blood S1P levels but do not have lymphopenia (73).

We found that S1P levels in lymph nodes, lymphatic fluid, and interstitial fluid were elevated in Sps2 knockout mice (64), which suggests an aberrant S1P gradient. This increase in S1P could downregulate S1PR1 expression on the lymphocytes required for their egress out of the lymph node. We also observed that mice with a global knockout of Sps2 have aberrant lymphatic sinuses that appear collapsed, with reduced numbers of lymphocytes. These suggest that Sps2 plays a role in regulation not only of blood S1P but also lymph node and lymph S1P levels, and consequently influences lymphocyte trafficking and lymphatic vessel network organization (64). However, using mice with a specific deletion of Sps2 in hematopoietic and endothelial cells, Schwab and colleagues found that lymphatic S1P levels were decreased, which they suggested was responsible for blocking lymphocyte egress from the lymph nodes and lymphocyte circulation (69). Comparing the results of the global knockout to the endothelial-specific knockout of Sps2 suggests that Sps2 expression in nonendothelial cells also contributes to regulation of S1P levels and the S1P gradient. Indeed, levels of S1P in tissues such as liver, lung, colon, and lymph nodes (64), where Sps2 is highly expressed (68), are also increased in Sps2 knockout mice.

Surprisingly, the role of SPNS2 in cancer progression, including its effect on tumor environment, has not yet been investigated. However, given its importance for lymphocyte egress that is responsible for cancer immune responses and lymphangiogenesis as discussed below, further studies to elucidate its role are warranted.

**TUMOR-INDUCED ANGIOGENESIS BY INSIDE-OUT SIGNALING OF S1P**

Angiogenesis, generation of new blood vessels from preexisting ones, is a hallmark of cancer and is a crucial component of the tumor microenvironment. Angiogenesis determines the growth and progression of cancer by providing oxygen, nutrition, and conduits for invading cells to metastasize. Recent growing evidence implicates inside-out signaling of S1P as one of the key regulators of angiogenesis. Expression of S1PR1 is upregulated in tumor vessels, and its downregulation was effective in inhibiting angiogenesis and tumor growth in vivo, suggesting that S1PR1 is also a critical component of tumor-induced angiogenesis (74).

Neutralization of extracellular S1P with an anti-S1P antibody significantly inhibited angiogenesis, tumor growth, and metastasis, further confirming the dominant role that extracellular S1P plays in this process (75). This study also raised an important question: which cells are producing S1P, the tumors themselves, the tumor microenvironment, or tumor-associated immune cells? Several previous studies demonstrated that cancer cells can secrete S1P (54, 76, 77). Moreover, data from preclinical mouse cancer models (37, 78) and human patient samples (35, 36, 79) suggest that the tumors themselves with upregulated SphK1 may be a key source of S1P. However, Hla and colleagues (51) reported that endothelial cells (human umbilical vein vascular endothelial cells and mouse embryonic endothelial cells) synthesize and release endogenous S1P more efficiently than human colon cancer cell lines. This raises the possibility that stromal cells may also supply S1P in the tumor microenvironment. In agreement, a recent study suggests that both tumor and systemic S1P regulate local tumor growth, whereas lung colonization/metastasis is controlled selectively via systemic S1P (78). Neutralization of systemic S1P with a specific monoclonal antibody (Sphingomab) suppressed lung metastasis (78), providing further optimism for the use of this strategy in the clinic.

Previous studies have shown that persistent activation of a master transcription factor signal transducer and activator of transcription factor 3 (STAT3), which is a major mechanism for cancer initiation, development, and progression (80), involved upregulation of S1PR1 (81). In a seminal study, it was demonstrated that STAT3 is a transcription factor for S1pr1, which is elevated in STAT3-positive tumors. Conversely, enhanced S1pr1 expression activates STAT3 and upregulates If6 gene expression, thereby accelerating tumor growth and metastasis in a STAT3-dependent manner. The S1PR1-STAT3 signaling axis is a major positive feedback loop for persistent STAT3 activation in breast cancer cells and the tumor microenvironment for malignant...
progression (81). This S1PR1-STAT3 axis also plays an important role in lymphomas (82) and is crucial for myeloid cell colonization at future metastatic sites in prostate and melanoma cancers (83). We have recently extended these observations and shown that S1P produced by upregulation of SphK1 during colitis and associated cancer is essential for production of the multifunctional NF-κB-regulated cytokine IL-6, persistent activation of the transcription factor STAT3, and consequent upregulation of the S1PR, S1PR1. Hence, we suggested that upregulated SphK1 links chronic intestinal inflammation to colitis-associated cancer by stimulation of S1PR1 leading to activation of the master transcription factors NF-κB and STAT3 in a malicious feed-forward amplification loop (84). Of particular relevance, inhibition of SphK1 or S1PR1 blocked the persistent activation of STAT3 and reduced both inflammatory mediators and cancer progression (84), and may be a useful approach to treat colon cancer patients with inflammatory bowel disease.

It has long been known that S1PR1 is important for angiogenesis and vascular maturation during development by regulating migration of mural cells around newly formed blood vessels (85). However, recent studies showed that S1PR1 is involved in termination of sprouting angiogenesis (86, 87) and that endothelial S1PR1 restricts sprouting angiogenesis by regulating the interplay between vascular endothelial (VE)-cadherin and VEGFR2 (88). Hence, S1P carried by blood flow closes a negative feedback loop that inhibits sprouting angiogenesis once the vascular bed is established and functional (87). Whether S1PR1 functions similarly in tumor-driven angiogenesis is still not clear. If so, this may have important clinical implications for cancer treatment, as it is possible that exaggerated VEGF signaling in tumor angiogenesis could be reduced by agonists that activate S1PR1 signaling. It is expected that further studies will answer this important question and might provide new avenues for cancer therapy.

INSIDE-OUT SIGNALING OF S1P IN TUMOR-INDUCED LYMPHANGIOGENESIS

In contrast to angiogenesis, only a few studies so far have examined the involvement of S1P signaling in lymphangiogenesis. S1P can induce in vitro lymphatic endothelial cell (LEC) tube formation via activation of S1PR1 (77, 89). Moreover, Ang2-induced in vitro lymphangiogenesis was suppressed by a SphK1-specific pharmacological inhibitor (90, 91), suggesting cross talk between Ang2 and S1P signaling pathways (32). Interestingly, LEC-specific deletion of SphK1 in the SphK2 knockout mouse, i.e., total knockout of S1P production specifically in LECs, inhibited lymphatic vessel maturation, indicating that SphKs and S1P in LECs are required for proper development of lymphatic vessels (92).

Tumor-associated lymphangiogenesis is usually evaluated by histological examination of microvessel density based on selective morphometric analyses (e.g., vessel counts, vascular morphology, etc.) (93). The strengths of the morphometric analysis are that it can evaluate the location of the vessels as well as the morphology of the vessels during tumor progression. The limitations include variable sites of tissue sectioning, variable immunostaining techniques, different vessel density quantification methods, and the lack of standardization in the estimation of angiogenesis and lymphangiogenesis (93). To compliment this approach and overcome some of these limitations, we have developed another approach using flow cytometry to quantify both blood endothelial cells and LECs from the same sample to simultaneously evaluate both angiogenesis and lymphangiogenesis. By combining flow cytometry and a matrigel plug assay, we found that exogenous S1P enhanced both angiogenesis and lymphangiogenesis, while specifically inhibiting SphK1 and completely blocking Ang2-induced angiogenesis and lymphangiogenesis in vivo (64).

Utilizing our newly established murine syngeneic orthotopic metastatic breast cancer model, we found that circulating S1P levels correlated with tumor burden (37). Interestingly, serum S1P levels were also significantly elevated in stage IIIA breast cancer patients who had developed lymph node metastasis compared with age/ethnicity-matched healthy volunteers. Importantly, inhibiting SphK1 significantly decreased S1P levels in serum and tumors in these mice, and both angiogenesis and lymphangiogenesis were suppressed, not only around the primary tumor but also in lymph nodes that were distant from the tumor (37). Furthermore, lymph node and lung metastases were significantly suppressed. These results indicate that S1P plays a key role not only in tumoral lymphangiogenesis but also in lymph node lymphangiogenesis, which may actively promote metastasis via the lymphatics.

CONCLUDING REMARKS

Over the past years, major progress has been made in our current understanding of the mechanisms by which S1P regulates cell growth and survival, migration, and angiogenesis by inside-out signaling through S1PRs in autocrine, paracrine, and/or endocrine manners. However, much less is known about how S1P is released from cells, particularly cancer cells. ABC transporters and Spns2 are the only known transporters that usher S1P through the cell membrane. It is assumed that S1P released from cancer cells functions predominantly to bind to S1PRs on neighboring blood and lymphatic endothelial cells to regulate angiogenesis and lymphangiogenesis to support tumor growth and/or metastasis. However, S1P can also affect the cancer cells themselves, and an area that still has not received much attention is the effects of S1P on the tumor microenvironment, particularly on trafficking and functions of infiltrating inflammatory cells.

Unmet challenges include deciphering the mechanisms that control release of S1P from cancer cells as well as identification of the transporters in erythrocytes and platelets and their roles in cancer development. How the S1P levels of plasma, lymph, and interstitial fluid are regulated is also still unclear. Much work remains to be done to identify the human transporters that export S1P in different tissues and disease states. It is of great importance to
develop methods to accurately measure levels of S1P in interstitial fluid. This is a critical issue, as it is generally assumed that the S1P levels are very low in interstitial fluid and this is a key determinant of the S1P gradient (low in tissues and high in blood and lymph). Finally, if SPNS2 is a unique transporter that is specific for S1P and dihydro-S1P, compounds that target SPNS2 might be of great value because they would suppress lymphocyte trafficking and induce reversible immunosuppression.

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