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Sphingolipid Signaling and Hematopoietic Malignancies: To the Rheostat and Beyond

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Abstract

Sphingosine-1-phosphate (S1P) is a bioactive lipid with diverse functions including the promotion of cell survival, proliferation, and migration, as well as the regulation of angiogenesis, inflammation, immunity, vascular permeability and nuclear mechanisms that control gene transcription. S1P is derived from metabolism of ceramide, which itself has diverse and generally growth-inhibitory effects through its impact on downstream targets involved in regulation of apoptosis, senescence and cell cycle progression. Regulation of ceramide, S1P and the biochemical steps that modulate the balance and interconversion of these two lipids are major determinants of cell fate, a concept referred to as the "sphingolipid rheostat." There is abundant evidence that the sphingolipid rheostat plays a role in the origination, progression and drug resistance patterns of hematopoietic malignancies. The pathway has also been exploited to circumvent the problem of chemotherapy resistance in leukemia and lymphoma. Given the broad effects of sphingolipids, targeting multiple steps in the metabolic pathway may provide possible therapeutic avenues. However, new observations have revealed that sphingolipid signaling effects are more complex than previously recognized, requiring a revision of the sphingolipid rheostat model. Here, we summarize recent insights regarding the sphingolipid metabolic pathway and its role in hematopoietic malignancies.

Keywords

cancer; ceramide; chemotherapy; leukemia; lymphoma; multiple myeloma; sphingolipid; sphingosine-1-phosphate

Introduction

Sphingosine-1-phosphate (S1P) is a lipid signaling molecule generated by sphingosine kinase (SK)-mediated phosphorylation of the long chain base sphingosine. Sphingosine is itself a degradation product of the central molecule of sphingolipid metabolism, ceramide. Between 1990 and 1992, several research groups reported that S1P exerts biological effects in mammalian cells, including the ability to stimulate calcium release from intracellular stores and to promote cell proliferation, migration and tumor cell invasiveness [1–3]. In 1993 a landmark study by Olivera and Spiegel demonstrated that SK activation and S1P formation contributed to the effects of growth factor tyrosine kinase-mediated mitogenic signaling pathways [4]. In 1989, ceramide generated from sphingomyelin breakdown through the actions of a neutral sphingomyelinase was shown to promote differentiation of HL60 leukemia cells [5]. In 1993 ceramide was shown to elicit apoptosis in leukemia cells, and in 1996–8 S1P was shown to counteract the pro-apoptotic effects attributed to ceramide [6–8].

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The notion that these two inter-related metabolites (i.e., S1P and ceramide) exert opposing effects on cell fate became recognized as the “sphingolipid rheostat”, adopted for many years as one of the central tenets of sphingolipid-mediated biology (Figure 1). The rheostat was a conceptually tidy notion that suggested ceramide-induced cell death is counteracted by the actions of its downstream metabolite S1P, thereby presenting a self-limiting biochemical model of internal feedback that has been useful for explaining many observed phenomena in the field. The possible role of S1P signaling and the sphingolipid rheostat in carcinogenesis and the potential of modulating this delicate balance for therapeutic purposes in cancer was recognized early and has been the subject of prolonged and intense investigation.

Between 1997–2000, the individual genetic components of S1P metabolism and signaling were identified, including genes encoding two SKs (SphK1 and SphK2) responsible for S1P biosynthesis, S1P lyase and S1P phosphatases responsible for its degradation and recycling, respectively, and five G protein coupled receptors that mediate S1P signaling events originating at the plasma membrane [9]. S1P was also shown to be a substrate for dephosphorylation by nonspecific lipid phosphatases, and more recently S1P cell export functions of ATP-binding cassette (ABC) and Spns2 transporter proteins have been revealed [10–13]. The elucidation of these critical regulators of S1P levels, transport and action has stimulated an explosion of research into S1P’s biological functions and regulation. Many studies have examined the potential oncogenic role of S1P signaling. In fact, as annual publications related to S1P have steadily increased (from 2 in 1991 to 380 in 2010, totaling 2800 as of this writing), between 10–29% of these publications in every year since 1994 have focused on the observed or potential role of S1P signaling in cancer.

Over the past two decades, S1P signaling has become increasingly recognized for its diverse roles in embryology and physiology, including essential functions in developmental angiogenesis and cardiac morphogenesis, and its influence over post-natal vascular homeostasis, lymphocyte trafficking and inflammatory pathways [9]. S1P acts as an intracellular signaling molecule that influences calcium mobilization, nuclear functions, gene expression and proteosomal degradation. It also serves as a ligand for a family of functionally distinct G protein coupled receptors (GPCRs) that act in autocrine and paracrine fashion to modulate cell migration, cell-cell interactions and generally promote cell survival in response to stressful conditions including ischemia, hypoxia, nutrient deprivation, inflammation, oxidant stress, oncogenic stress and DNA damage from radiation and chemotherapy agents. Many of these processes are related to carcinogenesis, cancer progression and therapeutic resistance.

In 2000, SphK1 was shown to behave as an oncogene in cell and animal models of tumorigenesis wherein it appeared to mediate the mitogenic effects of oncogenic Ras [14]. In 2003, SphK1 upregulation was confirmed in a variety of human tumors, whereas its pharmacological inhibition was cytotoxic to tumor cells [15]. Since that time, specific alterations in the metabolic and signaling components responsible for maintaining S1P homeostasis have been implicated in various cancers of adults and children and in cell and animal models of cancer. These molecular changes affect patient outcome and prognosis, tumor progression, and chemotherapy resistance [16]. New findings suggest that the rheostat model may no longer suffice to explain the effects of ceramide and S1P in all contexts of cancer, including the requirements of some tumors for C16 ceramides for survival, as well as examples of S1P-mediated apoptotic and autophagic cell death [17–19].

Multiple reviews have described the role of ceramide, S1P and the sphingolipid metabolic pathway in cancer [16, 20–24]. This article will review basic concepts pertaining to sphingolipid

structure, S1P signaling and S1P metabolism, and will then focus on current understanding of the role of S1P signaling specifically in the context of hematological malignancies.

Components of S1P Metabolism and Signaling

A sphingolipid consists of a long chain amino base (sphingoid base), most commonly sphingosine in mammalian cells, which forms the structural anchor of the molecule. This base can be modified by the addition of fatty acids of specific chain lengths at the free amino group, thereby generating a variety of distinct ceramide molecular species. In addition, the hydroxyl group at the C1 position of the long chain base can be modified by the addition of a polar head group or sugar residue, singly or in sequential reactions, thereby generating myriad complex sphingolipids found in the lipid rafts of the plasma membrane [25]. *De novo* sphingolipid biosynthesis involves the formation of a 3-ketosphinganine that is subsequently converted to dihydrosphingosine through the actions of a 3-ketosphinganine reductase. Dihydrosphingosine can be acylated by a family of ceramide synthases with specific fatty acid substrate preferences, thereby giving rise to the formation of various dihydroceramides. The dihydroceramides are then converted to ceramides by dihydroceramide desaturase, which introduces a double bond into the long chain base backbone, converting it from dihydrosphingosine to sphingosine. In contrast, sphingolipid recycling, which occurs by hydrolysis of the polar head group of membrane sphingolipids in which the long chain base has already been desaturated, directly produces ceramides. The most prominent example of this is the generation of ceramide from sphingomyelin by stress-activated sphingomyelinases.

The sphingolipid degradation pathway is initiated by the deacylation of ceramides by a family of pH-dependent ceramidases, thereby releasing the free long chain base. SphK1 or SphK2 can then phosphorylate free sphingosine, thereby yielding S1P or, in the case of other long chain bases, producing the corresponding long chain base phosphate [26]. SphK1 is primarily cytosolic. However, mitogenic signals including phorbol esters, tumor necrosis factor-α (TNFα), growth factor receptors, estrogens, cytokines, calcium and phospholipase D induce the phosphorylation of SphK1 on Ser225 by extracellular signal-regulated kinases (ERK)1/2, leading to its membrane translocation. This event, which is also facilitated by the calcium and integrin binding protein (CIB1), substantially increases SphK1 activity [27]. In contrast, SphK2 is primarily nuclear in its subcellular localization and has unique functions as a member and negative regulator of a histone deacetylase (HDAC) 1/2 complex that represses the expression of p21, c-Fos and potentially other targets [28]. There is also evidence that SphK2 can function as a pro-apoptotic Bcl-2 homology 3 (BH3)-only protein [29]. Once formed, S1P can be dephosphorylated by the actions of S1P phosphohydrolases and lipid phosphatases or irreversibly degraded by S1P lyase to yield a long chain aldehyde and ethanolamine phosphate [30, 31]. These enzymes are implicated in the regulation of cell fate through their impact on intracellular levels of S1P, sphingosine and ceramide [32–36]. An alternative pathway of ceramide metabolism involves its direct phosphorylation by the actions of ceramide kinase, thereby producing ceramide-1-phosphate, which has itself turned out to be an interesting signaling molecule involved in inflammatory signaling [37, 38].

Whereas ceramide appears to mediate its effects intracellularly, S1P has both intracellular functions and extracellular functions mediated through GPCRs. Currently, there are five known GPCRs belonging to the S1P group of receptors, formerly known as Endothelial Differentiation Gene (EDG) receptors and now designated $S1P_{1–5}$. Nearly every human cell type examined expresses one or more S1P receptor, and many cells express a combination of these. Ligand binding and activation of these receptors initiates multiple signaling pathways, including ERKs, phosphoinositide-3-kinase (PI3K), and cyclic AMP downstream mediators [39, 40]. Further, S1P receptors interact and exhibit cross-talk with other growth

factor receptors including those activated by vascular endothelial growth factor and platelet derived growth factor (PDGF), thereby increasing the complexity of S1P signaling and its ramifications for cell biology [41].

The specific functions and regulation of the S1P receptors and the enzymes affecting the sphingolipid rheostat have been described in detail elsewhere, as cited above. In the following sections, we will focus on describing the evidence supporting a role for S1P signaling and metabolism in the development, progression and acquisition of drug resistance of hematopoietic malignancies, including leukemia, lymphoma and multiple myeloma.

The Sphingolipid Rheostat in Leukemia Cell Lines

In the 1980s, HL60 leukemia cells were shown to generate ceramide by activating neutral sphingomyelinase in response to vitamin D3 treatment, thereby leading to cell differentiation. This was the first demonstration that intracellular ceramide generated by a "sphingomyelin cycle" could function as a lipid mediator [5]. Subsequently, ceramide was found to activate stress-activated protein kinases in HL60 cells, and to induce apoptosis via Bax translocation and inhibition of the antiapoptotic protein Bcl-xL [42, 43]. Cellular ceramide levels were shown to increase in leukemia cells in response to many cytotoxic factors including TNFα, dexamethasone, activators of Fas, chemotherapeutic agents, reactive oxygen species (ROS) and ionizing radiation (see Figure 1) [44–51].

Endogenous ceramide accumulation has been shown to originate from many sources in leukemic cells, including sphingomyelin breakdown (via the activation of sphingomyelinases) [5], *de novo* biosynthesis and the direct action of ceramide synthases [52], inhibition of ceramidase [53, 54], and most recently by inhibition of its incorporation into sphingomyelin in Jurkat cells [55]. Ceramide-mediated cell death has been attributed to its impact on numerous downstream targets in leukemia cells. Ceramide can induce apoptosis through activation of caspases in MOLT4 leukemia cells [56]. It activates a protein phosphatase 2A (PP2A)-type phosphatase in HL60 cells, leading to a block in transcriptional elongation of the TNF-α downstream target c-myc and resulting in inhibition of cell growth [57, 58]. Activation of stress-induced protein kinases including p38 and c-Jun N-terminal kinase (JNK) contribute to apoptosis in response to ceramide generation or exogenous treatment with ceramide analogs [59, 60]. Ceramide reduces survivin expression in human and rat natural killer large granular lymphocyte (LGL) cells [61]. In addition, the ability of ceramide to represses human telomerase (hTERT) promoter activity by HDAC1-mediated deacetylation of Sp3 was paralleled by findings of ceramide-mediated repression of hTERT in KG1 leukemia cells (see Figure 2) [62, 63].

In the early 1990s, the mitogenic effects of S1P and SK activation as downstream events in mitogenic signaling pathways were demonstrated, thereby revealing an interesting contrast between the pro- and anti-proliferative effects of these two sphingolipid metabolites [4]. In 1996, exogenous S1P was shown to induce calcium mobilization and activation of phospholipase C (PLC) in HL60 leukemia cells, and a pertussis-toxin sensitive receptor was shown to be involved [64, 65]. That same year it was shown that human erythroleukemia cells activate SK in response to phorbol esters in a protein kinase C (PKC)-dependent manner [66]. Later studies in the human acute leukemia Jurkat, U937 and HL60 cell lines showed that S1P has an inhibitory effect on apoptosis [67]. The effects of S1P were attributed to the inhibition of cytochrome c and Smac/DIABLO translocation to the cytoplasm from the mitochondria in the presence of S1P. Treatment of HL60 cells with vitamin D3 was found to activate SK in a PKC-dependent manner, and treatment with S1P protected HL60 cells from ceramide-induced apoptosis, whereas SK inhibition augmented it [68]. These studies indicated

the existence of a delicate balance in sphingolipid metabolism that can affect cell survival pathways in leukemia as well as other cell types (Figure 1).

Anoikis, which is a form of apoptotic cell death mediated by detachment from the substratum, was also shown to be prevented by SK activation in U937 cells, whereas SK inhibition induced by detachment promoted anoikis in HL60 cells [69, 70]. SK activation and S1P production was also implicated in chemoattractant signaling in differentiated HL60 cells [71]. Many subsequently observed effects of S1P and ceramide in the context of leukemia are consistent with the sphingolipid rheostat model [72]. Upon recognition that S1P serves as a ligand for EDG/S1P receptors, some of S1P's effects on leukemia cells were attributed to receptor signaling [73]. For example, it was soon established that HL60 cells preferentially express $S1P_3$, and that $S1P_3$ is downregulated during differentiation induced by dibutyryl cAMP, retinoic acid, and vitamin D3 [74].

Mast cells are bone marrow derived cells that mediate allergic responses, and rat basophilic leukemia (RBL)-2H3 cells serve as a model of mast cell function. Cross-linking of the high-affinity immunoglobulin E receptor on (RBL)-2H3 cells results in activation of SphK1, export of S1P through ABC transporters and activation of $S1P_1$ and $S1P_2$, leading to degranulation and chemotaxis [75]. These findings raise the possibility that S1P signaling could be involved in proliferative disorders of the mast cell including mast-cell leukemia and/or mastocytosis.

S1P Signaling in Leukemia

The previous studies using leukemia-derived transformed cell lines revealed many of the basic effectors of ceramide and S1P signaling in leukemia cells and established their generally opposing actions in the context of leukemia. More recent studies have examined the effects of sphingolipid signaling in human primary leukemia cells and animal models of the disease. Findings in these systems have provided more substantial evidence strengthening the physiological relevance of sphingolipid signaling in hematopoietic malignancies.

For example, microarray analysis demonstrated that an $S1P_5$ receptor is upregulated in human T-cell LGL leukemia [76]. LGL leukemia cells appear to derive from a clonal expansion of terminally differentiated, antigen-primed, competent cytotoxic T lymphocytes. As such, patients with LGL leukemia have autoimmune conditions such as rheumatoid arthritis [77]. Recent studies have implicated PDGF and IL-15 as key signaling factors responsible for the pathogenesis and activation of LGL [78]. Interestingly, multiple genes involved in the sphingolipid metabolic pathway were found to be differentially expressed in these tumors [79]. Among the genes identified in this study were SphK1, acid ceramidase, and neutral sphingomyelinases. Inhibition of acid ceramidase or SK, or treatment with FTY720, a sphingosine analog that functions as an immunomodulatory drug by binding to and promoting internalization of $S1P_1$, induced apoptosis or sensitized the cells to Fas-induced apoptosis. Thus, dysregulation of the S1P signaling may contribute to the effects of upstream growth factor activation in LGL, such as by facilitating the escape of activated T cells from Fas-mediated cell death.

A central role for SphK1 in the development of erythroleukemia was revealed when microarray analysis was used to compare gene expression in nontumorigenic and tumorigenic proerythroblasts from a transgenic mouse model of erythroleukemia [80]. Using this approach, SphK1 upregulation was found to be a recurrent oncogenic event underlying the tumorigenic phenotype in proerythroblasts. When erythroleukemic cells were forced to overexpress SphK1, they exhibited a proliferative advantage, increased clonogenicity, tumorigenicity when implanted in nude mice, and resistance to apoptosis in response to

serum-deprivation. This protection from stress was mediated through an ERK- and PI3K-dependent pathway. *In vitro* suppression of SK activity either by expressing a dominant negative SphK1 or through pharmacological inhibition blocked the proliferation and stress resistance of erythroleukemia cells.

In chronic myelogenous leukemia (CML), the tyrosine kinase ABL is in a constitutively active state, resulting in unrelenting growth signaling. The drug imatinib, which inhibits ABL tyrosine kinase activity, is the first example of a new class of chemotherapy agents that target a mutant enzyme that is characteristic of a particular cancer cell. This distinguishes imatinib from the many nonspecific agents that target DNA replication and cell cycle progression and thereby affect all or most rapidly dividing cell populations. The development of imatinib mesylate for CML has revolutionized CML treatment. However, resistance has been a problem in achieving complete killing of tumor cells [81]. A recent study examined the mechanisms for resistance to imatinib-induced apoptosis using a pair of isogenic human K562 CML cell lines, one of which is resistant to imatinib and the other sensitive. In these cells, ceramide and S1P were found to have opposing effects on imatinib-induced apoptosis [82]. Imatinib treatment induced the generation of endogenous C18 ceramide, resulting in enhanced apoptosis in the sensitive cell line, whereas decreased apoptosis was observed in imatinib-resistant cells that failed to generate C18 ceramide, exhibited elevated SphK1 expression and had an increased ratio of S1P/C18 ceramide. Importantly, partial knockdown of SphK1 in the resistant cells increased their sensitivity to imatinib. In a separate study, cell lines derived from CML patients in blast crisis were used to investigate the role of SphK1 in imatinib resistance [83]. Overexpression of SphK1 resulted in resistance to imatanib-induced cell death, concomitant with decreased levels of cytochrome c and Smac/DIABLO expression. Further, SphK1 activity was significantly decreased with imatanib treatment. Recently, continuous exposure of K562 cells to gradually increasing concentrations of imatinib was used to select an imatinib-resistant cell line. This line exhibited upregulation of both BCR-ABL and SphK1. SphK1 was found to be upregulated in an AKT2-dependent manner that promoted imatinib resistance. Both BCR-ABL upregulation and imatinib resistance could be reversed by SphK1 knockdown and by pharmacological inhibition of SK activity using the sphingosine analog N,N-dimethylsphingosine (DMS) [84]. These studies suggest that modulating the sphingolipid rheostat could be exploited to address the problem of drug resistance in long-term treatment of CML.

In an effort to investigate mechanisms of chemotherapy resistance in acute myeloid leukemia (AML) cells, the role of SphK1 was explored in AML cells demonstrating resistant to the chemotherapeutic agents doxorubicin or etoposide [83]. As in CML, elevated SphK1 expression levels correlated with survival and inhibition of apoptosis in AML, and these findings were associated with a block in mitochondrial cytochrome c translocation to the cytoplasm (see Figure 2).

The predominance of published studies analyzing S1P and ceramide signaling in cancer have focused on the intracellular events controlling their biosynthesis and degradation. However, recent reports have begun to establish a role for S1P receptors in hematopoietic malignancy as well. For example, the $S1P_1$ receptor has recently been implicated in myeloid tumor progression via a mechanism involving signal transducer and activator of transcription 3 (STAT3) signaling [85]. The STAT3 protein is a transcription factor that is activated by many cytokines and plays a central role in inflammation, cell growth and apoptosis [86]. The binding of IL-6 cytokine family members to the gp130 receptor results in STAT3 phosphorylation by Janus kinase 2 (JAK2), and constitutive activation of this signaling pathway has been implicated as a causative factor in cancer. STAT3 signaling has been shown to be important in determining sensitivity of leukemia cells to apoptosis [87].

Recently, STAT3 was shown to be involved in a feedback loop with $S1P_1$ [85]. $S1P_1$ was found to be a transcriptional target of STAT3, and STAT3 positive tumor cells exhibited elevated $S1P_1$ expression. In addition, $S1P_1$ signaling led to activation of STAT3 and increased IL-6 via upregulation of JAK2 activity, thereby resulting in an auto-stimulatory loop. Unlike transient activation of STAT3 by IL-6, $S1P_1$ signaling was shown to persistently activate this pathway through its influence on tumor cells and the tumor microenvironment (see Figure 2). Importantly, the effects of this auto-activating signaling loop promoted tumor growth and metastatic spread, whereas inhibition of $S1P_1$ signaling prevented these effects. These results provide new insight into how S1P signaling may contribute fundamentally to leukemogenesis. They simultaneously raise the possibility that S1P signaling may be an Achilles heel for some leukemias in which the pathway is dysregulated.

S1P Signaling in Lymphomas

In one of the earliest studies exploring how T lymphoma cells invade their microenvironment, investigators found that their ability to invade a fibroblast monolayer required the actions of Tiam1 and its downstream mediator Rac [88]. Serum lysophospholipid mediators S1P and lysophosphatidic acid (LPA) were shown to act on cell surface GPCRs, thereby mediating RhoA and PLC signaling pathways that stimulated the formation of pseudopodia and enhanced infiltration. These seminal findings were subsequently followed by many other published reports showing the migration of tumor cells, endothelial cells, neurons and bone marrow derived cells were orchestrated by similar mechanisms linking S1P and LPA signaling to cytoskeletal changes that promote cell movement [89].

Runx genes play roles in development, including the generation of hematopoietic stem cells [90]. Overexpression of Runx genes in transgenic mice can promote the development of leukemia and lymphoma in collaboration with c-myc. Further, lymphoid cells and fibroblasts overexpressing Runx genes exhibit reduced apoptosis and enhanced survival under the stress. Recently, investigators seeking to understand how Runx genes influence cell fate under stress performed gene array comparisons that identified significant differences in three key enzymes in sphingolipid metabolism in Runx-overexpressing cells [91]. Genes encoding two enzymes of sphingolipid biosynthesis whose actions reduce free ceramides (UDP-glucose ceramide glycosyltransferase and beta-galactoside alpha-2,3-sialyltransferase 5) were upregulated by Runx gene expression, whereas the S1P phosphatase 1 (Sgpp1) gene was down-regulated. These changes correlated with Runx-binding sites found in the promoters of the genes, and reduced ceramides and elevated S1P levels were observed in Runx expressing cells compared to control cells. Addition of exogenous S1P conferred improved survival in control cells, suggesting that some of the Runx growth advantage is imparted by elevated S1P.

In large B-cell lymphoma development, $S1P_2$ signaling appears to serve an anti-oncogenic role. It was observed that 54% of $S1P_2$ receptor null mice monitored over a two year time span developed germinal center derived DLBCL, compared to 3.6 % incidence in littermate control mice [94]. This finding is compatible with that of another study demonstrating that the tumor environment of $S1P_2$ knockout mice supported Lewis lung carcinoma or B16 melanoma cell tumor growth and angiogenesis better than control mice [95]. These studies illustrate that S1P signaling through $S1P_2$ is inhibitory to lymphoma and other cancer development and progression. $S1P_2$ has been shown to mediate the $G_{12/13}$ and Rho-dependent inhibitory effects of S1P on AKT, Rac, and cell migration, thereby negatively regulating angiogenesis [92, 93]. Whether the effect of $S1P_2$ on lymphomagenesis is an effect intrinsic to bone marrow-derived progenitors of lymphoma or rather due to an effect on cells

within the tumor niche involved in angiogenesis, inflammation and cancer surveillance remains to be determined (see Figure 2).

The sphingolipid rheostat has also been implicated in mantle cell lymphomas (MCL). The endocannabinoid analogue R(+)-methanandamide binding to the endocannabinoid receptor CB1 leads to increased ceramide synthase 3 and 6 transcription and a subsequent increase in synthesis of multiple forms of ceramide including C16, 18, 24 an 24:1 ceramides, leading to decreased cell viability [96]. Moreover, inhibition of the enzymes SphK1 and glucosylceramide synthase using specific inhibitors or siRNA prevented the further catabolism of ceramides and potentiated cell death in the presence of the R(+)-methanandamide. Interestingly, MCLs have been shown to express significant levels of $S1P_1$ as a distinguishing feature compared to other lymphomas (see Figure 2). [97]

Additional clinical studies are beginning to report interesting findings related to S1P signaling in human lymphomas. One study comparing Sphk1 expression in clinical tissue samples from 44 patients with non-Hodgkin's lymphoma (NHL) versus 25 patients with reactive lymphoid hyperplasias (nonmalignant conditions) demonstrated significantly higher levels of SphK1 mRNA and protein in varying grades of NHL compared to nonmalignant lymphoid hyperplasia, with a clear trend toward increasing expression levels correlating with higher grade lymphomas [98]. Although in some cases immunohistochemistry (IHC) showed SphK1 expression in nonmalignant tissues within the biopsy, in most cases the IHC confirmed expression within malignant cells.

The follicular lymphoma variant translocation 1 (FVT1) gene, which was identified through its involvement in an atypical follicular lymphoma translocation, turns out to be the principal 3-ketosphinganine reductase in mammalian cells [99]. This enzyme is required for synthesis of long chain bases through the *de novo* sphingolipid biosynthetic pathway. The expression of FVT1 was investigated in B-cell non-Hodgkin lymphoma biopsy material [100]. FVT1 expression was shown to be significantly reduced by germinal center-type diffuse large B-cell lymphoma (DLBCL) when compared with non-germinal center-type DLBCL, follicular lymphoma, and normal tonsil control samples. These findings suggest that bioactive sphingolipids may be important in the pathogenesis and treatment of some types of DLBCL, although it is not clear how flux through the *de novo* pathway impacts this process.

S1P Signaling in Multiple Myeloma

S1P has been shown to be protective against apoptosis in multiple myeloma cells, which were found to express $S1P_{1–3}$. Addition of exogenous S1P to these cells was shown to upregulate the anti-apoptotic protein Mcl-1, whereas pan-inhibition of the S1P receptors led to an attenuation of Mcl-1 expression [101]. Other studies in multiple myeloma cells have focused on the relationship between IL-6 and SphK1. IL-6 is an important target in multiple myeloma, since it confers an anti-apoptotic effect. It is now known that IL-6 partially mediates its anti-apoptotic effects via activation of SphK1 (see Figure 2)[102].

In 1994, 2-amino-2- 2- 4-octylphenyl ethyl - 1,3-propanediol hydrochloride (FTY720) from the fungus *Isaria sinclairii* was discovered to have potent immunosuppressive properties by preventing lymphocyte trafficking [103]. In 2003, FTY20 was found to act as a sphingosine analog that becomes phosphorylated *in vivo* and binds to S1P receptors, thereby linking S1P signaling to lymphocyte trafficking [104–106]. FTY720-P preferentially targets $S1P_1$ and $S1P_{3–5}$, leading to receptor activation and subsequent downregulation [107]. However, FTY720 has also been shown to exert anti-tumor effects and to promote apoptosis in cancer cells [108–110]. FTY720 has been shown to have growth inhibitory effects on chemotherapy-resistant multiple myeloma cells [111]. Similar to the action of ceramide on cytochrome c and Smac/DIABLO, FTY720 was able to induce translocation of these mediators of apoptosis.

FTY720 was effective in inducing apoptosis in both sensitive and resistant cells, and its function could be augmented by combination treatment with dexamethasone and anti-Fas antibodies [111].

However, it should be noted that FTY720 has pro-apoptotic effects on some leukemia and lymphoma cells that appear to be completely independent of FTY720's effect on S1P receptors and S1P-dependent signaling. These effects include its ability to activate PP2A and thereby promote the dephosphorylation of oncogenic c-KIT on tumor cells, resulting in reduced signaling through downstream targets such as AKT, STAT5 and ERK and causing apoptosis [112]. This example points to the importance of recognizing that sphingolipid analogs may have off-target pharmacological effects that can contribute significantly to their impact on tumorigenicity[113].

Targeting the Sphingolipid Pathway

Based on the fundamental role that sphingolipids play in regulating hematopoietic cell proliferation, conserved death pathways and drug resistance patterns, it is understandable that significant effort is being expended to harness the potential of this pathway for therapeutic benefit in cancer. Strategies have included development of small molecule inhibitors of SK, ceramidase and glucosylceramide synthase, as well as ceramide and long chain base analogs, S1P receptor antagonists and monoclonal antibodies against S1P. Testing of these therapeutic strategies is underway in clinical trials in cancer and other diseases, and reports are beginning to emerge in the literature [22, 114].

Studies targeting SK were among the earliest therapeutic strategies to be employed and have produced some promising results. As expected, inhibition of SK with DMS led to apoptosis of HL60 human leukemia cells [115]. A recent study on L-*threo*-dihydrosphingosine (Safingol), which acts an inhibitor of SphK1 and PKC, has shown promising results with advanced solid tumors [116, 117]. Safingol enhanced cellular toxicity in drug resistant cells when given in combination with doxorubicin [118]. In the most recent study using Safingol, a dose-dependent reduction in plasma S1P levels was achieved, with only reversible hepatotoxicity as a side effect. Although a Phase I clinical trial, stable disease and partial response was observed in several patients receiving safingol in combination with cisplatin [117]. Given a cohort of patients with advanced disease and documented resistance to cisplatin, these results are encouraging and reinforce the notion of manipulating the sphingolipid rheostat to overcome drug resistance.

Studies with a selective SphK1 inhibitor, BML-258, have shown promising results as well [119]. BML-258 decreased growth in human leukemia U937 and Jurkat cells with increased apoptosis in leukemic blasts. Similar results were seen in AML allografts.

Recent studies have shown that inhibition of SphK2 by 3-(4-chlorophenyl)-adamantane-1-carboxylic acid (pyridin-4-ylmethyl)amide (ABC294640) is able to induce cell death in kidney carcinoma, prostate, and breast adenocarcinoma cell lines [120]. Interestingly, in this report the major mechanism for tumor cell killing was found to be autophagy rather than apoptosis.

Although FTY720 has been considered primarily an immune modulator, multiple studies have also provided evidence that FTY720 can function as an anti-tumor agent. Effects on tumor growth, apoptosis and angiogenesis have been demonstrated in prostate, breast, hepatocellular, pancreatic, gastric, and hematopoietic cancers [121, 122]. The effects of FTY720 and FTY720P reveal the complexity inherent in targeting the sphingolipid metabolic pathway. Recent work by Yasui et. al., has shown that FTY720 treatment resulted in ~40% inhibition of purified SphK1 [111]. Interestingly, vinylphosphonate, the S enantiomer

of FTY720, produced an 80% reduction in SphK1 activity. In addition to catalytic inhibition, SphK1 underwent proteosomal degradation in the presence of FTY720 or (S)-FTY720 vinylphosphonate in hPASMC and MCF-7 cell lines. The proteosomal degradation of SphK1 led to apoptosis, as evidenced by caspase-3 activation.

The use of sphingosine has also been shown to have an effect on human leukemia cell lines. Using sphingosine in micromolar concentrations, CMK-7, HL60 and U937 leukemic cell lines were induced to undergo apoptosis with high frequencies [123]. Various long chain base analogs have been exploited to emulate the effects of sphingosine. For example, phytosphingosine derivatives that serve as SK inhibitors were recently described to promote apoptotic cell death in HL60 cells [124]. In addition, naturally occurring sphingadiene compounds found in soy were shown to induce apoptotic and autophagic cell death in colon cancer cells through an AKT-dependent mechanism and to suppress tumor formation in animal models [125]. Whether these strategies will be relevant to the treatment of hematopoietic malignancies remains to be tested.

The S1P monoclonal antibody, Sphingomab, has shown very promising results as a novel strategy for removing S1P and thereby preventing pro-angiogenic signaling and tumor-promoting effects associated with S1P signaling. Initial results have demonstrated excellent results against murine xenografts and allografts [114, 126].

Several recent studies using ceramidase inhibitors show promise as a strategy for raising ceramide levels and thereby inducing cytotoxicity and reducing tumor growth [127–132].

As interest in the field continues to grow, critical information including identification of the crystal structures of key enzymes and targets in the pathway should become available. Combined with advanced drug screening efforts, this information should facilitate the development of more specific small molecule modulators of the sphingolipid rheostat. The strategies that are now being tested, as well as more challenging ones such as achieving targeted S1P catabolism and ceramide synthesis within tumors and inhibiting S1P export to prevent autocrine and paracrine S1P signaling within tumors and their microenvironment, have not been fully explored and remain intriguing possibilities for therapeutic intervention.

Beyond the Rheostat

As the examples above have illustrated, the rheostat model appears to have substantial merit as a general framework in which to understand the mechanisms of sphingolipid signaling in cancer. The identification of molecular targets at the membrane, in the cytosol, in the nucleus and in the extracellular space that mediate the specific effects of S1P and ceramide on tumor cell fate have gradually populated the picture of sphingolipid activities in the malignant cell and its niche with details, in most cases without refuting the rheostat model.

However, some recent findings indicate that a rigid rheostat model is insufficient to explain the pleiotropic effects of sphingolipids in the malignant cell and its environment. For example, C16 ceramides generated by ceramide synthase 6 appear to potentiate the growth of some tumors, and inhibition of the biosynthesis of these lipids can promote the ER stress response [18]. In addition, the development of B cell lymphomas in $S1P_2$ knockout mice and the enhanced growth of tumor xenografts placed in the context of a $S1P_2$ knockout mouse niche as described above demonstrate that S1P signaling is not all tumor-promoting. The finding that SphK2 can function as a pro-apoptotic protein is also not consistent with the original rheostat model. Furthermore, autophagy, a conserved catabolic process that can facilitate tumor cell survival in the absence of nutrients but also can lead to cell death, is activated by both ceramide and S1P signaling [17]. In addition, S1P has been shown to promote apoptosis in certain contexts [19].

Other sphingolipid intermediates besides S1P and ceramide may have an impact on cell fate and carcinogenesis, including dihydrosphingosines, dihydroceramides, sphingosine and the long chain aldehyde product generated by S1P lyase, hexadecenal. Early studies showed that sphingosine induces dephosphorylation of the retinoblastoma gene product (pRb), thereby inducing G1 cell cycle arrest in the lymphoblastic leukemia cell line MOLT-4 [133, 134], whereas dihydrosphingosine and dihydryoceramide have been implicated in the pro-apoptotic effects of tocopherol gamma, a form of vitamin E that has chemopreventive activity [135]. Fenretinide, a retinoid chemotherapeutic agent was found to function as an inhibitor of dihydroceramide desaturase, resulting in dephosphorylation of pRb and G0/1 cell cycle arrest [136]. These findings raise the possibility that dihydroceramide, previously considered an inert sphingolipid intermediate, may function as a growth inhibitory molecule. We recently showed that hexadecenal, the long chain aldehyde produced by S1P lyase-mediated S1P cleavage, induces apoptosis and cell detachment through a JNK-dependent pathway that involves ROS generation [137]. JNK activation by hexadecenal resulted in c-Jun phosphorylation, cytochrome c release, Bax activation, Bid cleavage and increased translocation of Bim into mitochondria, thereby promoting apoptosis. Inhibition of JNK abrogated the cytoskeletal changes and apoptosis caused by trans-2-hexadecenal, whereas Rac1 and RhoA were not involved. Aldehydes are also well known to induce DNA damage, and we have observed that hexadecenal can indeed form adducts with DNA (our unpublished observations). Whether hexadecenal formation plays a role in genetic instability in cancer is not known, but this could potentially account for the unexpected finding that S1P lyase is upregulated in many cancers. We have also recently shown S1P lyase to be a regulator of acid sphingomyelinase, the G2 checkpoint and DNA repair, demonstrating the complex feedback mechanisms within the sphingolipid biochemical pathways that have ramifications with regard to modulation of specific targets [138].

While some of these new findings are not inconsistent with a rheostat model, they do significantly alter the number of factors that may weigh in on the balance of positive and negative forces exerted by sphingolipids on cell fate. Gene expression patterns and biochemical parameters of some tumors are not easily accommodated by a rheostat model. Some of these observations, such as upregulation of genes involved in S1P catabolism in cancer, may be explained by a reaction of the tumor microenvironment to S1P production by the tumor or by the niche in response to tumor signals. However, many instances cannot be explained conveniently by the conventional model and suggest that a revision of the rheostat will be required to encompass the full dynamic range of sphingolipid signaling in hematopoietic malignancies and other cancer contexts.

S1P Signaling in the Supportive Management of Hematological Malignancies

In treating patients with hematological malignancies, many of whom are children and young adults, there are other factors in addition to achieving cure that are important components of clinical management. As acute lymphocytic lymphoblastic leukemia cure rates in children reach 80% or greater and patients are expected to enjoy a long life expectancy, long-term side-effects such as late cardiotoxic effects of anthracyclines, chronic graft-versus-host disease (GVHD) and sterility become less tolerable sequelae of treament [139]. In this context, S1P signaling and inhibiting ceramide formation may be relevant, as they have been shown to play a protective role in the maintenance of viability of nonmalignant cells including protection of oocytes from chemotherapy- and radiation-induced apoptosis [140]. The protection of stem cells and gut endothelial cells from radiation has also been achieved by increasing S1P levels and inhibiting ceramide formation [141–143]. Studies have suggested that FTY720 may reduce GVHD without compromising malignant cell killing in the setting of bone marrow transplantation for leukemia [144]. S1P lyase activation sensitizes cells to

radiation and chemotherapy-induced apoptosis through a variety of mechanisms, whereas its inhibition appears to protect cells from DNA damage, ischemia, and other forms of injury [138, 145, 146]. Therefore, S1P lyase suppression could potentially be used in strategies to protect normal tissues and thereby improve the therapeutic index of chemotherapy agents and radiation.

Concluding Remarks

The sphingolipid metabolic pathway is essential to normal cellular function. However, hematopoietic malignancies and the tumor microenvironment exhibit changes in sphingolipid signaling and metabolism that affect cell growth, apoptosis, invasion, and resistance to chemotherapy. The complexity of the pathway, which includes multiple enzymes, receptors and downstream effectors, presents a challenge to the researcher and clinician. Many questions still remain to be answered. For example, why have mutations directly affecting the expression or function of genes involved in sphingolipid metabolism and signaling only rarely been identified in cancer, whereas expression changes in SphK1, S1P lyase and other components of S1P signaling appear to be common manifestations of cancer? Many of our model systems cannot distinguish between effects on tumor promotion secondary to environmental cues versus effects that are intrinsic to the tumor cell. The effects of modulating the sphingolipid pathway on critical malignant and nonmalignant cell populations such as cancer stem cells and tissue stem cells remain unknown. As each specific target of S1P or ceramide is revealed, it also begs the question whether other sphingolipids directly or indirectly exert opposing effects on that target. As we gain new information, including the crystal structures of key targets in the pathway, rational drug design and targeted manipulation of this pathway for therapeutic benefit will become ever more feasible. New cancer profiling approaches are likely to reveal many more specific instances in which molecular events affecting sphingolipid signaling and metabolism contribute to carcinogenesis, cancer progression and the development of drug resistance. As we develop more sophisticated and individualized tumor profiling technologies that can be applied in advance of and throughout the course of treatment, this information may be coupled to new treatment options targeting the components of the sphingolipid pathway for hematological malignancies as well as other forms of cancer.

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LIST OF ABBREVIATIONS

AML	Acute myelogenous leukemia
ABC	ATP-binding cassette
BH3	Bcl-2 homology 3
CIB1	calcium and integrin binding protein-1
CML	chronic myelogenous leukemia
JNK	c-Jun N-terminal kinase
DLBCL	diffuse large B-cell lymphoma
EDG	endothelial differentiation gene

ERK	extracellular signal-regulated kinase
FVT1	follicular lymphoma variant translocation 1
GPCR	G protein coupled receptor
GVHD	graft versus host disease
HDAC	histone deacetylase
hTERT	human telomerase
IHC	immunohistochemistry
JAK2	janus kinase 2
LGL	large granulocytic lymphoma
LPA	lysophosphatidic acid
MCL	mantle cell lymphoma
DMS	N,N-dimethylsphingosine
NHL	nonhodgkins lymphoma
PI3K	phosphoinositide-3-kinase
PLC	phospholipase C
PDGF	platelet derived growth factor
PKC	protein kinase C
PP2A	protein phosphatase 2A
ROS	reactive oxygen species
pRb	retinoblastoma gene product
STAT3	signal transducer and activator of transcription 3
SK	sphingosine kinase
S1P	sphingosine-1-phosphate
TNFα	tumor necrosis factor α

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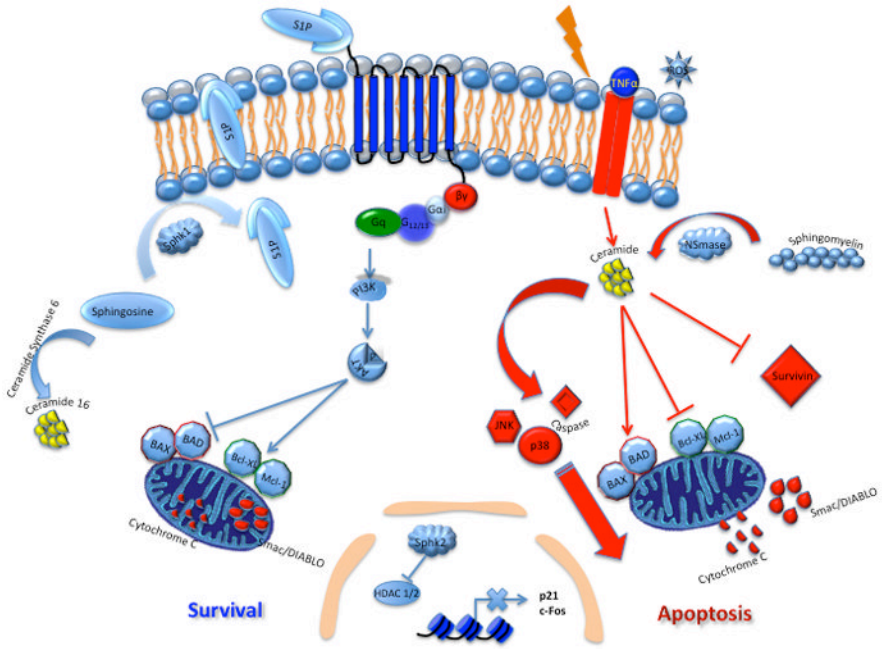

Figure 1. Sphingolipid rheostat

S1P is produced from the conversion of sphingosine to S1P via SK enzymes. S1P is then shuttled to the extracellular space, where it can activate five known GPCRs. Upon activation, downstream signaling events occur to produce myriad effects. Ceramide can be produced via sphingomyelinases (SMase) by conversion of sphingomyelin to ceramide. Radiation, ROS, ischemia and TNFα can also contribute to ceramide generation. The traditional ‘rheostat’ model suggests a balance of forces between S1P and ceramide, with S1P promoting survival and ceramide promoting apoptosis. Downstream mediators affected by the rheostat include Bad, Bax, Bcl-XL and Mcl-1. Ceramide is also shown here to affect signaling via JNK and p38. Ceramide can also affect caspase activation and survivin suppression. Contrary to the ‘rheostat’ model, ceramide 16 promotes growth and inhibits the ER stress response in some cancers. SphK2 is also shown here to repress HDAC1/2 with subsequent upregulation of p21 and c-fos transcription.

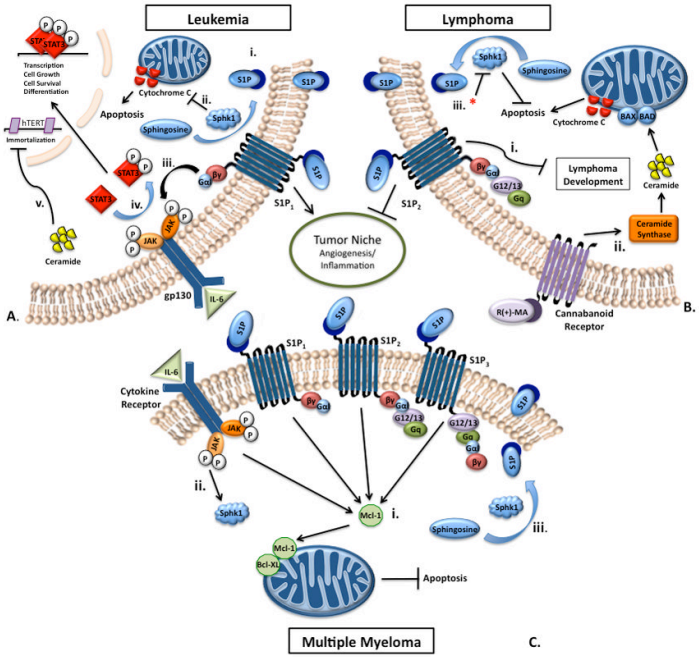

Figure 2. S1P signaling in hematopoeitic malignancies

A.) The role of S1P in leukemia. i.) Sphingosine is converted by SphK1 into S1P and is exported to the extracellular space, where it can then activate S1P receptors. ii.) Upregulation of SphK1 inhibits cytochrome c transport to the cytoplasm. iii.) S1P activates $S1P_1$, which persistently activates the STAT3 pathway. iv.) STAT3 is transiently activated by IL-6 and travels to the nucleus for transcription. v.) Ceramide represses human telomerase (hTERT) promoter activity through HDAC-1-mediated deacetylation of sp3. B.) S1P signaling and apoptosis in lymphomas. i.) $S1P_2$ exerts a negative effect on B-cell lymphoma development, as $S1P_2$ knockout mice exhibit enhanced lymphoma formation. ii.) R(+)-methanandamide treatment results in decreased cell viability through increased ceramide synthase activity in mantle cell lymphoma. iii.) When SK is inhibited by DMS or SphK1 is inhibited by siRNA (*) in the presence of R(+)-methanandamide, cytochrome c induced apoptosis is potentiated in mantle cell lymphoma. C.) S1P signaling and apoptosis in Multiple Myeloma. i.) Mcl-2 anti-apoptotic effect is activated by S1P through $S1P_1$, $S1P_2$, and $S1P_3$. ii.) IL-6 confers an anti-apoptotic effect through activation of SphK1. iii.) IL-6 activation of SphK1 mediates conversion of sphingosine into S1P to prevent apoptosis.