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Therapeutic applications of bioactive sphingolipids in hematological malignancies

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Mini Review

Sphingolipids are sphingosine-based lipid molecules that have important functions in cellular signal transduction and in a variety of cellular processes including proliferation, differentiation, programmed cell death (apoptosis) and responses to stressful conditions. Ceramides, dihydroceramide, sphingosine and sphingosine-1-phosphate are examples of those bioactive sphingolipids. They have a major impact on determination of the cell fate by contributing to the cell survival or cell death through apoptosis. Despite the number of carbon atoms in the fatty acid chain changes the physiological role; ceramides generally exert suppressive roles on the cell proliferation. There have been several enzymes identified in this pathway that are responsible for the conversion of ceramide into other sphingolipid derivatives. Those derivatives also have differential roles on those cellular processes. Sphingosine-1-phosphate is an example of such sphingolipid derivatives which has antiapoptotic effects. As they have significant impacts particularly on the cell death and survival, bioactive sphingolipids have a great potential to be targets in cancer therapy. Increasing number of studies indicates that sphingolipid derivatives are important in the progression of hematological malignancies, and they are also involved in the resistance to current chemotherapeutic options. This review compiles the current knowledge in this area for enlightening the therapeutic potentials of bioactive sphingolipids in various leukemias.

Sphingolipids are one type of lipids that are formed by the combination of a fatty acid and amino alcohol sphingosine with a changeable side chain. Different groups linked to the sphingosine backbone determine the type of the sphingolipid. Ceramide is the fundamental unit for the synthesis of other sphingolipids. They are important constituents of the eukaryotic plasma membranes with the exception of few bacterial species. Since their identification in 1876, sphingolipids have been considered to have mainly structural roles in the cells. However, arising evidence showed that sphingolipids are versatile macromolecules having important roles in a variety of processes including signal transduction, differentiation, proliferation and programed cell death.[1,2] Most widely studied bioactive sphingolipids include ceramide, ceramide-1-phosphate (C1P), dihydroceramide (dhCer), sphingosine and sphingosine-1-phosphate (S1P).[2] Glucosyl ceramide (GluCer) is an another intermediate of sphingolipid metabolism, which was implicated in the drug resistance and cellular trafficking.[3] As sphingolipids are involved in the regulation of essential pathways ensuring the homeostasis, deregulated or defective sphingolipid metabolism might be reflected as pathologic conditions. Indeed, there are numerous studies indicating the importance of sphingolipids in health and disease.[4]

This review will present general information about bioactive sphingolipids with an emphasis on the involvement of bioactive sphingolipids in hematological malignancies such as acute and chronic leukemias, and it will provide some future perspectives for their usage as the leukemia therapeutics.

Key words: bioactive sphingolipids, leukemia, drug resistance, apoptosis

Abbreviations: ALL: acute lymphoblastic leukemia; AML: acute myeloid leukemia; APL: acute promyelocytic leukemia; ATL: adult T-cell leukemia/lymphoma; C1P: ceramide-1-phosphate; CAPP: ceramide-activated protein phosphatase; CDK: cyclin-dependent kinase; CerK: ceramide kinase; CLL: chronic lymphoblastic leukemia; CML: chronic myeloid leukemia; dhCer: dihydroceramide; GluCer: glucosyl ceramide; PDGF: platelet-derived growth factor; PKC: protein kinase C; PP1: protein phosphatase-1; PP2A: protein phosphatase-2A; Rb: retinoblastoma; S1P: sphingosine-1-phosphate; SM: sphingomyelin; SMase: sphingomyelinase; TNF-α: tumor necrosis factor alpha; VEGF: vascular endothelial growth factor

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Types of Bioactive Sphingolipids

Ceramide

Ceramides are the central molecules of the sphingolipid metabolism, and they are involved in the regulation of numerous cellular processes including proliferation, differentiation, senescence, apoptosis and responses to stressful conditions. Structure of ceramides contains a sphingosine base and a fatty acid chain with varying number of carbons. Ceramide

levels are regulated in the cells by several mechanisms.[2] Generation of ceramides and their conversion to other sphingolipid derivatives are essential for this regulation. One of the mechanisms responsible for the generation of ceramides involves the activation of sphingomyelinase (SMase) enzyme, which catalyzes the hydrolysis of membrane phospholipid sphingomyelin (SM) to ceramide.[5,6] TNF-α, FAS ligand and oxidative stress are known to stimulate SMases for the production of ceramides[7–9]; therefore, this pathway is thought to be particularly important for the elevation of ceramide levels in the stress conditions. Ceramides can also be generated *de novo* from serine and palmitoyl CoA in endoplasmic reticulum.[10,11] These two compounds initially condense to form ketosphinganine in a reaction catalyzed by serine palmitoyltransferase. This intermediate is then reduced into dihydrosphingosine which would be subsequently converted to dihydroceramide (dhCer) by dihydroceramide synthase. Ceramide synthesis from dhCer is catalyzed by dihydroceramide desaturase in the last step of *de novo* ceramide production.[12] In addition to those pathways, recycling of complex sphingolipids can result in the production of ceramides by a process called the salvage pathway. A variety of enzymes including cerebrosidases, SMases, ceramidases and ceramide synthases are involved in the salvage pathway as a result of which sphingolipids are broken down into sphingosine that would be reutilized for the ceramide production.[13]

Current evidence indicates the involvement of ceramides in apoptosis, growth arrest, proliferation, survival and aging.[14] Ceramides interact with protein kinases and phosphatases for exerting regulative functions in the cellular processes stated previously. Protein phosphatase-1 (PP1) and protein phosphatase-2A (PP-2A) are activated by long-chain ceramides,[15] and hence, they are known as ceramide-activated protein phosphatases (CAPPs). Activated CAPPs are responsible for carrying the signal further to downstream targets including retinoblastoma protein, cyclin-dependent kinases (CDKs) and Bcl-2 family members.[14,16] Dephosphorylation of retinoblastoma (Rb) protein upon elevation of the cellular ceramide level is linked to the growth inhibition in lymphoblastic leukemia cell line.[17] Moreover, in another study, ceramide was shown to suppress cellular growth by negatively regulating cdk2 through the activation of phosphatases.[18] Intrinsic apoptotic pathway is induced by the ceramides through the regulation of cytochrome c release and the loss of mitochondrial membrane potential.[19] In addition to these downstream targets, ceramides are known to be interacting with Akt, protein kinase C (PKC), phospholipase D and cathepsin D.[20,21] Ceramides were also linked to the reduction of telomerase activity through the repression of telomerase reverse transcriptase promoter in lung carcinoma cell line.[22] Findings of some studies indicated that ceramides with different lengths of fatty acid chains have different roles in the cellular physiology. In the majority of head and neck squamous cell carcinomas, low levels of C_{18}-ceramide were detected, whereas C_{16}-ceramide was significantly upregulated.[23,24] Further studies confirmed that C_{18}-ceramide has apoptotic effects, whereas C_{16}-ceramide contributes to prosurvival.[25] In another study, C_2-ceramide was found to be unable to induce cell death in K562 chronic myeloid leukemia (CML) cells, whereas C_6-ceramide contributed apoptotic induction.[26] Investigations in neuroepithelioma cells have shown that C_6-ceramide is involved in the apoptotic induction, whereas long-chain ceramides that were accumulated upon the treatment with C_6-ceramide are ineffective in this manner.[27]

Dihydroceramide, ceramide-1-phosphate and glucosyl ceramide

DhCer is an intermediate in the *de novo* ceramide generation pathway. It is synthesized from dihydrosphingosine (sphinganine) in a reaction catalyzed by dhCer synthase,[28] and it is converted to ceramide by dhCer desaturase.[12] Initially, dhCer was thought not to be important in apoptosis and cell cycle arrest.[29,30] However, increasing number of studies provided evidence attributing new roles to dhCer in the cells. Induction of autophagy upon treatment with exogenous dhCer analogs is the first clue of dhCer as a bioactive sphingolipid. This effect of dhCer was demonstrated on both prostate and gastric cancer cells.[4,31] Besides its role in autophagy, dhCer is also thought to be important in growth suppression and hypophosphorylation of Rb protein.[32,33] Levels of dhCer were elevated after photodynamic therapy in mice squamous cell carcinoma,[34] and this event might indicate the importance of *de novo* ceramide generation pathway in the photodynamic therapy. Exogenously applied dhCer can be hydrolyzed by the enzymes ACER2/haCER2[35] and ACER3[36] to the dihydrosphingosine, which might then be responsible for the cellular effects thought be caused by the dhCer itself. This anticipation is supported by a recent study showing that dhCer and dihydrosphingosine levels are elevated in various tumor cells upon application of fenretinide, where dihydrosphingosine is likely to be the inducer of the cytotoxicity.[37]

C1P is produced by the phosphorylation of ceramide by the ceramide kinase (CerK), and the reverse reaction is catalyzed by C1P phosphatase.[38] Current evidence indicates that C1P has prosurvival functions including induction of DNA replication and suppression of acid SMase that is responsible for the synthesis of ceramide, and therefore, it blocks apoptosis.[39,40] In addition to the cell cycle regulation, C1P is involved in the mammalian inflammatory responses and in the process of neutrophil phagocytosis.[41,42]

GluCer is produced from ceramides by the catalysis of glucosylceramide synthase, and it is a precursor for the synthesis of complex glycosphingolipids.[43] As shown by the experiments carried on various cells, GluCer has proliferative functions, and it is thought to be important in the chemotherapeutic drug resistance.[44,45] GluCer levels were found to be increased in the resistant cancer cells.[46] Inhibition of the GluCer synthesis resulted in sensitization to drugs and cell cycle arrest providing supportive evidence to the roles of

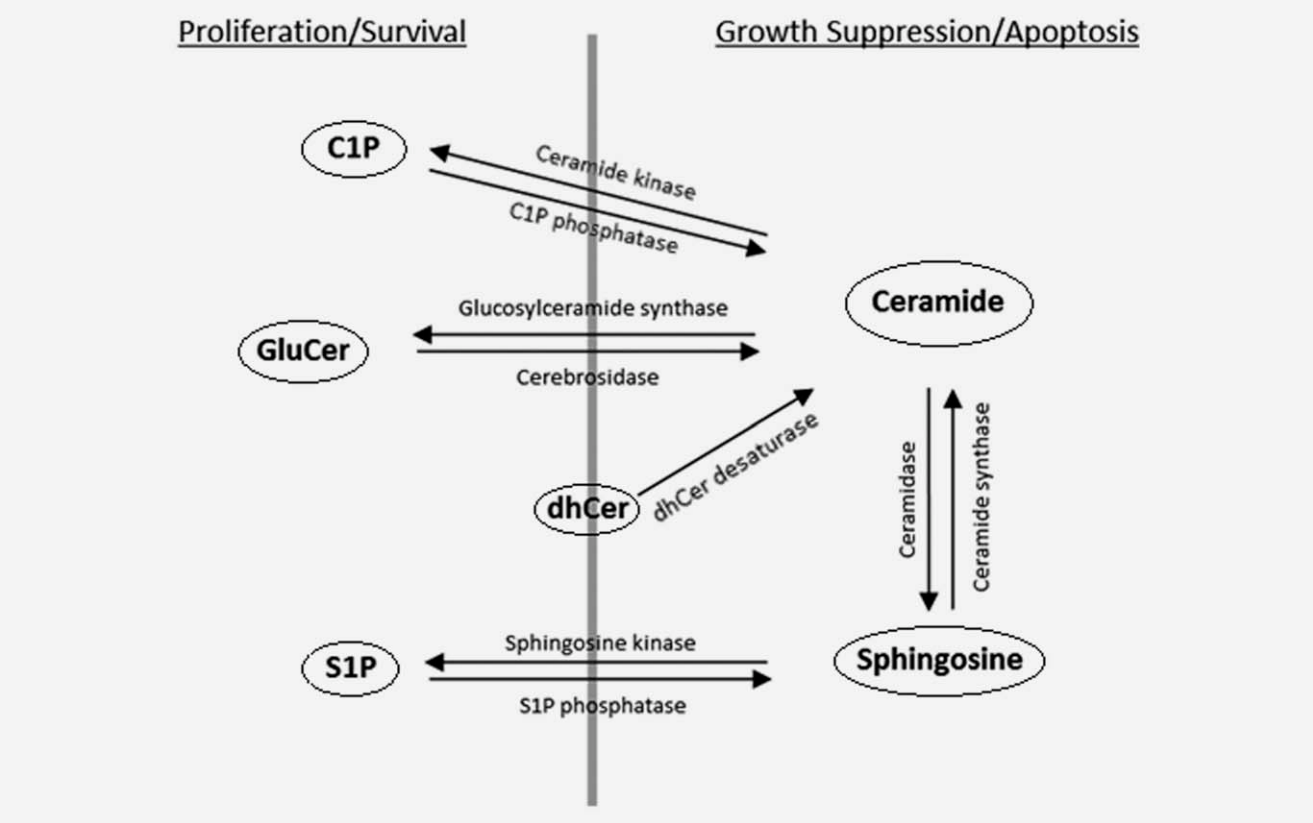

Figure 1. Bioactive sphingolipids and their effects on the cell growth and suppression. The balance of the levels of those sphingolipids is essential for the determination of cell fate either as death or survival. dhCer was placed to the middle because of the lack of definitive information showing its roles in cell growth and apoptosis.

GluCer in the development of chemotherapeutic resistance and in the cellular proliferation.[47–49] As ceramide exerts antagonistic roles to C1P and GluCer, maintenance of the homeostasis depends on the balance of those lipid species (Fig. 1). Deregulation of these pathways might possibly contribute to the progression of diseases such as cancer.

Sphingosine and sphingosine-1-phosphate

Ceramide is converted to sphingosine by the ceramidases, which are classified as acid, neutral and alkaline ceramidases according to their optimal pH and cellular locations for enzymatic reaction[50–52] (for more information, see the related reviews[2,53]). The reverse reaction in which ceramide is synthesized from sphingosine is catalyzed by ceramide synthase. However, under certain circumstances, some ceramidases were also shown to catalyze the reverse reaction to produce ceramides by using sphingosine and a fatty acid as substrates.[54,55] Sphingosine has a strong potential to induce apoptosis in leukemia cells and in a variety of other cell types. Degradation of the genomic DNA as a hallmark of apoptosis was documented in high proportions of the leukemic cells of different origins after exposure to sphingosine.[56–59] Similar observations were made for the effects of sphingosine on the cell death in multidrug-resistant cancer cell lines, suggesting that multidrug resistance mechanisms are ineffective for protection against the sphingosine-induced cell death.[60,61] Sphingosine is also effective for apoptotic induction in various cancer cells including epidermoid carcinomas, colonic carcinomas, melanomas and soft tissue sarcomas as shown by numerous other studies.[56,62,63] Sphingosine might be exerting its functions by interacting with several cellular components. PKC is a known target of sphingosine,[64] and because it can be considered as a survival protein, sphingosine-mediated inhibition of PKC is reflected as the apoptotic induction.[65] Moreover, sphingosine interacts with other antiapoptotic factors such as ERK and Akt/Protein kinase B.[58] Sphingosine-driven apoptotic induction is not only mediated by suppressing the antiapoptotic proteins. Sphingosine was also known to be responsible for cytochrome c release from mitochondria and activation of downstream caspases.[66–68] Beta subunits of integrin molecules are among the targets of sphingosine, and their maturation is inhibited by the sphingosine generated specifically by alkaline ceramidase 2.[69] In another study, this inhibition was shown to be followed by fragmentation of the Golgi complex and anoikis, which is a form of apoptosis occurring because of the insufficient adhesion.[70] This study is one of the emerging studies attributing roles to sphingosine in the cellular processes in which ceramides were thought to be

responsible. Similarly, sphingosine and its phosphorylated derivative S1P, both of which are synthesized from ceramides, were shown to be responsible for the regulation of cell death and survival of HeLa cells in another study.[35] In accordance with those findings, neurons and oligodendrocytes were documented to have an active sphingolipid metabolism by which exogenous C_2- and C_6-ceramides are immediately converted into sphingosine and S1P, which in turn determines the cellular fate.[71] Apoptosis of the Jurkat cells is induced by sphingosine converted from the ceramide by the acid ceramidase by a process involving cytochrome c release and activation of the executioner caspases.[66]

Sphingosine is phosphorylated by sphingosine kinase to produce S1P,[72,73] and S1P phosphatase simply cleaves the phosphate group of S1P liberating sphingosine in the reverse reaction.[74] S1P also acts antagonistically to the ceramide and enhances cell survival. Angiogenesis, migration, adhesion and inflammation are other cellular processes in which S1P has a role.[41,75] S1P has importance in the translocation of T and B cells from lymphoid organs to the bloodstream.[76] Level of S1P is elevated upon activation of sphingosine kinases by the growth factors and cytokines including VEGF and PDGF. S1P was also found to be important in the inflammatory responses by activating COX2 in the presence of TNF-α.[77] Unexpectedly, S1P induces growth arrest in keratinocytes, but this observation is not mechanistically related to cytotoxicity or apoptosis; in fact, S1P acts protective for the programed cell death in these cells.[78] S1P acts as a ligand to the cell surface receptors of lysophospholipid receptor family, which has five members identified up to date. Some of those receptors demonstrate expressional tissue specificity and provide different tissue-specific responses to S1P.

Types and Characteristics of Blood Cancers

Uncontrolled malignant growth of blood cells is known as leukemia. Blood cancers can be examined under two main classes as acute and chronic forms. Acute leukemia progresses when the regulation of hematopoiesis is lost at the very initial steps. In this case, malignant cells rapidly accumulate in the bone marrow and bloodstream and prevent the production and functioning of healthy cells. Acute leukemias comprise the form of blood cancer commonly seen in the children. In chronic leukemia, malignant cells are relatively differentiated, yet they are only partially functional. Their progression is slow and may require years to progress and become a life-threatening condition. In addition to these classifications, leukemias are subdivided into further types according to the affected cell lineage. Cancers of the cells having lymphoid origin that would normally differentiate into white blood cells are called as lymphoblastic/lymphocytic leukemias. Myeloid originated cells differentiate into erythrocytes, platelets and other white blood cells under normal physiological conditions; cancers of such cells are known as myeloid/myelogenous leukemia. The following sentences will briefly summarize the current knowledge about various leukemias, but the ones seeking for detailed information about the pathogenesis and progression pathways of those cancers are advised to consult the related review articles.

CML is the first leukemia whose progression is directly linked to a chromosomal aberration. The main driving force of the CML is the translocation between 9th and 22nd chromosomes resulting in the synthesis of BCR/ABL fusion protein showing constitutive tyrosine kinase activity.[79] Constitutive tyrosine kinase activity induces cell proliferation and prevention of apoptosis and results in the accumulation of malignant cells in the bone marrow and bloodstream. After its pathobiology is delineated, targeted chemotherapies were developed[80] for CML and survival times of the patients are greatly prolonged. Chronic lymphoblastic leukemia (CLL) is the most common form of the leukemia, and it is manifested by the accumulation of CD5-positive B cells in the circulation. Studies attempting to shed light on the molecular biology of CLL have revealed deregulation of Tcl1-Akt pathway, TNF-NFκB pathways and antiapoptotic pathways mediated by Bcl-2 in malignant cells.[81] CLL cells are found to be quiescent in the G_0 stage of the cell cycle; therefore, their accumulation is linked to the defective apoptotic mechanism.[82] Acute myeloid leukemia (AML) is one type of myeloid lineage-originated leukemia. Chemotherapy and radiation was shown to create predisposition for the progression of this leukemia.[83,84] In addition to those, some myelodysplastic disorders are known to turn into AML.[85] Acute lymphoblastic leukemia (ALL) is manifested by excess numbers of undifferentiated white blood cell progenitors in the bloodstream. Exact causes of ALL are not known, but some genetic aberrations were observed in the immature leukemic cells. Those aberrations include chromosomal translocations residing the genes encoding for transcription factors responsible for the hematopoiesis.[86] Besides those major structural changes, some single nucleotide polymorphisms were shown to be related to ALL.[87]

Bioactive Sphingolipids in Hematological Malignancies

Despite the advancements of the therapeutic options and the prolonged survival times in recent years, thanks to them; hematological malignancies are still far away from being eradicated because of the recurrence after the treatment in most cases. Because sphingolipids have important functions in cell cycle regulation and differentiation, considerable effort is being made to reveal the roles of bioactive sphingolipids in the progression or prevention of the blood cancers. Ceramide as the central component of the sphingolipid metabolism is one of the most widely studied sphingolipid species for that purpose. It is involved in a variety of cellular processes such as differentiation and programmed cell death, which are altered in the malignant transformation. Induction of ceramide synthesis and accumulation were documented in the leukemic cells undergoing apoptosis upon treatment with several chemotherapeutic agents. In a study with acute

promyelocytic leukemia (APL) and adult T-cell leukemia/lymphoma (ATL) cells, it was shown that cytotoxic levels of ceramides accumulate upon treatment with arsenic trioxide, suggesting that ceramides might be the mediator of the arsenic trioxide-dependent cell death.[88] Chemotherapeutic agent etaposide was shown to induce *de novo* ceramide generation pathway as a result of which cellular ceramide levels are increased and apoptosis is triggered in ALL cell line.[89] One study with sodium nitroprusside, which is an NO-donating apoptotic inducer, showed that ceramide generation takes place in NO-induced apoptosis of promyelocytic leukemia cells. This study also provided a link between the enzymes of apoptotic pathway and the enzymes responsible for the production of ceramides from SM, which might be interesting for future research to reveal the roles of sphingolipid species in NO signaling.[90] Cannabinoids are compounds having proapoptotic properties for the tumor cells. These compounds induce intrinsic apoptotic pathway, which was shown to be stimulated by the increased levels of ceramides in the Jurkat cell line.[91] Retinoids are also known with their apoptotic properties especially through p53-dependent cytotoxicity and increased level of ceramides in solid tumor samples. One study showed that retinoids induce apoptosis through increasing the cellular ceramide levels in ALL cells, whereas no cytotoxicity is observed in the nonmalignant cells.[92] Some other chemotherapeutic agents including fludarabine and histone deacetylase inhibitors were also found to induce leukemic cell death through a mechanism involving enhanced ceramide generation.[93–95] Cytotoxicity of resveratrol, a novel potent antineoplastic agent, also involves the accumulation of ceramides as documented by various studies.[96,97] By several other studies, direct incorporation of ceramides or ceramide analogs to the cell media was shown to suppress growth of various cancer cell lines.[26,98–102] In addition to their roles in chemotherapeutic cell death, ceramides were also shown to be associated with the photodynamic therapy-induced and gamma radiation-induced apoptosis in different leukemia cell lines.[103,104] As supportive to those observations, suppression of sphingomyelin synthase converting ceramide into SM was shown to potentiate the effects of photodynamic therapy.[105] However, according to the cell type used in the experiment, observations for roles of ceramides may differ. For instance, unlike the process in the Jurkat cells,[104] ceramides were found to be nonessential for the radiation-induced apoptosis in MOLT-4 cells.[106] Some experiments with ALL and AML cells have revealed that ceramides are also functional in cell cycle arrest besides inducing apoptosis.[107,108] Ceramides were also shown to be important second messengers in FAS-induced apoptosis.[109,110]

In addition to its roles in suppression of cell growth, ceramide metabolism was also implicated to be altered in the differentiation and chemotherapeutic resistance. In differentiation of AML blasts to macrophage-like and granulocyte-like cells, CerK that produces C1P from ceramide was shown to be differentially regulated, suggesting that CerK may have important functions in differentiation of leukemic cells.[111] Involvement of ceramides in differentiation was also addressed by several other studies some of which provide promising data for the usage of ceramides as a therapeutic option for the enhanced responses to the conventional chemotherapy.[112–114] Defective ceramide signaling and the loss of the balance between apoptotic and proliferative sphingolipids contribute to the chemotherapeutic resistance in the leukemic cells. Decrease of the ceramide level by its conversion into antiapoptotic GluCer and S1P was shown to be important for conferring chemotherapeutic resistance to leukemic cells in various studies.[115,116] P-glycoprotein (P-gp), an ATP-binding cassette transporter found in the cell membrane, increases cell survival through modulating sphingomyelin–ceramide pathway in addition to its known role in effusing the drug from the cell.[117] By further studies, evidence was provided linking P-gp and GluCer synthesis for chemotherapeutic resistance.[118,119] Moreover, defective ceramide metabolism was also shown to contribute to the resistance to radiation-induced cell death, suggesting an important role of ceramides in the apoptosis induced by radiation.[120,121]

There are few studies about dhCer as a bioactive sphingolipid in hematological malignancies compared to the ceramide. In one study, dhCer was shown to be unable to induce apoptosis in leukemic cells unlike the ceramides, which might indicate the importance of the double bond in the structure for growth suppressive actions.[122] Supporting to the findings of this study, incorporation of the synthetic dhCer to the B-CLL and ALL cells did not result in the increased amount of apoptosis in other studies.[101,123] Suppression of the enzyme sphingomyelin synthase, which is responsible for the conversion of ceramide into SM, caused the accumulation of dhCer and ceramide and eventually sensitized Jurkat T lymphoma/leukemia cells to photodynamic therapy, but dhCer might possibly be an intermediate compound for the subsequent synthesis of ceramides; therefore, apoptotic induction cannot be attributable to the dhCer directly in this scenario.[105] However, in another study, cytotoxicity caused by the anticancer agent 4-HPR was shown to be related with the increased amounts of dhCer in HL-60 cells.[37]

Sphingosine and S1P are other important bioactive sphingolipids in leukemic cells having proapoptotic and antiapoptotic properties, respectively. In various leukemic cell lines, it was shown that sphingosine and its methylated derivative induce apoptosis independent of the involvement of ceramide synthase.[56] In another study, sphingosine was shown to induce c-jun expression and apoptosis by a distinct mechanism than ceramide analogs.[124] S1P produced by the phosphorylation of sphingosine exerts antiapoptotic functions and thus possibly involved in chemotherapeutic resistance. In fact, apoptosis induced by the application of various chemotherapeutic drugs including imatinib and daunorubicin was suppressed by the S1P as shown in the various leukemia cell lines.[125,126] Because of its tumor-promoting properties, inhibition of S1P synthesis was shown to be potent for obtaining more effective therapeutic responses to conventional drugs in

various leukemia types and for overcoming multiple drug resistance.[126–131] Studies aiming to shed light on the importance of S1P have revealed that sphingosine kinase is activated by BCR/ABL, Il6 and vitamin D in the CML, multiple myeloma and AML cells, respectively.[132–134] Antagonistic function of S1P to apoptosis was found to be mediated by inhibition of the cytochrome c and Smac/DIABLO release from mitochondria in acute leukemia cells.[135] Possible chemotactic roles were also attributed to S1P for attracting the nearby phagocytic cells such as macrophages and primary monocytes for the engulfment of the apoptotic cell.[136]

Conclusion and Perspectives

Sphingolipids are important constituents of the cells with emerging roles in the regulation of numerous cellular processes. Loss of regulation of the sphingolipid metabolism is involved in the progression of malignancy and drug resistance. As different sphingolipids exert differential functions on the cell growth, one promising approach for eradication of the hematological malignancies is increasing the proapoptotic sphingolipids such as ceramides while suppressing the synthesis of the antiapoptotic ones such as glucosyl ceramide and sphingosine-1-phospate. A variety of studies have shown that this approach is feasible for obtaining better responses to the chemotherapy.[35,40,43] Usage of bioactive sphingolipids as a therapeutic option as independently or in combination with other drugs gained importance especially for the hematological malignancies in recent years, because leukemic cells are not eradicated completely in the patients despite highly specific drugs, causing relapse of the disease with the resistance to chemotherapy. In this manner, manipulation of sphingolipid metabolism might be a good opportunity to tackle the drug resistance commonly seen in many forms of hematological malignancies. However, because apoptotic sphingolipids such as ceramides may cause cytotoxicity in healthy cells too, future endeavor might be concentrated on delivering those species specifically to the malignant cells. For this reason, studies conducted in the cell lines should be carried further, and more *in vivo* experiments are needed to be done to reveal the actual potentials of bioactive sphingolipids as cancer therapeutics in leukemias. In the light of the extensive literature being accumulated in this area, responses to leukemia therapies would possibly be advanced in the near future by the involvement of bioactive sphingolipids.

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