Chapter 7

Animal Models for Studying the Pathophysiology of Ceramide

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Abstract

Bioactive sphingolipids play key roles in the regulation of several fundamental biological processes such as proliferation, apoptosis and transformation. The recent development of genetically engineered mouse (GEM) models has enabled the study of functional roles of sphingolipids in normal development and disease. In this chapter, we review the phenotypes of GEM models (knockout mice) that lack sphingolipid metabolism-related enzymes, discuss what we have learned from animal models and describe future directions of animal models in sphingolipid research.

Introduction

Sphingolipids constitute a class of lipids that share the presence of a sphingosine base in the backbone of their structures. In addition to their important structural functions, sphingolipids are currently considered key bioactive molecules that modulate cellular processes such as proliferation, differentiation, senescence, apoptosis and transformation. The recent development of genetically engineered mouse (GEM) models has contributed extensively to the field of sphingolipid research. In this review, we discuss biological functions that have been revealed using GEM for sphingolipid metabolism-related enzymes: sphingosine kinase 1 and 2, ceramidases, sphingomyelinases, sphingomyelin synthases and sphingosine-1-phosphate (S1P) lyase.

Sphingosine Kinase 1/2

Sphingosine kinases (SphKs), which are highly conserved enzymes found in mammals,[1-4] insects,[5] plants,[6] yeast,[7] worms[8] and slime molds,[9,10] catalyze the synthesis of sphingosine 1-phosphate (S1P) via the phosphorylation of sphingosine. To date, two distinct isoforms of SphK have been identified in mammals—SphK1 and SphK2. Two enzymes in mice contain five highly conserved regions (C1-C5) and an ATP binding site within a conserved lipid kinase catalytic domain.[1,2] Despite sharing two large conserved regions, these kinases have different kinetics of expression during development as well as different subcellular localizations.[2,11,12] Northern blot and quantitative PCR have revealed that SphK1 mRNA is high at embryonic day 7 (E7) and then decreases, but SphK2 mRNA expression remains high at later embryonic developmental stages.[2] SphK1 is predominantly localized to the cytoplasm but can be induced to localize to the inner leaflet of the plasma membrane.[13] SphK1 is distributed differently than SphK2 in the tissue, although both enzymes are widely expressed in most tissues.[2,14] Mice lacking the gene that encodes either *SphK1* or *SphK2* have no abnormal phenotypes, but SphK1/2 double knockout (KO) mice are lethal prior to

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E13.5 with severe vascular and neural tube defects, resembling S1P 1 receptor-null mice.[15,16] These results indicate that S1P signaling during embryonic development is critical for neurogenesis and angiogenesis. Further analysis of SphK-deficient mice revealed that SphK1$^{-/-}$SphK2$^{+/-}$ females—but not mutant males nor any other mutant female combination—were infertile due to severe defects in decidual cells and decidual blood vessels, leading to early embryonic lethality.[17] A function of SphK1 and 2 in immunoregulatory system is well studied. FTY720, a potent immunosuppressive agent that, when phosphorylated, functions as an agonist for S1P receptors, S1P1, S1P3, S1P4 and S1P5, caused lymphopenia in SphK1-null mice, but not in SphK2-null mice, indicating that SphK2 and not SphK1, is the primary kinase responsible for phosphorylating FTY720 in vivo.[15,18] Conditional deletion of SphK1 and SphK2 in mice prevented lymphocyte egress from the thymus and peripheral lymphoid organs via the reduction of plasma and lymph S1P levels.[19]

Recently, roles of the SphK1/S1P pathway in inflammation and cancer have been revealed. The SphK1/S1P pathway has been reported to regulate the cyclooxygenase-2 (COX-2)/prostaglandin E_2 (PGE_2) pathway and pro-inflammatory products in several cell lines, such as murine fibroblasts and human colon and lung cancer cells.[20,21] In mast cells, macrophages and neutrophils, SphK activity is stimulated by the ligation of plasma membrane receptors for C5a, Fc and formyl peptide (fMLP).[22-25] Two reports of studies using SphK KO mice suggested a role for SphKs in inflammation and neutrophil functions.[26,27] SphK1 KO mice exhibit normal inflammatory cell recruitment during thioglycollate-induced peritonitis and SphK1-null neutrophils respond normally to formyl peptide. In a collagen-induced arthritis model of rheumatoid arthritis, SphK1 KO mice develop arthritis with a normal incidence and severity.[26] Neutrophils isolated from the bone marrow of SphK1 KO mice or SphK2 KO mice have normal increases in intracellular Ca^{2+} stimulated by fMLP, platelet-activating factor, the anaphylatoxin C5a, or ATP and normal migration towards fMLP and C5a.[27] These findings do not support the hypothesis that SphKs play a critical role in inflammation. Further analysis using a dextran sodium sulfate (DSS) colitis model has revealed that SphK1 KO mice have significantly less severe DSS-induced acute colitis.[28] Very recently, SphK1 KO mice have been shown to have significantly less aberrant crypt foci (ACF) formation, which is a preneoplastic lesion of colon cancer induced by the rodent colon carcinogen azoxymethane (AOM).[29] In addition, SphK1 deficiency significantly protects against colon cancer development induced by AOM/DSS treatment in a colitis-induced colon carcinogenesis model. An AOM-induced colon carcinogenesis mouse or rat model has a similar spectrum of colonic lesions as various human colon neoplastic diseases including the ACF-adenoma-carcinoma sequence. SphK1 has been reported to play a critical role in intestinal tumor formation in an *Apc^{Min}* (Min) mouse model.[30] The Min mouse model was generated by random ethylnitrosourea mutagenesis. It carries a nonsense mutation at codon 850 of the *Apc* gene leading to a truncated *Apc* polypeptide and is relevant to human familial adenomatous polyposis coli. Deletion of the SphK1 gene in Min mice resulted in profoundly suppressed adenoma size but not its incidence. These findings suggest that SphK1/S1P signaling may play a pivotal role in inflammation and cancer.

Ceramidases

In the sphingolipid degradative pathway, ceramidases (EC 3.5.1.23) catalyze the hydrolysis of the *N*-acyl group of ceramide to yield sphingosine and fatty acids. Ceramidases have traditionally been classified according to the pH range (acid, neutral, or alkaline) that supports their optimal activity.

Acid Ceramidase

An acid ceramidase is encoded by the *Asah1* gene.[31-33] Mutations in the corresponding human gene cause Farber disease, a lysosomal storage disorder that results in the accumulation of ceramide. To date, 17 different mutations in the acid ceramidase gene have been found in people with Farber disease.[31,32,34-36]

An acid ceramidase-null mouse model was created and homozygosity for the mutant allele led to an early, embryonic lethal phenotype before E8.5.[37] Acid ceramidase homozygous embryos

could not survive beyond the 2-cell stage and underwent apoptotic death, suggesting that acid ceramidase is essential for embryo survival, removing ceramide from the newly formed embryos and preventing the default apoptosis pathway.[38]

Neutral Ceramidase

A neutral ceramidase in mice is encoded by the *Asah2* gene.[39-41] Orthologous genes have been identified in human,[42] rat,[43] zebrafish,[44] *Drosophila*[45] and bacteria.[46] The neutral ceramidase has been identified as a Type II integral membrane protein that can be cleaved to yield a soluble secreted protein.[47] The enzyme has also been found in the apical membranes of proximal and distal tubules, collecting ducts of kidney, endosome-like organelles of hepatocytes[43] and in the epithelia of the jejunum and ileum,[41,48] suggesting possible diverse physiological functions.

A complete null mouse for neutral ceramidase has been created by Dr. Proia's group.[49] It has been shown that neutral ceramidase-null mice are viable with a normal life span with no obvious abnormality. Neutral ceramidase-null mice are deficient in the intestinal degradation of ceramide, suggesting that neutral ceramidase is important for the catabolism of dietary sphingolipids and regulation of bioactive sphingolipid metabolites in the intestinal tract.

Sphingomyelinases (SMase) and Sphingomyelin Synthases (SMS)

Sphingomyelin is ubiquitously present in eukaryotic cells distributed in a gradient fashion from membranes of the endoplasmic reticulum, Golgi apparatus and lysosomes to the plasma membrane, which contains 70-90% of total cellular sphingomyelin. Sphingomyelinases (SMase, EC 3.1.4.12), which have been implicated in important and diverse cellular functions, catalyze the hydrolysis of sphingomyelin to ceramide and phosphocholine and are characterized by their optimal pH into acid, neutral and basic SMase species.[50,51]

On the other hand, sphingomyelin is synthesized by sphingomyelin synthase (SMS), which transfers the phosphorylcholine moiety from phosphatidylcholine onto ceramide, producing sphingomyelin and diacylglycerol.[52] SMS is implicated in NF-κB activation mediated by TNF-α and phorbol ester using cell culture systems.[53]

Acid Sphingomyelinase (ASMase)

The cellular glycoprotein acid sphingomyelinase (ASMase) has been shown to be located in the acidic lysosomal compartment and contributes to lysosomal sphingomyelin turnover.[54] The cDNA and gene encoding ASMase (designated *sphingomyelin phosphodiesterase 1*, *SMPD1*) were cloned in 1989 and 1992, respectively.[55,56] In humans, an inherited deficiency of ASMase activity results in Type A and B forms of Niemann-Pick disease.[57,58] More than 300 Niemann-Pick disease cases and a dozen distinct mutations in the ASMase gene have been reported. A mouse model of Niemann-Pick disease Type A has been generated by targeted disruption of the ASMase gene.[59] These ASMase-null (ASM KO) mice mimic the human disease phenotype inasmuch as they die by 8 months-of-age, display ataxia and tremors and show visceral symptoms similar to those observed in human patients. Recent evidence for the involvement of ASMase in membrane reorganization suggests that neurons from ASM KO mice have elevated sphingomyelin in detergent-resistant membrane microdomains, leading to an aberrant distribution of glycosyl phosphatidyl inositol-anchored proteins.[60] In addition, ASMase plays a role in cerebral ischemia. In wild-type, but not ASM KO mice, an experimental model of transient focal cerebral ischemia resulted in neuronal increases in ASMase, ceramide and the production of inflammatory cytokines. Wild type mice also had larger infarct size and worse behavioral outcomes than ASM KO mice.[61] Interestingly, increased lung ceramide was reported in cigarette-smoking patients with emphysema, so ceramide may be involved in disease prevention.[62] In addition, a recent paper described that membrane ceramide levels were elevated in respiratory tissue from two different cystic fibrosis mouse models and patients with cystic fibrosis. ASM heterozygous KO mice with a cystic fibrosis transgene or under pharmacological ASMase inhibition were more resistant to *Pseudomonas* and their baseline pulmonary inflammation was decreased,[63] suggesting reduced ASMase inhibited *Pseudomonas* infection and increased survival in cystic fibrosis mice. Interestingly, *Pseudomonas*

infection has been shown to be more lethal in ASM homozygous KO mice than in wild type mice, likely due to reduced bacterial internalization caused by an inability to form ceramide-enriched microdomains.[64] ASMase plays a key role in stress-induced apoptosis. Among a variety of other functions, p53 is required for many cells to enter apoptosis after a lethal DNA-damaging dose of ionizing irradiation. In the thymus of p53-null mice, apoptotic cells were dramatically reduced in ASM KO mice. In the small intestine of p53-null mice, a significant amount of apoptotic endothelial cells were observed after irradiation, an event not observed in ASM KO mice, suggesting that ASM KO mice were protected from gastrointestinal tract syndrome (fatal bleeding into the gastrointestinal tract after irradiation).[65]

Neutral Sphingomyelinase (nSMase) 1/2

Two mammalian neutral sphingomyelinases have been identified: nSMase 1 and nSMase 2 (or in *unigene* nomenclature—SMPD2 and SMPD3, respectively). nSMase 1 is ubiquitously expressed with the mRNA and protein being greatest in the kidney.[66] nSMase 2, which has a different domain structure, is expressed mainly in brain.[67]

nSMase 1 deficient mice were generated in 2002.[68] Interestingly, nSMase homozygous KO mice are phenotypically normal and show neither lipid accumulation nor detectable changes in sphingomyelin, despite a gross reduction of nSMase activity in all organs except in the central nervous system.

Two lines of nSMase 2 (SMPD3)-deficient mice were generated: chemical-induced (fro/fro) and targeted (KO) mice. The mutation, fragilitas ossium (fro), was discovered in a random-bred stock of mice after treatment with the chemical mutagen tris(1-aziridinyl) phosphine-sulphine.[69] The mutation in the mouse has been demonstrated to have clinical, radiographic and morphologic manifestations similar to those which arise in autosomal recessive forms of osteogenesis imperfecta occurring in humans.[70] Recently, positional cloning results revealed that this mutation is a deletion in *Smpd3*, the gene encoding nSMase 2 and nSMase activity was abolished in fro/fro mice.[71] At birth, affected mice are smaller than normal with deformities and multiple fractures of ribs and long bones. Cartilage formation is normal, but the matrix of developing bones is severely undermineralized. Mortality is elevated in the perinatal period (up to 30%), but the condition stabilizes in mutant mice that survive to weaning. Most fro/fro adults breed and have normal behavior and lifespan. In mutant mice, blood calcium is normal, parathyroid hormone is elevated and bone osteonectin is decreased by 30%. Severe tooth and alveolar bone abnormalities linked to impaired mineralization were also observed. Another line of nSMase 2 deficient mice was generated with a gene-targeting method.[72] nSMase KO mice developed a dwarf phenotype with severe retardation of late embryonic and postnatal growth, a new hypothalamic form of combined pituitary hormone deficiency. These mice showed no sphingomyelin storage abnormalities, unlike nSMase 1 KO mice which exhibited massive sphingomyelin storage in lysosomes of the reticuloendothelial system.

Sphingomyelin Synthases (SMS)

Two SMS genes, SMS1 and SMS2, have been cloned and characterized for their cellular localizations.[73,74] SMS1 is found in the trans-Golgi apparatus, whereas SMS2 is predominantly found at the plasma membrane. Using cell culture systems, SMS1 has been implicated in the regulation of lipid raft sphingomyelin and raft functions, such as FAS receptor clustering,[75] endocytosis and apoptosis.[76] SMS2 homozygous KO mice were generated.[77] SMS2 KO mice display no obvious abnormalities, grow into adulthood and breed normally in a conventional environment. Using macrophages extracted from these mice, SMS2 deficiency significantly attenuated NF-κB activation, suggesting that SMS2 is a modulator of NF-κB activation and may be important in inflammation during atherogenesis.

S1P Lyase

S1P lyase catalyzes the final step of sphingolipid catabolism, namely the irreversible degradation of S1P. The human S1P lyase gene, S1P lyase 1 (*SGPL1*), encodes a protein of 568 amino acids with a molecular weight of 63.5 kDa.[78] The murine ortholog carries the same name and its

amino-acid sequence is 84% identical and 91% similar to the human S1P lyase. In mice and rats, S1P lyase activity and expression are highest in the small intestines, colon, thymus and liver and lowest in the heart and brain, with the exception of the olfactory mucosa epithelium, where the enzyme is highly enriched.[79,80] Inhibition of S1P lyase was shown to prevent lymphocyte trafficking by disrupting S1P gradients in blood and tissues, demonstrating that S1P lyase can significantly effect S1P signaling.[81] In contrast, S1P lyase expression promotes apoptosis in human cells.[82] It has been also found that S1P lyase is down-regulated in human colorectal cancers and in Apc^{Min} mouse intestinal polyps, suggesting that S1P lyase loss of function may correlate with and/or contribute intestinal carcinogenesis.[83] Homozygous S1P lyase KO mice do not survive beyond 3-4 weeks after birth and they show significant growth failure and anemia.[84] Several congenital abnormalities were reported, including vascular abnormalities, which lead to fatal hemorrhage and anemia; skeletal defects, which involved improper palatal fusion; thoracic malformations of the sternum, ribs and vertebrae; and renal abnormalities. These results suggest that S1P lyase may have a role in the regulation of mammalian angiogenesis and other developmental processes.

The Other GEM for Sphingolipid-Related Enzymes

There are several GEM available for other important enzymes involved in the sphingolipid pathway, namely dihydroceramide desaturase 1, ceramide galactosyltransferase, ceramide glucosyltransferase and ceramide kinase (CEK).

Mice were generated lacking the gene for dihydroceramide desaturase 1 (*Des1*), which encodes the enzyme that converts metabolically inactive dihydroceramide into active ceramide.[85] Homozygous KO pups have no detectable DES1 protein and much less ceramide, but dramatically more dihydroceramide than wild type or heterozygous littermates. The homozygous null mice have incompletely penetrant lethality and surviving animals are small with a complex phenotype, including scaly skin and sparse hair, tremors, signs of growth retardation, including notably decreased mean body weight and length, total tissue mass, lean body mass, bone mineral content and density and liver dysfunctions (increased serum alkaline phosphatase, alkaline aminotransferase and total bilirubin).

Galactocerebroside is synthesized by addition of galactose to ceramide in a single step with UDP-galactose as the donor. The enzyme responsible for this reaction is UDP-galactose:ceramide galactosyltransferase. Mice lacking UDP-galactose:ceramide galactosyltransferase do not synthesize galactocerebroside or sulfatide, but they form myelin containing glucocerebroside, a lipid not previously identified in myelin.[86] These mice exhibit severe generalized tremoring and mild ataxia. With age, these mice develop progressive hindlimb paralysis and extensive vacuolation of the ventral region of the spinal cord.

Ceramide kinase (CEK) phosphorylates ceramide to form ceramide-1-phosphate. CER homozygous KO mice are viable and CER activity is completely abolished in these mice.[87] Neutrophils are strikingly reduced in the blood and spleen of CER homozygous KO mice and they develop more severe *Streptococcus pneumoniae* infections.

Glucosylceramide (GSL), the core structure of the majority of glycosphingolipids, is synthesized on the cytoplasmic face of the Golgi by glucosylceramide (GSL) synthase via the transfer of a glucose residue from UDP-glucose to ceramide.[88] Dr. Proia's group has disrupted the gene (*Ugcg*) encoding GSL synthase.[89] Disruption of GSL synthase causes embryonic lethality beginning about E7.5 with complete resorption of the embryo by E9.5 due to enhanced apoptosis in the ectoderm during gastrulation.

Conclusion

The past 20 years have witnessed tremendous progress in the field of sphingolipids, which has been made by the recent development of mass spectrometry coupled to synthesis of internal standards and development of gene targeting approaches, such as the KO mouse and RNA interfering techniques. KO mice phenotypes suggest multiple disease-related genetic and therapeutic possibilities involving the sphingolipid pathway and these mice are instrumental in analyzing

biological functions. Further studies with stress-induced models such as carcinogenesis models with genetically engineered mice could better elucidate roles of sphingolipid pathways in disease and aid in identifying and validating involved enzymes and mechanisms for further therapies.

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