EFFECTS OF SPHINGOLIPIDS ON INTESTINAL FUNCTIONS AND INTRACELLULAR OXIDATIVE STRESS IN CACO2-BBE AND CACO2-BBE/HT29-MTX CO-CULTURED CELLS

by

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A Thesis
Submitted to the Faculty of Graduate Studies of
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MASTER OF SCIENCE

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FACULTY OF GRADUATE STUDIES

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Effects of Sphingolipids on Intestinal Functions and Intracellular Oxidative Stress in CACO2-BBE and CACO2-BBE/HT29-MTX Co-Cultured Cells

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ABSTRACT

Sphingolipids are enriched in human milk, but their roles in intestinal function during infancy are poorly understood. This study investigated the impacts of sphingomyelin (SM), gangliosides (GG) and n-acetylneuraminic acid (NANA) on intestinal function and antioxidant properties *in vitro*. Different sources of SM (egg yolk, bovine milk and human milk), GG and NANA were applied to Caco2-BBE and a novel co-cultured Caco2-BBE and HT29-MTX cells at concentration of 0 (control), 40, 80 and 100 μg/ml. Cell proliferation, migration and barrier function were assessed by MTT assay, *in vitro* wound assay, and transepithelial electrical resistance (TEER), respectively. Total antioxidant capacity of SM, GG and NANA was determined by the oxygen radical absorbance capacity (ORAC) assay and intracellular oxidative stress were measured after challenging cells with H_2O_2. SM, GG and NANA at 40 μg/ml significantly increased cell proliferation in comparison to the control in co-cultured cells ($p<0.001$). After 24 hours of cell injury, the percentage of wound closure was three times higher with SM and GG, and twice higher with NANA compared to controls in Caco2-BBE cells. SM and GG significantly ($p<0.001$) improved barrier function of Caco2-BBE monolayer at 40 and 80 μg/ml. Mean ORAC values of egg yolk SM, bovine milk SM, GG and NANA were 359.7, 324.6, 100.0 and 56.5 μmol Trolox/g, respectively. Egg yolk and bovine milk SM but not human milk SM significantly decreased intracellular ROS in comparison to control. These results indicate that sphingolipids and NANA may play an important role in intestinal epithelial wound healing by modulating cell growth, cell proliferation and migration. SM might have antioxidant properties, thereby decreasing intracellular oxidative stress. Sphingolipids may protect the intestine against infection and/or improve

recovery of damaged enterocytes that often occur in premature infants. Further studies are necessary to determine the mechanisms of action of sphingolipids in gut health.

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LIST OF ABBREVIATION

ANSA	aniline naphthalene sulfonic acid
BBM	brush border membrane
CAT	catalase
CNS	central nervous system
Cer	ceramide
C1P	ceramide-1-phosphate
CCK	cholecystokinin
DCF	dichlorofluorescein
E.coli	*Escherichia coli*
GG	gangliosides
GC	gas chromatography
GC-MS	gas chromatography-mass spectrometry
GPx	glutathione peroxidase
GSLs	glycosphingolipids
HPLC	high performance liquid chromatography
ILBP	ileal lipid binding protein
I-FABP	intestinal fatty acid binding protein
L-FABP	liver fatty acid binding protein
LCFA	long chain fatty acids
MTT	3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide

NANA	n-acetylneuraminic acid
NEC	necrotizing enterocolitis
ORAC	oxygen radical absorbance capacity
PC	phosphatidylcholine
PE	phosphatidylethanolamine
PI	phosphatidylinositol
PKC	protein kinase C
PS	phosphatidylserine
ROS	reactive oxygen species
RNS	reactive nitrogen species
SM	sphingomyelin
SMase	sphingomyelinases
SK	sphingosine kinase
S1P	sphingosine-1-phosphate
SPP	sphingosine phosphate phosphatase
SOD	superoxide dismutase
TEER	transepithelial electrical resistance
TLC	thin-layer chromatography

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CHAPTER I

INTRODUCTION

Sphingolipids are found in all eukaryotic cells functioning as important structural components of plasma membrane and bioactive mediators in a variety of cell functions (Merrill et al., 1997). Recently, much attention has been paid to the role of sphingolipids and their metabolites in human health, especially in intestinal function. Although sphingolipids are not essential nutrients, it may provide a huge potential serving as bioactive nutrients against many gastrointestinal diseases.

Sphingolipids especially sphingomyelin (SM) and gangliosides (GG) are higher in human milk compared to infant formula and present a unique characteristic distribution during lactation. It is not known if the notable distinction between breast-fed and formula-fed infants in growth and development can be attributed to this difference. Also many studies show that SM and GG might benefit an infant's intestinal function by promoting gut maturation, protecting the intestine from various infections and enhancing nutrient uptake in the intestine. However, the mechanisms of how these sphingolipids function in the infant's intestine are not well understood.

Oxidative stress may be related to many illnesses in preterm infants. Studies have shown many antioxidants and antioxidative enzymes to be present in human milk, including Vitamin A, Vitamin C, Vitamin E, superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT). Considering high concentration of sphingolipids in human milk, these molecules may also have antioxidant capacity.

1. Sphingolipids

Structure

Sphingolipids are the most structurally diverse category of lipids in nature. At present, more than 300 different sphingolipids have been found and all contain a long-chain base (sphingoid or sphingoid base), which is the characteristic structural unit of sphingolipids. In 1884, the sphingolipid was named because of its enigmatic ("sphinx-like") properties. Currently, sphingolipids are still the most elusive and complicated group of lipids. This long chain base is variable and can be different form such as sphingosine, dihydrosphingosine, phytosphingosine and dehydrophytosphingosine (**Figure I-1**). They are all aliphatic amines that are 12-22 carbon atoms long, usually contain two or three hydroxyl groups, and often a distinctive *trans*-double bond in position 4. Sphingosine is the most common and abundant one found in animal tissues and it was the first one found to have a unique structural unit that distinguishes the sphingolipids from other complex lipids. Concentration of free sphingoid bases is low in cells and they are further linked to a long-chain fatty acid through an amide bond, thereby forming ceramide (Cer), which is central to sphingolipids metabolism (Vesper et al., 1999). However, it is rare to find Cer in tissue since it quickly converts to more complex forms of sphingolipids such as SM.

The fatty acids composition differs in chain length and hydroxylation in different tissues. Usually, 14-26 carbons exist in both of odd and even number (DeVries and Norton, 1974; O'Brien and Rouser, 1964). It is the most unlikely structural unit to be linked to the biological function of sphingolipids. Furthermore, addition of a polar head group such as phosphocholine, joined by phosphodiester bond, or carbohydrates, joined by glycosidic linkage, leads to SM and glycosphingolipids, respectively (**Figure I-2**).

Figure I-1. Structure of sphingoid backbone

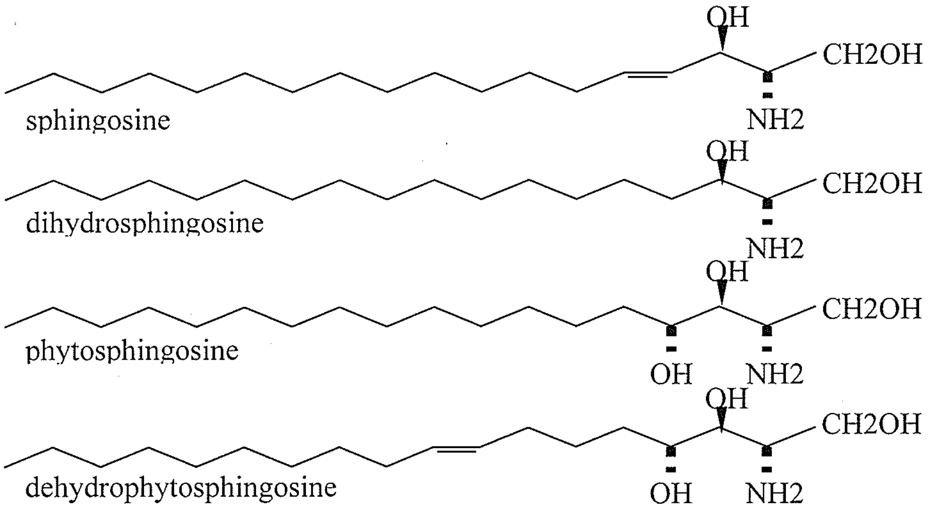
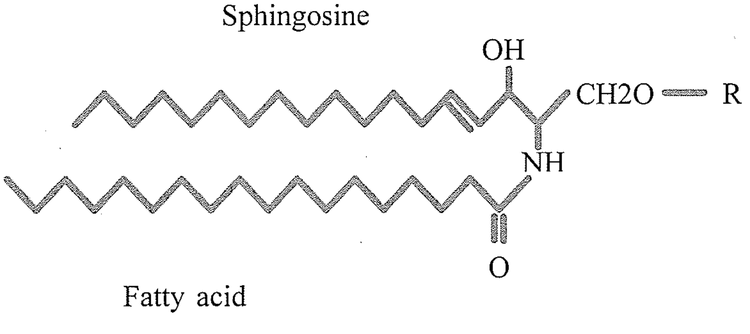


Figure I-2. General structure of sphingolipids



Substituent (R)	Sphingolipid
H	Ceramide
Phosphocholine	SM
Sugar (s)	Glycosphingolipid

Sphingomyelin

Since SM contains phosphocholine as the polar head group, it is also classified as both a phospholipids and a glycerophospholipids, and it counts for 7-14% of all phospholipids in liver, spleen and brain (Chatterjee et al., 2006). SM resembles phosphatidylcholine in the general properties and three-dimensional structure, and in having no net charge on the head group. Indeed, it can thus be viewed as sphingolipid analogue of phosphatidylcholine which is the primary phospholipid in mammalian cells. SM is present in the outer leaflet of plasma membrane of animal cells and the membranes of sub-cellular organelles such as the lysosomes and Golgi apparatus. Central nervous system (CNS) myelin, a membranous sheath that surrounds and insulates the axons of some neurons, contains around 65-80% of lipid due to especially prominence of SM, thus the name "sphingomyelin" (Oshida et al., 2003). In addition to CNS, SM is also enriched in plasma lipoprotein. Plasma SM in human (416 μg/ml) is higher than that of other species and primarily present in different lipoprotein (Nilsson and Duan, 2006). A total of 63–75% of plasma SM is in low density lipoprotein and very low density lipoprotein and 25–37% is in high density lipoprotein (Bagdade and Subbaiah, 1989). Moreover, the intestinal mucosa contains a relatively high level of SM, which counts about 10-12% of total lipids extracted from rat colonic mucosa and Golgi membranes of small intestine (Brasitus and Keresztes, 1983; Brasitus et al., 1988)

Gangliosides

GG are the most complex sphingolipids and have oligosaccharides as their polar head groups and one or more residues of sialic acid at the termini. Sialic acid refers to all derivatives of 5-amino-3,5-dideoxy-d-glycero-d-galacto-non-aulopyranosonic acid or neuraminic acid. Sialic acid gives GG a negative charge at pH 7 that distinguishes them from globosides. Variations in the structures of sialic acid also induce the diversities in GG (Rueda et al., 1998; Sonnino et al., 2007). There are three main forms of sialic acids in GG, the 5n-acetyl-, the 5n-acetyl-9 oacetyl- and the 5n-glycolyl derivates. Humans do not express 5-n-glycolyl derivates (Sonnino et al., 2007; Lloyd and Furukawa, 1998). The nomenclature of GG was developed by Svennerholm in 1964 and continues to be commonly used. The letter G is common to all GG and is followed by one of the next Latin letter initials: M, D, T, Q, P, H or S, referring to one, two, three, four, five, six or seven sialic acid residues which present in the molecule. For example, they are called monosialogangliosides, disialogangliosides, trisialogangliosides and so on. These two letters are further followed by a figure corresponding to a different number of sugar residues in the oligosaccharide chain. For example, one means four residues, two means three residues, three means two residues and four means one residue. An additional sub index letter including a, b, c and d indicates the pathway by which the molecule was biosynthesized (Rueda et al., 1998).

The majority of GG of normal mammals and birds belong to the a- and b-series, whereas GG in lower vertebrates and amphibians are almost exclusively from the c-series (Ando and Yu, 1979; Yu and Ando, 1980; Fredman et al., 1982; Hilbig and Rahmann, 1984). The c-series GG also occur in the serum of patients with neuroblastoma and type I diabetes mellitus (Yamanaka et al., 1987; Gillard et al., 1989) and have recently been found in small amounts in the human brain (Miller-Podraza et al., 1991).

GG are preferentially presented in cell surface with their polar head group facing the extracellular environment. Although having been first discovered and most concentrated in central nervous system (Fredman and Lekman, 1997), GG can be found in most vertebrate tissue and fluid such as skeletal muscle (Dasgupta et al., 1991), liver (Riboni et al., 1990), amniotic fluid (Rueda et al., 1993) and milk (Rueda et al., 1998). In addition, approximately 20% of intestinal brush border membrane (BBM) lipids are glycosphingolipids, predominantly GM3 (Bouhours and Bouhours, 1983). GG content and distribution are tissue and development stage specific (Suzuki, 1965; Svennerholm et al., 1989). Human milk is a good example that will be elucidated later in this chapter.

Absorption and digestion of sphingolipids

The digestion of sphingolipids is complicated and not completely understood. Several key enzymes are involved. After oral administration, sphingolipids are hydrolyzed to Cer, which is further degraded to free sphingosine and free fatty acid. The whole small intestine and colon are exposed to the metabolites. There are two alkali-sphingomyelinases (SMase) in human: one in the bile and the other in the intestinal mucosa. After a meal containing SM and other nutritional component such as fat or proteins, the released cholecystokinin (CCK) stimulates the contraction of the gallbladder and delivers bile alkali-SMase together with bile salts to the intestine. At the same time, CCK increases the secretion of pancreatic trypsin, which together with bile salt dissociates both intestinal alkali-SMase and neutral ceramidase from the mucosa into the lumen. Then SM is sequentially digested by alkali-SMase and neutral ceramidase in the presence of bile salt. The sphingosine produced is absorbed in the intestinal epithelial cells and degraded to free fatty acid or reincorporated into complex sphingolipids like Cer (Vesper et al., 1999). An animal study showed that digestion and uptake of sphingolipids occurred in any region of the intestine, but not efficiently (Schmelz et al., 1994).

Unlike SM, digestion and absorption of dietary GG are not completely understood. One animal study showed that dietary GG may reach the intestine, thus are absorbed by enterocytes, further altering the ganglioside level in the intestinal mucosa, plasma and brain (Park et al., 2005).

Metabolism of sphingolipids

Metabolism of sphingolids can be simplified as a "sphingomyelin cycle" (**Figure I-3**). Many bioactive metabolites of sphingolipids are involved in this cycle. More specifically, condensation of palmitoyl-coenzyme A with serine by serine palmitoyltransferase on the cytosolic side of the endoplasmic reticulum initiates the sphingolipids synthesis. In this first step, serine palmitoyltransferase is considered as the key enzyme to control ceramide synthesis. The production 3-keto-sphinganine is further reduced by 3-ketodihydrosphingosine reductase quickly and generates dihydrosphingosine (sphinganine). Then different ceramidases introduce various fatty acids ranging from carbon atoms 14 to 28 in chain length by Acyl-CoA to produce dihydroceramide which is then desaturated by dihydroceramide desaturase to insert a characteristic trans-double bond at position 4 to final form Cer. Cer can also be produced from SM through sphingomylinase and provides sufficient substrate for the synthesis of other complex sphingolipids. For example, SM is biosynthesized through the action of SM synthase transfer of phosphocholine from phosphatidylcholine to ceramide. Gangliosidease also uses Cer as substitute, sialyltransferases and glycosyltransferases are involved in this biosynthesis pathway. Another important metabolite sphingosine-1-phosphate (S1P) functioning as opposite of ceramides is produced by sphingosine kinase (SK) from sphingosine substrate which is also can be regenerated through sphingosine phosphate phosphatase (SPP). In sphingolipids metabolism, Cer is put in the central place and can be de novo synthesized, released from complex sphingolipids and cleaved from sphingosine, which is called salvage. It is important to maintain balance among all individual metabolites. Hindering this balance may lead to multiple metabolic diseases (Cowart et al., 2009). More important, sphingolipids and their metabolites are believed to be a new class of potential modulators of cell function in both of intracellular and extracellular circumstance and will be elucidated later in this chapter. More detailed sphingolipids metabolism is shown below (**Figure I-4**) (Cowart et al., 2009).

Biological functions of sphingolipids

To better understand the biological functions of sphingolipids, it is necessary to look at their specific location on cell membranes. It is well known that the basic structure of the cellular membrane is a fluid lipid bilayer. Glycerophospholipids, sphingolipids and cholesterol are the lipid components of cell membranes. Among these, sphingolipids are located only in the outer (exoplasmic) leaflet of the plasma membrane bilayer unlike glycerophospholipids including phosphatidylinositol (PI), phosphatidylserine (PS) and phosphatidylethanolamine (PE) which occur only in the inner (cytoplasmic) leaflet (Etemadi et al., 1980). In addition, cholesterol is believed to occur in roughly equal proportions in both leaflets. Up to now, at least 60 different sphingolipids in human have been identified on the cellular membranes, which indicate that sphingolipids are key constituents of cellular membranes. Furthermore, it is now acknowledged that they not only contribute to the formation of lipid bilayer, but also the maintenance of lipid rafts, which is lipid membrane microdomains. These lipid rafts are defined as highly dynamic, cholesterol and sphingolipid enriched microdomains participating cellular processes. The composition of these domains is different from the rest of the membrane and concentrating particular membrane proteins into a small region allows that region of the membrane to be specialized for some particular function. Compartmentalisation can be achieved by preventing free diffusion of the membrane proteins. As much as 50% of the plasma membrane may consist of such rafts and there are many different proteins in the lipid rafts including glycerophosphoinositol (GPI)-anchored proteins and tyrosine receptor kinases, which are involved in many cellular processes such as adhesion and differentiation. These provide much of the important biological properties of rafts, and are also essential to maintain their stability.

Sphingolipids are believed to function as "second messengers" to be involved in modulating growth factors, extracellular matrix proteins and various enzymes, thereby, affecting proliferation, differentiation, apoptosis and inflammatory response. For example, sphingosine has an inhibitory effect on protein kinase C (PKC) and phosphatidic acid phosphohydrolase while activating phospholipase D in various cell lines (Lavie et al., 1990; Natarajan et al., 1994; Yamada and Sakane, 1993). On the other hand, different studies confirmed that sphingolipids affect plasma lipoprotein constitution and kinetics as well as the development of atherosclerosis (Worgall et al., 2007). Furthermore, sphingolipids can serve as binding sites for some microorganisms, microbial toxins and viruses (Fantini et al., 1993).

2. Human milk

Dietary sources of sphingolipids

Sphingolipids are present in most foods. The total amount varies from low micromoles (vegetables and fruits) to several milimoles per kilogram (eggs, cheese and cream). The soybean is an exception that contains almost the same amount as that of some dairy products (Vesper et al., 1999). However, considerable variation existed in this database because of the different analysis methods used and different specific sphingolipids classes measured, such as, measurement of phosphorus content of SM, lipid bound sialic acid amount of GG and different analytic tools used. Based on Vesper's summary (1999), estimated sphingolipids consumption of adults in United States is 0.3-0.4 g per day and major food source is dairy products. In the case of infants, a newborn baby ingests 400-500 ml and a 4-month-old baby 550-1000 ml human breast milk per day, which represents approximately 80-200 mg SM and some glycosphingolipids (Nilsson et al., 2003). Recently, Yunoki et al. (2008) reported that the total sphingolipid intake in typical high- and low- calorie meal over two days were 292, 128 and 81, 45 mg/day, respectively. The major sphingolipids in the meals of this study were SM and cerebroside. Considering the differences between Japanese and North American diet, further study is needed.

Human milk sphingolipids

Human milk is the best source of nutrients for infants during the first four to six months of life. It is considered to contain all elements needed for normal growth and development of infants. Accurate and complete understanding of the components of human milk is required to know the nutritional requirements for newborns which depend on human milk naturally. This also helps to develop more beneficial infant formulas when human milk is unavailable.

The average lipid content of human milk is around 4.0 g/100ml with a big range during lactation in comparison to protein and lactose (Koletzko et al., 2001; Hamosh et al., 1985). Lipids in human milk consist of about 98% triglycerides, offering about 50% of the dietary calories, 1% phospholipids and 0.5% cholesterol (Robert, 1989).

Sphingomyelin

SM is the predominant human milk phospholipids, which accounts for around 40% of the total amount of phospholipids, significantly higher than other phospholipid classes, including phosphatidylcholine (PC) (28.4%), PE (27.7%), PS (8.8%) and PI (6.1%) (Koletzko et al., 2001). Most sphingolipids are present in the milk fat globule membrane. The daily intake of SM for suckling babies is calculated at 60-150mg per day (Zeisel et al., 1986). Dietary products also contain SM approximately 25% of the total phospholipids, which is lower than human milk (Palmquist, 2006).

SM and PC are the major sources of choline for infants. Choline is thought to be a key constituent of membranes in the brain and nervous tissue. It is considered as an essential nutrient especially for preterm infants, whose hepatic pathway for choline biosynthesis is not fully functional. According to several studies, the SM and PC concentration in human milk remains constant during lactation ranging from 100 to 200 μmol/l. There is no significant difference in these phospholipids between human milk and bovine milk, whereas several infant formulas showed significantly low level of SM, but not PC (Zeisel et al., 1986; Holmes-McNary et al., 1996). Another recent study showed that SM in human milk is 129.1 μmol/l in colostrums and 94.9 μmol/l in mature milk, but still significantly higher than infant formulas available at that time (Ilcol et al., 2005).

As shown above, SM content in human milk varies. This perhaps due to: different maternal dietary habits or different methods of measurement, some measured SM as choline content by using HPLC and gas chromatography-mass spectrometry (GC-MS), others measured SM as phosphorus containing content after thin layer chromatography and different dietary habit of mothers. There is an overall agreement that the level of SM in human milk is higher than that of SM in bovine milk based infant formula. Soy protein based infant formulas showed the lowest amount of SM (Zeisel et al., 1986).

Milk from mothers delivering term and preterm infants maintains same level SM (Holmes-McNary et al., 1996). Moreover, there was no difference between colostrum and mature milk (Sala-Vila et al., 2005). However, SM content in colostrum (36.0 ± 4.7% of total phospholipids) of Japanese mothers giving birth to preterm infants was lower than that in mature milk (42.3 ± 8.3% of total phospholipids). The same trend was not found in mothers with term infants (Shoji et al., 2006). This finding was not in agreement with the consistent amount of human milk SM in different stages during lactation regardless of milk from mothers delivering preterm infants or term infants in western countries. Another study in Japanese women showed a relatively lower level of SM (30.6 ± 6.6% of total phospholipids) in breast milk in comparison to other studies (Wang et al., 2000). Further studies are required to confirm SM content by considering various conditions, such as ethnic background, mother's diet and giving birth of preterm and full term babies. Summary of comparison of SM content in human milk, infant formula and bovine's milk are listed in **Table I-1**.

Gangliosides

Several studies showed the content and distribution of GG in human milk. Even though there were some variations, they all observed a selective change of relative concentration of GM3 and GD3 between colostrum and mature milk. GD3 is the predominant ganglioside in early lactation, whereas GM3 in predominant later on in lactation (Takamizawa et al., 1986; Rueda et al., 1998). In bovine milk, GD3 was the major GG followed by GM3. GD3 comprises 85% of the total GG of buttermilk (Hauttecoeur et al., 1985). Rueda et al. (Rueda et al., 1996) detected and compared the relative concentration of individual GG in human milk from mothers delivering term and preterm infants during lactation. They found that both GD3 and GM3 were higher in preterm than term colostrum and mature milk. GD3 is usually detected in developing tissues and biological fluid (Rueda et al., 1993; Asano et al., 1991), whereas GM3 is more abundant in mature tissues. This indicates that GD3, high in colostrum, appears to have a particular role in supporting growth and development of infant organs. In addition to GD3 and GM3, four other GG in human milk have been found, which were assumed to be c-series (described as GX1, GX2, GX3 and GX4), and have not shown the distinctive differences between colostrum and later milk. Only two of these four GG were observed in bovine milk and infant formulas (Pan and Izumi, 1999; Pan and Izumi, 2000). **Table I-2** summarized the amount and distribution of GG in human milk, infant formulas and bovine's milk.

3. Sphingolipids in intestinal function

Intestine maturation

A few studies showed that sphingolipids might influence intestinal maturation. Intestinal alkaline SMase and neutral ceramidase are key enzymes for the metabolism of SM and Cer. In both rat and human fetuses, expression of these two brush border membrane enzymes coincides with the differentiation and maturation of the intestinal villus (Nilsson and Duan, 1999). The presence of alkaline SMase and neutral ceramidase in the meconium of human newborns as well as significant level SM and sphingosine indicated that milk SM and its metabolites might have an influence on intestinal maturation, especially for preterm infants (Duan et al., 2007). One animal study showed that feeding milk containing 0.5% SM for one week promoted the maturation of intestine in suckling rats indicated by differences in lactase activity, distribution of vacuolated cells and auerbach plexus area (Motouri et al., 2003). The authors suggested that dietary SM during infancy might accelerate the enterocyte turnover rate and development of intestine (Motouri et al., 2003). More investigation is needed to bring more evidence.

Intestinal infection

Some microorganisms, microbial toxins and viruses can bind to cells via sphingolipids located on the cell membrane. This indicates a possible way for sphingolipids in human breast milk to protect infants from various infections. Human milk GG has already been demonstrated to have inhibitory action on *Escherichia coli*

Table I-1. SM content in human milk, infant formula and bovine milk

Human milk	Infant formula	Bovine's milk	Note	Reference
Foremilk: 163.4±11.3 µM Middle milk 167.8±9.8 µM Hind milk: 206.4 ± 9.4 µM	24.77 ± 28.78 µM	123µM	SM content of formulas varied widely and lower than that of human milk and bovine milk, especially soy protein based formulas	Zeisel et al., 1986
Preterm: 104 ± 9 µM (n=17) Term: 124 ± 9 µM (n=16)	Bovine-derived formulas were not significant different from human milk. Soy-derived formulas has less SM than did human milk (P<0.01) (data not shown)	Bovine milk SM concentrations were not significant different from those in human milk. (data not shown)	No significant difference between milk from mothers delivering preterm infants and term infants	Holmes-McNary et al., 1996
30.6 ±6.6% of total PL				Wang et al., 2000
Colostrums: 129 ± 13 µM (n=21) Mature milk: 94 ± 9 µM (n=95)	12.71± 8.86 µM (n=28)			Ilcol et al., 2005
Colostrums: 40.49 ± 3.57% of PL (n=30) Transitional milk: 39.20 ± 3.63% of PL (n=17) Mature milk: 41.03 ± 3.41% of PL (n=19)			No significant difference among colostrums, transitional milk and mature milk	Sala-Vila et al., 2005
Preterm: 42.3 ± 8.3% of PL (n=14) Term 36.0 ± 4.7% of PL(n=15)			SM content of mature milk in the preterm group was higher than that in term group (P<0.05).	Shoji et al., 2006
		839 and 1044 µg/g fat in different breed cows	Breeds, sampling day, sampling season may affect SM content, but supplemented diet may not.	Graves et al., 2007

Table I-2. GG content and profiles in human milk, infant formula and bovine milk

Human milk	Infant formulas	Bovine's milk	Note	Reference
11 mg/l	6 mg/l	11 mg/l	Total GG content in human milk and bovine milk was higher than that in formulas, however, they showed same pattern: GM 74%, GD 25%.	Laegreid et al., 1986
Range from 1.01 to 2.34 µmol LBSA/100ml during lactation (day 2 to 390)			Changes of GM3 and GD3 during lactation, molar ratio of GM3 to GD3 started from 0.25 to 19.82	Takamizawa et al., 1986
0.33 mg/g dried milk				Laegreid et al., 1987
			GM3 + GD3 > 95%	Rueda et al., 1995
Colostrum:9.51±1.61µg LBSA/ml (n=20) Mature milk: 9.07±1.15µg LBSA/ml (n=41)	1)14.14±1.35 µg LBSA/ml(n=3) 2)15.72±1.28 µg LBSA/ml (n=3) 3)12.56±1.30 µg LBSA/ml (n=3)	3.98±0.25		Pan and Izumi, 2000
1.79±0.28 µmol LBSA/100ml (3 to 4 days postpartum)		1.02±0.05 µmol LBSA/100ml (10 to 30 days postpartum)	GM3 29.2%, GD3 68.7%, GT3 0.5%, GM1 0.02% in human milk GM3 12.3%, GD3 69.8%, GT3 3%, GM10.4% in cow's milk	Iwamori et al., 2008

*LBSA: lipid bound sialic acid

(*E.coli*) and *Vibrio cholera* enterotoxins. Furthermore, human milk GG shows around five to ten times higher inhibitory activity than cow milk and infant formulas (Laegreid et al., 1986). GM1 is believed to contribute to this inhibitory action as the receptor for these enterotoxins, even though human milk contains trace amounts of GM1 (Laegreid et al., 1987; Otnaess et al., 1983).

Figure I-3. Sphingomyelin cycle

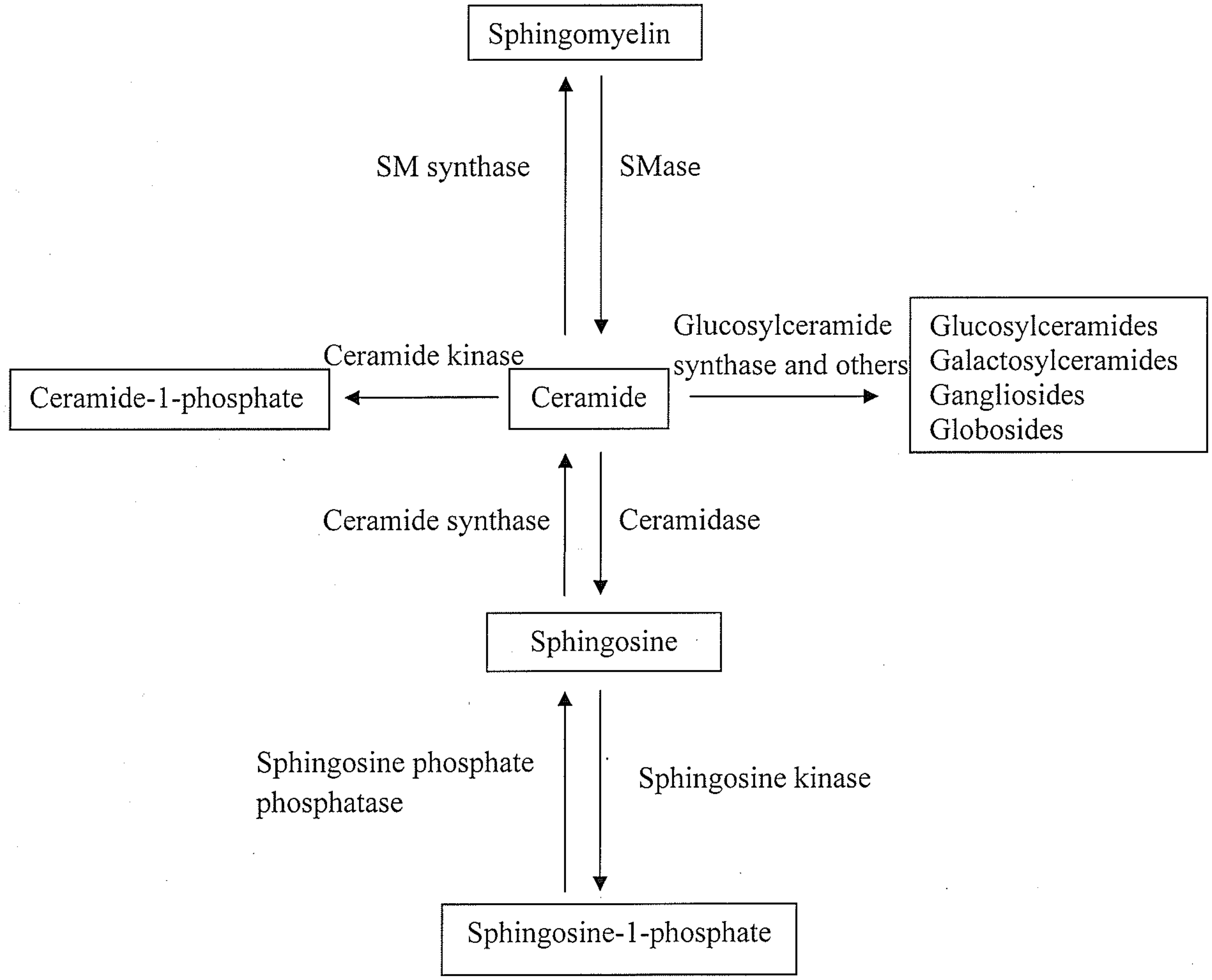
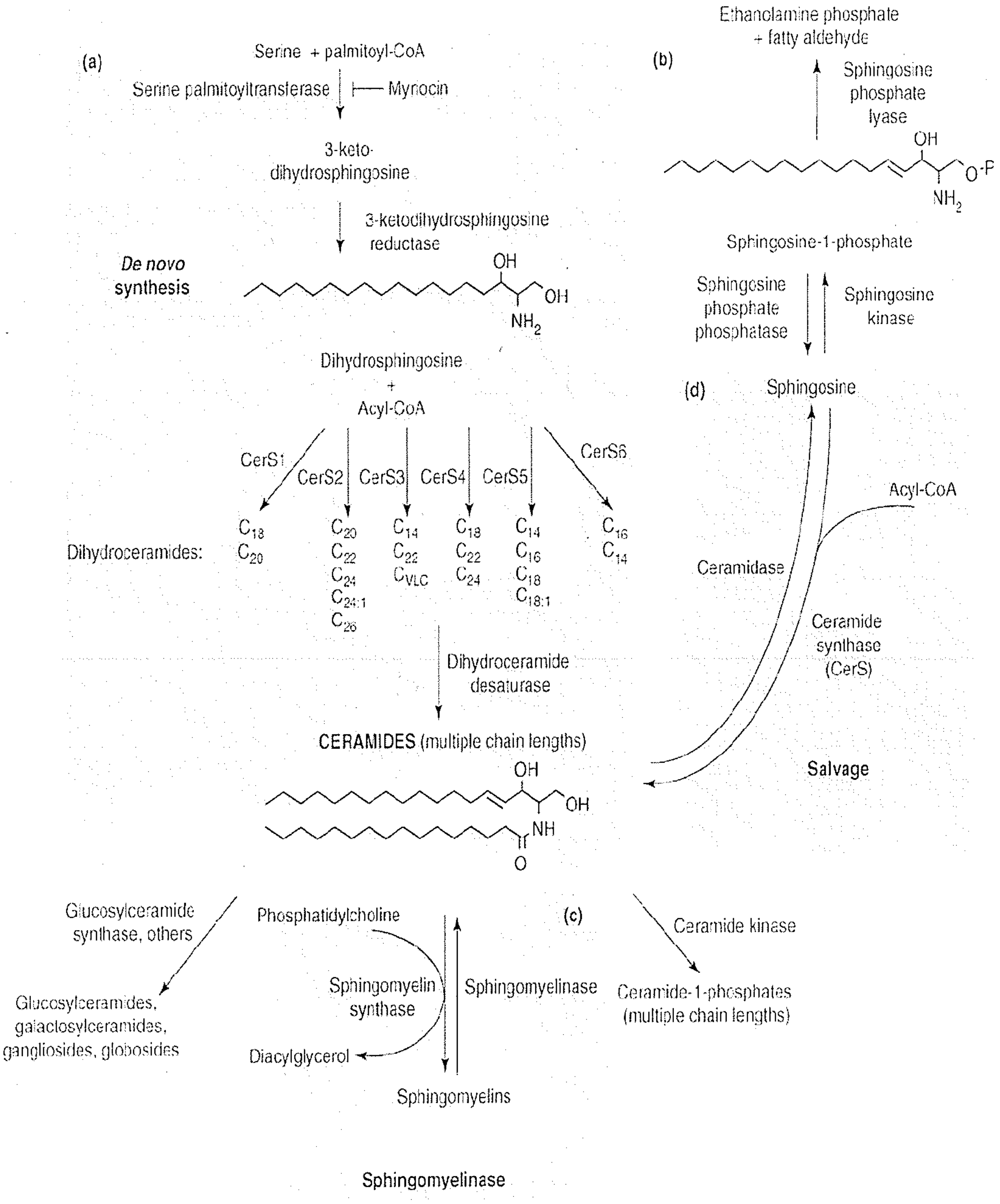


Figure I-4. Sphingolipids metabolism



Rueda et al. (1998) showed GG can modulate the intestinal microflora of neonate. They measured the fecal *E.coli* and bifidobacteria in two groups of preterm infants by providing GG supplemented formula or the standard formula without GG. After the infant's first month of life, the fecal *E.coli* counts in the GG group were lower than the control group, whereas the fecal Bifodibacteria counts in the GG group were higher than the control group. They also found that the colonization of Bifidobacteria flora was faster in infants fed with GG supplemented formula (Rueda et al., 1998). It seems that GG can suppress the growth of *E.coli* and promote the growth of Bifidobacteria, as a result, preferentially altering the gut microflora to beneficial types and provide greater disease protection in the preterm infants. Another study showed that GG might play a role against parasite infection (Suh et al., 2004). Female CD-1 mice fed with 1.0% (w/w) GG diet showed a significantly low output of cysts released in the faeces and trophozoites present in the small intestine. Results from this study showed that GG might have the protective effects against *G. muris* infection in vivo and affect the survival of *G. lamblia* trophozoites in vitro which may be directly toxic to parasites. It is hard to specify which structure of GG contributes to the specific biological effects, but it seems that the carbohydrate portions either resemble or are identical to those of free oligosaccharides with proven prebiotic capabilities (Rueda, 2007).

Studies showed that GG have been involved in the activation of T cells (Yuasa et al., 1990). They increased the number of intestinal IgA-secreting cells and luminal content of secretory IgA, induced an earlier development of cytokine-secreting cells and a higher number of Th1 and Th2 cytokine-secreting lymphocytes in lamina propria and Peyer's patch lymphocytes in weanling mice (Vázquez et al., 1999; Vázquez et al., 2001).

Based on these studies, dietary ganglioside may contribute to regulate intestinal immunity which partially explains the possible mechanism of protective effects against microorganisms in gastrointestinal tract (Rueda, 2007).

Intestinal nutrients uptake

Since the intestine is the most important organ for nutrients absorption, dietary modification may play important role in intestinal adaptation, which results in changes in nutrient absorption. Limited studies showed that dietary sphingolipids might influence intestinal uptake of lipid and glucose in animal studies. Weanling male Sprague-Dawley rats were fed with GG enriched diet or diet enriched with polyunsaturated long chain fatty acids (LCFA) (Birecki et al., 2006). After two weeks of feeding, the uptake of lipid in intestinal wall (Jd) or mucosa (Jm) was measured by the expression of three proteins including ileal lipid binding protein (ILBP), intestinal fatty acid binding protein (I-FABP) and liver fatty acid binding protein (L-FABP). It was observed that the uptakes of several LCFA were higher in GG group than in control or polyunsaturated LCFA group, and this effect was most pronounced in the jejunum. However, there was no difference in the abundance of I-FABP and L-FABP protein and mRNA among the three diet groups. The ILBP in the ileum was also similar among the diet groups. Thus, the enhanced intestinal uptake of LCFA due to dietary GG could not be explained by the change in mRNA level or abundance of related lipid binding protein. The same group (Drozdowski et al., 2007) also found that GG provided in the diet increased glucose uptake with no significant change in glucose transporters (SGLT-1, GLUT2 and GLUT5) at the protein or mRNA level. This may be result of physicochemical changes of intestinal mucosa with GG (Park et al., 2005). In contrast, dietary SM in mice was found to inhibit D-galactose while stimulating glycylsarcosine uptake via brush border membrane vesicles with increased expression of SGLT-1. However, a lower activity of sucrase and maltase was also detected in mice fed 0.05% SM (Barrenetxe et al., 2006). Therefore, the impact of sphingolipids on nutrient uptake needs to be further investigated.

Dietary SM influences cholesterol absorption in the intestine. Many studies demonstrated that dietary SM is able to lower the plasma cholesterol level and inhibit the cholesterol absorption of intestine (Eckhardt et al., 2002; Noh and Koo, 2004). This great inhibition ability explained an extreme case reported in 1991: an eighty-eight year old man who ate 25 eggs per day did not present with a high plasma cholesterol level, even though egg is the richest food source for cholesterol currently known(Kern, 1991). This might be the counteraction of SM in eggs that protects cholesterol from being absorbed.

Colon cancer

Much of initial interests of dietary sphingolipids for the intestine are about colon cancer. As discussed above, sphingolipids and metabolites, specifically, sphingosine, ceramide and sphingosin-1-phosphate affect cell growth, proliferation and apoptosis. Since the entire intestine is exposed to dietary sphingolipids and their bioactive metabolites during digestion, it is easy to hypothesize that dietary sphingolipids may influence human colon cancer. Female CF1 mice fed purified milk SM (0.1% w/w) after treated with 1,2-dimethylhydrazine had a significantly reduced amount of aberrant colonic crypt foci, which is the earliest morphological marker of colon carcinogenesis (Dillehay et al., 1994). This result agreed with another study showing SM decrease the risk of colon cancer in rodents (Schmelz et al., 1996). Numerous studies used HT 29 cell line (human adenocarcinoma cell line) as well as other cell lines, and have shown the benefits of dietary SM via inducing apoptosis or reducing the tumor number (Borek et al. 1991; Schmelz et al., 1998). No side effects have been found in feeding sphingolipids to animals in previous studies. Even though dietary habit is thought to be related to the risk or the benefit for colon cancer, the link between dietary sphingolipids and colon cancer has not been studied clinically or epidemiologically. More studies are needed to understand the effect of dietary sphingolipids on colon cancer.

4. Sphingolipids and oxidative stress

Oxidative stress in infants

The normal gestational period for human full-term infants ranges from 38-42 weeks. Preterm infants are babies born before 37 weeks of completed gestation. The weight of preterm infants at birth is usually less than 2,500 g, while very low birth weight premature infants weigh less than 1,500 g due to immaturity (Alexander et al., 2003). Preterm delivery is now thought to be a significant global health problem, with considerable variation within and across populations, ranging from 5%–10% of births in industrialized countries to as high as 25% of births in areas of Asia and Africa (Sangild, 2006). The rates of preterm birth have increased in many countries over the past 20 years. In Canada, preterm births increased from 6.3 % of live births in 1981 through 1983 to 6.8 % in 1992 through 1994, and the data from 2003 shows the rate has increased to 7.7% (Joseph, 1998). Birth before 37 weeks is the most common cause of neonatal mortality, morbidity and long-term disability. In industrialized countries, preterm birth is probably the most important risk factor for decreased neonatal survival and health.

Oxidative stress is where there is an imbalance between reactive oxygen species (ROS; O_2^-, H_2O_2, OH^-), reactive nitrogen species (RNS; NO and $ONOO^-$) and antioxidants, thereby disrupting redox signaling and control, consequently, damaging macromolecules such as protein, lipid and DNA. Oxidative stress is associated with many pathologic processes of human disorders (Shoji and Koletzko, 2007). This process is supposed to begin after delivery, but it can even affect the fetus when maternal pregnancy diseases occur, especially in the case of preterm delivery. Even though the contribution of oxidative stress to the pathogenesis and progression of neonatal diseases is not completely understood, there was a hypothesis that a so-called "oxygen radical disease of neonatology" exists. Accumulating evidence indicates that many serious illnesses during infancy are related to oxidative stress, including bronchopulmonary dysplasia, chronic lung disease, retinopathy of prematurity and intraventricular hemorrhage. However, the interaction between oxidative stress and the certain disease process in the perinatal period still needs further investigation.

There are some possible reasons to explain why oxidative stress is more severe in infants, especially preterm infants. First of all, the fetus transfers from an intrauterine hypoxic environment with a PO_2 of 20–25mmHg to an extrauterine normoxic environment with a PO_2 of 100mmHg. This four to five-fold increase is believed to induce an increased production of ROS (Shoji and Koletzko, 2007). Secondly, because of the surfactant deficiency at the gas-liquid interface, their lungs have difficulties in exchanging enough oxygen for distribution to the body's organs (Ward and Beachy, 2003). Then in order to treat the hypoxia, supplemental oxygen therapy is routinely administered to premature infants and oxygen supply is increased from 21% (room air) to 70 - 90% and may reach 100%. The increase in inspired oxygen concentration facilitates the exchange of oxygen and increases arterial oxygen supply, but may also cause hyperoxia which provides excessive amount of oxygen reaching body tissues. Thirdly, the preterm infants are more likely to suffer from inflammation and infection, and free radicals are more likely to be released during these processes. In addition, it is well accepted that preterm infants have a limited protection mechanism against oxidative stress, which increases their risk of experiencing oxidative stress. For instance, premature infants lack some antioxidant enzymes that protect them from attacks by free radicals.

Much research in the area of preterm birth affecting the development and health in infants has investigated lung and brain maturation, as well as the respiratory and cerebral defects associated with preterm birth. However, gut maturation and the gastrointestinal tract function also deserve attention because of the frequency of gastrointestinal tract diseases in the neonatal period of both preterm and full term infants. Infants, especially preterm infants, have fragile and immature gastrointestinal tract function in comparison to adults. An immature gastrointestinal tract usually gives rise to feeding intolerance. Moreover, because of the immature barrier function of the intestinal mucosa and the immune response, these premature infants are particularly susceptible to intestinal infection, which easily induces oxidative stress in intestine. The worst, and more frequent, situation is when necrotizing enterocolitis (NEC) occurs, which is the most critical disease in newborn babies. More than 90% of cases occur in preterm infants.

The possible causes include infection, oxidative stress and an impaired or limited protection system.

Antioxidant properties of human milk

Human milk is not only considered to be the best food for infants, but has also proved to be the dietary source of antioxidants, presenting a better antioxidant properties than infant formulas (Friel et al., 2002). Human milk fed infants had a lower level of oxidative stress markers, such as urinary malondialdehyde, than the level of these same markes in formula fed infants (Koletzko et al., 2003). In a very recent published work on so called "normal" preterm infants, who do not have any perinatal oxidative stress conditions, human milk fed preterm babies had decreased levels of general biomarkers of oxidative stress than formula fed infants (Ledo et al., 2009).

The relationship between total antioxidant capacity of human milk and the dietary intake of antioxidants in mothers during pregnancy and lactation has been well established. Colostrum, transitional and mature milk from mothers having high antioxidant intake had high antioxidant capacity measured by oxygen radical absorbance capacity (ORAC), in comparison to mothers with low antioxidant intake (Alberti-Fidanza et al., 2002). Various known antioxidants in human milk include enzymes (SOD, CAT, GPx), vitamins (Vitamin C, E, β-carotene and coenzyme Q), constituents of antioxidative enzymes (zinc, copper, selenium), isoflavones and some binding proteins like lactoferrin. Increased daily intake of Vitamin A, E and C of mothers' diet improving antioxidant capacity of their breast milk has been studied (Ortega et al., 1997; Ortega et al., 1998).

The total antioxidant property of human milk is also affected by many other factors like ethnic group and smoking habits (Ermis et al., 2005; Ortega et al., 1998).

Antioxidant properties of sphingolipids

Recently, along with the roles of sphingolipids in cell proliferation and apoptosis, it has been reported that sphingolipid metabolism is influenced by oxidative stress (endogenous prooxidants like ROS and RNS via regulation of several key enzymes such as SMase and ceramidase. Sphingolipids including Cer, sphingosine and S1P are also able to regulate cellular redox homeostasis through regulation of NADPH oxidase, mitochondrial integrity, nitric oxide synthase and antioxidant enzymes. In other words, sphingolipid metabolism and redox homeostasis are regulated in a bidirectional manner and these two pathways interact closely with each other (Won and Singh, 2006).

Several studies have showed the antioxidant activity of individual GG and a mixture of GG. Adult male Wistar rats received two injections of GM1 (50 mg/kg, i.p.) reduced spontaneous chemiluminescence and increased catalase activity in cerebral cortex ex vivo, but had no effect on total radical-trapping potential (TRAP), SOD or GPx activities (Fighera et al., 2004). GG reduced the free radicals formed during reperfusion of ischemic myocardium in isolated rat heart via direct scavengers of superoxide anions, hydroxyl radicals and hypohalite radicals, as shown by HPLC-electrochemical detection technique (Maulik et al., 1993). Another recent study used electron paramagnetic resonance spectroscopy to demonstrate that GG micelles inhibit iron-catalysed hydroxyl radical formation due to their iron chelation potential rather than their hydroxyl radical scavenging capacity. GT1b showed the better antioxidant ability than GM1 probably because of higher content of sialic acid residues (Gavella et al., 2007).

Since GG contain one or two moieties of sialic acids, it was expected that sialic acid also has antioxidant ability. As expected, NANA (Neu5Ac) was demonstrated to have hydrogen peroxide (H_2O_2)-scavenging activity by showing the conuntaction of cytotoxcity of H_2O_2 in EL-4 murine lymphoma cells and the direct chemical reaction in the culture media (Iijima et al., 2004; Iijima et al., 2007). In addition, NANA can reduce hydroperoxides by oxidizing lipids in the same way as it oxidizes H_2O_2. This reaction lowered the toxicity of lipid hydroperoxides toward cultured cells (Iijima et al., 2009). This antioxidant activity of NANA is ascribed to its α-ketocarboxylic acid structure, namely, pyruvic acid, oxalacetic acid, and α-ketoglutaric acid. For instance, pyruvic acid is the smallest α-ketocarbonic acid, and its carbonyl group is highly reactive towards H_2O_2.

Chapter II

RESEARCH PLAN

Rationale

Sphingolipids can be found in all eukaryotic cells and are important components of cell membranes. Sphingolipids and their bioactive metabolites are involved in modulating multiple cell functions by being involved in signaling pathways. Even though the intestine contains naturally-occurring sphingolipids comprising up to 20% of brush border membrane and sphingolipids can be easily found in our normal diet, the clear link between dietary sphingolipids and intestinal function has not been established.

Many studies have shown that dietary sphingolipids influence intestinal physiology and pathophysiology. Short term animal feeding studies and cell culture work demonstrated that dietary SM is related to a decreased risk for colon cancer by affecting cell growth, cell differentiation and apoptosis (Borek et al. 1991; Dillehay et al., 1994 Schmelz et al., 1996; Schmelz et al., 1998). However, not much is known regarding the nutritional aspects of sphingolipids in view of neonatal intestinal function. SM provided in the diet has shown to promote intestine maturation in suckling rats by increasing lactase activity and accelerating the turnover rate of enterocytes (Motouri et al., 2003). Dietary GG enhanced intestinal glucose and lipid uptake in weaning rats probably by changing physicochemical properties of intestinal mucosa (Birecki et al., 2006; Drozdowski et al., 2007; Park et al., 2005). In addition, preterm infants fed GG supplemented formula modified their intestinal microflora by lowering *E.coli* and increasing bifidobacteria. GG provided in the diet also changed the course of *G.Muris* infection by decreasing cyst output and the number of trophozoites attached in the small intestine (Suh et al. 2004). These results indicate that sphingolipids may have the potential to serve as bioactive nutrients against many gastrointenstinal diseases.

Sphingolipids exist in most of foods, especially dairy products and human milk, which contains relative high amount of sphingolipids as a form of SM and GG. Moreover, human milk GG shows characteristic selective changes of content and type during lactation, which may reflect the potential biological activities in the growth and development of infants (Takamizawa et al., 1986; Rueda et al., 1998; Pan and Izumi, 1999). Whether enrichment of these sphingolipids in human milk benefits intestinal function of infants requires further investigation.

Oxidative stress plays an important role in many illnesses during infancy. Intestinal function can be impaired in preterm infants as a result of a damaged defense system against oxidative stress and malnutrition. Previous researches showed that human milk possesses better antioxidant capacity than infant formula (Friel et al., 2002). Whether these sphingolipids also contribute to the antioxidant property has not been investigated.

Hypothesis

Therefore, it is hypothesized that

1. Sphingolipids will positively influence intestinal function, such as cell growth, cell proliferation, cell migration and cell barrier function
2. Sphingolipids will reduce intracellular oxidative stress in intestinal cells

Objectives

The overall objective is to study the roles of sphingolipids in intestinal function in a novel co-culture model. More specifically, the objectives are:

1. To test the effect of SM, GG and NANA on intestinal cell functions by measuring cell viability, cell proliferation, cell migration and cell barrier function
2. To test whether SM, GG and NANA has antioxidant properties by measuring intracellular ROS
3. To measure the total antioxidant capacity of SM, GG and NANA by ORAC assay
4. To compare if the various sources of SM have same effect on cell proliferation and antioxidant capacity.

Chapter III is written in the form of manuscript.

Chapter III

Effects of sphingolipids on intestinal functions and intracellular oxidative stress in Caco2-BBE and Caco2-BBE/HT29-MTX co-cultured cells

Introduction

Recently, sphingolipids have drawn a lot of attention due to their involvement in a variety of cell functions as bioactive mediators. These molecules are also emerging as important nutrients, especially for infants. Human milk contains sphingolipids as a form of SM and GG and is relatively high in these nutrients compared to bovine milk and bovine milk based infant formulas (Zeisel et al., 1986; Holmes-McNary et al., 1996; Ilcol et al., 2005; Pan and Izumi, 1999; Pan and Izumi, 2000). SM is the predominant phospholipid in human milk comprising about 40% of total phospholipids (Koletzko et al., 2001). GG is also abundant in human milk comprising about 10 µg/ml with characteristic changes during lactation, higher GD3 in colostrum and higher GM3 in mature milk (Pan and Izumi, 1999; Pan and Izumi, 2000; Takamizawa et al., 1986; Rueda et al., 1998). In addition, human milk also contains a large amount (0.7 mg/ml) of NANA, called sialic acid, which is a family of nine-carbon acidic monosaccharides attached to the terminal end of oligosaccharides in glycolipids and glycoprotein (Nakano et al., 2001). The highest concentration of sialic acid occurs in the central nervous system where it participates as an integral part of ganglioside structure in synaptogenesis and neural transmission (Wang et al., 2003). Studies have shown that infants fed human milk have a decreased incidence of intestinal infection and gastrointestinal disease (Morrow et al., 1999; Khadivzadeh and Parsai, 2004; Newburg and Walker, 2007). However, the strong correlations with sphingolipids and NANA and the necessity of these nutrients in infant nutrition need to be further studied.

The intestine contains naturally occurring sphingolipids comprising up to 20% of brush border membrane lipids. Studies on animals and infants have shown that dietary SM and GG play important roles in intestinal function. For example, SM and/or GG provided in the diet promoted intestinal maturation (Motouri et al., 2003), prevented intestinal infections and damage, and enhanced nutrients uptake in animals (Rueda et al., 1998; Laegreid et al., 1986; Suh et al., 2004; Birecki et al., 2006; Drozdowski et al., 2007). Whether SM and GG have similar effect or not, has not been tested in the same experimental conditions.

Preterm infants are at a high risk of suffering from oxidative stress after birth because of immature antioxidant defense system, exposure to high concentration of oxygen treatment, and increased oxidative stress due to inflammation and infections. It is believed that human milk is a better antioxidant than cow's milk and infant formulas (Aycicek et al., 2006; Friel et al., 2002) because human milk contains SOD, GPx, CAT and antioxidant vitamins (vitamin A, C and E). Our previous study showed that human milk and *in vitro* digested milk are equally efficient in reducing the formation of intracellular reactive oxygen species (ROS) in Caco2-BBE/HT29-MTX co-culture (Yao et al., 2009). Considering high content of SM, GG and NANA in human milk, these molecules may also have contributed to the above finding. However studies have not shown the antioxidant properties of sphingolipids.

Therefore, this study was planned to examine the roles of SM, GG and NANA in intestinal functions *in vitro* and to determine if these nutrients have antioxidant properties. We also tested the impact of different sources of SM on intestinal functions. This study was performed using two cell culture models: Caco2-BBE and Caco2-BBE/HT29-MTX co-culture.

Materials and methods

Materials and chemicals

Egg yolk SM (purity 98%) and bovine milk SM (purity 98%) were purchased from Avanti Polar Lipids, Inc (Alabaster, AL, USA). Human milk SM was prepared in our laboratory as described below. GG mixture (bovine gray matter, purity ≥ 98%, a mixture of 18% GM1, 55% GD1a, 15% GD1b, 10% GT1b, and 2% other GG) was obtained from Calbiochem (San Diego, CA, USA) and NANA (sheep submaxillary gland) from Sigma (St. Louise, MO, USA). Egg yolk SM was used in all of cell culture work. SM, GG and NANA were dissolved in cell culture medium containing 0.5% ethanol (w/w) and provided at the concentration of 0 (control), 40, 80, 100 μg /ml in cell cultures.

Cell culture

Two cell culture models were used in this study. Caco2-BBE and Caco2-BBE/HT29-MTX co-culture. Caco2-BBE cell line was purchased from American Type Culture Collection (Manassas, VA, USA) and HT29-MTX was kindly provided by Dr. T. Kissel (University of Marburg) and Dr. P. Artursson (University of Lund). Both cell lines were cultured in 25 cm^2 polystyrene flasks containing Dulbecco's Modified Eagle Medium (DMEM,GIBCO-BRL, Grand Island, NY, USA) supplemented with 10% fetal bovine serum, 1% penicillin and streptomycin, 200 mM L-glutamine, 1 mg/ml human transferrin and 100 mM sodium pyruvate at 37°C in 5% CO_2 and 80% humidity. All the experiments were carried out within 2 to 17 cellular passages unless otherwise specified. In case of co-culture, Caco2-BBE and HT29-MTX were mixed at a ratio of 7:3 as we and others have previously established (Yao et al., 2009; Hilgendorf et al., 2000; Mahler et al., 2009), which mimics the actual condition of human small intestine. Co-culture cells were seeded at a cell density of 2×10^6 and grown in 96-well plates in the culture medium as described before.

Sphingomyelin and gangliosides preparation from human milk

Human milk samples were obtained from five mothers with full term babies and collected by the mothers using a breast pump or by hand expression and transported on ice to the laboratory stored at – 80°C until analysis. Consent and ethical approval according to University of Manitoba Human Research Ethics Board was obtained from the women who participated in the study. The colostrum samples were collected during the first week of lactation and mature milk from the second to the third month during the lactation. An infant formula (Good Start, Nestle, North York, ON, Canada) was also included as a control.

The total lipids from human milk and infant formula were extracted by using Folch method (1957). The upper aqueous phase containing GG was collected and purified by using Sep-Pak C18 catridge (Waters Corp., Milford, MA, USA). The concentration of total GG was quantified as described by Suzuki (1964). The lower phase containing phospholipids was separated by thin-layer chromatography in a solvent system composed of chloroform: methanol: 2-propanol: 0.25% (w/v) KCl: triethylamine (30:9:25:6:18, by vol). Silica gel containing SM bands was eluted twice with chloroform:

methanol (2:1, v/v) and dried completely under N_2. An aliquot of SM extract was measured for the concentration of SM by measuring phosphate according to the method of Itoh et al. (1986).

Cell proliferation

Co-cultured cells were seeded and grown on 96-well black plates for 24 hours and then treated with cell medium containing SM, GG and NANA at the concentration of 0 (control), 40, 80, and 100 µg/ml. After cells were grown to confluence, cell proliferation was measured by using 3-(4,5-Dimethylthiazol-2-yl)-2,3-diphenyltetrazolium bromide (MTT) according to a protocol in the assay kit (ATCC, Manassas, VA, USA). The optical density (OD) was then measured at 570 nm by using SpectroMax Microtiter Plate Reader (Molecular Devices Co., Sunnyvale, CA, USA).

In vitro wound assay

Cell migration was measured based on cell closure of a defined injury area by using *in vitro* wound assay. Caco2-BBE cells were grown to complete confluence and then linear wounds were made with a sterile plastic pipette tip. Photography was taken immediately after as time 0 hour with an Axiovert 200 inverted microscope system (Zeiss; Göttingen, Germany) at ×40 magnification with a digital camera (QImaging Retiga EXi.; QImaging Designer, Surry, BC, Canada). Then cells were treated with SM, GG and NANA supplemented culture medium at a concentration of 40µg/ml for 24 hours. The same fields were photographed 24 hours post wound. The width of the wound was measured by using software, Northern Eclipse (Empix Imaging Inc., Mississauga, ON, Canada). The percentage of wound closure reflecting cell migration was calculated by subtracting the width of the scratch at 24 hours from the width of the scratch at 0 hour, divided by the width of scratch at time 0 hour multiplied by 100. The experiment was repeated five times for each nutrient treatment.

Cell barrier integrity

Cell monolayer integrity was assessed based on the measurement of transepithelial electrical resistance (TEER). Caco2-BBE cells were seeded on polycarbonate membranes with 0.4 μm pore size in 12 diameter transwell chamber (Corning Inc., Lowell, MA, USA). After 24 hours, cells were treated with SM, GG and NANA supplemented culture medium at the concentration of 0 (control), 40, 80 and 100 μg/ml. After cells were grown to confluence (14-21 days), TEER value was measured with an EVOMX hand-held electrometer by using REMS autosampler (World Precision Instruments Inc., Sarasota, FL, USA).

Intracellular oxidative stress

Intracellular oxidative stress was measured by dichlorofluorescein (DCF) assay. Co-cultured cells were seeded on 96-well black plates. After 24 hours, co-cultured cells were treated with SM, GG and NANA supplemented culture medium at the concentration of 0 (control), 40, 80, and 100 μg/ml. After cells were grown to confluence, they were challenged with 1 mM H_2O_2 for 30 min to generate ROS. Then 10μM CM-H_2DCFDA (Invitrogen, Eugene, OR, USA) was applied to each well to trap fluorescent DCF. Fluorescence intensity was measured by using Fluoroskan Ascent© Fluorometer

(Thermo Fisher Scientific Inc., Waltham, MA, USA) at a 485 nm excitation and a 527 nm emission filter.

Total antioxidant capacity

Total antioxidant capacity of egg yolk SM, bovine milk SM, GG and NANA was determine by measuring ORAC based on the method described by Glazer (1990). SM, GG and NANA were prepared at 100μg/ml. Fluorescein working concentration was 0.0816 μM and used as substrate. 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH) was prepared at 0.153 μM immediately before transferring to generate peroxyl radical. Rutin trihydrate working concentration was 20 μM and used as control. All above reagents and samples were prepared in 75 mM ORAC working solution (75 mM KH_2PO_4 and 75 mM K_2HPO_4). Briefly, around 200 μl of samples, ORAC working solution as blank control, rutin trihydrate and trolox standard were put in 96-well black flat bottom polystyrene microplate (Corning Inc., Lowell, MA USA) manually. Then, automatic transfer was done by using a Precision 2000 automated microplate pipetting system (Bio-Tek Instruments Inc., Winooski, VT, USA). Finally, fluorescence intensity was measured at 485/20 and 528/20 nm filter pair with FLx800 microplate fluorescence reader (Bio-Tek Instruments, Inc., Winooski, VT, USA) with KC4 3.0 software (version 29). The data was expressed as an ORAC value (μmol Trolox equivalents/g).

Statistics analysis

The effects of sphingolipids on intestinal function and antioxidant properties were analyzed by one-way analysis of variance using GLM procedure of SAS software (SAS, Version 9.1, Cary, NC). Multiple comparison was made by Duncan's multiple range test (Steel & Torrie, 1990). All results were expressed as mean ± SD, $p<0.05$ is considered significant.

Results

Concentration of SM and GG in human milk

Two colostrums and two mature milk samples were analyzed in triplet each and compared with an infant formula of chosen. The concentration of SM in infant formulas, colostrums and mature milks were 143.3, 305.9 and 250.2 μM, respectively (**Table III-1**). Human milk contained higher amount of SM than the infant formula chosen in this study. Colostrum has 1.2 times higher SM than mature milk. The concentration of total GG in colostrums and mature milks were similar, 11.5 μg/ml and 10.1 μg/ml, respectively which is 2.5 and 2.2 times higher, respectively, than the formula.

Effect of sphingolipids on cell proliferation

SM, GG and NANA significantly promoted cell proliferation of Caco2-BBE/HT29-MTX co-cultures, SM ($p<0.001$), GG ($p<0.0001$) and NANA ($p<0.0001$), respectively, (**Figure III-1**) in comparison to the control. Although there is a trend of increasing cell proliferation dose dependant of GG and NANA, no statistical differences were found in each nutrient concentration, except GG at 100μg/ml.

To determine if there is a variation from different sources of SM on cell proliferation, egg yolk SM, bovine milk SM and human milk SM were compared at the concentrations of 40 and 80μg/ml in Caco2-BBE/HT29-MTX co-cultures (**Figure III-2**).

Cell proliferation was significantly increased at both concentrations of SM, 40 μg/ml ($p<0.005$) and 80 μg/ml ($p<0.002$) regardless of its source.

Effect of sphingolipids on cell migration

The capacity of enterocytes migration is important for wound healing. To assess if SM, GG and NANA have beneficial effect on this, cell migration to close the wounded gap was monitored at 24 hours post injury in Caco2-BBE cells. SM, GG and NANA were significantly ($p<0.002$) effective on wound closure at 40 μg/ml compared to control (**Figure III-3B**). Although SM and GG showed higher cell migration than NANA, there was no statistical difference. Photographs of injury at time 0 and time 24 hours post injury are also shown in **Figure III-3A**.

Effect of sphingolipids on cell barrier function

The effect of SM, GG and NANA on enterocyte membrane integrity was measured on Caco2-BBE monolayers by using the TEER assay. SM ($p<0.0004$) and GG ($p<0.0001$) supplementation at 80 and 100 μg/ml significantly increased TEER values in comparison to control (**Figure III-4**). The increase of TEER value at 80μg/ml was 18.7% and 38.0% higher in SM and GG, respectively. NANA supplementation slightly increased TEER value with no statistical differences (**Figure III-4**).

Effects of sphingolipids on intracellular oxidative stress

To assess if SM, GG and NANA have antioxidant properties, intracellular ROS was measured after challenging the co-cultured cells with H_2O_2. The positive control cells, incubated with 1 mM H_2O_2, were 8% higher in ROS level than the negative control without H_2O_2. GG and NANA did not decrease intracellular ROS level at any concentrations (**Table III-2**)

To determine if different sources of SM on intracellular oxidative stress, egg yolk SM and bovine milk SM and human milk SM were compared at the concentrations of 40 and 80 µg/ml in Caco2-BBE/HT29-MTX co-cultures. In comparison to the positive control, egg yolk SM and bovine milk SM significantly ($p<0.0001$) decreased intracellular ROS level at both concentrations (**Table III-3**). No differences were found in human milk SM.

Antioxidant capacity of sphingolipids

Total antioxidant capacity of different sphingolipids was expressed as ORAC values. There were significant ($P<0.0001$) differences in ORAC values among different sources of sphingolipids. The ORAC value of egg yolk SM was the highest and was 1.1 times, 3.6 times and 6.4 times higher than bovine milk SM, GG, and NANA, respectively (**Table III-4**).

Table III-1. The concentration of sphingomyelin and total gangliosides in human milk and infant formula

	Sphingomyelin (μM)	Total gangliosides (μg/ml)
Infant formula	143.3	4.5
Colostrum	305.9	11.5
Mature milk	250.2	10.1

Values are mean (n=1-2) with triplet analysis for each nutrient.

Table III-2. Effect of gangliosides and n-acetylneuraminic acid on intracellular oxidative stress in Caco2-BBE/HT29-MTX co-cultures

Treatment	Emission intensity	
Medium - H_2O_2	6.4 ± 0.3	
Medium + H_2O_2	6.9 ± 0.5	
Concentration (μg/ml)	GG	NANA
40	6.7 ± 0.3	6.7± 0.2
80	6.5 ± 0.3	6.7± 0.3
100	6.8 ± 0.2	6.8 ± 0.3

Values are mean (n=8) ± SD. Intracellular production of ROS generated after challenging Caco2-BBE/HT29-MTX co-cultured cells with 1mM H_2O_2 were measured by DCF assay. Cells were incubated with GG and NANA at the concentration of 40, 80 and 100 μg/ml. Medium without H_2O_2 and with 1 mM H_2O_2 were used as negative and positive control, respectively. No significant effects of GG and NANA were identified at any given concentration.

Table III-3. Effect of different sources of sphingomyelin on intracellular oxidative stress in Caco2-BBE/HT29-MTX co-cultures

Treatment		Emission intensity	
Medium - H_2O_2		4.3 ± 0.1^b	
Medium + H_2O_2		4.9 ± 0.3^a	
Concentration (μg/ml)	Human milk SM	Egg yolk SM	Bovine milk SM
40	4.8 ± 0.3^a	4.5 ± 0.2^b	4.4 ± 0.3^b
80	5.0 ± 0.2^a	4.4 ± 0.2^c	3.8 ± 0.2^b

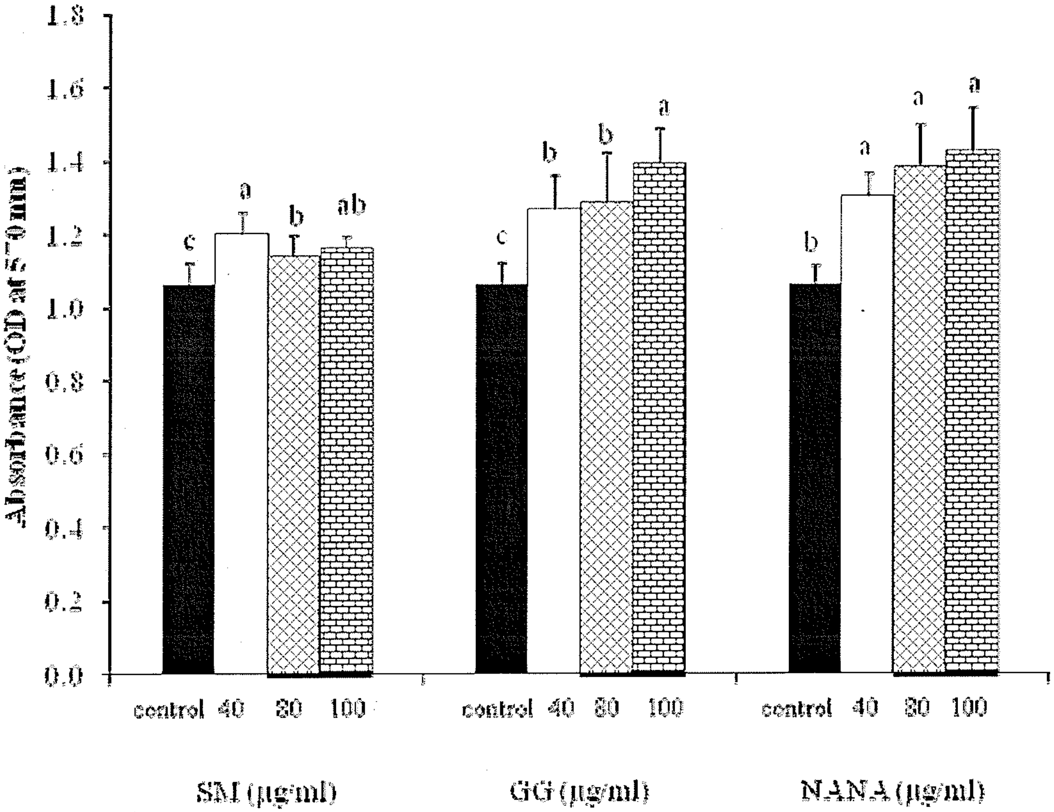
Values are mean (n=8) ± SD. Intracellular production of ROS generated after challenging Caco2-BBE/HT29-MTX co-cultured cells with 1mM H_2O_2 were measured by DCF assay. The cells were incubated with different sources of SM at the concentration of 40 and 80 μg/ml. Medium without H_2O_2 and with 1 mM H_2O_2 were used as negative and positive control, respectively. Significant differences were identified by one way analysis of variance: human milk SM, not significant; egg yolk SM, $p<0.001$; Bovine milk SM, $P < 0.0001$. Values having a different superscript letter within each nutrient and the controls are significantly different.

Table III-4. Antioxidant capacity of sphingolipids

Sphingolipids	ORAC (umol Trolox/g)
Egg yolk sphingomyelin	359.7 ± 19.6^{a}
Bovine milk sphingomyelin	324.5 ± 34.7^{b}
Gangliosides	100.0 ± 11.8^{c}
N-acetylneuraminic acid	56.5 ± 12.6^{d}

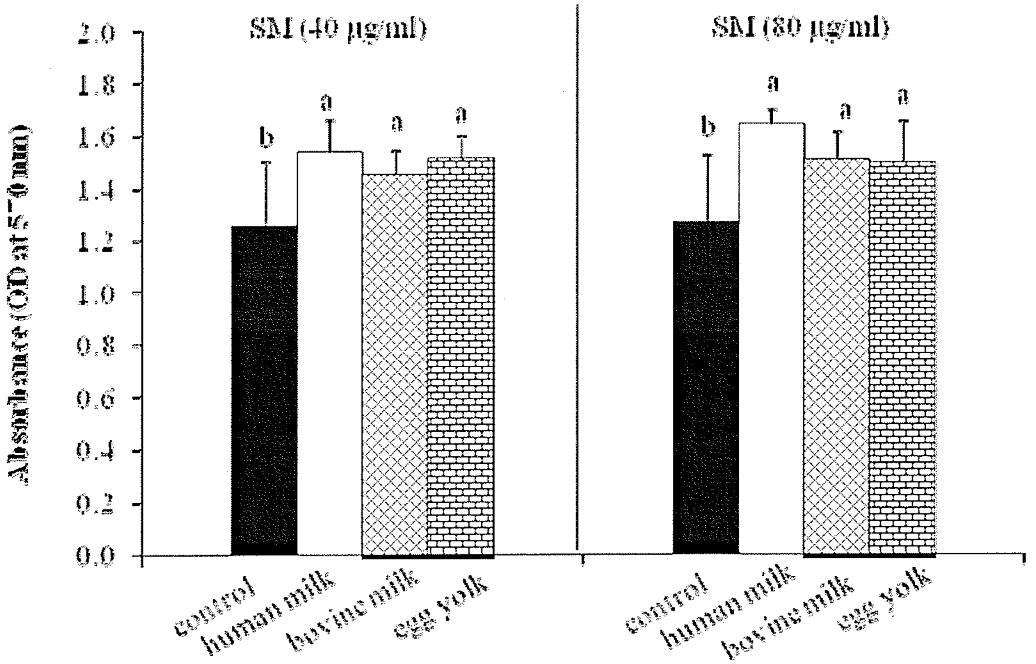
Values are means (n=9) ± SD. Significant differences were identified by one way analysis of variance. Values having a different superscript are significantly different at $p < 0.0001$.

Figure III-1. Effect of sphingolipids on cell proliferation of Caco2-BBE/HT29-MTX co-cultures



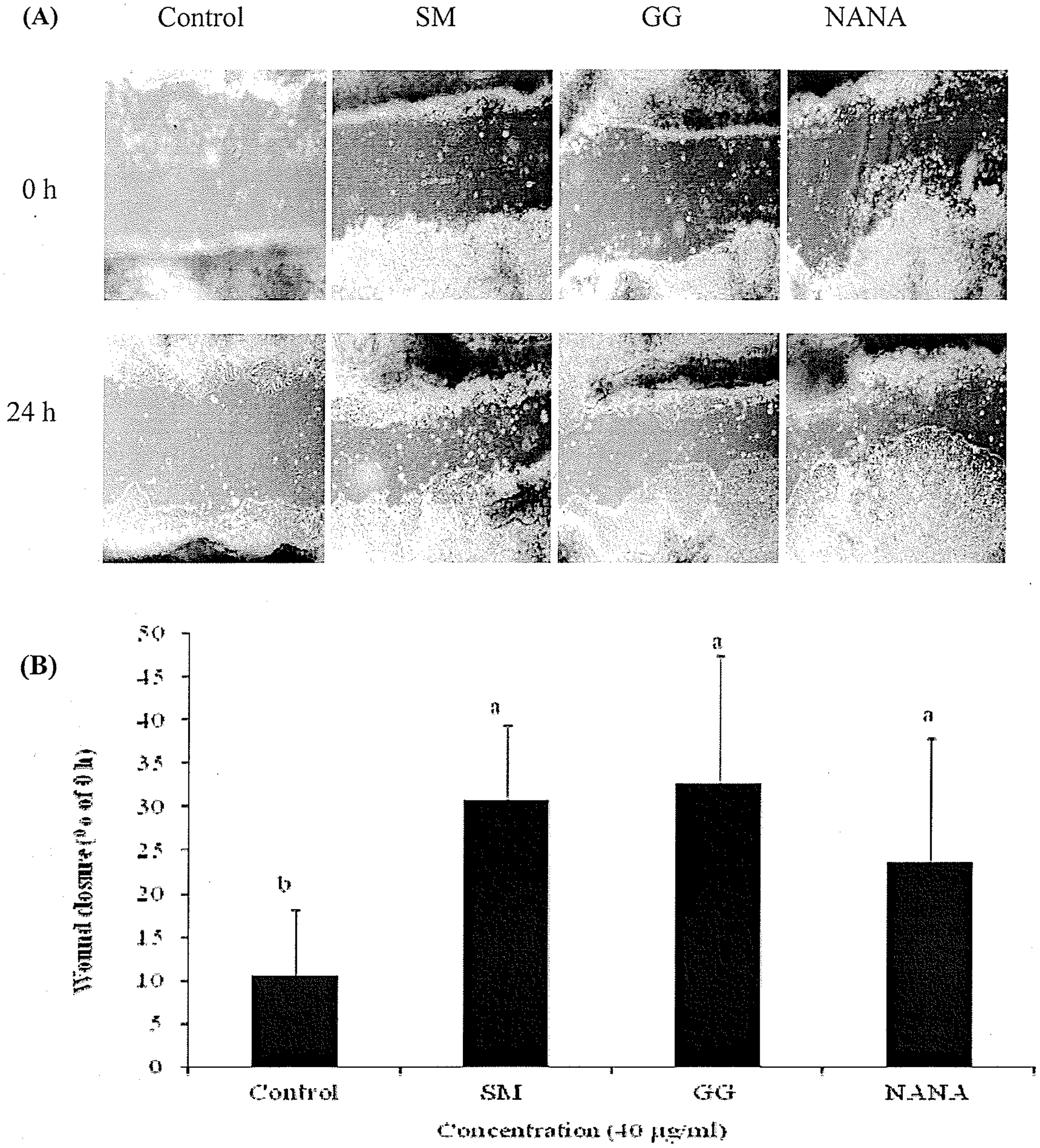
Co-cultured cells were treated with SM, GG and NANA at the concentration of 0 (control), 40, 80 and 100 µg/ml. Cell proliferation was determined by MTT assay. Values are mean (n=8) ± SD and indicate absorbance at 570 nm. Significant differences were identified by one way analysis of variance: SM, $p < 0.001$; GG, $p < 0.0001$; NANA, $p < 0.0001$. Values having a different superscript in each nutrient are significantly different from control.

Figure III-2. Effect of different sources of sphingomyelin on cell proliferation of Caco2-BBE/HT29-MTX co-cultures



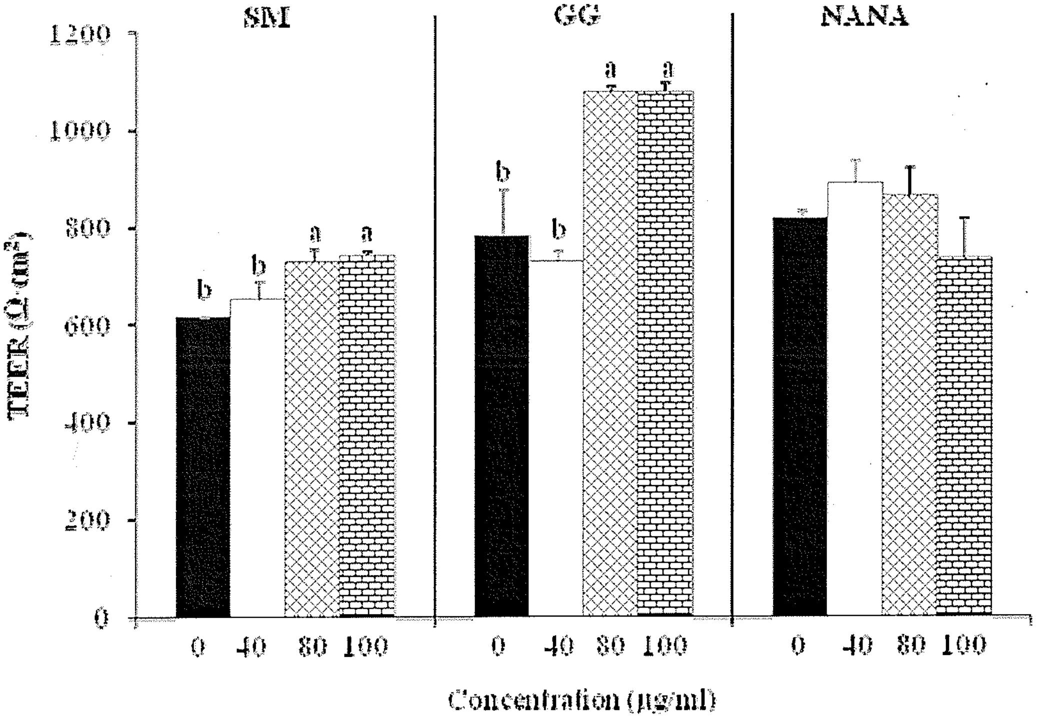
Co-cultured cells were treated with SM from human milk, bovine milk and egg yolk at a concentration of 0 (control), 40 and 80 µg/ml. Cell proliferation was determined by MTT assay. Values are mean (n=8) ± SD and indicate absorbance at 570 nm. Significant differences were identified by one way analysis of variance in each concentration: 40 µg/ml, $p < 0.005$; 80 µg/ml, $p < 0.002$.: Values having a different letter in each concentration are significantly different.

Figure III-3. Effect of sphingolipids on cell migration of Caco2-BBE cells



SM, GG and NANA were supplemented at 40µg/ml after cell injury. (A) Representative images of the *in vitro* wound assay at time 0 and 24 hours post-injury. (B) Values are mean (n=7-13) ± SD and present % of wound closure. Significant differences were identified by one way analysis of variance ($p<0.002$). Values having a different letter are significantly different.

Figure III-4. Effect of sphingolipids on cell barrier function of Caco2-BBE cell monolayers



Caco2-BBE cells were treated with SM, GG and NANA at the concentration of 0 (control), 40, 80 and 100 µg/ml. Cell barrier function was measured by TEER value. Values are mean (n=3) ± SD. Significant differences were identified by one way analysis of variance in each nutrient: SM, <0.0004; GG, p<0.0001; Values having a different letter in each nutrient are significantly different.

Discussion

SM, GG and NANA on cell functions

The present study examined the effects of SM, GG and NANA on intestinal cell functions and their antioxidant properties in Caco2-BBE and HT29-MTX co-cultures. SM and GG significantly enhanced cell proliferation, migration, and barrier function. NANA also promoted cell proliferation and migration.

Caco-2 is one of the best characterized and most extensively used intestinal cell lines during the last thirty years (Sambuy et al. 2005). Even though the parent cell line originated from a human colon adenocarcinoma, it presented many characteristics of human small intestine such as forming polarized monolayer, developing apical microvillus with absorptive enterocytes, expression of typical metabolic enzymes and transporters as well as dense paracellular and transcellular barrier. However, for the last one, the tight level may be more resembles colon than small intestine, resulting in poor permeability. The total GG content and individual GG composition of differentiated Caco-2 cells was proved to be similar to those of neonatal rat intestine, which makes them a best alternative in vitro model for studying physiological process in the small intestine and colon (Schnabl et al., 2009). Furthermore, several subclonal cell lines were established to improve the homogeneity and the stability of the parent cell. Caco2-BBE used in current study is one subclonal cell line with a more homogeneous expression of brush border than Caco-2.

HT-29 is another commonly used human colon cancer cell line (Sambuy et al. 2005). Unlike Caco-2, which is the only one known to differentiate spontaneously without synthetic or biological inducer in a long term (Schnabl et al., 2009), a goblet cell clone HT29-MTX was established when giving HT-29 methotrexate (MTX) which interferes with a metabolic pathway (Pinto et al., 1983; Lesuffleur et al., 1991). At 20 days of culture, scanning electron microscopy detected cell surface with goblet cells and dense mucus gel at HT29-MTX, however, the mucus gel was labile and brush border was observed after being extensively washed (Lesuffleur et al., 1990).

However, the limitation of using a sole cell line is obvious because human intestinal epithelium is actually composed of more than eight cell types, for instance, enterocyte, goblet cell, Paneth cell, lymphocyte, endocrine cell and undifferentiated cell (Kong et al., 1998; Schenk et al., 2008). A mixed-cell co-culture system of mucin-produing cell HT29-MTX and absorptive enterocyte Caco-2 was first used to test the permeability of several drugs in intestine (Walter et al., 1996).

In our study, we developed the co-culture model combining Caco2-BBE and HT29-MTX at the start-seeding ratio of 7:3 based on our previous experiments and other researches (Yao et al., 2009; Walter et al., 1996; Hilgendorf et al., 2000; Lesuffleur et al., 1991), which is to better mimic the physiological condition of human small intestine. The morphology and mucin production of our co-culture model were confirmed in our previous experiment.

The doses of SM, GG and NANA used in our study was based on animal feeding studies (Motouri et al., 2003; Schmelz et al., 1996; Barrenetxe et al., 2006; Zhang et al., 2008; Suh et al., 2004; Park et al., 2005; Drozdowski et al., 2007; Birecki et al., 2006; Vázquez et al., 1999; 2001) as well as in vitro studies (Brønnum et al., 2005; Ogushi et al., 2004). It was also estimated the level of daily intake in breast fed suckling babies as ranging from 80 -200 mg SM plus some glycoshingolipids and approximately

280 mg sialic acid per day (Vesper et al., 1999; Nilsson et al., 2003; Nakano et al., 2001; Wang et al., 2007). Generally, human milk contains around 10 μg/ml LBSA representing total GG content and around 100 μM SM. In addition, human milk contains approximately 0.7 mg/ml of sialic acid, which most of them are bound to oligosaccharides accounting for about 75% of the total sialic acid contained in human milk.

Among three nutrients, SM was the only one showed antioxidant properties. Most of these effects were achieved at a concentration of 40μg/ml except cell barrier function at 80μg/ml. Based on the discussion above, the effective doses we found in current study meet the physiological level of estimated daily intake level of suckling babies.

The mechanism of this positive effect of these molecules requires further study. It is possible that SM and GG might modulate the sphingolipids metabolites, for example, changing the balance of S1P and Cer, which has counter effect in cell proliferation. Several studies showed that both SM and GG are involved in cell proliferation (Gómez-Muñoz, 2004; Gómez-Muñoz et al., 2004; Kent et al., 2008; Maupas-Schwalm et al., 2004; Saqr et al., 1995; Fujimoto et al., 2005). Our data demonstrated that exogenous SM, GG and NANA stimulate co-cultured intestinal cells to proliferate and grow, which also implicates the beneficial effects in intestinal maturation in other studies.

Unlike the cells of most other adult tissues, where motility is seen only in response to injury or as a result of carcinogensis, the intestinal epithelium is in a dynamic equilibrium of cell production and cell death, thus constantly on the move. Therefore, enterocyte migration is important pathological function in intestine. Our data from in vitro wound scratch assay for the first time showed that closure of cell wound reflecting cell migration was mediated by SM, GG and NANA in injured Caco2-BBE cells. We also observed that cell migration of wounded skin fibroblast cells were significantly enhanced by SM treatment (data not shown). Since S1P has been shown to affect cell migration in various cell lines (Spiegel et al., 2003; Miller et al., 2008; Roztocil et al., 2009), it is possible that SM and GG might have influence on the production of this cell signaling molecule.

Intact intestinal mucosa functions as a defense barrier against infections, allergy and harmful dietary antigens. Disruption of intestinal epithelial barrier due to local and systemic insult is thought to be one risk factor for NEC, which is the most common life-threatening gastrointestinal disease in preterm infants. Some study showed that long chain polyunsaturated fatty acids support epithelial barrier integrity and reduce IL-4 mediated permeability *in vitro* (Willemsen et al., 2008). Conversely, rapid accumulation of ceramide in the membrane fractions containing occludin and claudin-4, representing tight junctions, which is hydrolyzed from sphingomyelin by exogenous SMase, impaired epithelial barrier function probably because of the changes in lipid composition of tight junctions (Bock et al., 2007). TEER value obtained in our study indicated that SM and GG significant improve barrier function in Caco2-BBE monolayers, which extend our understanding of the protection of sphingolipids on intestine.

Antioxidant properties of SM, GG and NANA

Human milk is able to significantly decrease oxidative stress in breast fed infants in comparison to formula fed infants (Friel et al., 2002; Koletzko et al., 2003; Ledo et al., 2009). Various components has been isolated and distinguished to contribute to the antioxidant property of human milk including enzymes (SOD, CAT, GPx), vitamins (Vitamin C, E, β-carotene and coenzyme Q), constituents of antioxidative enzymes (zinc, copper, selenium), isoflavones and some binding protein like lactoferrin. In addition to these nutrients, the present study showed that SM from bovine milk and egg yolk, but not GG and NANA, significantly reduced oxidative stress in the co-culture cells. This finding coincided with higher antioxidant capacity of SM from these sources measured by ORAC in present study. Only a few studies have reported the ORAC value of whole human milk (Alberti-Fidanza et al., 2002; Li et al., 2009). However, human milk SM did not show antioxidant properties as expected. Unfortunately, the ORAC value of human milk SM was not measured to confirm due to the lack of samples. Furthermore, to better investigate the antioxidant capacity of sphingolipids, we measured ORAC value of different sphingolipids.

Recently, it has been reported that oxidative stress (endogenous prooxidants like ROS and RNS) are involved in sphingolipids metabolism via regulation of several key enzymes such as SMase and ceramidase. It is also believed that sphingolipids and various metabolites are able to regulate cellular redox homeostasis through regulation of NADPH oxidase, mitochondrial integrity, nitric oxide synthase, and antioxidant enzymes. In another word, sphingolipid metabolism and redox homeostasis are regulated in a bidirectional manner and these two pathways are interacted to each other closely (Won et al., 2006). There are several studies demonstrated that the antioxidant activity of individual ganglioside and mixture of gangliosides *in vitro* and *ex vivo* (Fighera et al., 2004; Maulik et al., 1993; Gavella et al., 2007). In addition, NANA (Neu5Ac) was demonstrated to have hydrogen peroxide (H_2O_2)-scavenging activity and reduce–OOH by oxidizing lipids in the same way as it oxidizes H_2O_2 (Iijima et al., 2004, 2007, 2009). It is of interest to test if SM can stimulate or enhance the effect of antioxidative enzymes in human milk or the fatty acid of SM is prone to lipid peroxidation due to the double bonds in the molecule. It is still unclear why GG and NANA are not able to decrease intracellular ROS in our co-cultured cells. Because most of studies showing antioxidant capacity of GG were conducted on brain cells, difference in tissue distribution of GG in intestine maybe part of the reasons.

In conclusion, the results this study indicate that sphingolipids (SM and GG) and NANA play an important role in intestinal epithelial wound healing, perhaps by modulating cell growth, cell proliferation and cell migration. SM may have antioxidant properties, thereby decreasing intracellular oxidative stress. Considering high incidence of intestinal diseases in infants, sphingolipids may protect against intestinal infection and/or improve recovery of damaged enterocytes. Further research is needed to determine the mechanisms of action of sphingolipids and NANA in gut health.

Our present study needs to continue to examine the metabolism of these sphingolipids in co-cultured cells. Especially, whether exogenous sphingolipids alters the production of cell signaling metabolites, are able to incorporate into the micro domains of cell membrane, and change the lipid profile, thereby providing beneficial effects on cell functions. Also animal feeding study is needed to find consistent results as in vitro.

Although it is still too early to say, the present study provide the basis of potential use of SM, GG and NANA in improving infant health, especially intestinal function and antioxidant defense system in preterm infants. With more convincing data, our finding may lead to recommend pregnant and lactating women to consume diets rich in SM, GG and NANA during pregnancy and lactation to increase the level of SM, GG and NANA in breast milk. For example, eggs contain large amounts of SM (estimated at 2.2mmol/kg or 0.17% of wet weight), so do dairy products. On the other hand, researchers should continue to characterize the bioactive and specific components like SM, GG and NANA in human milk to make nutritional modification of infant formulas, as a result to develop the infant formula as close as to human milk. Since most of infant formulas are manufactured based on cow's milk, how to increase sphingolipids content in cow's milk is also an important challenge in dairy industry. However, attempting to increase SM content of bovine milk by feeding SM enriched food failed (Graves et al., 2007). Therefore, it is maybe the only way to artificially supplement infant formula with SM to provide as sufficient as human milk SM to infants.

Study limitations and future research

Several limitations exist in current study. Firstly, with regard to the measurement of concentration of sphingolipids in human milk, we only collected two colostrums and two mature milks. In case of this, we did not have enough samples to do statistical analysis to confirm whether there is significant difference in SM and GG content between human milk and infant formula. Even though it is of importance to clarify the physiological level of sphingolipids for further understanding their biological function, controversial or at least inconsistent data are still emerging in the literature. Secondly, due to the very small amount of GG in human milk, we could not extract and accumulate enough human milk GG to conduct experiments as expected, therefore lacking an important part of effects of different sources of GG on intestinal functions, even though we assumed the variation due to sources might be minor. Thirdly, we did not analyze fatty acid composition of human milk SM and GG. Forth, little is known about the uptake and metabolism of sphingolipids in cultured cells which is the critical base for underlying mechanism. Lastly, when we supplemented fetal bovine serum (FBS) to cell culture medium, cholesterol, bile acid and some free fatty acid presented in FBS, even in a very small amount, might aggregate with SM and GG to form micelles. This may influence the uptake of sphingolipids by cultured cells, interfere with our results. To choose serum free medium should be better for our current study.

For future study, we should fulfill the above issues, for instance, to explore the possible mechanism of effects of sphingolipids on intestinal functions by investigating the metabolism of exogenously addition of sphingolipids in co-cultured cells. Moreover, weaning animal feeding with human milk shingolipids supplemented diet or human milk directly will be vital to deeply understand the roles of sphingolipids in infants' intestinal function.

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APPENDIX

OTHER EXPERIMENTAL METHODS AND RESULTS

Lipid analysis of human milk

Extraction and separation of total lipid from human milk

The purpose of this experiment is to extract sphingolipids from human milk for further study. Human milk samples stored at -80˚C were thawed at room temperature. 4 ml human milk and 0.5 g formula powder was homogenized in 4 ml 0.025% $CaCl_2$ for one minute. 20 ml of chloroform: methanol (2:1, vol/vol) were added to extract total lipids. Upper phase containing GG were transferred to 8 ml tube for ganglioside purification. The lower phase containing phospholipids was transferred to a pre-weighed 25 ml tube and dried under nitrogen gas. The samples were re-dissolved in chloroform: methanol (2:1, vol/vol) at a final concentration of 1 mg/20 μl.

Phospholipids separation

To collect SM from total lipid extract, individual phospholipid were separted on silica gel H coted thin layer chromatography by using a solvent system, chloroform 45 ml, methanol 13.5 ml, 2-propanol 37.5 ml, triethlamine 27 ml, 0.25% KCl 9 ml. 0.25% KCl. In present study, 90 μl samples were applied on the plate along with a phospholipid standard. The SM band was visualized with 0.1% aniline naphthalene sulfonic acid (ANSA) under UV light. Typical phospholipid separation is shown in **Figure A-2.**

Sphingolimyelin elution from silica gel

0.5 g silica gel powder scraped off from TLC plate was eluted with 10 ml chloroform: methanol (2:1, vol/vol) and 2 ml distilled water. This mixure was sonicated in the water bath for 10 minutes without any shaking and vortexing, and centrifuged at 3000 rpm for 10 minutes. The upper phase was discarded. The organic lower phase was collected. The above step was repeated once more and then all collected organic phase was combined, dried down under nitrogen gas, and re-dissolved in 5 ml methanol. After one more centrifugation at 3000 rpm, the supernatant was collected and dried down again, and then finally dissolved in 300 μl chloroform: methanol (2:1, vol/vol). This SM sample was stored at fridge until use.

Microscale phosphorus analysis

In order to quantitate the concentration of SM in human milk, phosphorus assay was performed according to the method of Itoh et al. (1985). Briefly, standard solution was prepared as below: 1 ml 0.15 M KH_2PO_4 and 99 ml 0.25 M sucrose. The reducing reagent consisted of 2.5 g sodium bisulfite, 0.5 g sodium sulfite and 0.042 g ANSA in in 250 ml water, which was filtered into a brown bottle. The composition of color producing reagent was 3 ml 5% ammonium molibdate, 30 ml water and 3 ml reducing reagent. These reducing reagent and ammonium molibdate were stored in the fridge and mixed together just before use. 0, 5, 10, 20, 40, 80, 100, 160 μl standard solution was used to make standard curve. Scraped silica gel containing SM fraction was digested with 0.2 ml $HClO_4$ at 180°C in the oven over night. After cooling, 0.8 ml color producing reagent was added, vortexed thoroughly, and then heated for 10 minutes in boiling water bath. The developed pigment was extracted with 1 ml n-butyl acetate: n-butanol (85:15, vol/vol). The organic phase was clearly separated from the aqueous phase and silica gel powder, and the absorbance was measured at 790 nm by using Ultra Spec 4000.

Ganglioside purification and separation

The purpose of this experiment is to quantitate total milk GG. Two C18 cartridges (Sep-Pak, Waters Corp., Milford, MA) were used for sample purification as follows: Firstly, the cartridges were washed by 10ml methanol, 10ml chloroform: methanol (2:1, vol/vol) and 10ml methanol. Then total milk GG were loaded and purified by passing the upper phase of lipid extract through prewashed C18 cartridges. After washingwith 20 ml of distilled water to remove salts and water-soluble contaminants, then GG were eluted with 10 ml methanol and then 20 ml chloroform-methanol (2:1, vol/vol), dried down under nitrogen gas without heat, and then re-dissolved with 300 µl of chloroform-methanol (2:1, vol/vol).

To identify individual GG pattern in human milk, 40 µl of purified GG were seperated on HPTLC plate (Whatman Inc., Clifton, NJ) in a developing system of 27.5ml chloroform, 22.5 ml methanol and 5 ml 0.2% $CaCl_2$. 10 µl GG mixture (mouse brain; Alexis, San Diego, CA) and GM3 (bovine brain; Alexis, San Diego, CA) were used as standard. After developing around 40-50 minutes, individual GG band was visualized after spraying with 3% resorcinol-HCl followed by charring the plate at 170 °C for approximate 10 minutes until the color developed. Typical GG separtaitons on HPTLC plate is in **Fugure A-3.**

Total GG analysis

Measurement of total GG content was performed and expressed as GG-bound sialic acidas described by Suzuki (1964). Commercial NANA, 250μg/ml (Sigma-Aldrich, St. Louis, MO) was used as a standard. 0, 10, 20, 40, 80, 120 μl of NANA was added in 8 ml screw top test tubes, then 500, 490, 480, 460, 420 and 380 μl of distilled water was added, mixed well. 30ul of purified GG was dried under nitrogen gas and dissolved with 0.5 ml distilled water. 0.5ml 3% resorcinol-HCl was added to each sample and standard and then incubated in the oven 150-160°C around 10-15 minutes develop color. The purple-blue color developed was extracted with 275 μl butyl acetate : butanol (85:15, vol/vol). The top phase (blue phase) was transferred to BrandTech UV micro cuvettes and the absorbance was measured at 580nm by using Ultra Spec 4000 within one hour.

Cell culture conditions

Seeding co-culture

Caco2-BBE cells (ATCC) and HT29-MTX cells (donated by Dr. Thomas Kissel, University of Marburg and Per Artursson, University of Lund) were maintained in an incubator at 37°C, an atmosphere of 5% CO_2 and 95% air and 80% humidity. 500 ml Dulbecco's Modified Eagle's Medium (DMEM, Gibco) was supplemented with 5ml 100 U/ml penicillin/ 100 μg/ml streptomycin solution, 10 ml 2 mM L-glutamine solution, 5 ml 1 mM sodium pyruvate solution, 5 ml 10 μg/ml human transferring solution, and 60 ml 10% fetal bovine serum. Fresh supplemented culture medium is changed every 2 days until cells grow to confluence. One flask of Caco2-BBE cells and one flask of HT29-MTX cells were transferred to a sterile hood. Old medium was removed and 1 ml EDTA was added to each flask. The flask was shook gently and then EDTA was removed. Cells were incubated with 1 ml of EDTA and 1 ml trypsin until most of cells fall off from the bottom. 8 ml medium was added to wash all the cells off from the bottom and resuspend cells. The cell suspension was spun down for 3 minutes. The old medium was aspirated and 10 ml fresh medium was added to resuspend cells again. 10 μl homogenized cells were counted by using hemocytometer in a diagonal frames with sixteen grids and averaged. Based on the total cell numbers, two cells were mixed at a ratio of 7:3 and reach the cell density at 2×10^6 cells/ml. Then the cell mixture was seeded in 96-well plate or other plate for further experiment.

Cell viability assay (Live dead assay)

For the live/dead assay, the co-culture was grown in 96-well black well plates. 24 hours after seeding, the co-culture cellswere treated with SM, GG and NANA containing culture medium (containing 0.5% ethanol) at the concentration of 0 (control), 40, 80, and 100 μg/ml and three columns were treated with normal culture medium containing 0.5% ethanol) as controls. To measure cell viability calcein AM (Invitrogen, Eugene, OR) and ethidium bromide (EB) (Fisher Scientific, Ottawa, ON) was used to measure live and dead cells. Because cell membrane permeant calcein AM can be hydrolyzed into highly negatively charged green fluorescent calcein by endogenous esterase (Neri et al., 2001)), the viable cells were labeled with evenly distributed intracellular green fluorescence, which can be visualized at 485/527 nm filter pair. In contrast, EB is only able to enter the cells with damaged membrane and undergo 20 times enhancement of a red fluorescence once binding to nucleic acid (Gray et al., 1987).

Therefore, the dead cells were labeled with red fluorescence detected at 527 /590 nm filter pair. Before assay, 5 μl DMSO was added to 50 μg calcein AM to be 10 mM and , another 2.5 μl DMSO was added to 2.5 μl 20 mM ethidium bromide to dilute the concentration to 10 mM EB. Then 5 ml culture medium was added 5 μl 10 mM calcein AM, another 5 ml culture medium was added to 5 μl 10 mM EB, mixed together to get the mixture of calcein AM and EB. Old medium was aspirated from one control column, 100 μl formaldyhyde was added as positive control, covered with parafilm, incubated for 30 minutes. Then medium in each well was aspirated. PBS was used three times to wash cells. 100μl prepared dye mixture was applied to each well except one control column. 100μl normal culture medium was added to this column as a blank control. The dye was aspirated after incubation for 30 minutes followed by washing with PBS three times. The fluorescence intensity was measured by using Fluoroskan Ascent© Fluorometer (Thermo Fisher Scientific Inc.) at 485/527 nm and 527 /590 nm filter pair were chose. The number of live cells is proportional to emission detected at 527 nm and the number of dead cells is proportional to emission detected at 590 nm.

MTT [(3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide)]assay

For the MTT assay, the co-culture was grown in 96-well black well plates. 24 hours after seeding co-cultured cells, cells were treated with SM, GG and NANA supplemented media at 0 (control), 40, 80, and 100 μg/ml. Only one column was treated with normal culture medium containing 0.5% ethanol as control and another column was also only added 100 μl culture medium containing 0.5% ethanol (without cells) as blank control. Co-cultured cells were maintained in the incubator as described before completely confluent for assay. To assess cell proliferation, MTT assay kit (ATCC) was used according to a protocol provided by manufacture. Firstly, 10 µl MTT reagent was added to each well followed by 4 hours incubation at 37°C. Once the purple precipitate was clearly visible under the microscope, 100 µl detergent reagent was added to each well followed by another 4 hours incubation in the dark at room temperature. The optical density (OD) was measured at 570 nm by using SpectroMax Microtiter Plate Reader (Molecular Devices Co., Sunnyvale, CA, USA). Because the yellow tetrazolium MTT is only reduced by metabolically active cells, in part by the action of dehydrogenase enzymes, to generate reducing equivalents such as NADH and NADPH, the resulting intracellular purple formazansolubilized was quantified by spectrophotometric, which can be used as the indicator of cell proliferation (Mosmann et al., 1983).

Cell migration (in vitro wound assay)

To investigate the effects of SM, GG and NANA on cell migration and closure of a defined area of injury on a confluent monolayer, *in vitro* wound assay was conducted on Caco2-BBE cells. For this assay, Caco2-BBE cells were grown on tissue culture coated cover slip to complete confluence. Linear wounds were made with a sterile plastic pipette tip and then photographed immediately indicating time 0 hour by using an Axiovert 200 inverted microscope system (Zeiss, Göttingen, Germany) at a ×40 magnification with a digital camera (QImaging Retiga EXi.; QImaging Designer, Surry, BC, Canada). Cells were treated with SM, GG and NANA supplemented culture medium (containing 0.5% ethanol) at 40µg/ml for 24 hours. The same fields were photographed again at time 24 hours. The width of the wound was measured by using software , Northern Eclipse (Empix Imaging Inc., Mississauga, ON, Canada). The mean percentage of wound closure after 24 hours was calculated by subtracting the width of the scratch at time 24 hours from the width of the scratch at time 0 hour, divided by the width of scratch at time 0 hour multiplied by 100. The experiment was repeated five times for each Sphingolipids treatment.

Transepithelial Electrical Resistance (TEER) assay

The purpose of this experiment was to examine monolayer barrier integrity after treatment by measuring TEER value. For this assay, CaCo2-BBE cells were seeded on polycarbonate membranes with 0.4 µm pore size in 12 diameter transwell chamber (Corning Incorporated, Lowell, MA, USA). After 24 hours after seeding, cells were treated with SM, GG and NANA supplemented culture medium (containing 0.5% ethanol) at 0 (control), 40, 80 and 100 µg/ml. Then cells were grown to confluence (14-21 days), fresh supplemented culture medium were changed every two days. Before the assay, the plate was taken out and put in room temperature for at least 30 minutes. Because electricity conductivity is affected by the temperature of fluid, TEER value was highly sensitive to environmental factors. In view of this, it is important to maintain the same condition such as room temperature and humidity at each measurement. The plate had to be placed at the giving direction provided by the manufactory. Then TEER value were measured with an EVOMX hand-held electrometer by using REMS autosampler (World Precision Instruments Inc., Sarasota, FL)

DCF assay

The purpose of this experiment was to monitor intracellular oxidative stress status challenged by hydrogen peroxide after treatment by measuring the production of intracellular ROS. A molecular probe, 5-(and-6)-chloromethyl-2, 7-dichlorodihydrofluorescein diacetate acetyl ester (CM- H_2DCFDA) was employed in this assay. For this assay, the co-culture was grown in 96-well black well plates. After 24 hours of seeding, co-cultured cells were treated with SM, GG and NANA supplemented culture medium (containing 0.5% ethanol) at 0 (control) 40, 80, 100 μg/ml. In each plate, four columns were treated with normal culture medium (containing 0.5% ethanol) as control groups for further assay. Then cells were grown until confluence (7-10 days) and fresh supplemented culture medium were changed every two days. Before assay, 10 μl 10 mM H_2O_2 was added to each well except for two control columns, followed by 30 minutes incubation at 37°C, resulting in the generation of oxidative stress. Old medium was aspirated and PBS was used to wash cells for three times. 100 μl of 10 μM CM-H_2DCFDA (Invitrogen, Eugene, OR) was applied to each well except for another two control columns. Therefore, except for all the treatment groups with H_2O_2, four control columns were: with H_2O_2 and CM-H_2DCFDA, without H_2O_2 or CM-H_2DCFDA, with H_2O_2 but without CM-H_2DCFDA, without H_2O_2 but with CM-H_2DCFDA. Fluorescence intensity was measured by using Fluoroskan Ascent© Fluorometer (Thermo Fisher Scientific Inc.) at a 485 nm excitation and a 527 nm emission filter. Because the determination of intracellular oxidative status was based on the oxidation of CM-H_2DCFDA to yield an intracellular-trapped fluorescent compound DCF in the presence of ROS, the emission of DCF can be measured to indicate the oxidative activity (Shanker et al., 2004).

ORAC assay

The oxygen radical absorbance capacity (ORAC) assay is a preferred and common used method to determine the antioxidant property in foods (Huang et al., 2002). We measured ORAC value of egg yolk SM (Avanti Polar Lipids, Inc), bovine milk SM (Avanti Polar Lipids, Inc), GG (bovine brain mixture; Calbiochem) and NANA (from sheep submaxillary gland; Sigma). The following equipments were used in ORAC assay: an FLx800 microplate fluorescence reader (Bio-Tek Instruments, Inc., Winooski, VT, USA) with KC4 3.0 software (version 29), a Precision 2000 automated microplate pipetting system (Bio-Tek Instruments, Inc., Winooski, VT, USA). Trolox ranging from 6.25 to 50 μM were used for the standard curve. Fluorescein working concentration was 0.0816 μM and used as substrate. 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH) was prepared at 0.153 μM immediately before transferring to generate peroxyl radical. Rutin trihydrate working concentration was 20 μM and used as control. SM, GG and NANA were prepared at 100μg/ml and measured nine times at one experiment. All above reagents and samples were prepared in 75 mM ORAC working solution (75 mM KH_2PO_4 and 75 mM K_2HPO_4). Briefly, around 200 μl of samples, ORAC working solution as blank control, rutin trihydrate and trolox standard were put in 96-well black flat bottom polystyrene microplate (Corning Incorporated, Corning, NY) manually. Then automatic transfer was done by the program set up in Precision Power V2. Finally fluorescence intensity was measured at 485/20 and 528/20 nm filter pair. ORAC value was expressed as μmol Trolox equivalents per gram.

Table A-1. Fatty acid composition of infant formula and human milk phospholipids

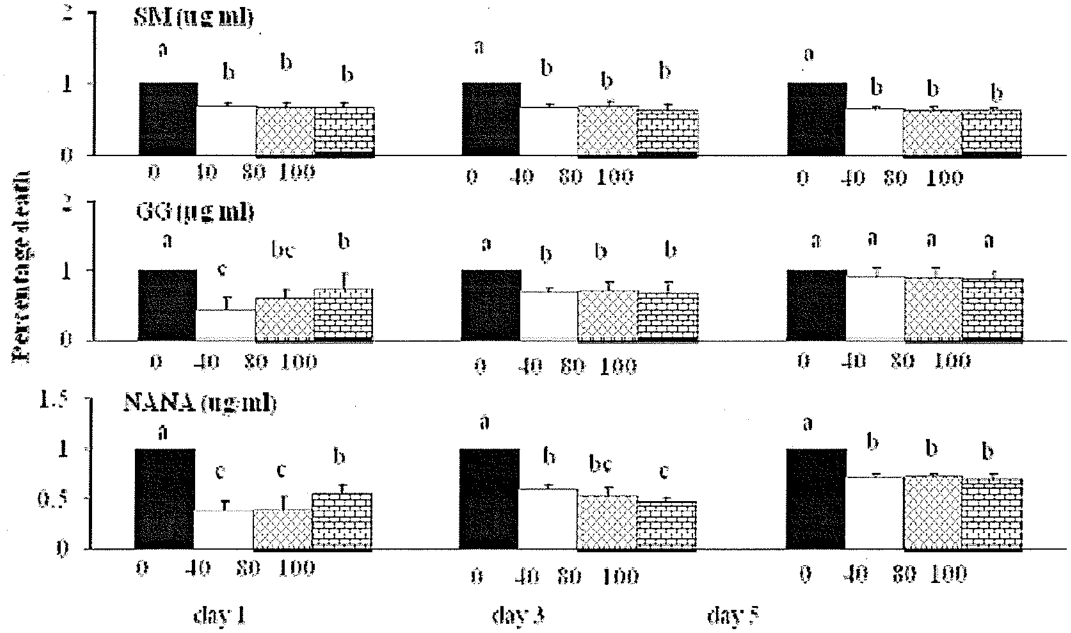
Fatty Acid	Formula (n=5)	Human milk (n=3)
10:0	0.80 ± 0.43	0.98 ± 0.26
12:0	9.23 ±1.10	4.67 ± 2.05
14:0	4.49 ± 0.25	5.82 ± 1.94
14:1	0.01 ± 0.01	0.24 ± 0.02
15:0	0.04 ± 0.03	0.35 ± 0.04
16:0	22.80 ± 1.80	21.94 ± 1.15
16:1	0.16 ± 0.03	2.39 ± 0.12
17:0	0.09 ± 0.03	0.42± 0.09
17:1	0.03 ± 0.03	0.33± 0.03
18:0	4.09 ± 0.25	7.72 ± 1.85
18:1	33.40 ± 2.84	35.32 ± 2.81
18:2n6	20.05 ± 1.04	12.03± 1.27
18:3n6	0.03 ± 0.03	0.12± 0.04
18:3n3	0.02 ± 0.06	0.04 ± 0.02
20:0	0.32 ± 0.03	0.21 ± 0.01
20:1	0.16 ± 0.01	0.50 ± 0.04
20:2n6	0.04 ± 0.06	0.26 ± 0.03
20:3n6	0.07 ± 0.07	0.30 ± 0.14
20:4n6	0.63 ± 0.50	0.46 ± 0.17
20:3n3	0.02 ± 0.02	0.09 ± 0.03
20:5n3	nd[2]	0.09 ± 0.04
22:0	1.72 ± 2.43	0.38± 0.17
22:1	0.01 ± 0.01	0.11± 0.03
22:4n6	0.49 ± 0.98	0.13 ± 0.07
22:5n3	nd	0.15± 0.06
22:6n3	0.22 ± 0.16	0.26± 0.11
24:0	0.10 ± 0.05	0.08 ± 0.03
24:1	0.02± 0.03	0.05 ± 0.04
Σ SAT	43.67 ± 1.87	42.58 ± 3.33
MUFA*	33.79 ± 2.87	38.92 ± 2.74
Σ n-3*	0.26 ± 0.14	0.64 ± 0.20
Σ n-6*	21.31 ± 1.35	13.31 ±1.68

1. values are mean ± SD
2. nd: not detected
3. * represents fatty acid composition of human milk is significantly different from infant formula at the level of $p<0.05$
4. Σ SAT, total saturated fatty acids; Σ n-3, total omega 3 fatty acids; Σ n-6, total omega 6 fatty acids

Table A-2. Infant formulas used in fatty acid analysis

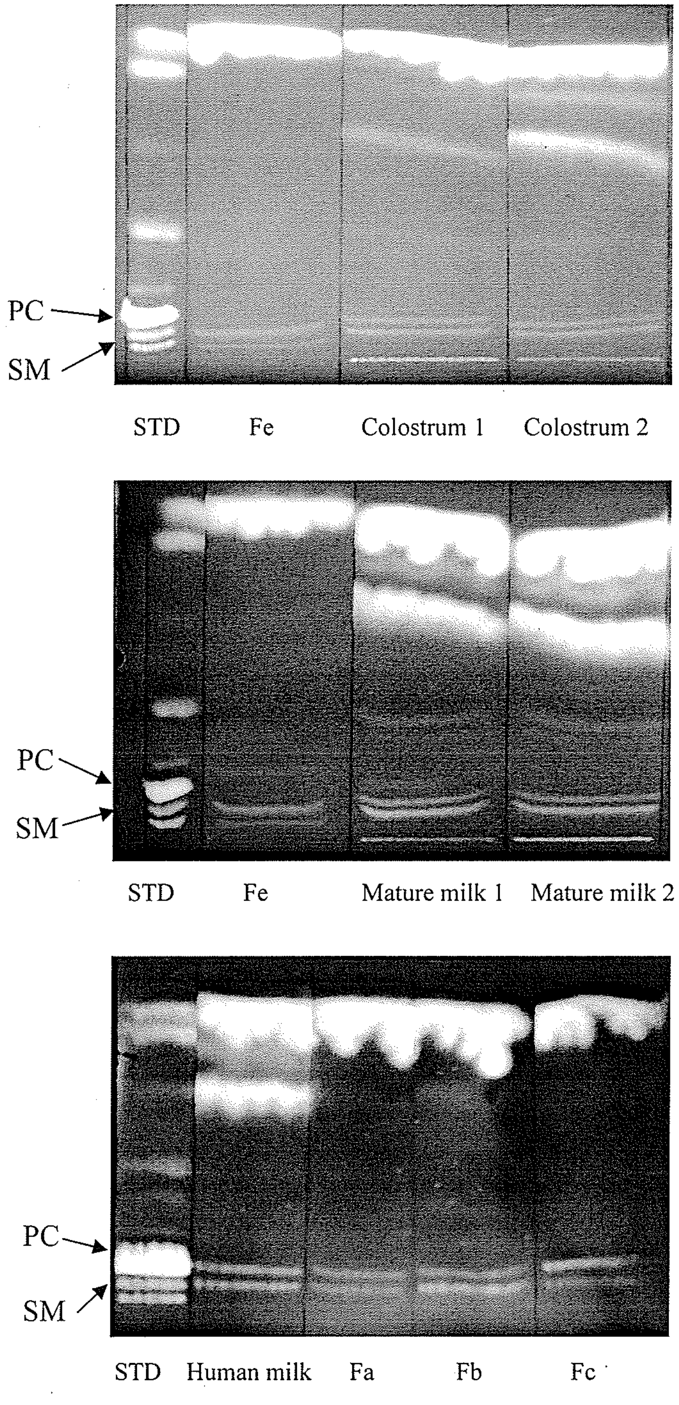
	Brand	Age	Description	Weight
Fa	Enfamil	N/A	Thickened and enriched with DHA & ARA	730g
Fb	Nestle	0-12+ month	Good start, easier to digest, iron fortified infant formula enriched DHA (an omega 3 fatty acid) and ARA (an omega 6 fatty acid).	730g
Fc	Parent's choice	0-12 month	Infant formula with iron milk-based with a blend of lipids (DHA+ARA).	900g
Fd	Nestle	0-12+month	Alsoy with omega 3 and omega 6 is an iron fortified soy based, DHA and ARA enriched starter infant formula specially designed for younger babies who are avoiding cow's milk or milk products. It is also lactose free and vegetarian.	730g
Fe	Nestle	0-12+month	Good start is the only iron fortified routine infant formula specially designed to be easier to digest and to help reduce your child's risk of developing allergies.	900g

Figure A-1. Effects of sphingolipids on cell viability of Caco2-BBE/HT29-MTX co-cultures



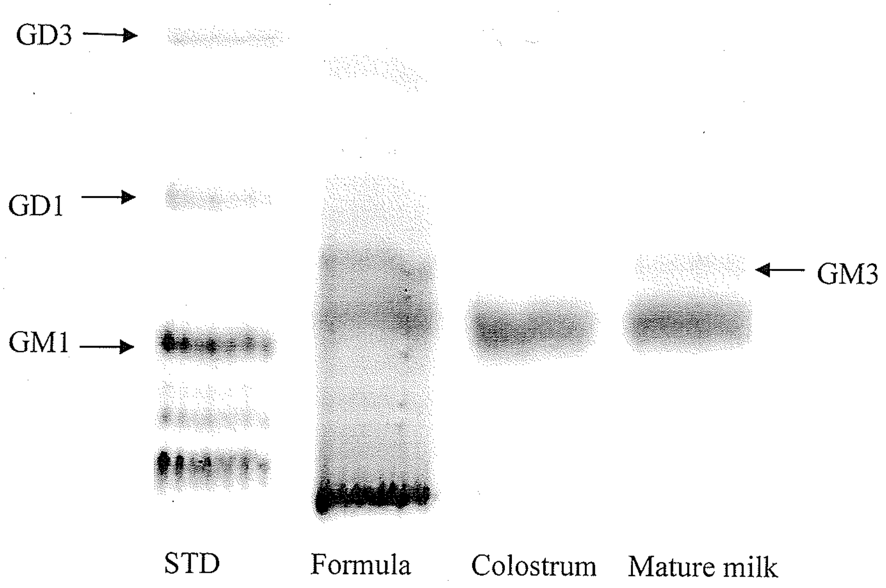
Co-cultured cells were treated with SM, GG and NANA at a concentration of 0 (control), 40, 80 and 100 µg/ml. Live/dead assay using calcein AM and ethidium bromide was performed to quantify the proportion of dead cells at day 1, 3 and 5 after treatment. Values are mean (n=8) ± SD and normalized to control. Significant differences were identified by one way analysis of variance at each nutrient on each culture day (P < 0.001 except GG at day 3). Values having a different superscript are significantly different from control.

Figure A-2. Phospholipids separation by TLC



STD: standard (OVO3); PC: phosphotidylcholin; SM: sphingomyelin; Fa, Fb, Fc and Fe refer to different infant formulas (details shown in Table 8)

Figure A-3. Individual gangliosides identification by HPTLC



STD: standard (GG mixture and GD3)