Uptake and fate of ganglioside GD3 in human intestinal Caco-2 cells

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Schnabl KL, Larcelet M, Thomson AB, Clandinin MT. Uptake and fate of ganglioside GD3 in human intestinal Caco-2 cells. Am J Physiol Gastrointest Liver Physiol 297: G52–G59, 2009. First published May 7, 2009; doi:10.1152/ajpgi.90599.2008.—Ganglioside GD3 is a glycosphingolipid found in colostrum, developing tissues, and tumors and is known to regulate cell growth, differentiation, apoptosis, and inflammation. Feeding a GD3-enriched diet to rats increases GD3 in intestinal lipid rafts and blood. The mechanism, efficiency, and fate of ganglioside absorption by human enterocytes have not been investigated. A model to study GD3 uptake by human intestinal cells was developed to test the hypothesis that enterocyte GD3 uptake is time and concentration dependent, with uptake efficiency and fate influenced by route of delivery. Caco-2 cells were exposed to GD3 on the apical or basolateral membrane (BLM) side for 6, 24, and 48 h. GD3 uptake, retention, transfer, and metabolism was determined. GD3 uptake across the apical and BLM was time and concentration dependent and reached a plateau. GD3 uptake across the BLM was more efficient than apical delivery. Apical GD3 was metabolized with some cell retention and transfer, whereas basolateral GD3 was mostly metabolized. This study demonstrates efficient GD3 uptake by enterocytes and suggests that the route of delivery influences ganglioside uptake and fate.

THE INTESTINAL BARRIER is comprised of specialized epithelial cells that form tight junctions and selectively allow movement of nutrients from the intestinal lumen into the circulation, while impeding penetration of harmful entities (18). The ability of nutrients to enter the enterocyte and cross the intestinal barrier is an important consideration when designing therapeutic agents for gastrointestinal disorders or for intravenous delivery to other tissues. In vitro intestinal barrier model systems such as the enterocyte-like human colon cancer Caco-2 cell line provide an efficient method to screen compounds for their ability to cross the intestinal barrier (32, 48). Caco-2 cells spontaneously differentiate into small intestinal-like enterocyte monolayers upon reaching confluence (10, 48), and following a 20-day postconfluent culture time they develop brush border microvilli, tight junctions, cell polarity, and expression of typical small intestinal microvillus hydrolases and nutrient transporters (11, 23, 45). Primary cultures of enterocytes derived from healthy intestine tissue exhibit limited survival time in culture, and ethically it is often difficult to obtain significant amounts of human intestine tissue for research. Thus the human intestine Caco-2 cell line remains the model of choice for studying nutrient bioavailability.

Gangliosides are sialic acid-containing glycosphingolipids concentrated predominantly in lipid rafts of the outer plasma membrane where the oligosaccharide portion is exposed toward the cell surface and the hydrophobic ceramide is inserted into the surface layer of the membrane (60). Schwarzmann (55) has previously presented the chemical structures of the various gangliosides. In rat intestine, gangliosides make up 34% of glycosphingolipids with the predominant ganglioside of mature intestine, GM3, localized in the brush border membrane (BBM) and the predominant ganglioside found in neonatal intestine, GD3, found in the basolateral membrane (BLM) (20, 43). The concentration of gangliosides is lowest in the proximal intestine and increases along the length of rat intestine toward the distal segment (14). Gangliosides are also found in subcellular organelles of glycosphingolipid metabolism (endosomes, endoplasmic reticulum, Golgi apparatus, lysosomes), plasma lipoproteins, and the milk fat globule membrane (13, 50, 60). Present in small amounts in the diet (61), dietary gangliosides are incorporated into tissues and exert potent effects on cellular functions (31, 43). Depending on location, concentration, and form, individual gangliosides play important roles in recognition, attachment, and translocation of cells, microbes, and macromolecules across membranes and regulate cell signaling and protein function (2, 17, 22, 27, 33, 50). For example, ganglioside GD3 is abundant in colostrum, developing tissues, and several tumors (4, 15, 50). GD3 suppresses inflammation and is involved in cell proliferation and differentiation during development (44, 50). GD3 also induces apoptosis, sensitizes tumor cells to anticancer drug therapy (36, 62), and suppresses immune system tumor killing (31). As protective mechanisms and to ensure proper function, cells regulate ganglioside levels in the body by intracellular compartmentalization, rapid turnover, and chemical modification (5).

To date there are no studies that have specifically examined the mechanism or efficiency of ganglioside uptake and transfer into and across the intestine. It is known that gangliosides form aggregates in aqueous environments such as the intestinal unstirred water layer and, depending on composition, form stable micelles or unilamellar vesicles (53, 55). Cell culture studies suggest that exogenously administered gangliosides are taken up by a wide range of cells via two different mechanisms: 1) molecules dissociate from micelles, diffuse through the aqueous phase, and insert into plasma membranes with or without a protein carrier; and 2) micelles are taken up by receptor-mediated endocytosis (55). Pagano's vesicle-sorting theory (42) suggests that absorbed gangliosides have three fates: transport back to the plasma membrane immediately after being endocytosed, endocytosis to the Golgi apparatus for glycosylation to form more complex ganglioside species, and transport by the endosome to the lysosome for degradation into bioactive mediators. Recently, glycolipid transport proteins
have been identified in the cytosol that may play a role in nonvesicular transport of glycolipids within cells (30). Considering that rats fed a diet enriched in ganglioside GD3 have elevated levels of GD3 in intestinal mucosal lipid rafts and plasma (43), it is reasonable to assume that gangliosides are absorbed by the intestine; that is, GD3 both is taken up across the BBM into the enterocyte and is transferred across the BLM into the blood. The capability of tumor cells to shed gangliosides as micelles, monomers, or membrane vesicles suggests a mechanism exists for removal of cell surface gangliosides (26).

A schematic summarizing proposed mechanisms of ganglioside absorption by the intestine and intracellular fate of absorbed ganglioside is shown in Fig. 1. The pathway(s) of absorption and fate of absorbed gangliosides remain uncertain; Clarke (13) has previously summarized what is known on this topic.

It is not known whether ganglioside uptake and transfer involve a carrier(s), whether the processes are efficient, and what the fate is for absorbed ganglioside. Furthermore, the uptake and transfer of gangliosides delivered on the BLM side of the intestine have not been investigated. It is essential to understand ganglioside absorption mechanism and fate to effectively deliver enteral or intravenous gangliosides to the gut to enhance development or treat intestinal diseases. Thus it is highly relevant to study absorption of ganglioside GD3 by human intestinal Caco-2 cells.

In this study, we ascertained the GD3 concentration range that is not toxic to enterocytes and compared the time frame, concentration effect, mechanism, and efficiency of GD3 absorption from both the apical and BLM side of human intestine Caco-2 cell monolayers. Moreover, the fate of absorbed ganglioside was investigated by determining the proportion of GD3 retained, metabolized, or transferred across the enterocyte following GD3 uptake. It was hypothesized that enterocyte uptake of GD3 would be efficient from both the apical and the basolateral side and that route of delivery would influence GD3 uptake efficiency and fate. We found that GD3 uptake into enterocytes was both time and concentration dependent and reached a plateau. Finally, this study demonstrated that the fate of intestinally absorbed ganglioside depends on the route in which it was delivered.

**MATERIALS AND METHODS**

**Materials.** Zeta dairy lipid powder was supplied by Fonterra (Cambridge, New Zealand; average molecular weight 1.542 g/mol). All cell culture plates and flasks were obtained from Costar (Cambridge, MA). Trypsin-EDTA, antibiotic-antimycotic, and fetal bovine serum (FBS) were ordered from Gibco (Invitrogen Canada, Burlington, ON, Canada). High-performance thin-layer chromatography (HPTLC) plates were purchased from Whatman (Clifton, NJ). Earle’s and Hank’s minimum essential media and all other chemicals were purchased from Sigma (St. Louis, MO) and were declared to be cell culture grade and high purity.

**Cell culture.** The human colon cancer cell line Caco-2 was obtained from American Type Culture Collection (Manassas, VA) and was grown (passage 50–65) under standard incubator conditions (humidified atmosphere, 5% CO₂, 37°C) as adherent monolayer cultures in T flasks containing Earle’s minimum essential medium (EMEM). The cell culture growth medium (pH 7.2) was supplemented with 10% (vol/vol) fetal bovine serum, 1% (vol/vol) antibiotic-antimycotic, 26 mM sodium bicarbonate, 10 mM HEPES, and 1 mM pyruvic acid. Medium was changed every 2–3 days, and cell density and viability were assessed with a hemocytometer by the Trypan blue dye exclusion method.

**Isolation and purification of gangliosides.** Total lipids were extracted from a GD3-enriched zeta dairy lipid powder by the Folch method (19). In short, 0.5 g of zeta lipid 2 was dissolved in 30 ml of chloroform-methanol 2:1 (vol/vol) and homogenized for 30 s. After vortex, the tubes were placed on a shaker overnight and 6 ml of double-distilled water (ddH₂O) was added to give a 5:1 ratio of chloroform-methanol 2:1 (vol/vol) to ddH₂O. To extract gangliosides, the ganglioside-containing upper aqueous phase was collected and the lower organic phase was washed twice with Folch upper phase solution (chloroform-methanol-water, 3:48:47 by volume). The pooled upper phase ganglioside-containing fractions were purified by passage through Sep-Pak C18 reverse-phase cartridges (Waters, Milford, MA) prewashed with 10 ml methanol, 20 ml chloroform-methanol 2:1 (vol/vol) and 10 ml of methanol as described by Williams and McCluer (63). Once the upper phase extract was loaded onto the column and washed with 20 ml of ddH₂O to remove salts and water-soluble contaminants, gangliosides were eluted from the column with 5 ml methanol and 20 ml chloroform-methanol 2:1 (vol/vol) and collected. The eluate was dried under N₂ gas (Praxair, Mississauga, ON, Canada) and the ganglioside powder was redissolved in 2 ml chloroform-methanol 2:1 (vol/vol). Gangliosides were stored at −20°C.

Individual gangliosides were separated on silica gel HPTLC plates (Whatman) with ganglioside standards GM3, GD3, and bovine brain ganglioside mixture (Alexis, San Diego, CA) in a solvent system of chloroform-methanol-0.2% (wt/vol) CaCl₂-2H₂O (55:45:10, by volume). To visualize ganglioside bands, HPTLC plates were sprayed with 0.1% (wt/vol) ANSA (8-anilino-1-naphthalene-sulfonic acid) and viewed under ultraviolet light. The GD3 ganglioside bands were scraped off HPTLC plates and pooled together in a glass tube.
Gangliosides were eluted from silica by vortex, sonication, and shaking overnight in 10 ml of chloroform-methanol (2:1 vol/vol). Tubes were centrifuged for 10 min at 1,000 rpm to spin down the silica. The chloroform-methanol (2:1 vol/vol) phase was collected and combined with a 10 ml chloroform-methanol (2:1 vol/vol) wash and a 5-ml methanol wash of the silica. Extracted GD3 was stored at −20°C until analysis.

Gangliosides were isolated from Caco-2 cells by sonication in Tris-mannitol lysis buffer and extraction in chloroform-methanol (2:1 vol/vol). Extraction and purification were completed as previously described.

**Intestinal cell sensitivity to gangliosides.** Human intestine Caco-2 cells were seeded at a density of 400,000 cells/ml on a 24-well plate. At confluence, cells were switched into EMEM-4 (4% FBS) and treated with a range of GD3 concentrations (0–64 µg/ml; 0–42 µmol/l) similar or higher than the amount in human milk on the apical side for 24 h under standard incubator conditions. Cell necrosis was accessed by use of a lactate dehydrogenase (LDH) release cytotoxicity assay kit (Promega, Madison, WI). In brief, cell lysates and supernatants were incubated with an enzyme-substrate solution for 30 min at room temperature and the absorbance of colored formazan product was measured at 490 nm with a microtiter plate reader (Molecular Devices, Sunnyvale, CA). Absorbance background reading under kit conditions was less than 0.075. Data were expressed as percentage LDH release from cells. The concentration of GD3 chosen for absorption experiments was based on intestinal cell sensitivity to gangliosides.

**Ganglioside uptake by human intestinal cells.** Polarized, differentiated monolayers of Caco-2 cells grown on Transwell plates were used as a model to study apical and basolateral GD3 uptake (Fig. 2). A triple-radiolabeled ganglioside would have provided additional information regarding the metabolic fate of the ganglioside and its distinct sialic acid, fatty acid, and carbohydrate moieties; however, this was not the intent of this study. Triple-radiolabeled gangliosides are not commercially available and the location of the tracer would not necessarily correspond to sites where the intact ganglioside would be present.

**Schematic of intestinal epithelial monolayers grown on Transwell plates.** Efficiency of ganglioside uptake is influenced by ganglioside structure, route of delivery, serum, temperature, pH, divalent cations, cell type, morphology, and density (53). Temperature, pH, and cell density were fixed for GD3 absorption experiments in the present study, whereas serum and divalent cation levels were low. Caco-2 cells were seeded at a density of 2.5 × 10⁵ cells/ml on 0.4 µm pore inserts in 24-well Transwell plates (Corning, Acton, MA). The apical (upper) and basolateral (lower) compartments contained 100 and 600 µl of EMEM-10, respectively. Caco-2 cells were grown to confluence and were differentiated 20 days prior to delivery of gangliosides. After 20 days, Caco-2 cells were differentiated and polarized with intact tight junctions, as confirmed by alkaline phosphatase activity and transepithelial resistance measurements (data not shown). On day 21, Caco-2 cells were transferred into Hanks’ MEM-4, a low-serum, low-calium medium containing no antibiotics to limit serum and calcium interference with ganglioside uptake and antibiotic effects on tight junction permeability. Serum present in the media aided in GD3 solubilization and micelle formation during sonication. Caco-2 cells were incubated under standard conditions in the absence (control, MEM-4 alone) or presence of purified ganglioside GD3 in MEM-4 for 6, 24, and 48 h at a concentration of 5 or 10 µg/ml. The time frame for exposing human intestine Caco-2 cells to ganglioside GD3 was chosen to be 6–48 h because previous work on ganglioside GM1 uptake by cultured cells predicts slow and incomplete ganglioside absorption from the apical side (1, 34). The GD3 concentration chosen for absorption experiments is physiological: close to the concentration of gangliosides in mature human milk (9.07 µg sialic acid per milliliter) and high enough to exert biological effects without exhibiting toxicity (8, 59). The ganglioside-treated cells received GD3 on either the BBM (apical) side or the BLM side of Caco-2 cell monolayers. GD3 delivery on the apical and BLM side of Caco-2 cells was used as an in vitro model reflecting enteral and intravenous nutrient delivery, respectively. After GD3 incubation, apical and BLM supernatants were collected and cells were washed with PBS for 20 min. Tris buffer containing EDTA wash was added to remove cell surface bound ganglioside and to help loosen cells from the inserts. Caco-2 cells from each Transwell insert were trypsinized, pooled together in Tris-mannitol buffer, and sonicated to form cell lysates. Cell lysates were saved for protein and ganglioside analysis.

**Ganglioside analysis.** Total and individual gangliosides were measured with a colorimetric sialic acid assay as ganglioside bound N-acetyl neuraminic acid. An aliquot of purified ganglioside sample was dried under N₂ gas and dissolved with 0.5 ml of ddH₂O and 0.5 ml of resorcinol-HCl in screw-capped Teflon-lined tubes. The purple-blue color developed by reacting resorcinol reagent with ganglioside sialic acid at 160°C for 8 min was extracted into butylacetate-butanol (85:15 vol/vol) solvent. Optical density was read by a spectrophotometer at 580 nm. For quantitative analysis, N-acetyl neuraminic acid (also known as sialic acid) was used as a standard.

**Calculating uptake, retention, transfer, and metabolism of ganglioside GD3.** The following equations were used to calculate the percentage and amount of ganglioside GD3 taken up, retained, transferred, and metabolized by human intestinal Caco-2 cells following BBM or BLM GD3 delivery. For all calculations the values obtained from the control were subtracted off the test groups to correct for small levels of ganglioside present in the serum and cells.

\[
\% \text{ uptake} = \frac{\text{(amount delivered} - \text{amount remaining)}}{\text{amount delivered}} \times 100\% \quad (1)
\]

\[
\% \text{ retention} = \frac{\text{amount cell}}{\text{amount uptake}} \times 100\% \quad (2)
\]

\[
\% \text{ transfer} = \frac{\text{amount receiving compartment}}{\text{amount uptake}} \times 100\% \quad (3)
\]
% metabolism = 
\[ \frac{\text{amount uptake} - \text{amount retained} - \text{amount transferred}}{\text{amount uptake}} \times 100\% \]  

(4)

Statistical analysis. Sample size determination for a one-tailed comparison at 80% power to detect a four- to fivefold increase in GM1 levels in skin fibroblasts (0.2 to 0.9 nmol/mg protein, \( P < 0.05 \)) requires a sample size of 3 (34). All data are expressed as means ± SE for a sample size of \( n = 4 \) (i.e., from 4 different cell passages) for intestinal cell sensitivity experiments and \( n = 3 \) or 4 (3–4 different cell passages) for ganglioside GD3 absorption experiments. Significant differences between the control and experimental group were determined by a one-way ANOVA and a Tukey’s test using SAS statistical software version 9.1. Differences between all treatments were considered statistically significant at an alpha of \( P < 0.05 \).

RESULTS

Sensitivity of human intestinal Caco-2 cells to ganglioside GD3. To determine the GD3 concentration range that does not exhibit toxicity to the enterocyte for absorption experiments, human intestinal Caco-2 cells were exposed to a range of ganglioside GD3 concentrations (0–64 \( \mu \)g/ml; 0–42 \( \mu \)mol/l) for 24 h and cell necrosis was assessed by measuring percentage LDH release from cells. Exposure of Caco-2 cells to 64 \( \mu \)g/ml GD3 induced significant release of LDH from cells (Fig. 3).

Although GD3 was not tested at higher concentrations, a crude mixture of gangliosides containing 80% GD3 continues to induce enterocyte toxicity at concentrations as high as 775 \( \mu \)g/ml, although the mixture was less potent than pure GD3 at the same concentration (data not shown). Knowing that the concentration of gangliosides in mature human milk is 9.07 \( \mu \)g sialic acid/ml (8) and in human serum is 10.5 nmol sialic acid/ml (59), a concentration of 10 \( \mu \)g/ml (6.49 \( \mu \)mol/l) was chosen as the physiological concentration of GD3 for absorption experiments. A concentration of 10 \( \mu \)g/ml exerts anti-inflammatory effects on infant bowel, without exhibiting toxicity (data submitted for publication).

Time-dependent uptake of ganglioside GD3 by human intestinal Caco-2 cells. Human intestinal Caco-2 cells were exposed to 10 \( \mu \)g/ml (6.49 \( \mu \)mol/l) ganglioside GD3 on the apical and BLM side for 6, 24, and 48 h at 37°C, to determine whether uptake of exogenous ganglioside GD3 is time dependent. The time course of GD3 uptake was rapid and linear for the first 6 h, slowed from 6 to 48 h, and reached a plateau (Table 1 and 2). Percentage ganglioside uptake was significantly higher at 24 and 48 h for apical and basolateral GD3 delivery compared with 6 h (Tables 1 and 2). After 6 h, 30% more ganglioside GD3 was taken up by Caco-2 cells when delivered from the basolateral side than when delivered from the apical side (Tables 1 and 2). Moreover, even though both routes of delivery reached a plateau within 24 h, basolateral ganglioside delivery produced a higher steady-state concentration of GD3 (Tables 1 and 2). Therefore, ganglioside GD3 uptake was faster and more efficient when GD3 was delivered on the BLM side of Caco-2 cell monolayers compared with apical delivery (Tables 1 and 2).

Concentration-dependent uptake of ganglioside GD3 by human intestinal Caco-2 cells. Human intestinal Caco-2 cells were exposed to 5 (3.25 \( \mu \)mol/l) or 10 \( \mu \)g/ml (6.49 \( \mu \)mol/l) ganglioside GD3 on the BLM side for 6 h at 37°C, to determine whether uptake of exogenous ganglioside GD3 is concentration dependent. The amount of ganglioside GD3 taken up from the BLM side by human intestinal Caco-2 cell monolayers was twice as high when the concentration of GD3 added was doubled (Fig. 4). Concentration-dependent uptake of ganglioside GD3 from the apical side was not measured.

Fate of ganglioside GD3 taken up by human intestinal Caco-2 cells. Changes in cellular retention, metabolism, and transfer of ganglioside GD3 taken up by human intestinal Caco-2 cells over time was followed to determine the fate of GD3 taken up from the BBM or from the BLM side. The metabolic fate of GD3 was not directly measured, and therefore it was assumed that GD3 that was not transferred or retained was metabolized or modified. The amount of GD3 taken up from the BBM side was not significantly higher at 24 or 48 h compared with 6 h (Table 1).

After 6-h apical exposure to GD3, 96% percent of GD3 taken up was metabolized with small amounts retained in the cell membrane and trace amounts transferred across the BLM (Table 1). After 24 h apical exposure to GD3, the percentage GD3 metabolized dropped 32% and significantly larger amounts of GD3 were retained in the cell membrane and transferred across the BLM (Table 1). The amount of GD3 taken up from the basolateral side was significantly higher at 24 and 48 h compared with 6 h (Table 2). Similar to apical GD3, BLM GD3 uptake following 6 h exposure was almost completely destined for metabolism with trace amounts retained in the membrane or transferred across the apical membrane (Table 2). In contrast to apical GD3, BLM GD3 uptake following 24 h exposure was completely metabolized with significant turnover of cell membrane GD3 and transfer of small insignificant amounts of GD3 across the apical membrane (Table 2).
Human intestinal Caco-2 cells were exposed to 10 μg/ml GD3 for 6, 24, and 48 h on the brush border membrane (BBM) side of cell monolayers. For BBM delivery, 24 μg of GD3 was delivered to the top compartment of 24 pooled Transwells (1 μg/100 μl/Transwell). Values are means ± SE for 3 different cell passages (n = 3). *P < 0.05; †P < 0.01 and ‡P ≤ 0.001 compared with 6 h.

**DISCUSSION**

Human intestine Caco-2 cell monolayers were grown on Transwell inserts as a model for enteral (BBM) and intravenous (BLM) ganglioside absorption. The physiological concentration range of ganglioside GD3 is identified for the enterocyte along with the time frame, efficiency, mechanism, and fate of GD3 taken up from the apical vs. basolateral side of the enterocyte.

Using a range of ganglioside GD3 concentrations, we have demonstrated that ganglioside GD3 may exhibit toxicity to enterocytes after 24 h exposure at concentrations greater than 32 μg/ml (Fig. 3). Although apoptosis was not directly measured, we observed that cells were confluent following ganglioside treatment with only trace cells detached, thus suggesting that apoptosis was limited. Knowing that the concentration of gangliosides in mature human milk is 9.07 μg sialic acid/ml (8), this observation suggests that ganglioside GD3 may be administered enterally at concentrations several times higher than that present in human milk in the absence of toxicity.

This study is the first to examine the time frame and efficiency for apical ganglioside GD3 uptake. Results demonstrate that apical GD3 uptake by human enterocytes begins rapidly within 6 h and reaches a plateau after 24 h. Drop in apical GD3 uptake after 48 h may be explained by disruption of ganglioside concentration gradients by ganglioside shedding, since 10 μg/ml GD3 is not toxic to other epithelial cell lines after 48 or 72 h (data not shown). Dietary sulfatide, an acidic glycosphingolipid, has been shown to be absorbed in rat ileum with small amounts incorporated in 2 h, larger amounts incorporated in 6 h, and most sulfatide disappearing from the intestine by 24 h (7). When present for 24–72 h at 37°C in culture, GM1 is incorporated into fibroblasts in the range of a few nanomoles per milligram cellular protein (1, 34). Compared with glycolipid uptake studies using other cell lines, the time frame for GD3 uptake by intestinal cells in the present study was similar. In contrast to GM1, GD3 was incorporated in the range of a few micromers per mg cell protein. Given that the amount of GM1 given to cells was similar or higher (1–150 μg/ml) than the amount of GD3 used in this absorption study (5–10 μg/ml), the present study may suggest that GD3 is more bioavailable than GM1 (1, 34, 46, 53).

In the present study, apical GD3 uptake efficiency reached a maximum of 62% and incorporated 19 μg of GD3 after 24 h. Ganglioside GM1 is a microbial receptor and modulates ion-sensitive surface proteins whereas GD3 is implicated in intracellular signal regulation (27, 46). It seems likely that most GM1 remains near the cell membrane surface for membrane protection, whereas GD3 is taken up into the intestinal cell and alters cell signaling. An apical ganglioside uptake efficiency of 62% by intestinal cells in vitro is similar to uptake of other lipids, since cholesterol and sphingomyelin, other amphiphilic lipids, have apical uptake efficiencies of 50 and 22–43%, respectively, for rat intestine in vivo (3, 54). The present study shows that apical GD3 uptake by intestine cells is time dependent, fast, and efficient, with clinical potential for enteral delivery of GD3 to the intestine and perhaps other tissues via the circulation.

In vitro absorption of GD3 from the basolateral side of intestine has not been previously investigated. In rats, intravenous ganglioside studies indicate that the plasma half-life for GM1 and GM3 is 1.4 and 1.8 h, respectively, with plasma gangliosides reaching steady state in 12–24 h (16). In rats, 75% of the radiolabel for GM1 and 38% of the radiolabel for GM3 was present in the liver after 3 h (16). Most of the GM1 was metabolized to GM2 and GM3 whereas GM3 was not metabolized (16). Analysis of tissue distribution revealed that 19% of
GM1 and 9.4% of GM3 was localized to the kidney, lung, and brain (16), whereas at most 6% of GM1 and 47% of GM3 could have been distributed to the intestine. In the present study, basolateral uptake of GD3 was linear and rapid for the first 6 h with an uptake efficiency of 74%. It is reasonable to observe higher uptake in vitro because there are multiple tissues where GD3 may distribute in vivo, leaving less available for the intestine. As the GD3 concentration increased from 5 to 10 μg/ml on the basolateral side, twice as much GD3 was taken up, indicating that uptake is concentration dependent. Incorporation of gangliosides into other cell lines is also concentration dependent (37).

Basolateral GD3 uptake was faster and more efficient than apical GD3 uptake by human intestinal cells. This finding is expected since Caco-2 cells express digestive enzymes for glycosphingolipids on the BLM that may not be present on the BLM (29, 56, 58). This study suggests that basolateral GD3 uptake by intestine cells is time dependent, concentration dependent, fast, and efficient with clinical potential to deliver GD3 to the intestine intravenously from the basolateral side of the cell.

Few studies have followed uptake of a fixed ganglioside concentration over time. In the present study, both apical and basolateral membrane uptake of GD3 followed zero-order kinetics, a saturable process, and reached a plateau, suggesting the presence of a carrier or passive ganglioside uptake dependent on a concentration gradient. Digestion of gangliosides is, however, slow and incomplete and likely requires several periods of mixing (segmented motility) for optimal digestion and absorption. A weakness of an in vitro model is that the motility component and stirred water layer are not accounted for. Several lines of evidence suggest that uptake of gangliosides into the cell membrane involves action of specific proteins. Proteins in BBM caveolae such as caveolin-1 appear to be involved in ganglioside uptake by endocytosis (12). Gangliosides added exogenously to epithelial cell cultures are taken up by apical membranes but do not pass the tight junction to the BLM of the cell (57). Pretreatment of cells with trypsin reduces ganglioside uptake (9), and recovery of ganglioside uptake ability requires de novo synthesis of proteins (28). Finally, ganglioside shedding from membranes is an active process that appears to be regulated indicating specific protein activity (25).

Several proteins have been implicated in binding and transfer of sphingolipids at the cell membrane surface including prosaposin, glycolipid transfer protein, lipoprotein receptors and several proteins involved in cholesterol absorption (26, 35, 40, 47, 51). Prosaposin is retained at the outer side of cell membranes in association with gangliosides (26). In vitro, prosaposin promotes transfer of gangliosides from liposomes to membranes in a concentration-dependent manner and catalyzes ganglioside transfer between different vesicles (26). Both gangliosides and prosaposin function through the formation and modulation of lipid rafts, and thus it has been hypothesized that a possible function of prosaposin in the cell membrane is to regulate formation and modulation of lipid rafts by insertion or removal of specific gangliosides (26). It is not known whether prosaposin is present in the brush border or basolateral membrane of enterocytes (26). Glycolipid transfer protein (GLTP) is a soluble protein that selectively accelerates intermembrane transfer of glycolipids in vitro (26). GLTP transfers glycolipids with shorter sugars more efficiently (26), which is consistent with the observation that GD3 and GM3 appear to be taken up more readily than GM1, which contains two additional sugar residues. On the basis of currently published results, prosaposin and GLTP are good candidate proteins potentially involved in regulating the uptake and shedding of gangliosides.

Evidence suggests that lipoprotein receptors may play a role in glycolipid uptake. Rensen et al. (49) demonstrated that lipoproteins are loaded with glycolipids and that recognition and uptake of lipoproteins by tissues was dependent on the dose of glycolipid incorporated into the lipoprotein. Sulfatides are acidic glycosphingolipids similar to gangliosides and have been shown to inhibit scavenger receptor uptake of LDL by macrophages (64). Lipopolysaccharide is a glycolipid that when injected intravenously into experimental animals binds quickly to circulating lipoproteins and slowly clears from the circulation by tissues that express lipoprotein receptors (38). Sphingomyelin carried in lipoproteins is transferred to cells primarily via scavenger receptor class B type I and to a lesser extent LDL receptor (40). Scavenger receptors of class B type I are expressed at high levels in the intestine including Caco-2 cells (6) and may be responsible in part for uptake of gangliosides from the apical and basolateral side of intestine.

Three cholesterol transporters have been implicated in the transport of sphingolipids, the Niemann-Pick C1-like 1 protein (NPC1L1), scavenger receptor class B type I (SR-B1), and the family of ATP-binding cassette (ABC) proteins. The NPC1L1 protein is an endosomal-lysosomal glycoprotein involved in cellular transport of glycosphingolipids from endosomes to lysosomes (51). Recently, expression of NPC1L1 was demonstrated in human small intestine and Caco-2 cells (52). The protein was most highly expressed in microvilli of the jejunum luminal plasma membrane and colocalized with SR-B1 and ABC cholesterol transporters (52). Of the ABC transporter family, ABCA1 and multidrug resistance protein P glycoprotein (MDR1 Pgp) are most likely to be involved in sphingolipid uptake and transfer. ABCA1 is expressed in small intestine and is involved in ATP-dependent export of lipids into HDL (41). The MDR1 Pgp is found in the apical membrane of mucosal cells and luminal membrane of endothelial cells and has been shown to translocate sphingomyelin and glucosylceramide (47). In summary, there are a substantial number of potential proteins involved in ganglioside transport, necessitating further research in this area of intestinal cell biology.

The fate of gangliosides taken up by human intestine has not been studied. To access the fate of ganglioside GD3 taken up by human intestine cells, the amount of GD3 retained in the cell, transferred across the membrane, and metabolized by intestine cells was calculated. In the present study, we demonstrate that apical GD3 was mostly metabolized after 6 h with small amounts retained by enterocytes or transferred across the BLM. After 24 h, additional GD3 taken up by cells was retained or transferred, resulting in a significant drop in the percentage of GD3 targeted for metabolism. Thus apical GD3 that was taken up by intestinal cells was prioritized to meet the apparent needs of the enterocyte first. GD3 may be metabolized to release bioactive metabolites or may be incorporated into the plasma membrane as an intact or modified form. Once the needs of the enterocyte were met, extra GD3 that entered the intestinal cell was perhaps transferred across the BLM for
transport in the circulation to other tissues. In contrast to apical GD3, basolateral GD3 was completely targeted for metabolism, likely into ceramide, sphingosine, or a free fatty acid. Extra GD3 taken up by enterocytes was not retained in the cell but metabolized with trace amounts moving across the apical membrane. Metabolism and removal of extra GD3 by enterocytes may be a protective mechanism since high levels of GD3 trigger apoptosis (24). Alternatively, cellular GD3 may have been acetylated. Acetylation has recently been shown to be a mechanism that tumor cells use as protection from apoptosis (24). Since the assay used for ganglioside analysis in this study measures sialic acid, any GD3 that was acetylated at the sialic acid residue would have been classified as being metabolized.

The influence of route of delivery on fate of GD3 may be explained by the distinct protein and lipid composition of the BBM and BLM. The BBM is enriched in lipid rafts, organized regions within the membrane that are enriched in cholesterol, glycosphingolipids, and signaling proteins (17). Gangliosides may be taken up by cells via endocytosis in lipid rafts and targeted to different intracellular pools by caveosomes, which can bypass lysosomal degradation (39). Moreover, glycosphingolipids have been found in chylomicrons and may pass through chylomicron formation pathways targeted for transfer of glycosphingolipids into plasma lipoproteins (40). The BLM does not contain lipid rafts but has abundant lipoprotein receptors. LDL uptake occurs via clathrin-dependent receptor-mediated endocytosis, a pathway that leads to lysosomal degradation (21). Thus some gangliosides may bypass metabolism when delivered on the apical side of the enterocyte. The difference in GD3 fate following uptake from different sides of the intestine cell suggests that route of delivery may be an important factor to consider when using gangliosides for different therapeutic purposes.

A limitation of the present study is that sialic acid was used as a measure of ganglioside GD3. Consequently, modifications such as acetylation of the sialic acid molecule could not be accounted for and would have been included as metabolized GD3. Moreover, because of the small amounts of gangliosides in cells it was not possible to distinguish between different ganglioside structures. Bile acids, lecithin, free fatty acids, and may have enhanced solubilization and uptake of gangliosides. Bile salts, lecithin, and fatty acids are amphiphilic and carry a negative charge at a pH similar to gangliosides. The net effect of all these factors on BBM morphology, permeability etc., is uncertain and may have affected ganglioside absorption. However, we believe that the net impact of these factors, if present, was likely small. Finally, gangliosides are susceptible to degradation and we could not distinguish intact vs. modified ganglioside. Future studies could overcome these challenges by using radiolabeled ganglioside in combination with a sensitive analytical technique such as tandem mass spectrometry.

In summary, the present study demonstrates that ganglioside GD3 uptake by human intestinal cells is time and concentration dependent with uptake occurring at either the apical or basolateral side of the enterocyte. Assessment of ganglioside metabolism is necessary to determine how much ganglioside is further converted.

REFERENCES


