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Effect of dietary glucosylceramide from sea cucumber on plasma and liver lipids in cholesterol-fed mice

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Abstract

Various physiological functions of dietary glucosylceramides (GlcCer), such as preventing colon cancer and improving the skin barrier function, have been reported. One of the potential GlcCer sources used as a foodstuff is sea cucumber. In this study, our objective was to determine the effect of dietary GlcCer prepared from sea cucumber on plasma and liver lipids in cholesterol-fed mice. ICR mice were fed four different diets (control diet, sea cucumber GlcCer supplemented diet, high cholesterol supplemented diet and high cholesterol + sea cucumber GlcCer supplemented diet). Dietary GlcCer decreased total cholesterol significantly in ICR mice. The mRNA expression of LDL receptor was increased significantly while the gene CYP7A1 involved in bile acids formation was decreased significantly comparing with control (diet without cholesterol). These results suggested that modulation of cholesterol homeostasis gene in liver was due to cholesterol lowering effect of dietary GlcCer.

Keywords glycosylceramide, sphingolipids, sea cucumber, cholesterol, lipid metabolism, mice.

Introduction

The role of functional foods in preventing various chronic diseases (e.g. cardiovascular disease, allergies, cancer) has been focused increasingly. Sphingolipids are highly bioactive compounds that participate in the regulation of cell growth, differentiation, diverse cell functions, and apoptosis [1, 2]. The nutritional and food functional importance of sphingolipids have been also disregarded for decades. It has been reported that dietary supplementation with sphingolipids has diverse physiological effect, such as improving skin barrier function [3, 4], protecting the colon against cancer [5, 6] and inhibiting inflammation [7, 8]. Sphingolipids are found in egg, milk, meat, fish, soybean and so on [9]. Dietary sphingolipids can be hydrolyzed by digestive enzymes in small intestine, although it is relatively hard to hydrolyze and to absorb compared with glycerolipids [10-12]. On the other hand, it has been reported that sphingomyelin (SM), which is a major phosphosphingolipid in animals, inhibits luminal absorption of cholesterol [13, 14]. One potential mechanism for this suppression may be associated with SM that may decrease micellar solubilization and transfer of cholesterol from the micellar matrix to the intestinal cells. In addition, it seems that free sphingoid bases liberated in intestinal tract may be important for inhibitory effect of dietary sphingolipids on cholesterol absorption [15]. Plasma cholesterol level is dependent on several parameters, including endogenous synthesis, secretion, and catabolism of the various plasma lipoproteins. Other major contributors to the amount of cholesterol entering the body each day include the amount of cholesterol in the diet and the rate by which the dietary cholesterol is absorbed [16, 17]. For example, a 90% reduction of cholesterol absorption in moderately hypercholesterolemic subjects has been shown to reduce plasma cholesterol and LDL levels by 35% [18].

The physiologically active substances including glucosylceramide (GlcCer) and some related compounds have been extracted from a variety of sea cucumber species [19, 20]. Dry sea cucumber contains ~200 mg GlcCer per 100 g dry weight [21]. GlcCer used for food ingredient has been isolated from some plant sources, but their content are very low (1-40 mg/100 g dry weight) [22]. Thus, sea cucumber might be suitable for one of dietary source of GlcCer. However, the sphingoid base structures in sea cucumber are more complicated than those in mammals [23] and there is little information about food function of these sphingoid bases that are not found in mammals. The aim of the present study was to evaluate the effect of dietary GlcCer from sea cucumber on plasma and liver lipids in cholesterol-fed mice.

Materials and methods

Preparation of GlcCer

GlcCer were prepared from sea cucumber by a silica gel column after lipid extraction and saponification as described previously [6, 21]. Their purities were above 96% determined by HPLC equipped with an evaporative light-scattering detector [22].

Animals and diets

All animals were treated in accordance with the guidelines for the regulation of animals drafted by the experimentation committee of Kyoto University, Japan. Four-week old male ICR mice (Japan SLC, Inc, Hamamatsu, Japan) were housed at 25^{o}C with a 12-h

light-dark cycle and acclimatized with a commercial diet (MF, Oriental Yeast, Kyoto, Japan) for one week. Four groups of 8 mice each were submitted to feeding for 2 weeks with semisynthetic diets (Table 1). Four groups were control diet (C), sea cucumber GlcCer supplemented diet (S), high cholesterol supplemented diet (HC), and high cholesterol plus sea cucumber GlcCer supplemented diet (HCS). During the feeding period, each group of mice was housed with free access to the diet and water. The body weight and the food intake were measured every day. All prepared diets were stored at 0^{o}C and replaced daily.

Sampling procedures

At the end of the feeding experiment, mice were sacrificed after blood collection under light ether anesthesia. Blood was centrifuged at 1,000 g for 15 min at 4˚C to separate plasma. Plasma samples were stored at -80^{o}C until lipid analysis. The liver, spleen and small intestine were taken, weighed, frozen in liquid nitrogen and kept at –80˚C. A portion of the liver was soaked in RNA later and kept at –80˚C for mRNA expression experiment.

Lipid determination

Triacylglycerols and total cholesterol of plasma and liver were colorimetrically determined by commercially available enzyme kits (Wako Pure Chemical, Osaka, Japan) according to manufacturer's protocol. For liver lipid analysis, the total lipids were extracted with 2 ml of a mixture of chloroform and methanol (2:1, v/v) from 0.5

ml of 25% liver homogenate. The total lipids were dissolved in 1 ml of Triton X-100 before colorimetric assays of the triacylglycerols and cholesterol [24].

Determination of mRNA expression of enzymes related to lipid metabolism

Total RNA was extracted from the liver of mouse using an RNeasy Mini Kit (QIAGEN, Valencia, CA, USA) according to the manufacturer's instructions. To quantify mRNA expression level, real-time quantitative RT-PCR was performed in a BIO-RAD Thermal Cycler (Bio-Rad, Hercules, CA, USA) using SYBR Green PCR regents. The following primers were used: *Fas*, 5′-ACCATGCCAACCTGGTAAAA-3′ (sense), 5′-CAGTGTTCACAGCCAGGAGA-3′ (anti-sense); *Srebp-1c* 5′-GGCTGGCCAATGGACTACTA-3′ (sense), 5′-GGCTGAGGTTCCAAAGCAGA-3′ (anti-sense); *Cyp7al*, 5′-AGACCGCACATAAAGCCCGG-3′ (sense), 5′-CTTTCATT-GCTTCAGGGCTC-3′ (anti-sense); *HmgcoAred*, 5′-TACAACGCCCACGCAGCA-3′ (sense), 5′- ACCAACCTTCCTACCTCAGCAA-3′ (anti-sense), and *Ldlr*, 5′-AGCCATTTTCAGTGCCAATC-3′ (sense), 5′-GAGGAGGGCTGTTGTCTCAC-3′ (anti-sense). The primer pair of *Gapdh* was 5′-TGGGATCGAGTGAAGGACCT-3′ (sense), 5′-CTCCTCCTGCCACTTCTTCTG-3′ (anti-sense). The reaction solution (20 μl final volume) contained 6 μl sample, 10 μl SYBR Green dye (Bio-Rad Laboratories Inc., Hercules, CA), and 2 μl each primer. The thermal cycling conditions were as follows: 48^{o}C for 30 min to prevent carrying over of DNA, an initial denaturation of 95^{o}C for 10 min, followed by 40 cycles of denaturation at 95^{o} C for 15 s and an annealing temperature of 55^{o}C for 1 min.

Statistical analyses

Data are presented as mean ± SD and analyzed by Student's t test or one-way ANOVA with Fishier's PLSD test to identify significant differences between the dietary groups. A level of $p < 0.05$ was considered significant.

Results

Dietary sea cucumber GlcCer did not affect the weight of body (Fig. 1). Daily food consumption was similar among the four groups: 36.5 ± 4.8, 33.9 ± 5.5, 37.3 ± 5.1, 35.5 ± 5.2 g/day/eight mice for C, S, HC and HCS groups, respectively. Based on these data, calculated daily intake of cholesterol in HC and HCS groups were approximately 2.3 and 2.2 mg/day/mouse. Liver and spleen weight was increased significantly in case of high cholesterol diet (Table 2). Contrary, the increase of liver and spleen weights was significantly suppressed by dietary GlcCer.

Sea cucumber GlcCer was used to evaluate the effect of GlcCer on plasma and liver triacylglycerol (TG) and cholesterol concentrations in mice. Dietary sea cucumber GlcCer without cholesterol supplement increased plasma TG and decreased plasma total cholesterol (TC) significantly comparing with control group, but liver TG and TC did not alter significantly (Table 3). Although HCS did not change plasma TG and TC comparing with HC group, HCS decreased liver TC significantly comparing with HC group (Table 3).

The hepatic expression of five genes was studied by using real-time RT-PCR on liver samples fed the experimental diet without cholesterol (Fig. 2). The mRNA

expressions of genes such as *Fas* and *Srebp-1c* involved in fatty acid and TG synthesis were tended to increase by dietary sea cucumber GlcCer but not significantly. The mRNA expression of *Ldlr* was significantly increased while *HmgcoAred* showed trend of increase comparing with control (diet without cholesterol). The gene *Cyp7a1* involved in bile acids formation was decreased significantly comparing with control.

Discussion

In our results, dietary sea cucumber GlcCer decreased plasma cholesterol concentration in mice. This cholesterol-lowering effect is possibly, at least in part, mediated through inhibition of intestinal absorption of both cholesterol and, eventually, would lead to protection of the liver from cholesterol-induced steatosis. In agreement with this prediction, dietary GluCer significantly suppressed the increase of liver weight caused by high-cholesterol diet. Intestinal absorption of cholesterol depends on bile acids and is favored by the presence of TG-derived fatty acids in the intestine that forms mixture of micelles with bile acids in which cholesterol is solubilized [13]. It has been reported that dietary SM inhibits luminal absorption of cholesterol [14]. The formation of stable cholesterol and SM (or sphingosine) complexes could be the cause of reduced intestinal absorption of cholesterol. Because of the diversity in chemical structure among the various sphingolipid species, a wide range of physical and chemical properties are expected and, thus, the present results might be due to not only specific complex formation with bile acids or disturbance of bile acids micelles in the intestinal lumen.

It was reported that short-term dietary supplements of GlcCer significantly increased serum SM levels without influence on cholesterol levels in rats [27]. It is

known that two types of cholesterol-raising fatty acids in the diet, saturated fatty acids and trans fatty acids, increase the serum low density lipoprotein cholesterol concentration [28, 29]. The increase of cholesterol by the sphingolipid-rich diet is more likely caused by the fatty acids derived from sphingolipids digestion. However, dietary sphingolipids are relatively hard to hydrolyze and to absorb compared with glycerolipids [10-12]. Indeed, it was also reported that long-term (through two generations) dietary supplements of sphingolipids could significantly decrease cholesterol (30%) but not SM levels in rats [30].

A reduction in the cholesterol pool in the liver leads to a reduction in bile acid synthesis as reflected by a reduced expression in the liver of bile acid synthesis gene *Cyp7a1*, concomitant with an increased expression of genes involved in hepatic cholesterol synthesis (*HmgcoAred*) and hepatic cholesterol uptake from plasma (*Ldlr*). To maintain its lipid homeostasis, the liver might compensate for the decrease sphingolipid-mediated dietary and biliary cholesterol and fatty acids supply from the intestine by increasing its endogenous cholesterol and fatty acid synthesis, as reflected in the trend of increased hepatic mRNA concentrations of *HmgcoAred, Ldlr, Fas* and *Srebp-1c*. A major regulator of fatty acid synthesis is *Srebp-1c* and it was reported that cholesterol feeding resulted in a large increase in the expression of *Srebp-1c* mRNA in the liver of mice [31].

In summary, sea cucumber GlcCer supplemented diet significantly decreased plasma cholesterol in ICR mice. It also decreased liver cholesterol. Further study is needed to identify the mechanisms of action by sea cucumber sphingoid bases on intestinal or liver physiology in order to layout the scientific basis for their use in the prevention of chronic diseases.

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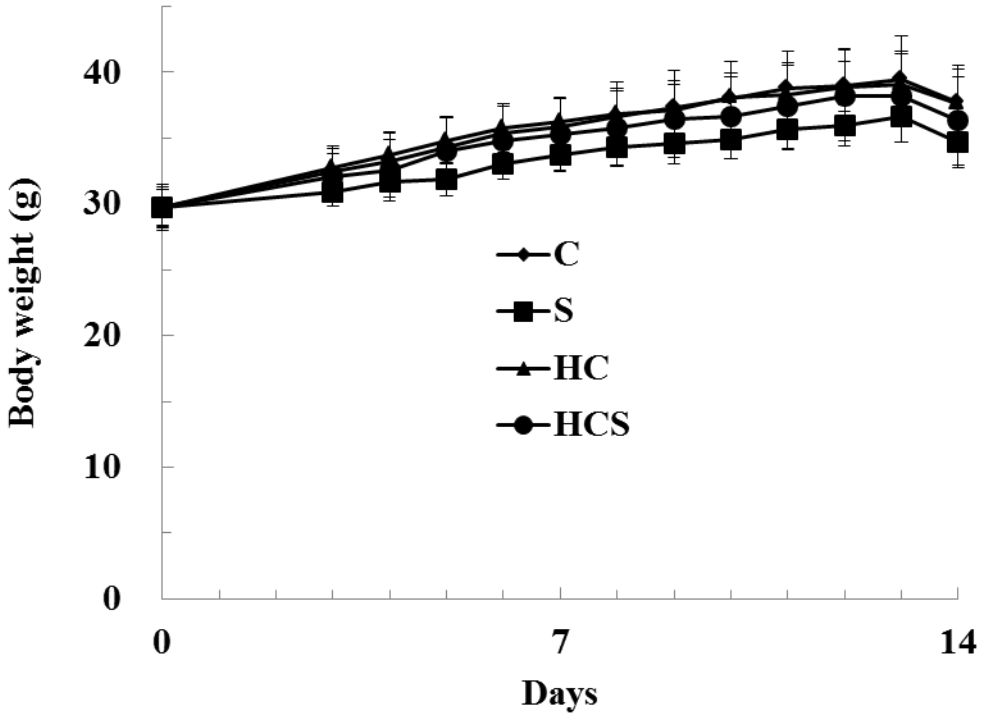
Figure legend

Figure 1. Body weight of mice during the experimental period.

Figure 2. Effect of sea cucumber sphingolipid on the expression level of *Cyp7al*, *HmgcoAred*, *Ldlr*, *Fas* and *Srebp-1c* mRNA in mouse liver. Mouse was fed sea cucumber sphingolipid supplemented diet for 2 weeks. Expression of *Cyp7al*, *HmgcoAred, Ldlr, Fas and Srebp-1c* was determined by real-time quantitative RT-PCR analysis. Data were normalized to GAPDH mRNA levels and are shown as the means ±SD. *p<0.01 and **p <0.05 vs control by Student's-t test


Body weight (g)
40
30
20
10
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Days
C
S
HC
HCS

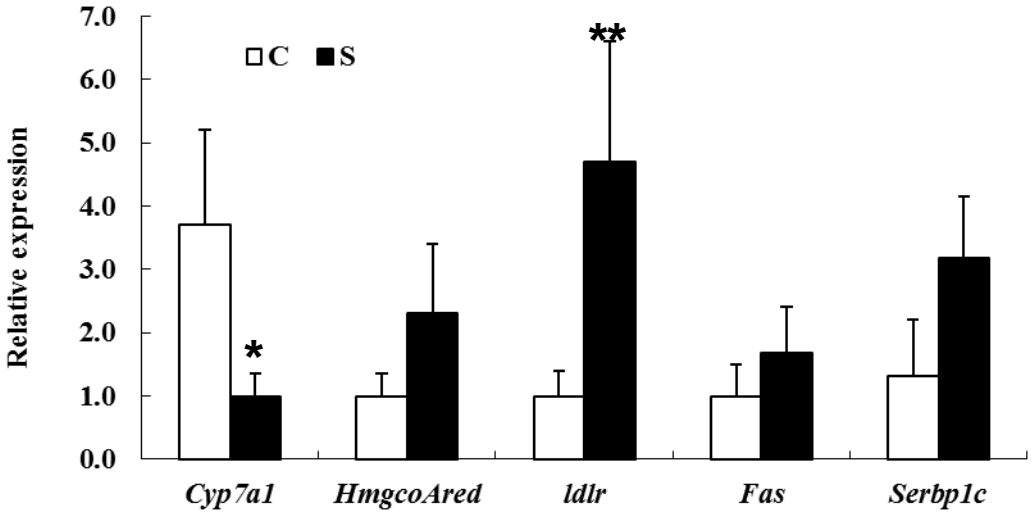
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
Relative expression
C
S
*
**
Cyp7a1
HmgcoAred
ldlr
Fas
Serbp1c

Table 1 Composition of the diets in experiment

Ingredient	C	S	HC	HCS
		g/kg diet		
Cornstrach	397.5	397.5	397.5	397.5
Casein	200.0	200.0	200.0	200.0
Dexrinized cornstrach	132.0	132.0	132.0	132.0
Sucrose	100.0	95.0	92.5	87.5
Soybean oil	70.0	70.0	70.0	70.0
Cellulose	50.0	50.0	50.0	50.0
Mineral mix	35.0	35.0	35.0	35.0
Vitamin mix	10.0	10.0	10.0	10.0
L-Cystine	3.0	3.0	3.0	3.0
Choline bitartrate	2.5	2.5	2.5	2.5
Cholesterol			5.0	5.0
Sodium cholate			2.5	2.5
Sea cucumber SL		5.0		5.0

C, Control diet; S, Sea cucumber sphingolipid supplemented diet; HC, High cholesterol supplemented diet; HCS, High cholesterol + sea cucumber sphingolipid supplemented diet

Table 2 Effects of dietary sphingolipids on weight of body, liver and spleen for 2 weeks of experimental period

Organs	C	S	HC	HCS
	g			
Body	37.69 ± 2.83	34.72 ± 1.96	37.64 ± 2.62	36.31 ± 3.33
Liver	1.44 ± 0.22[a]	1.40 ± 0.11[a]	2.35 ± 0.36[c]	1.91 ± 0.16[b]
Spleen	0.12 ± 0.01[a]	0.13 ± 0.02[a,b]	0.20 ± 0.05[c]	0.16 ± 0.03[b]

C, Control diet; S, Sea cucumber sphingolipid supplemented diet; HC, High cholesterol supplemented diet; HCS, High cholesterol + sea cucumber sphingolipid supplemented diet

Values in rows with different letters are significantly different by Fisher's PLSD test ($p<0.05$).

Table 3 Plasma and liver lipids of the animals fed different diets for 2 weeks of experimental period

	Lipids	C	S	HC	HCS
Plasma (mg/dL)	TG	114 ± 40^{b}	161 ± 25^{c}	54 ± 13^{a}	74 ± 17^{a}
	TC	153 ± 31^{b}	114 ± 24^{a}	216 ± 44^{c}	$179 \pm 43^{b,c}$
Liver (mg/g)	TG	39.8 ± 17.0	47.7 ± 27.9	22.0 ± 15.5	38.0 ± 22.3
	TC	3.8 ± 0.6^{a}	2.9 ± 0.7^{a}	36.6 ± 4.7^{c}	32.2 ± 6.9^{b}

C, Control diet; S, Sea cucumber sphingolipid supplemented diet; HC, High cholesterol supplemented diet; HCS, High cholesterol + sea cucumber sphingolipid supplemented diet; TG, Triacylglycerol; TC, Total cholesterol

Values in rows with different letters are significantly different by Fisher's PLSD test ($p<0.05$).