ABSTRACT

Previous in vivo studies including those with knockout mice suggested an essential role in the intestinal absorption of cholesterol. To characterize the mechanism of cholesterol uptake mediated by NPC1L1, an in vitro system reflecting the function of this transporter needs to be established. In the present study, we constructed NPC1L1 overexpressing CaCo-2 cells as an in vitro model and characterized the transport properties of NPC1L1. Immunohistochemical staining revealed that CaCo-2 cells express NPC1L1 on the apical membrane. It was also demonstrated that the uptakes of both cholesterol and β-sitosterol are increased by NPC1L1 overexpression. In addition, the uptake of cholesterol was increased in a dose-dependent manner by an increase in the content of taurocholate in micelles, whereas micellar phosphatidylcholine showed a negative correlation with cholesterol uptake. Furthermore, it was confirmed that sterol uptake increased by NPC1L1 overexpression was inhibited by ezetimibe. We could thus establish an in vitro intestinal model to study the mechanism of NPC1L1-dependent sterol uptake and to screen drug candidates whose target is NPC1L1.

A high plasma cholesterol level is one of the risk factors for lifestyle-related diseases such as arteriosclerosis and cardiovascular diseases. Cholesterol homeostasis depends on the balance between de novo syntheses and catabolism, excretion into the bile, and intestinal absorption from the diet. Up to now, many drugs including fibrates and statins have been developed and used to reduce plasma cholesterol levels. Among these drugs, ezetimibe is a novel drug that selectively blocks the absorption of cholesterol in the small intestine (van Heek et al., 2001; Patel et al., 2003). The pharmacological target of ezetimibe was demonstrated recently. In 2004, it was reported that Niemann-Pick C1-like 1 (NPC1L1) knockout mice exhibit a reduction in intestinal cholesterol absorption, and the degree of this reduction is almost the same as that observed in wild-type mice treated with ezetimibe (Altmann et al., 2004; Davis et al., 2004). In addition, it was demonstrated that the intestinal absorption of sitosterol was reduced in NPC1L1 knockout mice (Davis et al., 2004). The fact that NPC1L1 is highly expressed in the small intestine, particularly in the jejunum where sterol absorption predominantly takes place, and the fact that this transporter is localized on the apical surface of intestinal cells in rodents and humans (Altmann et al., 2004; Davis et al., 2004; Sane et al., 2006) are consistent with the finding that this transporter is involved in the intestinal absorption of cholesterol.

In contrast with these in vivo observations, few in vitro experimental models to examine the intestinal absorption of sterols have been established. In the present study, we constructed CaCo-2 cells stably expressing rat NPC1L1 as an in vitro model and characterized the transport properties of NPC1L1-mediated transport of sterols. In particular, we have focused on whether the uptakes of cholesterol and sitosterol depend on NPC1L1 expression and the concentration-dependent effect of ezetimibe on the NPC1L1-mediated uptake of sterols. In addition, we were interested in the effect of the micellar component (content of bile acids and phospholipids in micelles) on the uptake of cholesterol, because in vivo cholesterol absorption is affected by the micellar component.

Materials and Methods

Materials. [7α,3H]Cholesterol (7.0 Ci/mmol) was purchased from GE Healthcare (Little Chalfont, Buckinghamshire, UK); [22,23,3H]β-Sitosterol (50 Ci/mmol) was purchased from American Radio-

ABBREVIATIONS: NPC1L1, Niemann-Pick C1-like 1; PCR, polymerase chain reaction; BSA, bovine serum albumin; TBS-T, Tris-buffered saline containing 0.05% Tween 20; ABCG5, ATP-binding cassette, subfamily G, member 5; ABCG8, ATP-binding cassette, subfamily G, member 8; ANOVA, analysis of variance.
labeled Chemicals (St. Louis, MO). Cholesterol was purchased from Wako Pure Chemicals (Osaka, Japan). Sodium taurocholate, 1α-phosphatidylcholine, and β-sitosterol were purchased from Sigma-Aldrich (St. Louis, MO). Ezetimibe was purchased from Sequoia Research Products Ltd (Pangbourne, UK). CaCo-2 cells were purchased from Cell Bank, RIKEN BioResource Center (Ibaraki, Japan). pcDNA3.1 (+) vector was purchased from Invitrogen (Carlsbad, CA). HindIII and XbaI restriction enzymes were purchased from Takara (Shiga, Japan). Anti-HA tag antibody [HA-probe (Y-11); sc-805] was purchased from Santa Cruz Biotechnology, Inc. (Heidelberg, Germany). All other chemicals used were commercially available and of reagent grade.

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NPC1L1 cDNA was amplified by PCR from total RNA of rat intestine. The complete NPC1L1 cDNA (GenBank accession number Y843786) was amplified with the HindIII site at the 5’-end and with the XbaI site and HA tag (YPYDVPDYA) sequence attached at the 3’-end by PCR and then inserted into the pcDNA3.1 (+) vector plasmid. NPC1L1 in pcDNA3.1 (+) vector was transfected into CaCo-2 cells grown on a six-well plate with FuGene 6 (Roche Diagnostics, Indianapolis, IN) according to the user’s manual. Then, CaCo-2 cells were selected by culturing in the presence of 500 μg/ml G418 sulfate (Sigma-Aldrich).

CaCo-2 cells stably transfected with rat NPC1L1-HA cDNA (NPC1L1 cells) were cultured in Eagle’s minimum essential medium (Sigma-Aldrich) with 10% fetal bovine serum (Biowest, Miami, FL), 100 U/ml penicillin and streptomycin (Invitrogen), 1% nonessential amino acids (Invitrogen), and G418 sulfate (500 μg/ml) at 37°C in an atmosphere supplemented with 5% CO2. Cells were plated on 35-mm dishes at a density of 5.0 × 104 cells/dish and cultured for 14 days. After 14 days, cells were harvested with ISOGEN (Nippongene, Tokyo, Japan), and the prepared RNA was reverse-transcribed with ReverTra Ace (Toyobo Engineering, Osaka, Japan). Quantitative real-time PCR was performed using 2× Brilliant SYBR Green QPCR Master Mix (Stratagene, La Jolla, CA) and Chromo4 (Bio-Rad, Tokyo, Japan) at 95°C for 10 min followed by 40 cycles at 95°C for 15 s, 50°C for 30 s, and 72°C for 40 s. For standard curves, serial dilutions of plasmid vectors containing full-length human ABCG5 (GenBank accession number NM022436), human ABCG8 (GenBank accession number NM022437) and human NPC1L1 (GenBank accession number Y843786) were used as PCR templates. Primers for human ABCG5 (sense primer: 5‘-ACCCAAAAGGAAAGGGGAAGG-3’; antisense primer: 5‘-CAGGCCTCGCATGCTGTGTT-3’) human ABCG8 (sense primer: 5‘-GGGTGAGCGAGGAGACTGAG-3’; antisense primer: 5‘-TACACGCTGTCTTTTCAACAGG-3’), human NPC1L1 (sense primer: 5‘-GGTATCCTGAGGGAAGTC-3’; antisense primer: 5‘-AGGTATGAGGGAAGGTGAC-3’) and β-actin (sense primer: 5‘-CCGGAACGAAAGACTGACAG-3’; antisense primer: 5‘-GGTGTTGGAAGGTGACG-3’) were used.

**Western Blot Analysis.** For Western blotting, the cell pellet was resuspended in 1 ml of buffer A (50 mM Tris-HCl, pH 7.4, containing 2 mM EDTA, 2 mM EGTA, 2 mM phenylmethylsulfonyl fluoride, 5 mg/ml leupeptin, 1 mg/ml pepstatin, and 5 mg/ml aprotinin) with mild sonication. After centrifugation (1500g for 15 min), the supernatant was recentrifuged (20,000g for overnight). The radioactivity in the supernatant was recentrifuged (20,000g at 37°C for several hours).

**Micellar Sterol Preparations.** Cholesterol or β-sitosterol was mixed with (or without) ezetimibe diluted in ethanol and, the solvent was evaporated with mild heating under Nz gas. Then, the transport buffer (118 mM NaCl, 23.8 mM NaHCO3, 4.83 mM KCl, 0.96 mM KH2PO4, 1.2 mM MgSO4, 12.5 mM HEPES, 5 mM glucose, and 1.53 mM CaCl2 adjusted to pH 7.4) was added to prepare the medium for the transport experiments containing cholesterol or β-sitosterol (1 μM), taurocholate (2, 4, or 5.5 mM), phosphatidylcholine (10, 50, or 200 μM), ezetimibe (0, 1, 5, 15, 50, or 150 μM), and [7(n)-3H]cholesterol or [22,23-3H]β-sitosterol (0.04 μCi/ml). The micellar solution was thoroughly vortexed and stirred at 37°C for several hours.

**Micellar Sterol Uptake Assay.** Cells were seeded on 12-well plates at a density of 1.2 × 104 cells/well and cultured for 14 days to allow them to differentiate. During that period, medium was replaced every 2 to 3 days. After 14 days, cells were washed twice with the transport buffer (118 mM NaCl, 23.8 mM NaHCO3, 4.83 mM KCl, 0.96 mM KH2PO4, 1.2 mM MgSO4, 12.5 mM HEPES, 5 mM glucose, and 1.53 mM CaCl2 adjusted to pH 7.4) and then preincubated in the transport buffer for 30 min. After preincubation, mixed micelles containing [7(n)-3H]cholesterol or [22,23-3H]β-sitosterol were added, and cells were incubated for the indicated time. After the incubation, cells were washed with the ice-cold transport buffer and disrupted with 0.2 N NaOH for overnight. The radioactivity in the cell lysate was measured by a liquid scintillation counter to determine cellular cholesterol uptake. For normalization, the protein concentration of each culture was determined by the method of Lowry et al. (1951), with BSA as a standard.

**Results**

Expression and Localization of NPC1L1 in CaCo-2 Cells. To construct NPC1L1-overexpressing CaCo-2 cells, the expression vector containing rat NPC1L1 cDNA was transfected into CaCo-2 cells. The selected CaCo-2 cells over-expressing NPC1L1 were visualized by a confocal laser microscope (Olympus, Tokyo, Japan).

**Quantification of the Expression Levels of ABCG5, ABCG8, and NPC1L1.** Cells were plated on 35-mm dishes at a density of 5.0 × 104 cells/dish and cultured for 14 days. After 14 days, cells were harvested with ISOGEN (Nippongene, Tokyo, Japan), and the prepared RNA was reverse-transcribed with ReverTra Ace (Toyobo Engineering, Osaka, Japan). Quantitative real-time PCR was performed using 2× Brilliant SYBR Green QPCR Master Mix (Stratagene, La Jolla, CA) and Chromo4 (Bio-Rad, Tokyo, Japan) at 95°C for 10 min followed by 40 cycles at 95°C for 15 s, 50°C for 30 s, and 72°C for 40 s. For standard curves, serial dilutions of plasmid vectors containing full-length human ABCG5 (GenBank accession number NM022436), human ABCG8 (GenBank accession number NM022437) and human NPC1L1 (GenBank accession number Y843786) were used as PCR templates. Primers for human ABCG5 (sense primer: 5‘-ACCCAAAAGGAAAGGGGAAGG-3’; antisense primer: 5‘-CAGGCCTCGCATGCTGTGTT-3’) human ABCG8 (sense primer: 5‘-GGGTGAGCGAGGAGACTGAG-3’; antisense primer: 5‘-TACACGCTGTCTTTTCAACAGG-3’), human NPC1L1 (sense primer: 5‘-GGTATCCTGAGGGAAGTC-3’; antisense primer: 5‘-AGGTATGAGGGAAGGTGAC-3’) and β-actin (sense primer: 5‘-CCGGAACGAAAGACTGACAG-3’; antisense primer: 5‘-GGTGTTGGAAGGTGACG-3’) were used.
expressing rat NPC1L1 are referred to as “NPC1L1 cells,” whereas pcDNA3.1 vector-transfected cells are referred to as “control cells.”

To confirm the expression and cellular localization of exogenous rat NPC1L1 in CaCo-2 cells, Western blotting and immunohistochemical staining were performed. In the Western blot analysis, NPC1L1 expression was detectable as a 160-kDa band as previously reported (Fig. 1) (Iyer et al., 2005). To measure the expression level of endogenous human NPC1L1 in control cells and NPC1L1 cells, quantitative reverse transcriptase-PCR was performed. Compared with control cells, no increase of endogenous human NPC1L1 was observed in NPC1L1 cells. In addition, we analyzed mRNA levels of ABCG5 and ABCG8, which form a heterodimer on the brush-border membrane of enterocytes to exclude sterols (Berge et al., 2000), by quantitative reverse transcriptase-PCR. In both control and NPC1L1 cells, ABCG5 and ABCG8 mRNAs were hardly detectable and the relative expression levels of ABCG5 and ABCG8 were much less than that of NPC1L1. This result suggests that, in our cell lines, ABCG5/ABCG8 may minimally affect the efflux of sterol, if at all.

Furthermore, the cellular localization of NPC1L1 was analyzed by immunohistochemical staining. As shown in Fig. 2, the exogenous NPC1L1 was localized on the apical membrane. These results indicate that rat NPC1L1 is expressed in CaCo-2 cells and is localized on the apical membrane.

**Overexpression of NPC1L1 Increases Micellar Cholesterol Uptake.** To analyze the function of NPC1L1 in CaCo-2 cells, a micellar cholesterol uptake assay was performed. Figure 3 shows the time profiles for the uptake of cholesterol by control and NPC1L1 cells. The uptake of cholesterol by NPC1L1 cells was ~3-fold higher than that by control cells. For example, at 120 min, cholesterol uptake by NPC1L1 cells was 87.6 ± 0.8 pmol/mg protein and that by control cells was 29.3 ± 1.9 pmol/mg protein from the medium containing 2 mM taurocholate and 50 μM phosphatidylcholine as the micellar component. These results suggest that, in CaCo-2 cells, cholesterol uptake was stimulated by NPC1L1 overexpression.

**Cholesterol Uptake in CaCo-2 Cells Is Influenced by the Micellar Content of Taurocholate and Phosphatidylcholine.** In the small intestine, the concentrations of micellar bile acids and phospholipids may be altered by the dietary intake, and it is possible that the cholesterol absorption is affected by the micellar composition. To analyze the effect of micellar compositions on cholesterol uptake, a cholesterol uptake assay was performed with several concentrations of micellar taurocholate and phosphatidylcholine. In NPC1L1 cells, the cholesterol uptake by 4 mM taurocholate micelles or 5.5 mM taurocholate micelles was 163 ± 28 or 498 ± 5% of that by 2 mM taurocholate micelles, respectively (Fig. 4A). For phosphatidylcholine, cholesterol uptake by 10 μM phosphatidylcholine micelles or 200 μM phosphatidylcholine micelles was 220 ± 15 or 38.1 ± 1.9% of that by 50 μM phosphatidylcholine micelles, respectively (Fig. 4B). A similar trend was seen in control cells (data not shown). These results indicate that taurocholate and phosphatidylcholine, respectively, increase and decrease the micellar cholesterol uptake in a dose-dependent manner.

**Micellar Sitosterol Uptake Is Increased by NPC1L1 Overexpression.** In NPC1L1 knockout mice, the absorption of plant sterols, such as sitosterol and campesterol, was reduced as well as cholesterol (Davis et al., 2004). This result suggests that plant sterol absorption also depends on the function of NPC1L1. In the present study, we examined whether micellar β-sitosterol was taken up by NPC1L1. Figure 5 shows the time profiles for the uptake of β-sitosterol into control and NPC1L1 cells. NPC1L1 cells took up more β-sitosterol (35.2 ± 1.6 pmol/mg protein at 120 min) than control cells (21.6 ± 1.6 pmol/mg protein at 120 min) from the
micelles, which are composed of 2 mM taurocholate and 50 μM phosphatidylcholine (Fig. 5). In addition, compared with the result in Fig. 3, β-sitosterol uptake (35.2 ± 1.6 pmol/mg protein) was ~40% of the cholesterol uptake (87.6 ± 0.8 pmol/mg protein) at 120 min. These results indicate that, although β-sitosterol uptake is mediated by NPC1L1, cholesterol is a better substrate than β-sitosterol.

Ezetimibe Inhibits NPC1L1-Mediated Sterol Uptake. To further characterize the NPC1L1-mediated uptake of sterols, a sterol uptake assay was performed in the presence of ezetimibe. As shown in Fig. 6, A and B, ezetimibe inhibited cholesterol and β-sitosterol uptake in a dose-dependent manner. The $K_i$ values obtained for each sterol uptake were determined by nonlinear regression analysis of the NPC1L1-mediated uptake. The $K_i$ values for cholesterol and β-sitosterol uptake were 20.3 ± 7.1 and 12.1 ± 3.3 μM, respectively. These results suggest that ezetimibe inhibits both cholesterol and β-sitosterol uptake mediated by NPC1L1.

**Discussion**

It has been reported that, in NPC1L1 knockout mice, the intestinal absorption of cholesterol was significantly reduced and the degree of this reduction was similar to that observed in ezetimibe-treated wild-type mice (Altman et al., 2004; Davis et al., 2004). These results suggest that NPC1L1 plays a critical role in the uptake of cholesterol from the intestinal lumen into enterocytes. To clarify the mechanism of cholesterol uptake mediated by NPC1L1, an in vitro system reflecting the function of this transporter needs to be established.

In the present study, we constructed NPC1L1-overexpressing CaCo-2 cells as an in vitro model to study the transport properties of NPC1L1 and found apical localization of NPC1L1 in CaCo-2 cells (Fig. 2). This result is different from that reported by Davies et al. (2005), who indicated intracellular localization of NPC1L1 in CaCo-2 cells. It is possible that the difference in the cellular localization of NPC1L1 may be accounted for by considering the confluence of the cultured cells. Indeed, using NPC1L1-transfected hepatoma cells, Yu et al. (2006) demonstrated that more apparent plasma mem-
brane localization of NPC1L1 was observed in confluent cells than in proliferating cells. We observed apical localization of NPC1L1 in 14-day-old confluent CaCo-2 cells (Fig. 2), whereas Davies et al. (2005) observed intracellular localization in 2-day-old CaCo-2 cells. Together with these facts, it is possible that NPC1L1 can be localized on the apical membrane in highly confluent CaCo-2 cells. In addition, it is possible that the localization of NPC1L1 is affected by the kind of cell lines used for transfection. It has also been reported that NPC1L1 is expressed in the intracellular compartment in HepG2 cells (Davies et al., 2005), whereas plasma membrane localization of NPC1L1 was observed in Chinese hamster ovary (Iyer et al., 2005) and HEK293 cells (Garcia-Calvo et al., 2005).

We could also demonstrate that cholesterol uptake depends on the expression of NPC1L1 in CaCo-2 cells (Fig. 3). This result is consistent with the in vivo observation that, compared with wild-type mice, intestinal cholesterol absorption was decreased in NPC1L1 knockout mice (Altmann et al., 2004; Davis et al., 2004) and the in vitro observation that cholesterol uptake in NPC1L1 knockout CaCo-2 cells was less than that in control CaCo-2 cells (Sane et al., 2006).

The cellular localization and transport function were also determined in NPC1L1 cells cultured on transwell membrane inserts, because CaCo-2 cells become confluent and mimic the intestinal barrier if they are cultured on porous filters. Immunohistochemical staining indicated that the introduced NPC1L1 was expressed on the apical membrane in CaCo-2 cells grown on transwell filters (Supplemental Fig. A). In addition, the uptake of cholesterol into CaCo-2 cells grown on transwell filters from the apical side, but not from the basal side, was significantly increased by NPC1L1 overexpression (Supplemental Fig. B), which is consistent with the apical localization of NPC1L1 (Supplemental Fig. A). Because these results are consistent with observations in CaCo-2 cells grown on plastic dishes (Figs. 2 and 3), we used CaCo-2 cells grown on plastic dishes in further analysis.

In the present study, we have used NPC1L1 cells to characterize the mechanism of cholesterol uptake. Initially, we focused on the micellar component of the uptake of cholesterol mediated by NPC1L1. It is well established that cholesterol is dissolved with bile acids in the intestinal lumen. Bile acids form mixed micelles with digested food products and other bile components, including cholesterol, phospholipids and fatty acids. The concentrations of intestinal bile salts under fasting conditions are 3 to 6 mM (Horton and Dressman, 2001), which are easily affected by the dietary intake. Considering a recent report suggesting that micellar solubilization is essential for cholesterol absorption in humans and that the amount of micellar cholesterol depends on the luminal bile acids (Woollett et al., 2006), alterations in the micellar concentration or composition may affect cholesterol absorption in the intestine. Furthermore, dietary components also affect cholesterol absorption. For instance, dietary sphingosine from plants has been shown to inhibit cholesterol absorption by a specific physicochemical interaction with cholesterol (Garny et al., 2005). Based on these results, an in vitro model is needed to be established to evaluate the effects of micelle composition or dietary components on the absorption of cholesterol. The results of the present study indicated that, in NPC1L1 cells, micellar taurocholate increased cholesterol uptake in a dose-dependent manner (Fig. 4A). This result is consistent with previous report that patients with a hereditary defect in bile acid synthesis exhibit a reduction in cholesterol absorption and that bile acid supplementation resulted in a significant recovery (Woollett et al., 2006). In contrast with bile acids, micellar phosphatidylcholine showed a negative correlation with cholesterol uptake (Fig. 4B). The suppression of cholesterol absorption by phosphatidylcholine has also been observed in previous experiments with isolated brush-border membranes (Proulx et al., 1984). The present in vitro results showing that cholesterol uptake in NPC1L1 cells is affected by micelle composition are consistent with the previous in vivo observations.

We then focused on the mechanism of uptake of sitosterol. It has been reported that ~50% dietary cholesterol is absorbed in the small intestine, whereas only 5 to 15% of dietary plant sterols are absorbed (von Bergmann et al., 2005). This low absorption of plant sterols may be accounted for by the action of ABCG5 and ABCG8. Earlier in vivo studies have indicated that ABCG5 and ABCG8 are able to pump out plant sterols from enterocytes into the intestinal lumen (Hui and Howles, 2005). In addition, in NPC1L1 knockout mice, it has been reported that the absorption of plant sterols is much lower than that in wild-type mice (Davis et al., 2004). In our in vitro study, we found that NPC1L1 cells absorbed more β-sitosterol than control cells (Fig. 5). Moreover, our study also revealed that, despite the minimal expression of ABCG5 and ABCG8 in NPC1L1 cells, the uptake of β-sitosterol mediated by NPC1L1 was less than that of cholesterol (Figs. 3 and 5). Taking our results into consideration, it is possible that both the lower uptake of plant sterols, compared with that of cholesterol, is accounted for by the lower uptake mediated by NPC1L1 in addition to the excretion mediated by ABCG5/ABCG8.

Finally, we examined the effect of ezetimibe, a novel drug that selectively inhibits intestinal sterol absorption without affecting cholesterol synthesis or bile acid excretion (van Hek et al., 2001; Patel et al., 2003). Ezetimibe is used as monotherapy and/or in combination therapy with statins for the treatment of primary hypercholesterolemia. In the present study, it was demonstrated that ezetimibe reduced the NPC1L1-mediated uptake of cholesterol in a dose-dependent manner (Fig. 6A), which is consistent with previous in vivo observations. Furthermore, the fact that ezetimibe inhibits the uptake of β-sitosterol mediated by NPC1L1 (Fig. 6B) provides the molecular basis for the treatment of sitosterolemia with ezetimibe (Salen et al., 2004). Recently, it was reported that some patients exhibit no response to ezetimibe therapy. After sequence analyses of genomic DNA from these patients, nonsynonymous polymorphisms in the NPC1L1 gene were found (Wang et al., 2005). For further characterization of the mechanism of action of ezetimibe, in vitro analyses of these polymorphisms would be useful.

Collectively, the results of the present study indicate that in CaCo-2 cells, NPC1L1 is expressed on the apical membrane and is able to take up both cholesterol and sitosterol. In addition, NPC1L1-dependent cholesterol uptake is affected by the composition of mixed micelles. The results of the β-sitosterol uptake assay showed that, by the uptake of a lower amount of plant sterols than cholesterol, NPC1L1 may regulate the absorption of plant sterols in our body in conjunction with the action of ABCG5/ABCG8. Furthermore, NPC1L1-mediated uptake of sterols is sensitive to ezetimibe.
This is the first report of the development of an intestinal model for NPC1L1-dependent sterol uptake, and this in vitro model will be useful for estimating the effect of drugs or natural products on intestinal sterol absorption and for screening new drug candidates whose target is NPC1L1.

References


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