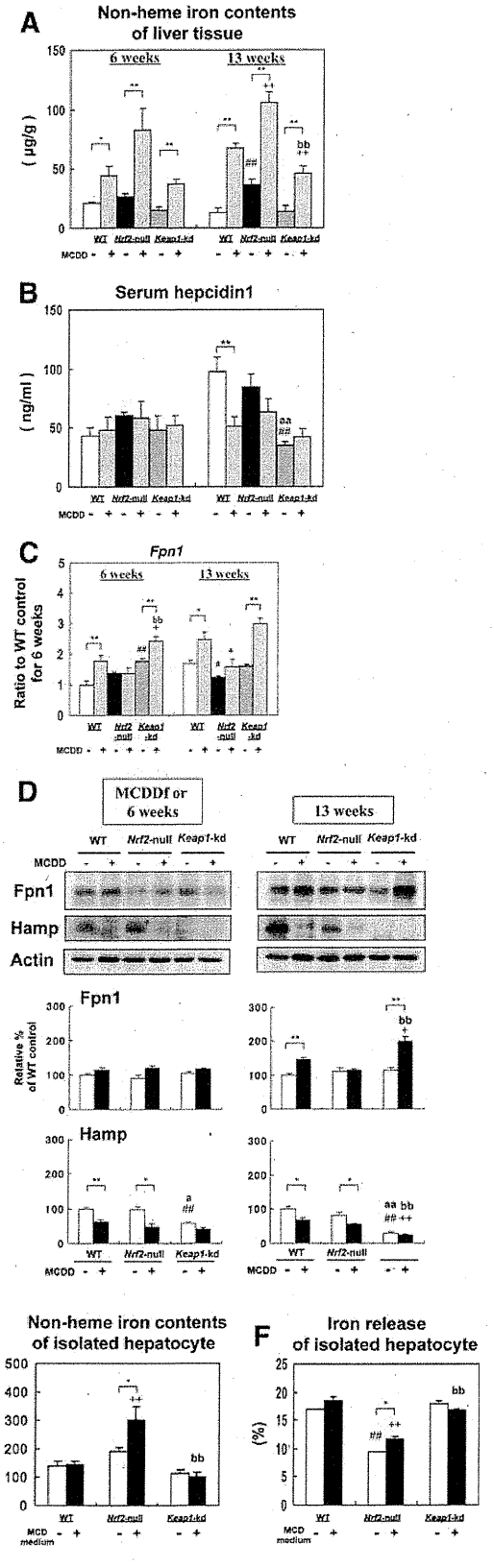

◀ **Fig. 4** The role of Nrf2 on iron metabolism in liver and isolated hepatocyte. **a** Non-heme iron contents of liver tissue in the WT, *Nrf2*-null and *Keap1*-kd mice fed an MCDD for 6 or 13 weeks. Data are given as mean ± SE (n = 8–15/group). $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, significantly different from the WT with control feeding; $^{a}P < 0.05$, $^{aa}P < 0.01$, significantly different from the *Nrf2*-null with control feeding; $^{\dagger}P < 0.05$, $^{++}P < 0.01$, significantly different from the WT with MCD feeding; $^{b}P < 0.05$, $^{bb}P < 0.01$, significantly different from the *Nrf2*-null with MCD feeding; *brackets* $^{*}P < 0.05$, $^{**}P < 0.01$, significantly different between the two groups. **b** Serum hepcidin 1 in the WT, *Nrf2*-null and *Keap1*-kd mice fed an MCDD for 6 or 13 weeks. Data are given as mean ± SE (n = 7–8/group). **c** Steady-state Fpn1 mRNA levels of the WT, *Nrf2*-null and *Keap1*-kd mice fed a control or MCDD for 6 or 13 weeks. Data are given as mean ± SE (n = 6–7/group). **d** Immunoblot analysis of Fpn1 and hepcidin gene (Hamp) proteins in livers of the WT, *Nrf2*-null and *Keap1*-kd mice fed a control diet or an MCDD for 6 or 13 weeks. *Bar graph* shows quantitation of optical density of the immunoblots. Data are given as mean ± SE (n = 8/group). **e** Non-heme iron contents and **f** iron release of isolated hepatocyte from the WT, *Nrf2*-null and *Keap1*-kd mice with or without an MCD medium for 24 h. The means of three independent experiments are shown with the SEM

Nrf2-null mouse livers, while it suppressed iron deposition in *Keap1*-kd mouse livers (Fig. 1a, b). Non-heme iron levels in the liver tissue were measured to directly evaluate liver tissue iron levels. Liver tissue non-heme iron levels after 13 weeks of control diet ingestion were significantly higher in *Nrf2*-null mouse livers than in WT mouse livers (Fig. 4a). MCDD treatment increased liver tissue non-heme iron levels in WT, *Nrf2*-null and *Keap1*-kd mice. Following 13-week MCDD treatment, non-heme iron levels were significantly higher in *Nrf2*-null mouse livers than in WT mouse livers and were significantly lower in *Keap1*-kd mouse livers. These results are consistent with the results of tissue staining using Berlin blue (Fig. 1a, b).

Iron uptake by hepatocytes takes place primarily via the transferrin receptor (TfR), and iron excretion from hepatocytes is primarily mediated by Fpn1. Moreover, hepcidin 1 binds to Fpn1 and induces its internalization and degradation. This restricts iron release from liver iron stores. The liver Fpn1 expression level was analyzed using quantitative PCR and immunoblot analysis (Fig. 4c, d). *Fpn1* mRNA levels rose significantly in WT and *Keap1*-kd mouse livers following MCDD treatment, while elevation was not observed in *Nrf2*-null mouse livers following MCDD treatment. The elevation in *Fpn1* mRNA level following MCDD treatment was generally greater in *Keap1*-kd mouse livers than in WT mouse livers (Fig. 4c). Fpn1 protein expression level following control diet ingestion showed no significant difference among WT, *Nrf2*-null and *Keap1*-kd mouse livers. There was also no significant difference in this parameter after 6-week MCDD treatment. However, following 13-week MCDD treatment, Fpn1 expression increased significantly in WT and *Keap1*-kd mouse livers (Fig. 4d). The increase of Fpn1 expression was more significant in the *Keap1*-kd mouse livers than in the WT

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mouse livers. These results suggest that MCDD treatment induces activation of iron excretion from the liver in *Keap1*-kd mice but not in *Nrf2*-null mice.

Regarding the serum hepcidin 1 levels (Fig. 4b), at 6 weeks after the start of either the control or MCD diet, the levels were not significantly different among the WT, *Nrf2*-null and *Keap1*-kd mice. At 13 weeks after the start of a control diet, the levels were lower in the *Keap1*-kd mice compared with WT and *Nrf2*-null mice. At 13 weeks after the start of MCDD, the levels were decreased significantly in WT mice but not in *Nrf2*-null and *Keap1*-kd mice. The hepatic Hamp levels (Fig. 4d) were significantly lower in the *Keap1*-kd mice compared with WT and *Nrf2*-null mice at 6 and 13 weeks after the start of a control diet. After MCDD treatment for 6 and 13 weeks, the levels were significantly decreased in the livers of WT and *Nrf2*-null mice but not in *Keap1-kd* mice (Fig. 4d). Taken together, the expression levels of hepcidin 1 and Hamp were not increased in the serum and livers of *Nrf2*-null mice fed MCDD. Therefore, in this study, the results of the increased hepatic iron contents associated with the loss of Fpn1 induction in *Nrf2*-null mice fed MCDD would not be attributed to an increase in the expression levels of hepcidin 1 and Hamp.

For a more detailed analysis of iron metabolism in hepatocytes, we isolated hepatocytes from WT, *Nrf2*-null and *Keap1*-kd mouse livers, and changes in iron metabolism depending on Nrf2 expression level were analyzed using primary hepatocyte cultures (Fig. 4e, f). To reproduce in vivo environments, hepatocytes from WT, *Nrf2*-null, and *Keap1*-kd mice were divided into an ordinary medium culture group and an MCD medium culture group. Analysis of intracellular iron levels revealed significantly higher iron levels in *Nrf2*-null mouse hepatocytes in the MCD medium culture group (Fig. 4e). An analysis of iron kinetics using radioisotope-labeled iron showed that *Nrf2*-null mice excrete significantly less iron from hepatocytes compared to WT and *Keap1*-kd mice (Fig. 4f). However, no increase in RI excretion was noted in *Keap1*-kd mouse hepatocytes as compared to that in WT mouse hepatocytes (Fig. 4f).

Effects of sulforaphane (an Nrf2 activator) in alleviating MCDD-induced steatohepatitis

Whether or not activation of Nrf2 can suppress steatohepatitis progression was examined from a therapeutic perspective. SFN, an Nrf2 activator, was administered together with MCDD to evaluate the effects of this treatment in WT mice. Histopathological changes of the liver were compared between WT mice treated with MCDD alone and WT mice treated with MCDD + SFN for 13 weeks (Fig. 5a). Figure 5b shows pathological changes resulting from this treatment, with NAS serving as an indicator. HE staining revealed no significant change in fat droplet deposition. Inflammatory cell infiltration decreased in the SFN-treated group. Evaluation of hepatic fibrosis using Sirius red and MT staining showed that fiber extension (stretching) was less evident in the SFN-treated group, thus indicating suppression of fibrosis. Immunostaining with 4-HNE also revealed suppressed iron deposition and suppressed chromatic response to 4-HNE in the SFN-treated group, while no marked change was noted in the *Keap1*-kd mouse livers (Fig. 5a). Analysis of NAS also revealed no effect of SFN in alleviating fat deposition, although inflammation and fibrosis were significantly alleviated in the SFN-treated group. Analysis of NAS revealed no significant differences in iron deposition.

Next, we measured liver tissue neutral fat, MDA and non-heme iron levels in the livers with or without SFN treatment for 13 weeks (Fig. 5c). Neutral fat level generally decreased following SFN treatment, although this change was not statistically significant. The MDA level improved significantly following SFN treatment. Thus, oxidative stress was suppressed by SFN treatment. The non-heme iron level decreased slightly yet significantly following 13-week SFN treatment, thereby indicating significant suppression of iron accumulation. GSH levels showed no elevation following SFN treatment (data not shown). α-Sma expression was suppressed following 13-week SFN treatment, thus indicating the suppression of stellate cell activation (Fig. 5d).

Changes in Nrf2 expression level following SFN treatment were analyzed, and an increase in expression (activation) was noted early (2 days after the start of treatment) compared to that found in the group treated with MCDD alone (Fig. 5e). However, there was no difference in the Nrf2 expression level at 6 weeks after the start of treatment between the MCDD + SFN treatment group and the MCDD alone treatment group. At 13 weeks, Nrf2 expression was no longer apparent in both the MCDD + SFN treatment group and the MCDD alone treatment group. These results indicate that activation of Nrf2 by MCDD + SFN treatment occurs earlier than that following MCDD alone, but the difference in Nrf2 activation levels between these two groups eventually decreases. Changes in liver tissue Fpn1 expression following SFN treatment were analyzed using immunoblot analysis. Fpn1 expression increased both in the groups with and without SFN treatment, and did not differ depending on whether or not SFN was administered together with MCDD (data not shown).

Discussion

The results of this study have demonstrated the importance of hepatic Nrf2 and its downstream signaling in the

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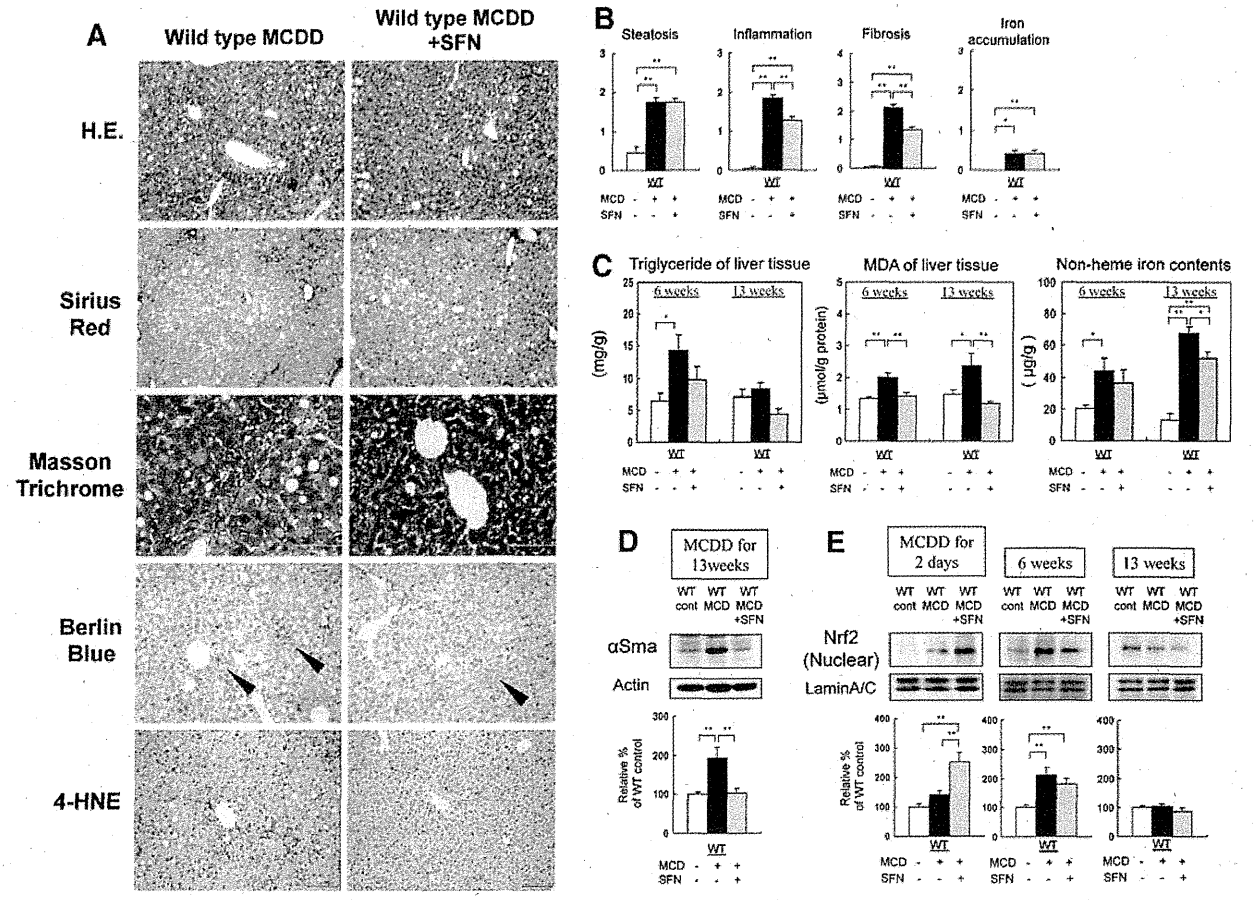

Fig. 5 Sulforaphane inhibits hepatic oxidative stress and counteracts inflammation and fibrosis in steatohepatitis induced by an MCDD. **a** H&E-, Sirius red-, Masson trichrome-, Berlin blue- and 4-HNE-stained sections of representative liver specimens from the WT mice fed an MCDD with or without SFN for 13 weeks (*bars* 100 μm). **b** NAFLD activity score (NAS) in liver samples fed a control diet or an MCDD with or without SFN for 13 weeks. Values are mean ± SE (n = 10–20/group). $^{*}P < 0.05$, $^{**}P < 0.01$, significantly different between the two groups. **c** Triglyceride, malondialdehyde (MDA) and non-heme iron contents of liver tissue in the WT mice fed a control diet or an MCDD with or without SFN for 6 or 13 weeks. Data are given as mean ± SE (n = 8–15/group). **d** Immunoblot analysis of α-Sma proteins in livers of the WT mice fed a control diet or an MCDD with or without SFN for 13 weeks. *Bar graph* shows quantitation of optical density of the immunoblots. Data are given as mean ± SE (n = 8/group). **e** Immunoblot analysis of Nrf2 proteins in nuclear fraction of livers of the WT mice fed a control diet or an MCDD with or without for 2 days or 6 or 13 weeks. Data are given as mean ± SE (n = 8/group)

development of nutritional steatohepatitis, using a mouse model induced by an MCDD. The sustained activation of Nrf2, which is found in the livers of *Keap1*-kd mice, exhibits a protective role against the exacerbation of the pathological state of the steatohepatitis. Moreover, the Nrf2 activation induced by a potent Nrf2 activator, SFN, partially prevents the progression of the pathological state of the steatohepatitis.

Nrf2 serves as an oxidative stress sensor and functions as a comprehensive host defense factor [11, 12, 14]. It has been reported that oxidative stress plays an important role in both the onset and progression of NASH [1, 2, 5–8]. Important sources of oxidative stress involved in NASH are thought to be ROS and radicals from fatty acid β-oxidation, inflammatory cytokines and/or iron accumulation [1, 2, 4, 20, 23]. In this study, increased accumulation of MDA and 4-HNE, i.e., increased oxidative stress in the livers, was noted in *Nrf2*-null mouse livers, while this accumulation did not occur in *Keap1*-kd mouse livers. γ-Gcs, Nqo1 and Gsta1, which are known as antioxidative stress response genes regulated by Nrf2 and factors involved in direct elimination of ROS [11, 12], were not induced in *Nrf2*-null mouse livers administered MCDD, while they were markedly induced in *Keap1*-kd mouse livers (Fig. 3b). The differences in these defense systems against oxidative stress should lead to the observed resistance in *Keap1*-kd mice, although, on the other hand, lead to the susceptibility in *Nrf2*-null mice in terms of the onset and progression of steatohepatitis induced by MCDD. Moreover, in this study, expression levels of factors involved in inflammation

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(inflammatory cytokines such as Tnf-α and Mcp-1) and factors involved in fibrosis (fiber growth factors such as α-Sma, Tgf-β1, and α1-procollagen) were upregulated in *Nrf2*-null mice, while they were down-regulated in *Keap1*-kd mice. Both experimental and clinical studies have shown that prolonged exposure to oxidative stress results in the progression of hepatic inflammation and fibrosis [3, 8]. To be consistent, this study has shown that the enhancement of the antioxidative defense systems induced by sustained Nrf2 activation leads to a marked suppression against the progression of hepatic inflammation and fibrosis.

In this study, excessive iron accumulated in *Nrf2-null* mouse livers; this is an interesting source of oxidative stress in mouse models of MCDD-induced steatohepatitis (Figs. 1a, 4a). Fpn1 is the only transporter known to be involved in the excretion of iron from liver tissue [24]. A recent study demonstrated that regulation of macrophages by Fpn1 involves Nrf2 [18]. The present study also examined the iron balance in liver tissue and hepatocytes. Iron staining using Berlin blue (Fig. 1a, b) and measurement of the non-heme iron level (Fig. 4a) in the mouse liver tissue following MCDD treatment revealed a significantly higher iron level in *Nrf2*-null mice liver tissue and a lower level in *Keap1*-kd mice. However, the expression levels of hepcidin 1 and Hamp, which are inhibitory molecules of Fpn1, were not significantly different between these two kinds of mice fed MCDD (Fig. 4b, d). Furthermore, primary hepatocyte cultures were examined, and we found that hepatocytes from *Nrf2*-null mice showed reduced excretion of radioisotope-labeled iron. Only hepatocytes from *Nrf2*-null mice showed iron accumulation following exposure to MCD medium (Fig. 4e, f), thereby suggesting that Nrf2 is an indispensable factor involved in excretion of iron from hepatocytes in mouse models of MCDD-induced steatohepatitis.

In macrophages reported by Itoh et al. [18], activation of Nrf2 resulted in increased expression of Fpn1 and enhanced excretion of radioisotope-labeled iron. In the liver tissue, however, an increase in Fpn1 expression was not potent even in *Keap1*-kd mice in which Nrf2 was permanently highly expressed, and no increase in RI excretion was noted in *Keap1*-kd mouse hepatocytes as compared to that in WT mouse hepatocytes (Fig. 4c, d, f). To date, only Fpn1 has been identified as a transporter for excreting iron from hepatocytes, but we cannot rule out the presence of unknown factor(s) involved in this process.

Initial treatment of NASH uses diet and exercise therapy; no first-line drug therapy for NASH has been established. Recently, large-scale interventional studies have demonstrated the effectiveness of vitamin E known as an antioxidant in adults with NASH [25]. In the present study, SFN (an Nrf2 activator) was administered together with MCDD to evaluate its effect in alleviating steatohepatitis, with an expectation of steatohepatitis alleviation through drug-induced Nrf2 activation. SFN is known to be contained in broccoli sprouts and other plant foods, and was recently shown to serve as a potent Nrf2 activator [21]. Treatment with SFN did not alleviate fat droplet deposition in histopathological specimens, but did suppress inflammation and fibrosis (Fig. 5a, b). Additionally, oxidative stress was suppressed by SFN treatment (Fig. 5a, c). These effects may be attributable to the enhanced oxidative stress scavenging potential following hepatic Nrf2 activation. The effect of SFN in suppressing liver tissue iron accumulation was not as evident as that seen in *Keap1*-kd mice, which express excessive Nrf2. SFN rapidly induced Nrf2 activation over approximately 2 days, in contrast to activation by MCDD alone, which occurred over approximately 1 week (Fig. 5d). Considering that Nrf2 expression permanently occurs in excess in *Keap1*-kd mice, prior activation of Nrf2 may be important in suppressing the progression of steatohepatitis. Thus, SFN is promising for preventing the progression of steatohepatitis.

In conclusion, this study using mouse models of MCDD-induced steatohepatitis revealed that Nrf2 plays an

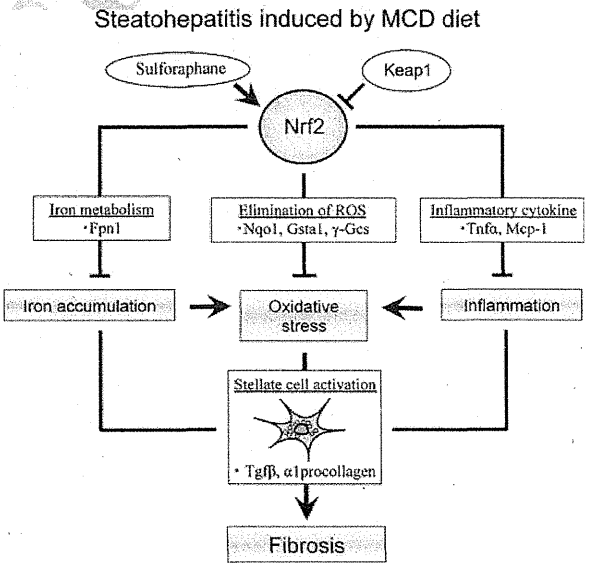

Fig. 6 Schematic summary for the protective role of Nrf2 against oxidative stress-induced liver injury in steatohepatitis induced by an MCDD. Examination of the pathophysiological background suggests that Nrf2 is a comprehensive factor that defends the host from oxidative stress in various aspects and stages. These effects include not only host defense against oxidative stress by direct elimination of active oxygen through stimulation of antioxidative stress response genes (a primary role of Nrf2 according to a conventional view), but also regulation of iron metabolism in new hepatocytes (shown in the present study), among other effects. It appears likely that suppression of oxidative stress by Nrf2 leads to suppression of stellate cell activation, thus allowing suppression of hepatic fibrosis progression

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important role in regulating the onset and progression of this disease. Examination of the pathophysiological background for these effects of Nrf2 suggests that Nrf2 is a comprehensive factor that defends the host from oxidative stress in various aspects and stages (Fig. 6). The alleviation of steatohepatitis following treatment with SFN suggests the feasibility of drug therapy using Nrf2 activators. Nrf2 activation by pharmaceutical intervention could be a new option for prevention and treatment of steatohepatitis.

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Conflict of interest The authors declare that they have no conflict of interest.

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Orexin 2 receptor as a potential target for immunotoxin and antibody-drug conjugate cancer therapy

MASATO KISHIDA[1], KAZUNORI ISHIGE[2], TOMOHISA HORIBE[3], NORIKO TADA[3], NOBUTAKA KOIBUCHI[4], JUNICHI SHODA[2], KIYOSHI KITA[1] and KOJI KAWAKAMI[3]

[1]Department of Biomedical Chemistry, Graduate school of Medicine, The University of Tokyo, Tokyo 113-0033; [2]Department of Gastroenterology and Hepatology, Institute of Clinical Medicine, University of Tsukuba, Tsukuba 305-8575; [3]Department of Pharmacoepidemiology, Graduate School of Medicine and Public Health, Kyoto University, Kyoto 606-8501; [4]Department of Pharmacology and Molecular Therapeutics, Kumamoto University Graduate School of Medical Sciences, Kamamoto 860-8556, Japan

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Abstract. Targeting tumor-specific receptors is a promising approach for cytotoxic agents. The orexin 2 receptor (OX2R) has reportedly been expressed in a few types of cancer, but not in normal, cells. This study aimed to explore and assess the expression levels of OX2R in a wide range of cancer cell lines and clinical samples to identify its localization. To analyze OX2R expression, we developed a polyclonal antibody specific to OX2R by immunizing two rabbits with a peptide cocktail. A total of 36 cancer cell lines were employed for reverse transcription polymerase chain reaction (RT-PCR) and western blot analysis, and 221 samples from various tissue arrays were used for the immunohistochemistry of OX2R expression. OX2R was identified in three cancerous cell lines, from the gallbladder, squamous cell carcinoma of the head and neck (SCCHN) and glioblastoma. With clinical samples of tissue arrays, 69/221 (31.2%) samples reacted positively with the OX2R antibody. We confirmed its presence on the cell membrane. In conclusion, OX2R was identified on several cancer cells as well as clinical samples. Further studies with larger numbers of clinical samples are required to confirm the statistical significance of the presence and relationships of OX2R with tumor histology. Results of the current study suggested that OX2R is a potent target for immunotoxin or antibody-drug conjugate (ADC) cancer therapy on OX2R-positive cancer cells.

Correspondence to: Dr Koji Kawakami, Department of Pharmacoepidemiology, Graduate School of Medicine and Public Health, Kyoto University, Yoshidakonoecho, Sakyoku, Kyoto 606-8501, Japan
E-mail: kawakami.koji.4e@kyoto-u.ac.jp

Key words: orexin 2 receptor, immunotoxin, antibody-drug conjugates, molecular target therapy

Introduction

Cytotoxic drugs are still widely used to treat malignant tumors via tumor cell death by apoptosis (1). One of the most representative cytotoxins is cisplatin, which reacts with target genomic DNA to form DNA adducts (2). Cytotoxins are typically non-selective and this lack of selectivity occasionally results in significant toxicity to normal cells. Moreover, treatment of cancer patients with chemotherapeutic agents frequently allows tumors to acquire a multidrug-resistant (MDR) phenotype (3,4). Overexpression of MDR1, other ATP-dependent transporters, amplification of drug-inactivating enzymes, mutations or modifications of drug targets, alterations in DNA repair machinery and increased resistance to apoptosis cause this resistance (3-5). Toxicities of chemotherapy, along with drug resistance, are major therapeutic limitations that result in poor clinical outcomes in cancer patients.

Targeting of low extracellular pH, elevated enzymes in tumor tissues, the hypoxic environment inside the tumor and tumor-specific antigens expressed on tumor cell surfaces were previously investigated as possible strategies for improved therapeutic outcomes (6-9). Singh *et al* highlighted recent trends in pro-drug and conjugate rationale and a design for cancer treatment, by analyzing comparative accounts of the advantages and disadvantages associated with each approach (10). A variety of receptors related to cellular growth factors or cytokines on tumor cells have been shown to be overexpressed (11), and we believe that targeting these receptors is a promising strategy. For example, the transfection of the tumor necrosis factor (TNF) receptor gene in cancer cells, or the exposure of cancer cells to certain reagents, may increase the expression of TNF receptors, resulting in the enhancement of the cytotoxic effect of TNF (12). Identifying new receptors on tumor cells, in addition to further investigation of therapeutic strategies, is still crucial for cancer treatment.

In the process of finding potential candidate receptors in cancer cells, the orexin 2 receptor (OX2R, also known as hypocretin receptor 2) was found to be a noteworthy target. The orexin family comprises orexins-A and -B, and their corresponding receptors are OX1R and OX2R, respectively. These

two receptors belong to the seven-transmembraned G-coupled receptor superfamily (13). It has become clear that orexin receptors regulate narcolepsy (14,15). Immunohistochemistry (IHC) analyses have demonstrated that certain peptides that bind to orexin receptors were selectively expressed in the hypothalamus, particularly in the lateral and medial hypothalamic regions (16).

At present, the presence of orexin receptors reported in human cancer cells is limited. The expression of OX1R has been found in cell lines from human colon cancer (17). However, the study pertaining to the expression of OX2R is limited to clinical samples of cortisol-secreting adrenocortical adenomas (18). Moreover, the role of orexin receptors in cancer cells is as yet unknown. Thus, identifying the location of orexin receptors remains a challenge.

OX2R was selected as a possible candidate since the expression levels of OX2R in normal cells are limited (16) and, thus, OX2R may be a cancer cell-specific target. Although a high expression of OX2R has been identified in hypothalamic samples, systemic administration of antitumor drugs targeting OX2R may not interact with the hypothalamus due to the presence of the blood-brain-barrier. In addition, orexin receptors are a well known target in the field of neuroscience, since they closely correlate with narcolepsy and other diseases (14,15). Investigating other functions of OX2R would be helpful in understanding the roles of orexins.

In the present study, following the screening of OX2R expression in a variety of cancer cell lines, we investigated cancer tissue array samples to further examine OX2R expression. In addition, we examined the possibility of OX2R as a target for immunotoxin or antibody-drug conjugate (ADC) cancer therapy.

Materials and methods

Materials. Hepatocellular carcinoma tissue array, ARY-HH0075 was purchased from Folio Biosciences (Columbus, OH, USA). Digestive system disease tissue array, DID381 was purchased from US Biomax (Rockville, MD, USA). Human cancer tissue array, VA2 was purchased from SuperBioChips (Seoul, Korea). All other reagents were of reagent grade quality. All tissue samples were collected under the ethical standards with the donor's complete informed consent under the control of manufacturers.

Polyclonal antibody against OX2R. Two New Zealand white rabbits were immunized with three specific peptides of OX2R to obtain a polyclonal antibody for OX2R. Three peptides (CRNWSSASELNETQE, FAHTEDRETVYAWF and C-AVAAEIKQIRARRK) were selected; GenBank reference: CAI19665.1. The immunization period was 64 days and immunization was performed four times during this period. Rabbit serum (10 ml) were purified with a peptide affinity column. The antibody titer of ELISA for CRNWSSASELNETQE was estimated to be 1 in 4,000 and for C-AVAAEIKQIRARRK to be 1 in 16,000. All procedures were performed by Japan Bio Services (Saitama, Japan).

Reverse transcription polymerase chain reaction (RT-PCR). Total RNA was extracted according to the manufacturer's instructions (TRIzol, Invitrogen, Carlsbad, CA, USA). Following quantification of the extracted RNA, first strand complementary DNA (cDNA) of each cancer cell line was synthesized.

OX2R expression was measured by RT-PCR, using 1 μg RNA and oligo (dT) as reverse transcription primers. A control reaction, which omitted reverse transcriptase was included to check for the presence of genomic DNA. OX2R was amplified using a Thermal Cycler (Applied Biosystems, Foster City, CA, USA) in a 20-μg reaction medium containing 0.1 μg of AmpliTaq Gold® DNA Polymerase (Applied Biosystems), using the following cycling conditions: 95°C for 10 min, followed by 35 cycles of 95°C for 30 sec, 57°C for 30 sec and 72°C for 60 sec, followed by a 10 min extension at 72°C. The sequences for the sense and anti-sense primers of OX2R and β-actin used were: 5'-caccgtgttcccaggcttag-3', 5'-ttctggctcggatctgcttt-3', 5'-cactgtgttggcgtacaggt-3' and 5'-tcatcaccattggcaatgag-3'. Amplicons were separated by electrophoresis in 2% agarose gel, stained with ethidium bromide and viewed under UV illumination. Gene sequence of OX2R was performed by Invitrogen Japan (Tokyo, Japan).

Western blotting. Proteins (30 μg) were evaluated by the bicinchoninic acid (BCA) protein assay (Thermo Fisher Scientific, Waltham, MA, USA), loaded onto a NuPAGE 10% Bis-Tris gel (Invitrogen) and blotted onto a Polyscreen polyvinylidene fluoride (PVDF) transfer membrane (ParkinElmer, Waltham, MA, USA) at 100 V for 1 h in a transfer buffer. The PVDF membranes were incubated with primary monoclonal antibody at a 1:2,000 dilution and with α-tubulin antibody (ab24246, Abcam, Cambridge, MA, USA) at a 1:20,000 in phosphate-buffered saline (PBS)-0.1% Tween (PBS-T) and 1% bovine serum albumin (BSA) overnight at 4°C. The membranes were washed, incubated with a secondary anti-rabbit horseradish peroxidase-conjugated antibody (GE Healthcare, Fairfield, CT, USA) at a 1:20,000 dilution for 90 min at a room temperature and washed for 60 min with PBS-T. Antibody complexes were visualized using enhanced chemiluminenscense (ECL) plus western blotting reagent (GE Healthcare). The images were scanned by LAS-3000 UVmini (Fuji Film, Tokyo, Japan).

IHC. IHC was performed using tissue arrays. Tissue arrays were deparaffinized by xylene treatment and washed with an alcohol gradient (from 99 to 50%) and PBS. Arrays were incubated with polyclonal antibody against OX2R at a 1:1,000 dilution overnight at 4°C. Slides were developed with alkaline phosphatase (AP)-goat anti-rabbit IgG (Invitrogen) and stained by BM purple AP substrate (Roche Diagnostics, Rotkreuz, Switzerland). Slides were also lightly counterstained with hematoxylin and eosin. Negative controls in this study were prepared by omitting the primary antibody step.

Immunohistochemical scoring. Samples were scored independently by an experienced histopathologist. Immunostaining was scored as ++/+++ (strong), + (moderate) or - (negative).

Results

OX2R mRNA expression in cancer cells assessed by RT-PCR. One of the main objectives of this study was to confirm the expression levels of OX2R in cancer cells. We investigated 36 cancer cell lines originating from 10 different organs, ranging

Table I. Cell lines used for RT-PCR and western blotting screening.

Organ	Cell line
SCCHN	YCUT231, YCUT891, YCUM 862, YCUM911, YCUMs861, YCUL891, Wmm-scc
Glioblastoma multiforme	SN19, U251tg, A172, U373, SF295
Breast	BT-20, MDA-MB-231, ZR-75-1, T47D
Liver	Hep3B, HepG2, PLC/PFR-5, HuH-7
Gallbladder	TGBC1, TGBC2, TGBC14, TGBC44, NOZ, OCUG
Cholangiocarcinoma	MzChA1,MzChA2, OZ, HuCCT-1
Pancreas	PANC1, SU.86.86
Uterine cervix	Hela, HT-29
Oesophageal carcinoma	OE19
Colon	DLD-1

RT-PCR, reverse transcription polymerase chain reaction; SCCHN, squamous cell carcinoma of the head and neck.

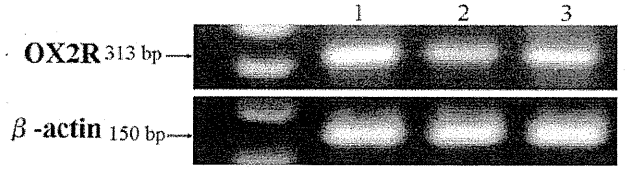

Figure 1. RT-PCR analysis of OX2R mRNA on cancer cells. Total RNA isolated from cancer cell lines (lane 1, TGBC2; lane 2, SF295 and lane 3, YCUM862) was reverse transcribed to cDNA and then assessed by PCR using specific primers as described in Materials and methods. β-actin was used as an internal control. Arrows show the location of amplified PCR products of OX2R and β-actin. OX2R, orexin 2 receptor; RT-PCR, reverse transcription polymerase chain reaction; cDNA, complementary DNA.

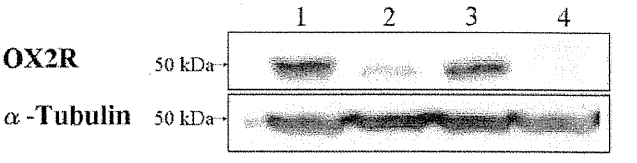

Figure 2. Expression levels of OX2R protein on cancer cells, which were found to be positive for OX2R mRNA upon RT-PCR analysis. Total cell lysates from cancer cells (lane 1, TGBC2; lane 2, SF295 and lane 3, YCUM862) were examined by western blot analysis as described in Materials and methods using specific antibody against OX2R. The extract from the OE19 cell line (lane 4), which was negative for OX2R mRNA expression in RT-PCR analysis was used as a negative control sample. α-tubulin was used as the loading control. Arrows show the location of the molecular weight for OX2R and the α-tubulin protein. OX2R, orexin 2 receptor; RT-PCR, reverse transcription polymerase chain reaction.

from glioblastoma to uterine cervix, and screened all 36 cell lines to assess this objective (Table I). Total RNA isolated from cancer cell lines was transcribed to cDNA, and then assessed by PCR as described in Materials and methods. Primer sequences and RT-PCR conditions were optimized by referring to other investigations analyzing the expression of OX2R (19,20) and, through several trials, to obtain a clear single band.

Of the 36 cell lines, three cell lines, originating from the gallbladder (TGBC2), glioblastoma (SF295) and squamous cell carcinoma of the head and neck (YCUM862), expressed OX2R mRNA (Fig. 1). In addition, we performed gene sequences of PCR products to confirm its homology to OX2R. The homology between PCR products and OX2R was 98%. We also screened for the expression of OX1R mRNA, but this was not identified in any of the cell lines tested in this study (data not shown).

OX2R protein expression in cancer cells assessed by western blotting. To investigate the expression of OX2R protein on cancer cells, we performed western blot analyses on the same cancer cell lines. Since antibodies against OX2R available for both western blotting and IHC were limited, we produced a polyclonal antibody against OX2R. We immunized two rabbits with three peptides specific to OX2R and purified serum as described in Materials and methods. Fig. 2 shows that all 3 cancer cell lines originated from the gallbladder (TGBC2), glioblastoma (SF295) and squamous cell carcinoma of the head and neck (YCUM862), which were positive for OX2R mRNA expressed the protein of OXR2. Conversely, OX2R was not detected in the OE19 cell line, which did not express the mRNA of OX2R in RT-PCR analysis and was used as a negative control (Fig. 2). This result indicates that the three cell lines express OX2R.

OX2R expression in clinical tissue array samples. Based on the results of the mRNA and protein expression analysis, we performed an IHC analysis to analyze localization of OX2R on multiple organs and its frequency of expression among clinical cancer using the polyclonal antibody against OX2R shown at the western blot analysis stage. We prepared 221 clinical samples from the three tissue arrays purchased, which enabled us to obtain insights on the objectives described previously. Details of tissue arrays and protocols are described in Materials and methods.

As shown in Table II, 69 of the 221 samples expressed OX2R, including 8 samples with strong staining and 61 samples with moderate staining. The remaining 152 samples did not react with the OX2R antibody. We observed that the expression of OX2R was located on the cell membrane, where receptors should be located, as shown in Fig. 3. These results suggest that 31.2% of clinical cancer samples express OX2R on their

Table II. Summary of OX2R immunohistochemistry analyses in a variety of cancers using three tissue arrays.

Organ	No. of patients	Staining intensity			Positive samples (%)
		-	+	++/+++	
Liver	80	71	7	2	11
Stomach	34	16	17	1	53
Colon	20	13	7	0	35
Salivary gland	15	8	6	1	47
Larynx	15	7	7	1	53
Rectum	9	6	2	1	33
Lung	9	8	1	0	11
Esophagus	6	6	0	0	0
Small intestine	6	0	5	1	100
Nasopharynx	6	4	2	0	33
Pancreas	6	3	3	0	50
Gallbladder	5	2	3	0	60
Tongue	2	2	0	0	0
Maxilla	2	0	1	1	100
Bile duct	2	2	0	0	0
Mandible	1	0	1	0	100
Lower lip	1	1	0	0	0
Tonsil	1	1	0	0	0
Anus	1	1	0	0	0
Total	221	151	62	8	

OX2R, orexin 2 receptor.

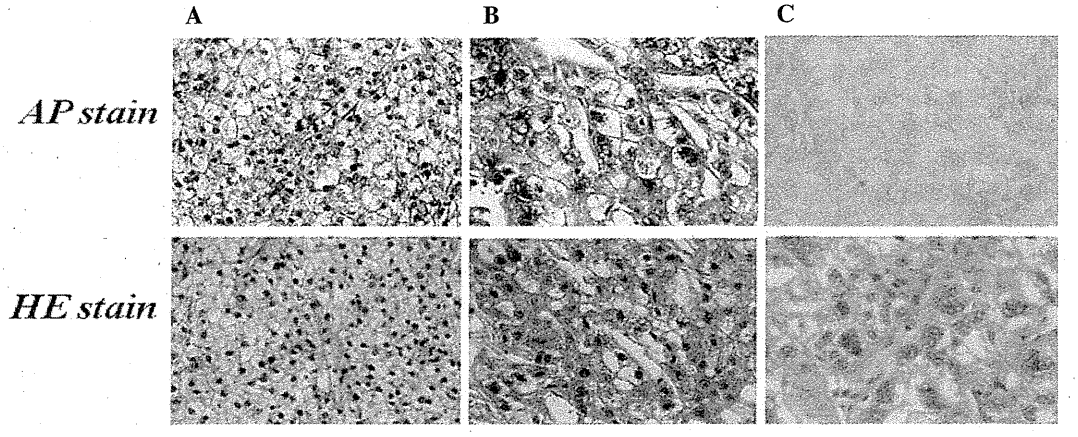

Figure 3. Immunohistochemistry analysis for OX2R expression in various types of cancer. Tissue specimens on arrays were stained with hematoxylin and eosin (H&E) or with alkaline phosphatase (AP), which binds specific polyclonal antibodies to OX2R. (A) Images from sample Folio ARY-HH0075 C6 (hepatocellular carcinoma; original magnification, x200). (B) The magnified immunohistochemical image shows the staining for OX2R is significantly limited on the cell membrane. (Folio ARY-HH0075 G3 sample; original magnification, x400). (C) Images of Folio ARY-HH0075 C4 as a negative control (original magnification, x400). OX2R, orexin 2 receptor.

cell membrane. It is also assumed that cancer organs such as the stomach, salivary gland and larynx expressed OX2R more frequently than other organs.

Discussion

This study showed the presence of OX2R on several clinical cancer samples including salivary gland, stomach and small intestine cancer. We demonstrated the existence of OX2R on cell lines as well as on clinical samples using tissue arrays. These results suggest that OX2R is overexpressed in certain types of cancer and may have biological roles in cancer cells. To reveal the functions of OX2R in cancer cells, we conducted additional experiments to confirm the reaction of OX2R-positive cells with a ligand, orexin B. We observed minimal growth promotion of OX2R-positive cancer cells despite increasing ligand

doses (data not shown). Thus, it is suggested that orexin B does not affect the growth of OX2R-expressing cancer cells and that traditional molecular-targeted approaches, such as anti-OX2R antibodies, may not work effectively. Reacting specifically to OX2R and exhibiting toxicity only inside the OX2R-expressing cells may be criteria for drug candidates to target OX2R as a potential mode of action.

Based on insights revealed in this study, we believe that antibodies or ligands conjugated to natural toxins or chemicals, termed immunotoxins or ADCs (21-23), are likely to be an ideal strategy to target receptors such as OX2R. Immunotoxin or ADCs is likely to be an appropriate approach to treat OX2R-positive cancer cells, since antibodies or ligands that bind OX2R themselves are often non-cytotoxic, as previously noted. Immunotoxin is a rationally designed anticancer agent with potent toxins that target cell-surface antigens or receptors on cancer cells. An immunotoxin strategy enables the OX2R antibody to add specific cytotoxicity to cancer cells by fusing the OX2R antibody to toxins. This strategy may also avoid toxicity to the hypothalamus where OX2R is normally expressed with the function of the blood-brain-barrier to maintain homeostasis (24,25). In addition to the direct killing of cancer cells, antibody-dependent cell-mediated cytotoxicity (ADCC) occurs if the antibody to OX2R is conjugated with toxic moiety.

OX2R immunotoxin is an alternative option for patients with resistance to current antibody therapies, since its anti-tumor effect varies from current molecular-targeting drugs. Moreover, dual-specific immunotoxin enables the enhancement of this type of multi-anti-tumor effect. Conjugating the OX2R antibody and kinase inhibitors may elicit both the ADCC and multi-kinase inhibition effects.

In this study, the presence of OX2R on a variety of types of cancer was suggested. However, further studies are required to confirm the presence of OX2R, since the number of clinical samples in the same organs are limited. At present, additional IHC experiments are underway using a larger number of clinical samples to confirm statistical significance of the presence of OX2R and the correlation with stage and cancer type. Therefore, further investigations on OX2R and new immunotoxin/ADC approaches may offer new therapeutic options for cancer patients.

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NPC2 Regulates Biliary Cholesterol Secretion via Stimulation of ABCG5/G8-Mediated Cholesterol Transport

YOSHIHIDE YAMANASHI,*,‡ TAPPEI TAKADA,* TAKASHI YOSHIKADO,* JUN-ICHI SHODA,§ and HIROSHI SUZUKI*

Departments of *Pharmacy and ‡Pharmacology and Pharmacokinetics, The University of Tokyo Hospital, Faculty of Medicine, The University of Tokyo, Tokyo; and §Division of Sports Medicine, Graduate School of Comprehensive Human Sciences, Tsukuba University, Ibaraki, Japan

BACKGROUND & AIMS: Biliary cholesterol secretion helps maintain cholesterol homeostasis; it is regulated by the cholesterol exporter adenosine triphosphate–binding cassettes G5 and G8 (ABCG5/G8) and the cholesterol importer Niemann-Pick C1-like 1 (NPC1L1). We studied another putative regulator of cholesterol secretion into bile, Niemann-Pick C2 (NPC2)—a cholesterol-binding protein secreted by the biliary system—and determined its effects on transporter-mediated biliary secretion of cholesterol. **METHODS:** Mice with hepatic knockdown of Npc2 or that overexpressed NPC2 were created using adenovirus-mediated gene transfer; biliary lipids were characterized. The effects of secreted NPC2 on cholesterol transporter activity were examined in vitro using cells that overexpressed ABCG5/G8 or NPC1L1. **RESULTS:** Studies of mice with altered hepatic expression of NPC2 revealed that this expression positively regulates the biliary secretion of cholesterol, supported by the correlation between levels of NPC2 protein and cholesterol in human bile. In vitro analysis showed that secreted NPC2 stimulated ABCG5/G8-mediated cholesterol efflux but not NPC1L1-mediated cholesterol uptake. Consistent with these observations, no significant changes in biliary cholesterol secretion were observed on hepatic overexpression of NPC2 in ABCG5/G8-null mice, indicating that NPC2 requires ABCG5/G8 to stimulate cholesterol secretion. Analyses of NPC2 mutants showed that the stimulatory effect of biliary NPC2 was independent of the function of lysosomal NPC2 as a regulator of intracellular cholesterol trafficking. **CONCLUSIONS: NPC2 is a positive regulator of biliary cholesterol secretion via stimulation of ABCG5/G8-mediated cholesterol transport.**

Keywords: ABC Transporter; Bile Acids; Liver; Phospholipids.

BASIC—LIVER, PANCREAS, AND BILIARY TRACT

Cholesterol homeostasis is regulated by a balance of de novo synthesis, catabolism in the liver, intestinal absorption, and secretion into bile. Physiologically, the amount of cholesterol secreted into bile each day is similar to the amounts synthesized in the liver and absorbed from the intestine,[1] indicating that biliary secretion is an important factor regulating whole-body cholesterol levels. In addition, it has been reported that abnormalities in biliary cholesterol secretion are associated with hepatobiliary diseases, because excess biliary cholesterol is involved in the formation of cholesterol gallstones,[2,3] a major risk factor for gallbladder cancer.[4] Therefore, proper regulation of biliary cholesterol secretion is important from both a physiologic and pathophysiologic point of view.

Recent studies have indicated that hepatic cholesterol transporters, such as heterodimer of adenosine triphosphate–binding cassette G5 (ABCG5) and G8 (ABCG8)[5] and Niemann-Pick C1-like 1 (NPC1L1),[6] regulate biliary cholesterol secretion, in addition to their roles in intestinal cholesterol absorption. Indeed, mice lacking ABCG5/G8 showed a drastic reduction in biliary cholesterol concentration,[7] suggesting that ABCG5/G8 plays a key role in cholesterol efflux from hepatocytes into bile. By contrast, NPC1L1 is believed to be involved in biliary cholesterol reabsorption in humans, as suggested by analysis of transgenic mice with liver-specific expression of human NPC1L1.[8] Because the expression of ABCG5/G8[9] and NPC1L1[10] is positively and negatively regulated, respectively, by cellular cholesterol levels, strict control of biliary cholesterol secretion is necessary to regulate cholesterol levels in the body.

Besides regulation of expression levels of hepatic cholesterol transporters, modulation of their cholesterol transport activity is another important factor influencing biliary transport. Although the detailed molecular mechanisms have not been elucidated, it has been reported that bile components affect the activity of ABCG5/G8 and NPC1L1. Indeed, in vitro studies have shown that ABCG5/G8 transport activity is stimulated by the addition of bile acids and phospholipids.[11] Also, NPC1L1-mediated cholesterol uptake was positively and negatively correlated with the concentration of bile acids and phospholipids, respectively.[12] In addition to biliary lipids,

Abbreviations used in this paper: ABCG5, adenosine triphosphate–binding cassette G5; ABCG8, adenosine triphosphate–binding cassette G8; Ad-GFP, green fluorescent protein–expressing adenovirus; Ad-tTA, tetracycline-responsive transcriptional activator-expressing adenovirus; MOI, multiplicity of infection; NPC1, Niemann-Pick C1; NPC1L1, Niemann-Pick C1-like 1; NPC2, Niemann-Pick C2; pfu, plaque forming unit.

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a variety of proteins are also secreted into bile.[13] However, little is known about the possible effects of these biliary proteins on the activity of cholesterol transporters and, consequently, biliary cholesterol secretion.

Recently, it was reported that Niemann-Pick C2 (NPC2)[14] is expressed in the liver and secreted into bile.[15] NPC2 has been shown to bind unesterified sterols with nanomolar affinity[16] and to be localized to lysosomes, where it acts cooperatively with Niemann-Pick C1 (NPC1), a homologue of NPC1L1, as an intracellular regulator of cholesterol trafficking from lysosomes to the plasma membrane and other organelles.[17,18] However, unlike lysosomal NPC2, no physiologic function for secreted NPC2 has been identified. This evidence, along with the fact that NPC2 can bind cholesterol in an extracellular environment as well as in lysosomes,[16,19] led us to hypothesize that biliary NPC2 influences the activity of cholesterol transporters and regulates biliary cholesterol secretion.

To elucidate the physiologic function of biliary NPC2, we performed a series of in vitro and in vivo experiments using adenoviruses to generate knockdown and overexpression conditions. Our results show a novel function of NPC2 by which biliary-secreted NPC2 increases cholesterol secretion into bile via stimulation of ABCG5/G8-mediated cholesterol efflux. This finding was supported by analysis of human bile.

Materials and Methods

Adenoviruses

Adenoviruses expressing the short hairpin RNA sequence for knockdown of the mouse Npc2 gene (accession no. BC007190) (Ad-shNpc2 #1 and #2) and adenoviruses expressing human NPC2 complementary DNA (cDNA) (accession no. BC002532) attached to a Myc tag and 6• His tag sequences (Ad-NPC2-Myc-His), human ABCG5 cDNA (accession no. NM022436) attached to a Myc tag sequence (Ad-Myc-ABCG5), and human ABCG8 cDNA (accession no. NM022437) attached to an HA tag sequence (Ad-HA-ABCG8) were constructed and purified as described in the supplementary material. In addition, shControl-expressing adenovirus, designed not to interfere with any genes (Takara Bio, Inc, Shiga, Japan), green fluorescent protein–expressing adenovirus (Ad-GFP),[20] and tetracycline-responsive transcriptional activator-expressing adenovirus (Ad-tTA) were also purified by the same method. Then, titers of the purified viruses (plaque-forming units [pfu] per milliliter) were determined using an Adeno-X Rapid Titer Kit (Takara Bio, Inc.) and the multiplicity of infection (MOI) was determined by normalizing the virus titer to cell count in each experiment.

Infection of Recombinant Adenoviruses and In Vivo Experiments

ddY mice (30–35 g) purchased from Nihon SLC (Shizuoka, Japan) and Abcg5/g8-deficient mice (B6; 129S6-Abcg5/Abcg8tm1Hobb/J) (30–35 g) purchased from Jackson Laboratory (Bar Harbor, ME) were anesthetized by ether inhalation; the jugular vein was exposed, and then recombinant adenovirus was intravenously injected. At 4 days (for experiments using Ad-shNpc2) or 3 days (for experiments using Ad-NPC2-Myc-His) after adenovirus infection, mice were anesthetized by intraperitoneal injection of urethane. The cystic duct was ligated and a common bile duct fistula was created using a Teflon catheter (UT-03; Unique Medical Co, Ltd, Tokyo, Japan) to collect hepatic bile specimens. Intravenous infusion of tauroursodeoxycholate was performed according to methods described in a previous report.[21] Hepatic bile specimens were collected, followed by liver, intestine, and brain samples. In each experiment, bile flow was not significantly different among groups.

To determine messenger RNA (mRNA) levels of Npc2 in collected tissues, quantitative real-time polymerase chain reaction was performed as described in the supplementary material. To determine protein levels of NPC2 in the liver and bile, 100 μg liver homogenate and 10 μL bile specimens were used for Western blot analysis as described in the supplementary material.

Concentrations of lipids in each specimen were quantified as described in the supplementary material. Biliary lipid secretion rates were calculated by multiplying the concentration of each lipid component by bile flow, which is normalized by 30 g body weight.

Protocols were performed according to accepted criteria for humane care of experimental animals and approved by the review board of our institution for animal studies.

Cholesterol Efflux Assay

LLC-PK1 cells were seeded on 12-well plates at a density of 1.0 • 10^5 cells/well. Two days later, cells were infected with Ad-tTA (10 MOI), Ad-Myc-ABCG5 (5 MOI), and Ad-HA-ABCG8 (5 MOI) to overexpress ABCG5/G8. For control experiments, cells were infected with Ad-tTA (10 MOI) and Ad-GFP (10 MOI). Then, 3 hours after infection, 1 μCi [^{3}H]cholesterol was added to the culture medium in each well and incubated for 24 hours. Cells were then washed twice and preincubated for 30 minutes with transport buffer. After preincubation, cells were treated with 800 μL transport buffer containing indicated amounts of recombinant NPC2 protein or bovine serum albumin together with the indicated concentrations of taurocholate and phosphatidylcholine for 3 hours and the radioactivity of the transport buffer in each well was then measured using a liquid scintillation counter. To measure cellular cholesterol content, cells were washed twice with ice-cold transport buffer and disrupted with 0.1N NaOH and 0.1% sodium dodecyl sulfate overnight, and the radioactivity in the cell lysate was then measured. The efflux ratio was calculated by

dividing the radioactivity in the transport buffer by the sum of that in the transport buffer and cell lysate.

Collection of Human Gallbladder Bile Specimens

Experiments using human samples were conducted according to a study protocol approved by the Institutional Review Board of the University of Tokyo and Tsukuba University upon obtaining informed consent from all subjects. Gallbladder bile specimens were obtained from 15 patients after surgical treatment at Tsukuba University and used for Western blot analysis to determine the expression of biliary NPC2 protein and for the quantification of biliary lipids as described in the supplementary material.

Results

Biliary Cholesterol Secretion Is Reduced in Hepatic Npc2-Knockdown Mice

To test the hypothesis that biliary NPC2 affects cholesterol secretion into bile, Npc2-knockdown mice were created by intravenous administration of adenoviruses (Ad-shNpc2 #1 and Ad-shNpc2 #2) expressing short hairpin RNA against Npc2. Following infection with Ad-shNpc2 #1 and #2, the hepatic expression of Npc2 mRNA was significantly reduced to 53% and 19% of basal levels, respectively, whereas expression of Npc2 mRNA in the intestine and brain was not affected (Figure 1A). Corresponding to the decreased mRNA level, hepatic and biliary Npc2 protein levels were significantly reduced in Npc2-knockdown mice compared with control mice (Figure 1B). By contrast, expression of the Abcg5 and Abcg8 proteins, which are cholesterol exporters expressed in the liver, was not different among control and Npc2-knockdown mice (Figure 1B). We then compared the biliary lipid composition of these mice. Figure 1C shows that the biliary cholesterol secretion in Npc2-knockdown mice was reduced to approximately 59% and 33% of that in control mice, while levels of bile acids and phospholipids, which are not NPC2-binding molecules,[22] remained unaffected. We also confirmed that the molar ratio of biliary cholesterol secretion was selectively decreased by Npc2 knockdown (Supplementary Table 1). These results suggest that hepatic and/or biliary NPC2 play important roles in the secretion of cholesterol from hepatocytes into bile.

Recombinant NPC2 Protein Can Stimulate ABCG5/G8-Mediated Cholesterol Efflux

Because Npc2-knockdown mice showed a reduction in biliary cholesterol secretion (Figure 1C), we surmised that biliary NPC2 facilitates cholesterol secretion from hepatocytes into bile. It is known that the ABCG5-ABCG8 heterodimer (ABCG5/G8) plays a key role in biliary cholesterol secretion,[23,24] so we hypothesized that secreted NPC2 may stimulate the ABCG5/G8-mediated cholesterol efflux. To verify this hypothesis, we constructed LLC-PK1 cells transiently overexpressing ABCG5/G8 due to infection with Ad-Myc-ABCG5 and Ad-HA-ABCG8. Protein expression and localization at the apical membrane of the introduced ABCG5 and ABCG8 cDNA products were confirmed by Western blot analysis (Figure 2A) and immunohistochemical staining (Figure 2B), respectively. Using this cell system, we performed a cholesterol efflux assay and analyzed the effects of adding recombinant NPC2 protein to the assay buffer. As a negative control, we used bovine serum albumin because it has been reported that albumin is secreted into bile[25] and binds to cholesterol.[26] The results in Figure 2C show that the addition of NPC2 protein leads to cholesterol efflux only in ABCG5/G8-overexpressing cells. In addition, we found that NPC2 protein can stimulate ABCG5/G8-mediated cholesterol efflux in the presence of various concentrations of taurocholate and phosphatidylcholine (Figure 2D), both of which are major components of bile. Further in vitro experiments were performed with 5 mmol/L taurocholate.

Because we found that the concentration of NPC2 protein in gallbladder bile was approximately 10 to 80 μg/mL in humans (Figure 7), we next analyzed the concentration dependence of NPC2 protein on its stimulatory effects. As shown in Figure 2E, NPC2 increased cholesterol efflux in a concentration-dependent manner only in ABCG5/G8-overexpressing cells and saturation of the stimulatory effect was not observed within the physiologically relevant concentration of NPC2 (• 100 μg/mL). Furthermore, despite the weak effects of cholesterol-preincubated NPC2 on ABCG5/G8 activity (Figure 2F), the addition of taurocholate restored the stimulatory effects of preincubated NPC2 on ABCG5/G8-mediated cholesterol efflux (Figure 2F). Taken together with the physiologic fact that biliary-secreted bile acids and phospholipids form micelles and facilitate the dissolution of cholesterol in bile, these results suggest that secreted NPC2 has the potential to increase ABCG5/G8-mediated cholesterol efflux under physiologic conditions.

Because NPC1L1, which plays a key role in the cellular uptake of cholesterol, is also expressed on the bile canalicular membrane of hepatocytes,[8] it is possible that biliary-secreted NPC2 may affect NPC1L1-mediated cholesterol reuptake from bile to hepatocytes. To examine this possibility, a cholesterol uptake assay was performed using NPC1L1-overexpressing Caco-2 cells.[12,27-29] Although cholesterol uptake was increased by overexpression of NPC1L1, no significant alteration in transport activity was observed following the addition of recombinant NPC2 protein (Supplementary Figure 1). This result implies that secreted NPC2 protein has only a minor effect on NPC1L1-mediated cholesterol uptake.

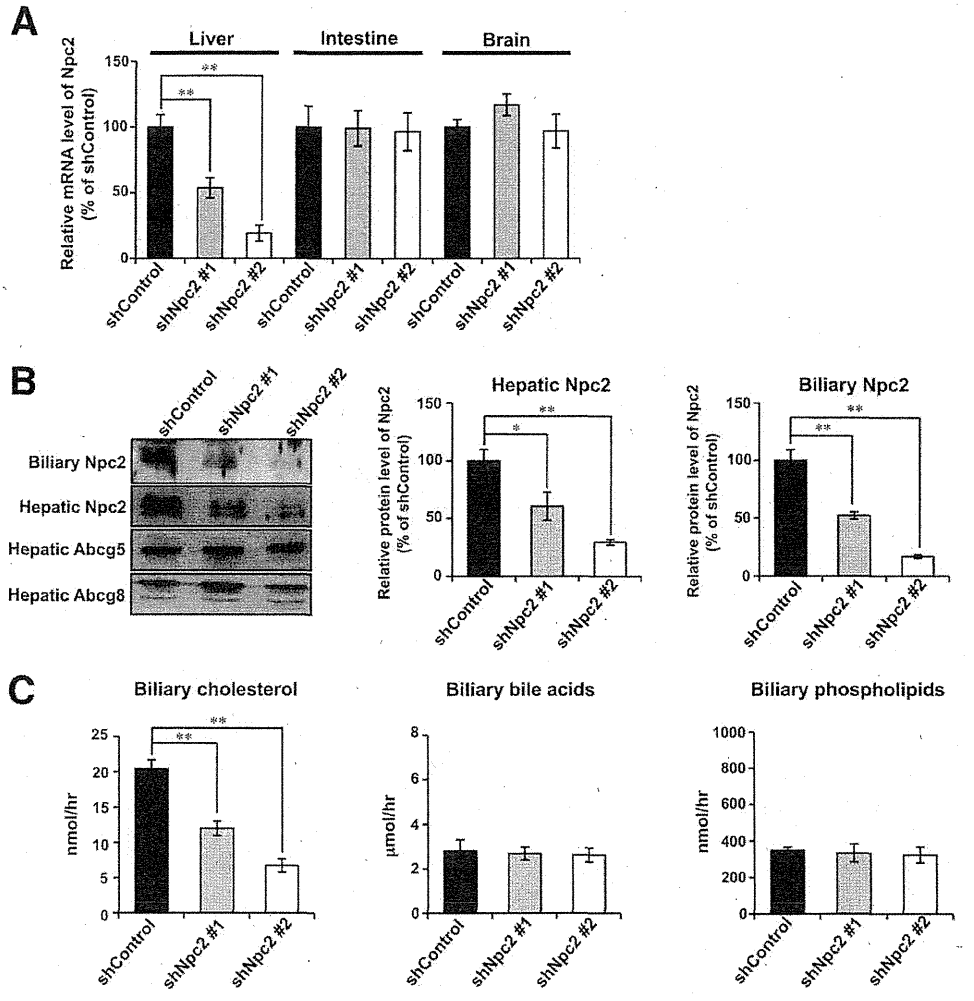

Figure 1. Effect of hepatic Npc2 knockdown on biliary cholesterol secretion in mice. Mice were infected with the indicated Ad-shNpc2 ($1.5 \cdot 10^9$ pfu) or shControl-expressing adenovirus ($1.5 \cdot 10^9$ pfu). Four days after adenovirus infection, liver, intestine, brain, and bile specimens were collected. (A) Relative mRNA levels of Npc2 were analyzed by quantitative real-time polymerase chain reaction and normalized to β-actin in each specimen. (B) Protein expression of biliary Npc2, hepatic Npc2, Abcg5, and Abcg8 was measured by Western blot analysis. Relative protein levels of hepatic and biliary Npc2 were calculated as the band density in each specimen. (C) Secretion rates of biliary cholesterol, bile acids, and phospholipids were quantified as described in the supplementary material. Each column and vertical bar represents the mean • SE of 4 determinations. *Significantly different by analysis of variance followed by Dunnett's test ($P \cdot .05$). **Significantly different by analysis of variance followed by Dunnett's test ($P \cdot .01$).

Increased Hepatic and Biliary NPC2 Facilitates Cholesterol Secretion Into Bile

To show the stimulatory effects of NPC2 protein on cholesterol secretion in vivo, we analyzed biliary cholesterol in hepatic NPC2-overexpressing mice. Infection with Ad-NPC2-Myc-His increased the amount of total NPC2 protein (introduced NPC2-Myc-His protein plus endogenous Npc2 protein) in the liver and bile, whereas expression of hepatic Abcg5/g8 protein was unchanged (Figure 3A). Biliary cholesterol secretion was significantly increased in response to increased hepatic and biliary NPC2 levels, without significant effects on biliary bile acids and phospholipids (Figure 3B). Taken together with the observation that Npc2-knockdown mice have reduced cholesterol levels in bile (Figure 1C), these results suggest that NPC2 can stimulate cholesterol secretion into bile in vivo.

The Stimulatory Effect of Biliary NPC2 on Cholesterol Secretion Is Independent of Its Function in Intracellular Cholesterol Trafficking

Because NPC2 is involved in intracellular cholesterol trafficking from lysosomes to the plasma membrane and other organelles,[18] it was possible that changes in biliary cholesterol secretion in hepatic Npc2-knockdown mice (Figure 1) and NPC2-overexpressing mice (Figure 3) were a secondary effect of alteration in intracellular cho-

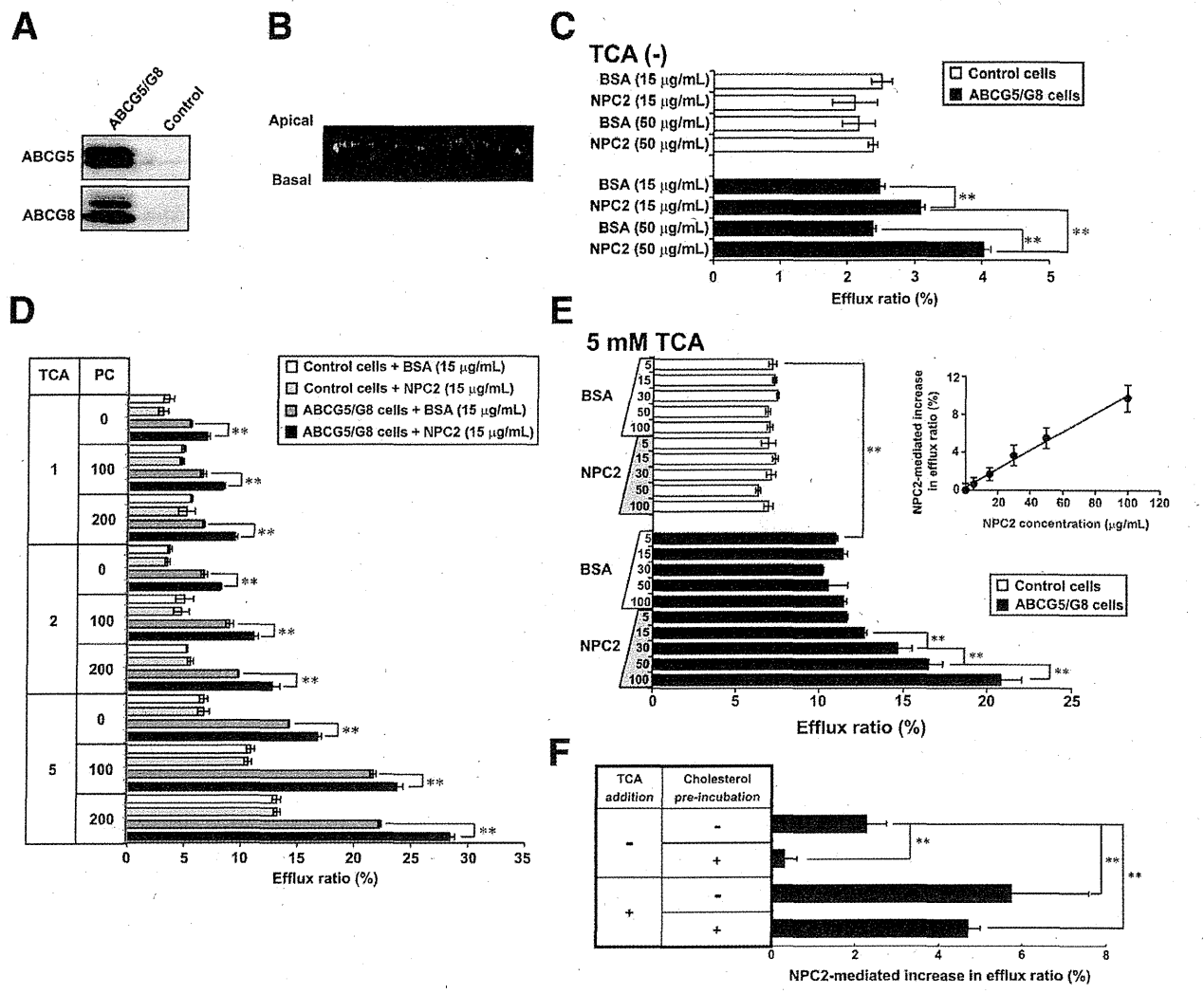

Figure 2. Effect of recombinant NPC2 protein on ABCG5/G8-mediated cellular cholesterol efflux. (A) Expression of Myc-ABCG5 and HA-ABCG8 24 hours after infection with Ad-Myc-ABCG5 and Ad-HA-ABCG8 was measured by Western blot analysis. (B) Cellular localization of Myc-ABCG5 (red) and HA-ABCG8 (green) 24 hours after infection was determined by immunohistochemical staining. Nuclei were stained with TO-PRO-3 iodide (blue), and codistribution of Myc-ABCG5 and HA-ABCG8 is shown in yellow. The panel shows the Z-sectioning image. (C) Cells were treated with transport buffer containing the indicated concentrations of recombinant NPC2 protein or bovine serum albumin (BSA) in the absence of taurocholate (TCA), and the cholesterol efflux ratio was determined. (D) Cells were treated with transport buffer containing recombinant NPC2 protein (15 μg/mL) together with the indicated concentrations of TCA and phosphatidylcholine (PC), and the cholesterol efflux ratio was determined. (E) Cells were treated with transport buffer containing the indicated concentrations of recombinant NPC2 protein or BSA together with 5 mmol/L TCA, and the cholesterol efflux ratio was determined. The NPC2-mediated increase in efflux ratio was calculated by subtracting the cholesterol efflux ratio in the absence of NPC2 from that in the presence of NPC2. (F) A cholesterol efflux assay with cholesterol-preloading recombinant NPC2 protein (50 μg/mL) was performed and the NPC2-mediated increase in efflux ratio was calculated. Each experiment was performed in 3 replicates, and results were confirmed by 2 independent sets of experiments. Each column and horizontal bar represents the mean • SD. **Significantly different by analysis of variance followed by Dunnett's test (P • .01).

lesterol trafficking but not an alteration in the NPC2-stimulated cholesterol efflux. To discriminate between these 2 possibilities, we constructed 2 NPC2 mutants (D72A and V96F); intracellular cholesterol trafficking activity in both mutants was reduced to less than 15% of that of wild-type, whereas cholesterol-binding activity was nearly normal in NPC2 D72A but disrupted in NPC2 V96F.[16] We first assessed the stimulatory effects of these 2 mutants on ABCG5/G8-mediated cholesterol efflux in vitro. Figure 4 shows that purified NPC2 WT and D72A protein increased cholesterol efflux mediated by ABCG5/G8, whereas NPC2 V96F had little effect on this process. This result suggests that the cholesterol-binding activity of NPC2 is necessary for the stimulatory effects of secreted NPC2 on ABCG5/G8-mediated cholesterol transport.

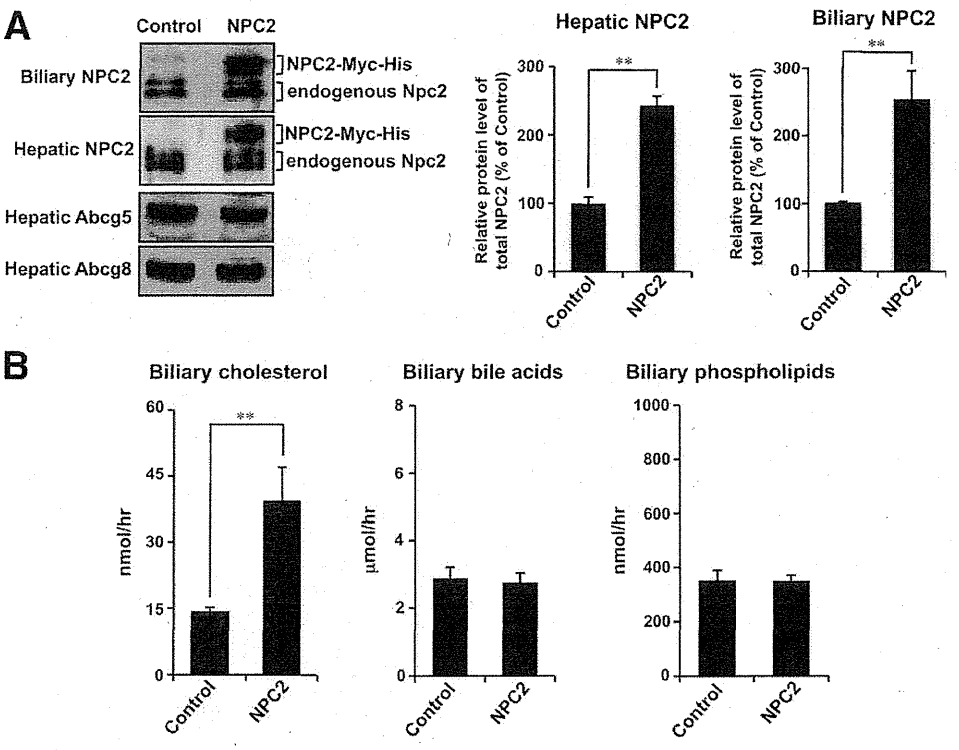

Figure 3. Changes in biliary cholesterol secretion following hepatic NPC2 overexpression in mice. Mice were infected with Ad-tTA (2 • 10^9 pfu) together with Ad-NPC2-Myc-His (2 • 10^9 pfu) or Ad-GFP control (2 • 10^9 pfu). Three days after adenovirus infection, liver and bile specimens were collected. (A) Expression levels of biliary NPC2, hepatic NPC2, Abcg5, and Abcg8 were analyzed by Western blot analysis. The Myc tag and 6• His tag increased the molecular weight of the NPC2-Myc-His protein compared with endogenous Npc2 protein. Relative protein levels of biliary and hepatic NPC2 were calculated by totaling band densities of NPC2-Myc-His protein and those of endogenous Npc2 protein normalized to the affinity to the anti-NPC2 antibody (details are described in the supplementary material). (B) Secretion rates of biliary cholesterol, bile acids, and phospholipids were quantified as described in the supplementary material. Each column and vertical bar represents the mean • SE of 5 determinations. **Significantly different by Student t test (P • .01).

Subsequently, to elucidate the in vivo effects of these NPC2 mutants on biliary cholesterol secretion, we introduced each NPC2 mutant into mice by adenovirus-mediated expression. After we confirmed that expression levels of hepatic Abcg5/g8 were similar and that a significant increase in total NPC2 protein level was observed in the liver and bile of each Ad-NPC2-Myc-His–infected mouse (Figure 5A), bile components of these mice were quantified. As shown in Figure 5B, biliary cholesterol secretion in mice overexpressing NPC2 D72A was significantly higher than that in control mice, whereas the overexpression of NPC2 V96F did not affect biliary cholesterol, both of which are consistent with our in vitro observations (Figure 4). By contrast, the concentrations of biliary bile acids and phospholipids did not differ under each set of conditions (Figure 5B). We also confirmed selective increases in the molar ratio of biliary cholesterol in NPC2 WT- and NPC2 D72A-overexpressing mice (Supplementary Table 1). Because a stimulatory effect on biliary cholesterol secretion was observed in NPC2 D72A, which had little effect on intracellular cholesterol trafficking,[16] the actions of secreted NPC2 as a stimulator of cholesterol secretion should be independent of the activity of lysosomal NPC2 to stimulate the intracellular transfer of cholesterol.

ABCG5/G8 Is Required for NPC2-Mediated Stimulation of Biliary Cholesterol Secretion

To examine whether stimulation of cholesterol secretion by biliary NPC2 is physiologically dependent on the ABCG5/G8-mediated pathway, we introduced NPC2 adenoviruses into Abcg5/g8-deficient mice. Although the difference in biliary cholesterol secretion between Abcg5/g8 (• /•) mice and Abcg5/g8 (• /•) mice[7] was lessened by adenovirus infection, the results shown in Figure 6 reveal a stimulatory effect of NPC2 only in Abcg5/g8 (• /•) mice (Figure 6B and Supplementary Table 1), despite the similarly increased expression of NPC2 protein in the liver and bile of both genotypes (Figure 6A). These results indicate that secreted NPC2 requires ABCG5/G8 to stimulate the biliary secretion of cholesterol.

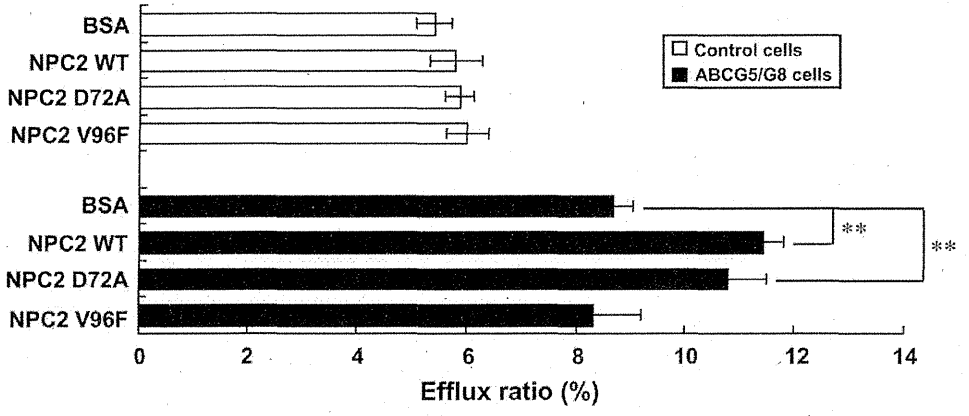

Figure 4. Comparison of stimulatory effects among NPC2 mutants on ABCG5/G8-mediated cellular cholesterol efflux. The effects of each NPC2 mutation on ABCG5/G8-mediated cholesterol efflux were analyzed using ABCG5/G8-overexpressing LLC-PK1 cells and recombinant NPC2 mutants. Cells were treated with transport buffer containing the indicated types of recombinant NPC2 or bovine serum albumin (BSA; 15 μg/mL) together with taurocholate (5 mmol/L), and the cholesterol efflux ratio was determined. This experiment was performed in 3 replicates, and results were confirmed by 2 independent sets of experiments. Each column and horizontal bar represents the mean • SD of 3 determinations. **Significantly different by analysis of variance followed by Dunnett's test (P • .01).

Positive Correlation Between NPC2 Protein and Cholesterol Levels in Human Bile

Following the observations that Npc2 levels in gallbladder bile are positively correlated with those in hepatic bile in mice (data not shown) and that ABCG5/G8 is expressed not only in hepatocytes but also in bile duct and gallbladder epithelial cells,[2,3] we further examined the relationship between NPC2 protein levels

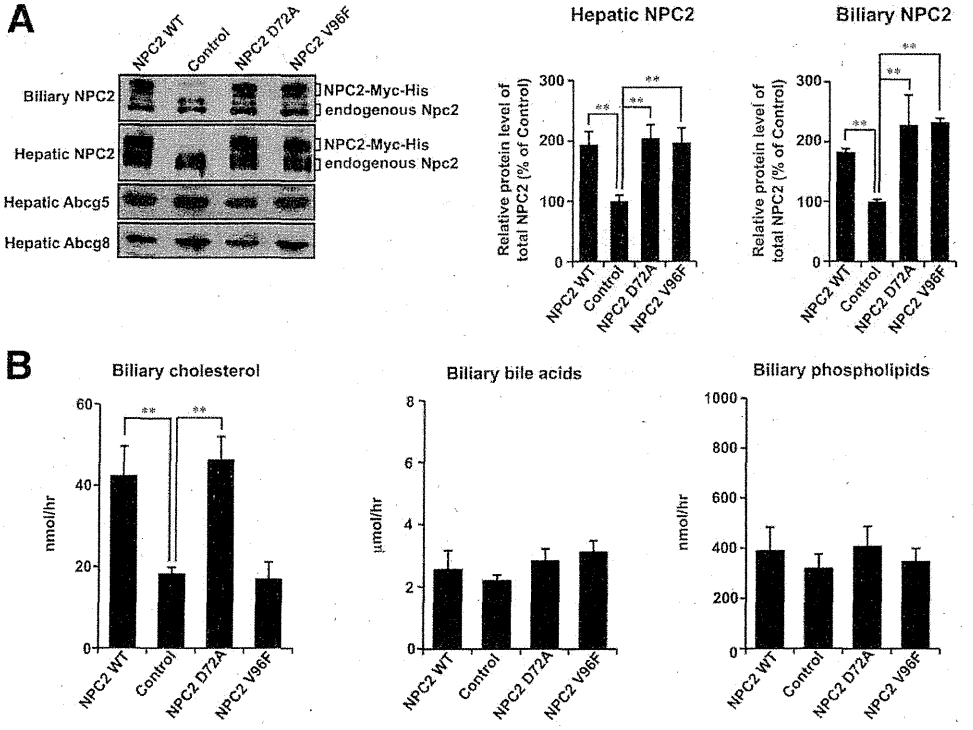

Figure 5. Comparison of biliary cholesterol secretion among mice overexpressing NPC2 mutants. Mice were infected with Ad-tTA (2 • 10^9 pfu) together with the indicated types of Ad-NPC2-Myc-His (2 • 10^9 pfu) or Ad-GFP control (2 • 10^9 pfu). Three days after adenovirus infection, liver and bile specimens were collected. (A) Expression levels of biliary NPC2, hepatic NPC2, Abcg5, and Abcg8 were analyzed by Western blot analyses as described in the legend for Figure 3A. (B) Secretion rates of biliary cholesterol, bile acids, and phospholipids were quantified as described in the supplementary material. Each column and vertical bar represents the mean • SE of 4 determinations. **Significantly different by analysis of variance followed by Dunnett's test (P • .01).

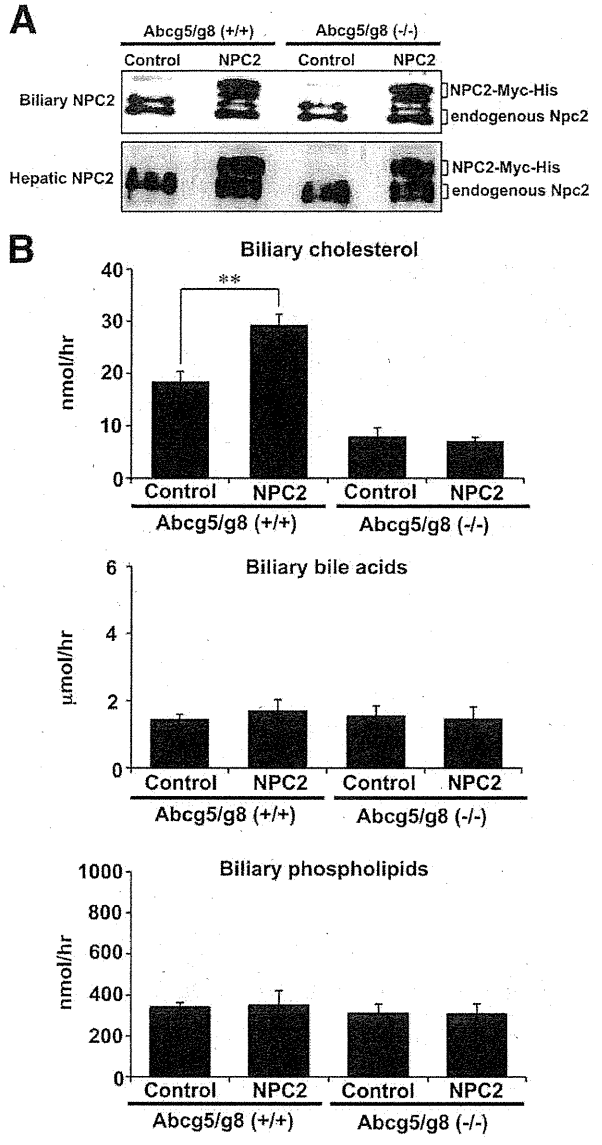

Figure 6. Effect of hepatic NPC2 overexpression in Abcg5/g8-deficient mice. Abcg5/g8(• /•) and Abcg5/g8(• /•) mice were infected with Ad-tTA (1 • 10^9 pfu) together with Ad-NPC2-Myc-His (1 • 10^9 pfu) or Ad-GFP control (1 • 10^9 pfu). Three days after adenovirus infection, liver and bile specimens were collected. (A) Expression levels of hepatic NPC2 and biliary NPC2 were analyzed by Western blot analysis. The Myc tag and 6• His tag increased the molecular weight of NPC2-Myc-His protein compared with endogenous Npc2 protein. (B) Secretion rates of biliary cholesterol, bile acids, and phospholipids were quantified as described in the supplementary material. Each column and vertical bar represents the mean • SE of 5 determinations. **Significantly different by analysis of variance followed by Dunnett's test (P • .01).

and cholesterol concentrations in human gallbladder bile. Figure 7 shows that biliary cholesterol concentration was positively correlated with biliary NPC2 content (R • 0.91, P • 1.3 • 10^{-6}). In addition, using partial correlation analysis, we confirmed that this positive correlation remained significant even if it was adjusted for the hydrophobicity of bile and the concentrations of biliary bile acids, phospholipids, and total proteins in each specimen (R • 0.70, P • .02). This positive correlation may be accounted for, at least partly, by the present finding that biliary NPC2 acts as a stimulator of cholesterol secretion into bile.

Discussion

The major contribution of the present study was to identify the physiologic function of biliary NPC2 as a positive regulator of cholesterol secretion into bile, and this novel function of NPC2 is accounted for by the stimulation of ABCG5/G8-mediated cholesterol efflux. It is known that the 2 half ABC transporters, ABCG5 and ABCG8, which act on the bile canalicular membrane,[23] are essential for the biliary secretion of cholesterol; Abcg5/g8-deficient mice have a significantly reduced biliary cholesterol output.[7]

In the present study, in addition to ABCG5/G8, NPC2 was found to be essential for cholesterol secretion, because hepatic Npc2-knockdown mice exhibit a significant reduction in biliary cholesterol secretion. Although it has been reported that Npc2-knockout mice have an accumulation of cholesterol and sphingomyelin in their livers[30] and this accumulation may affect biliary cholesterol secretion,[31] transient knockdown of hepatic Npc2 had little effect on hepatic levels of these lipids (Supplemen-

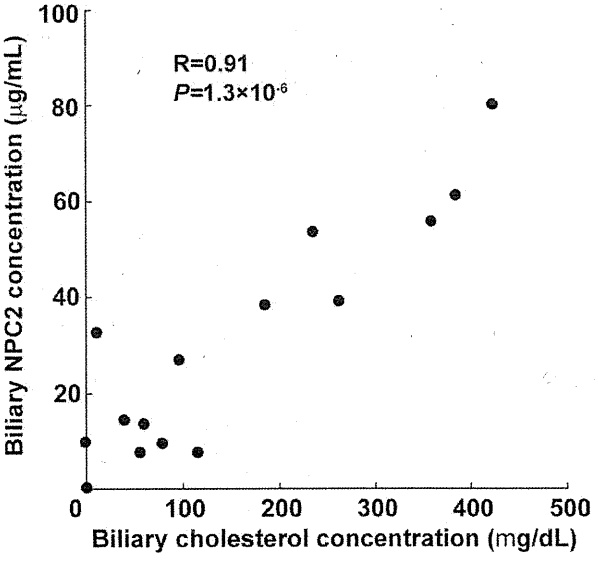

Figure 7. Correlation between cholesterol and NPC2 protein in human gallbladder bile. Five microliters of human gallbladder bile specimens was analyzed. Concentrations of biliary NPC2 were determined by Western blot analysis using recombinant NPC2 as a standard. Concentrations of cholesterol in each specimen were quantified as described in the supplementary material. Correlation analysis was performed using Pearson's method. R value represents Pearson's correlation coefficient.

tary Figure 2). In addition, bile acid composition and the hydrophobic index of bile, which can also affect the efficiency of biliary cholesterol secretion,[32] were unaffected by the transient knockdown and overexpression of hepatic and biliary NPC2 (Supplementary Figures 3 and 4). Together with the fact that NPC2 has little effect on the expression levels of hepatic Abcg5/g8 protein and biliary secretion of bile acids and phospholipids (Figures 1, 3, and 5), these results suggest that the reduction of biliary cholesterol secretion in Npc2-knockdown mice is a direct effect of reduced biliary (and hepatic) Npc2 protein.

Our in vitro studies showed that secreted NPC2 has the ability to act as a cholesterol acceptor in ABCG5/G8-mediated efflux (Figure 2C). However, considering that NPC2 binds cholesterol with 1:1 stoichiometry and that the cholesterol concentration in bile is approximately 0.03 to 10 mmol/L, which is estimated to be more than 100-fold higher than that of biliary NPC2 (0.3–3 μmol/L) (Figure 7), the significant reduction in biliary cholesterol secretion in Npc2-knockdown mice (Figure 1) cannot fully be accounted for by NPC2 action as a cholesterol acceptor. In general, most biliary cholesterol is dissolved in mixed micelles, which are mainly composed of bile acids and phospholipids, suggesting that these micelles are physiological solubilizers of cholesterol in bile. Based on the observation that the on-rate and off-rate of cholesterol from NPC2 are very rapid[16] and the fact that NPC2 can stimulate cholesterol transfer from one liposome to another,[33] we presume that NPC2 can accept cholesterol to accelerate the transfer of cholesterol from ABCG5/G8 proteins to micelles. Indeed, when ABCG5/G8-overexpressing cells were incubated with secreted NPC2 protein together with 5 mmol/L taurocholate and 200 μmol/L phosphatidylcholine, NPC2-mediated increases in the cholesterol efflux ratio (6.2% at 15 μg/mL NPC2 protein) (Figure 2D) were drastically higher than those when ABCG5/G8-overexpressing cells were incubated with NPC2 protein alone (0.6% at 15 μg/mL NPC2 protein) (Figure 2C). Within the physiologic range for NPC2 protein, the NPC2-mediated increase in cholesterol efflux was not saturated (Figure 2E), which is consistent with the linear correlation between NPC2 and cholesterol levels in human bile specimens (Figure 7). In addition, while cholesterol-preincubated NPC2 protein lost the ability to increase cholesterol efflux via ABCG5/G8, incubation with taurocholate restored the NPC2-mediated stimulation of cholesterol efflux (Figure 2F), suggesting that cholesterol can be transferred from NPC2 to taurocholate. These results support the hypothesis that NPC2 functions physiologically as a stimulator of cholesterol transfer from hepatocytes to biliary micelles, in addition to its function as a cholesterol acceptor.

In bile formation, cholesterol secretion is preceded by ABCB11-mediated bile acid secretion and ABCB4-mediated phospholipid secretion.[34] Because of its hydrophobicity, cholesterol requires mixed micelles, which are primarily composed of bile acids and phospholipids, to be solubilized in bile. Therefore, the functions of ABCB11 and ABCB4 are likely to be indirectly involved in the regulation of biliary cholesterol secretion.[34] In the present study, we showed that biliary secretion of bile acids and phospholipids was not affected by biliary NPC2 levels (Figures 1, 3, 5, and 6 and Supplementary Table 1), suggesting that NPC2 may only have a minor effect on the transport activity of ABCB11 and ABCB4. Taken together with the fact that NPC1L1-mediated cholesterol uptake was not affected by the presence of secreted NPC2 protein (Supplementary Figure 1), NPC2 is assumed to be a direct and selective stimulator of biliary cholesterol efflux.

Conversely, the transport activity of ABCB11 and ABCB4 may affect the activity of biliary NPC2, because an in vitro cholesterol efflux assay showed that the NPC2-mediated increase in cholesterol efflux was enhanced by the increased concentrations of taurocholate and phosphatidylcholine (Supplementary Figure 5). Additionally, in NPC2-overexpressing mice, the stimulatory effect of biliary NPC2 on cholesterol secretion was reduced by depletion of the endogenous bile acids pool, which was accompanied by a reduction in biliary bile acids and phospholipids (Supplementary Figure 6). Furthermore, this reduced activity of NPC2 was restored by tauroursodeoxycholate infusion, which can stimulate biliary secretion of bile acids and phospholipids[21] (Supplementary Figure 6). These results suggest that the activity of biliary NPC2 may be positively regulated by bile acids and phospholipids. Taken together with the observations that hepatic overexpression of ABCB11 increases biliary cholesterol secretion[35] and that Abcb4-deficient mice have reduced secretion of cholesterol into bile,[36] it appears that, through the secretion of biliary bile acids and phospholipids, ABCB11 and ABCB4 may play an important role in cholesterol secretion by indirectly increasing the activity of biliary NPC2, in addition to improving the cholesterol dissolution.

Our studies of Abcg5/g8-deficient mice (Figure 6) suggest that NPC2 stimulates ABCG5/G8-dependent cholesterol secretion but does not affect the ABCG5/G8-independent pathway.[37–39] Together with the established idea that biliary cholesterol secretion is primarily regulated by ABCG5/G8[40,41] and our findings that there is a strong positive correlation between biliary NPC2 and cholesterol levels in bile (Figure 7), NPC2- and ABCG5/G8-mediated biliary cholesterol secretion are assumed to share the same process, which would require an intimate functional association between these proteins. Selective regulation of cholesterol efflux by biliary NPC2 protein (Figures 1C, 3B, 5B, and 6B and Supplementary Table 1) may be accounted for by this functional interaction.

In conclusion, using a series of in vivo and in vitro analyses, we identified the physiologic function of biliary NPC2 as a positive regulator of biliary cholesterol secretion mediated by the ABCG5/G8-dependent pathway, which is also supported by the positive correlation between NPC2 protein and cholesterol levels in human bile. Because biliary cholesterol secretion is one of the key factors involved in maintaining whole-body cholesterol levels and is associated with hepatobiliary diseases,[2,3] our findings have both physiologic and pathophysiologic importance. This is the first report on the function of biliary NPC2 and provides new insights into the physiology of biliary cholesterol secretion.

Supplementary Material

Note: To access the supplementary material accompanying this article, visit the online version of Gastroenterology at www.gastrojournal.org, and at doi: 10.1053/j.gastro.2011.01.050.

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