Localization of Xenobiotic Transporter OCTN1/SLC22A4 in Hepatic Stellate Cells and Its Protective Role in Liver Fibrosis

doi: 10.1016/j.xphs.2016.02.023

Localizatoin of xenobiotic transporter OCTN1/SLC22A4 in hepatic stellate cells and its protective role in liver fibrosis

Yaliang Tang[1], Yusuke Masuo[1], Yoshio Sakai[2], Tomohiko Wakayama[2], Tomoko Sugiura[1], Ryuichi Harada[1], Azusa Futatsugi[1], Takuya Komura[2], Noritaka Nakamichi[1], Hirotaka Sekiguchi[3,4], Keita Sutoh[3,4], Koji Usumi[3], Shoichi Iseki[2], Shuichi Kaneko[2] and Yukio Kato[1]

[1]Faculty of Pharmacy and [2]School of Medicine, Institute of Medical, Pharmaceutical and Health Sciences, Kanazawa University, Kanazawa 920-1192, [3]Life Science Institute Co. Ltd., 2-6-6 Nihombashi-Horidomecho, Chuo-ku, Tokyo 103-0012 and [4]L•S Corporation Co. Ltd., 3-10-1 Ningyocho-Nihonbashi, Chuo-ku, Tokyo 103-0013, Japan

Corresponding author:

Prof. Yukio Kato, Ph.D

Faculty of Pharmacy, Institute of Medical, Pharmaceutical and Health Sciences, Kanazawa University, Kakuma-machi, Kanazawa 920-1192, Japan

Tel/Fax: (81)-76-234-4465 / Email: ykato@p.kanazawa-u.ac.jp

Number of figures and tables: 7 (6 figures and 1 table)

List of abbreviations: SLC, solute carrier; ECM, extracellular-matrix; NPC, nonparenchymal cells; NASH , non-alcoholic steatohepatitis; HSCs, hepatic stellate cells; OCTN1, Carnitine/organic cation transporter 1; ERGO, ergothioneine; ROS , reactive oxygen species; DMN, dimethylnitrosamine; ConA, concanavalin A; SIN-1, 3-morpholinosydnonimine; a-SMA, alpha-smooth muscle actin; 4NHE, 4-hydroxy-2-nonenal; [^{3}H]ERGO, [^{3}H]Ergothioneine; ERGO-d9, Deuterium-labeled L-ergothioneine; HFD, high-fat diet; MDA, malondialdehyde; SOD, superoxide dismutase; NOX, NADPH oxidase; siOCTN1, siRNA of OCTN1; siControl, control siRNA; TNF, tumor necrosis factor 1; TGF, transforming growth factor; IL, interleukin;

Keywords: Transporters; Membrane transporter; Organic cation transporters (OCT); Antioxidants; Induction

Conflict of interest: The authors report no conflict of interest.

Financial support: A Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan, a grant from Takeda Science Foundation (Osaka, Japan), a grant from Shimadzu Foundation (Kyoto, Japan) and a grant from Hoansha Foundation (Osaka, Japan)

Xenobiotic transporters play key roles in disposition of a certain therapeutic agents although limited information is available on their roles other than pharmacokinetic issues. Here, suppressive effect of multispecific organic cation transporter OCTN1/SLC22A4 on liver fibrosis was proposed in liver injury models. After injection of hepatotoxins such as dimethylnitrosamine (DMN) or concanavalin A, hepatic fibrosis and oxidative stress, evaluated in terms of Sirius red and 4-hydroxy-2-nonenal staining, respectively, were more severe in liver of *octn1/slc22a4* gene knockout (*octn1*$^{-/-}$) mice than that in wild-type mice. DMN treatment markedly increased α-smooth muscle actin (α-SMA) and F4/80, markers of activated stellate and Kupffer cells, respectively, in liver of *octn1*$^{-/-}$, but had less effect in wild-type mice. Thus, *octn1/slc22a4* gene deletion results in more sever hepatic fibrosis, oxidative stress and inflammation. DMN-treated wild-type mice showed increased Octn1 staining and hepatic concentration of its food-derived antioxidant ergothioneine. The up-regulated Octn1 was colocalized with α-SMA. Functional expression of Octn1 was demonstrated in activated human hepatic stellate cell lines, LI90 and LX-2. Provision of ERGO-rich feed ameliorated DMN-induced liver fibrosis and oxidative stress. Overall, Octn1 is up-regulated in activated stellate cells, resulting in increased delivery of its substrate antioxidant ergothioneine, and has protective effect against liver fibrosis.

Introduction

Liver fibrosis is a complex process that includes apoptosis of hepatocytes, infiltration of inflammatory cells, induction of inflammatory cytokines and proliferation of extracellular-matrix (ECM)-producing nonparenchymal cells (NPCs) [1, 2]. The resultant enhancement of ECM production can disrupt the balance between synthesis and degradation of ECM, progressively worsening the fibrosis. Liver fibrosis can silently progress, leading to liver cirrhosis and hepatocellular carcinoma, and is observed in various chronic hepatic diseases that develop following inflammatory stimuli such as viral infection, alcohol, drugs and non-alcoholic steatohepatitis (NASH) [3, 4]. Notwithstanding the pivotal role of fibrosis in hepatic diseases, few therapeutic agents that can directly suppress hepatic fibrosis are available.

Solute carrier SLC22A4 was first identified as carnitine/organic cation transporter 1 (OCTN1) in human fetal liver, but is expressed ubiquitously in organs [5, 6]. OCTN1/SLC22A4 is multispecific transporter which accepts various types of therapeutic agents as substrates [5, 6]. Metabolome analysis using cell lines transfected with *SLC22A4* gene *in vitro* and *octn1/slc22a4* gene knockout (*octn1*$^{-/-}$) mice *in vivo* have demonstrated that OCTN1 most efficiently transports a naturally occurring

food-derived antioxidant, ergothioneine (ERGO) [7, 8]. ERGO is highly hydrophilic, and its uptake through plasma membranes is primarily mediated by OCTN1, as evidenced by the fact that food-derived ERGO is detected at μM to sub-mM level in almost all organs in wild-type mice, but minimally detected in *octn1*$^{-/-}$ mice [8]. After oral ingestion, ERGO is efficiently absorbed in the gastrointestinal tract by OCTN1, then almost completely taken up by the liver and apparently not metabolized in the body [8, 9]. Thus, ERGO is mainly delivered to the liver after oral ingestion. Interestingly, ERGO is minimally taken up into liver parenchymal cells, but selectively taken up into NPCs [9]. This is consistent with the minimal immunoreactivity of hepatocytes with OCTN1 antibody [9]. Thus, OCTN1 could be unique drug transporter expressed in NPCs in the liver.

Hepatic NPCs contribute to production and secretion of multiple cytokines and inflammation-related substances, and are constantly exposed to oxidative stress [10, 11]. In particular, hepatic stellate cells (HSCs) are activated by oxidative stress to collagen-producing myofibroblasts, which promote liver fibrosis by producing ECM [12, 13]. Oxidative stress may also injure parenchymal cells, leading to further

progression of liver injury. It was recently reported that antioxidant and radical-scavenging enzymes can ameliorate hepatic fibrosis and injuries [14, 15]. Thus, proper control of the oxidative stress-antioxidant balance may inhibit the onset and/or progression of hepatic fibrosis.

Because ERGO is a stable antioxidant that can directly scavenge reactive oxygen species (ROS) [16, 17], it may have a protective role against oxidative stress after its uptake into NPCs. Nevertheless, pathophysiological role of ERGO and its transporter OCTN1 is minimally understood. Information on localization and function of the drug transporter in disease-related cells like NPCs may give an insight in novel drug delivery and pharmacotherapeutics. The purpose of the present study is to test the hypothesis that OCTN1 plays a role in suppression of oxidative stress and hepatic fibrosis by mediating delivery of its food-derived antioxidant substrate ERGO into hepatic NPCs. To examine this hypothesis, hepatic fibrosis models were constructed by two different hepatotoxins, dimethylnitrosamine (DMN) and concanavalin A (ConA) in wild-type and *octn1*$^{-/-}$ mice. DMN is widely used to produce model of hepatic fibrosis since the model can reproduce most of the features of human hepatic fibrosis [18, 19, 20]. On the other hand,

immunological responses are involved in pathogenesis of hepatic fibrosis in human, and administration of ConA leads to activation of immune system, resulting in hepatic fibrosis, which reproduces one of major pathology in human [21, 22]. NASH model was also constructed to evaluate OCTN1 expression during chronic liver injury.

Materials and methods

Materials

DMN and ConA were purchased from Wako Pure Chemical Industries (Osaka, Japan) and Sigma-Aldrich, Inc. (St. Louis, MO), respectively. N-Morpholinosydnonimine hydrochloride (SIN-1) was purchased from Dojindo Laboratories (Kumamoto, Japan). Mouse monoclonal antibodies against human α-smooth muscle actin (α-SMA) and 4-hydroxy-2-nonenal (4HNE), and rat monoclonal antibodies against F4/80 were obtained from Dako North America Inc. (Carpinteria, CA), Wako Pure Chemical Industries and Abcam Inc. (Cambridge, MA), respectively. [^{3}H]Ergothioneine ([^{3}H]ERGO; 293 Ci/mol) was purchased from Moravek Biochemicals (Brea, CA), diluted with transport buffer and stored at -30 ˚C. Its quality

was verified by checking the difference in the uptake between HEK293 cells stably transfected with *SLC22A4* gene and vector alone. Deuterium-labeled L-ergothioneine (ERGO-d9) was obtained from Tetrahedron (Vincennes, France). All other chemicals were commercial products of analytical grade.

Animals

Mice were used for all experiments at 6-8 weeks of age. The *octn1*$^{-/-}$ mice [8] and littermates were of a mixed genetic background (C57BL/6J and 129Sv/Ev), produced by intercrossing *octn1*$^{+/-}$ mice. They had free access to food (Sankyo-labo, Toyama, Japan), which contained approximately 0.2 μg ERGO/g chaw and water, except during the oral administration study of ERGO-rich feed. The present study was carried out in accordance with the Guide for the Care and Use of Laboratory Animals in Kanazawa University.

Models of Liver Fibrosis

Liver fibrosis was induced in female mice by intraperitoneal administration of

DMN at 10 μg/g body weight (1% w/v dissolved in saline solution) for the first three consecutive days of the week under light ether anesthesia as described previously [23]. The ConA-induced hepatitis model was constructed in male mice by injection of ConA (20 μg/g body weight) dissolved in PBS via the jugular vein once a week for 2 weeks [24]. Control mice received an equal volume of vehicle only. To develop NASH model, male mice were started at 7 weeks of age on an atherogenic high-fat diet (HFD) composed of cocoa butter, cholesterol, cholate, and corticotropin-releasing factor-1 (Oriental Yeast Co., Tokyo, Japan) for 14, 24 or 36 weeks.

Oral Administration of ERGO-rich Feed

Powdered extract of golden oyster mushroom containing 1% w/w ERGO (aminothioneine®, L•S Corporation, Tokyo) and ERGO-free feed (basal diet®, TestDiet, St. Louis, MO) which contains less than 0.01 μg ERGO/g chaw according to our LC-MS/MS determination. These were mixed in the ratio of 95:5 to prepare ERGO-rich diet containing 0.05% w/w of ERGO. C57BL/6J female mice were fed with ERGO-free or ERGO-rich diet for 8 weeks, and DMN treatment was performed in the final week.

Mice were then sacrificed, and liver was excised.

Histology and Immunohistochemistry

The liver samples were harvested from mice and immersion-fixed overnight in PBS containing 4% paraformaldehyde (PFA). The fixed liver was sequentially cryoprotected in 10, 20 and 30% sucrose in PBS, embedded in optimal cutting temperature compound and frozen at -30°C. Free-floating sections were cut at 10 μm using a Leica cryostat. Tissue sections were stained with picro-Sirius Red solution (0.1% Direct Red 80 dissolved in saturated picric acid) to visualize collagen deposition. The staining signals were visualized using a BIOREVO BZ-9000 microscope (Keyence, Osaka, Japan) and quantified using Dynamic cell count BZ-H1C software (Keyence). For quantification, the number of pixels showing the red color of stained collagen fibers was extracted after establishing a color threshold, and the percentage of those fibers in the liver was calculated by dividing the total red-colored area by the total area of the liver. Frozen sections were also heated at 92 ˚C for 15 min in 20 mM Tris buffer (pH 9.0) for antigen activation. Following successive pretreatments with 0.3% Tween

20/PBS for 20 min, 0.3% H_2O_2/PBS for 30 min and blocking solution (1% BSA, 5% skim milk and 1.5% goat serum in PBS) for 30 min, the sections were incubated overnight with anti-OCTN1, anti-α-SMA, anti-F4/80 or 4HNE antibody at 4˚C, followed by washing with PBS and further incubation with biotinylated anti-rabbit IgG, anti-mouse IgG or anti-rat IgG, repectively (Vector Laboratories, Burlingame, CA), for 1 h at room temperature. The immunoreaction product was visualized by incubating the sections successively with VECTASTAIN Elite ABC Standard Kit (Vector Laboratories) for 30 min and Peroxidase Substrate Kit including 3',3'-diaminobenzidine tetrahydrochloride (ImmPACT DAB, Vector Laboratories, Burlingame, CA) for approximately 10 sec. Finally, the specimens were mounted in VECTASHIELD mounting medium (Vector Laboratories), and examined with a BIOREVO BZ-9000 microscope.

Flow Cytometry of Isolated NPCs

Hepatic NPCs were isolated from two mice for each group as described previously [8], then fixed and permeabilized with kit reagents (BD Biosciences), and

stained with anti-OCTN1 and α-SMA antibodies for 30 min at room temperature. Cells were then washed with Perm/Wash Buffer (BD Biosciences) and incubated with secondary antibodies for 30 min at room temperature. Cells were further washed with Perm/Wash Buffer, and analyzed on a FACSCalibur flow cytometer (BD Biosciences).

Quantification of Malondialdehyde (MDA) and ERGO in mice organs

MDA concentration in the liver was determined using the TBARS Assay Kit (Cayman Chemical, Ann Arbor, MI) according to the manufacturer's recommendations. ERGO concentrations in blood, liver and kidney were determined using HPLC [8].

Cell Culture

LI90, human hepatic stellate cells, were obtained from Japanese Collection of Research Bioresources (Osaka, Japan) and grown in Dulbecco's modified Eagle's medium (Sigma-Aldrich) supplemented with 10% FBS, 1% sodium pyruvate, 100 unit/mL benzylpenicillin and 0.14 mg/mL streptomycin in a humidified atmosphere of 5% CO_2 in air at 37˚C. LI90 cells were activated with peroxynitrite donor SIN-1 (0.1

mM) for 24 hr in serum-free DMEM according to previous report [25]. For knockdown of OCTN1 with small interfering RNA (siRNA), cells were transiently transfected with siRNA (BONAC Corporation, Kurume, Japan) for OCTN1 by lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA) in Opti-MEM according to the manufacturer's instructions. Culture medium was replaced with DMEM supplemented with 10% FBS at 24 h after the transfection, followed by further culture for 48 hr. Another human hepatic stellate cell line, LX-2 was purchased from Merck Millipore Japan (Tokyo, Japan). Cells were grown in Dulbecco's modified Eagle's medium (Sigma-Aldrich) supplemented with 10% FBS, 100 unit/mL benzylpenicillin and 0.14 mg/mL streptomycin in a humidified atmosphere of 5% CO_2 in air at 37˚C. LX-2 cells were activated by treatment with 10 ng/mL TGF-β in 1% FBS DMEM for 24 hours according to previous report [26, 27].

Immunocytochemistry

Cells were grown on cover glass (12 mm □; thickness, 0.12-0.17 mm; Matsunami Glass Inc., Osaka, Japan) and fixed with 3% formaldehyde in PBS,

permeabilized with methanol for 5 min and incubated with PBS containing 3% skim milk and 5% goat serum for 30 min at room temperature. Cells were then incubated overnight with anti-OCTN1 and anti-α-SMA antibodies at 4˚C. Cells were washed with PBS and further incubated with Alexa Fluor 488 goat anti-rabbit IgG and Alexa Fluor 594 goat anti-mouse IgG (Molecular Probes Inc., Eugene, OR) for 30 min at room temperature. Attached cells were sealed onto the slides using Vectrashield mounting medium with DAPI (Vector Laboratories) and examined with a BIOREVO BZ-9000 microscope.

Uptake study

LI90 cells grown on collagen-coated 6-well plates were washed with the transport buffer and incubated with [^{3}H]ERGO (7.9 μM) for 60 min [9]. The cells were washed three times with ice-cold buffer and dried at room temperature, followed by addition of 0.2 M NaOH. For determination of radioactivity, the solubilized sample was neutralized with 5 M HCl, and the associated radioactivity was measured with a liquid scintillation counter, LSC-5100 (Aloka, Tokyo, Japan) with Clearsol I (Nacalai Tesque,

Inc., Kyoto, Japan) as the scintillation fluid. LX-2 cells were grown on poly-L-lysine coated 12-well plates, washed with the transport buffer and incubated with ERGO-d9 (10 µM) for 120 min. A mixture of ERGO-d9 (10 µM) and unlabeled ERGO (0-300 µM) was incubated for 120 min for kinetic analysis. The cells were washed three times with ice-cold buffer, dried at room temperature and burst with distilled water. ERGO-d9 was quantified by LC-MS/MS. Uptake was normalized both by cellular protein and medium concentration, and represented as distribution volume (µL/mg protein).

LC-MS/MS analysis

Quantification of d9-ERGO was performed using a triple quadruple mass spectrometer with electrospray ionization (ESI) coupled to a liquid chromatography system (LCMS-8040; Shimadzu, Kyoto, Japan). Chromatography was performed by means of a step-gradient elution (flow rate, 0.4 ml/min) as follows: 0–0.5 min, 5% A/95% B; 0.5–2.0 min, 5% A/95% B to 25% A/75% B; 2.0–3.0 min, 25% A/75% B to 70% A/30% B; 3.0–4.0 min, 70% A/30% B; 4.0–4.1 min, 70% A/30% B to 5% A/95% B; 4.1-6.5 min, 5% A/95% B (A, water containing 0.1% formic acid; B, acetonitrile

containing 0.1% formic acid) using an SeQuant® ZIC®-cHILIC Column (100 Å, 3 μm, 2.1 mm × 150 mm; Merck) at 40 °C. MS–MS detection was performed in positive electrospray ionisation mode using multiple reaction monitoring (MRM) acquisition mode. The MRM was set at 239.15 to 127.00 m/z for d9–ERGO, 253.10 to 159.10 for cimetidine (internal standard).

Quantitative RT-PCR

Tissue samples were immediately transferred to RNA*later* (Sigma-Aldrich, St. Louis, MO) and incubated at 4°C overnight, followed by preservation at -30°C until RNA extraction. Total RNA was extracted according to the standard ISOGEN procedure (Nippon Gene, Toyama, Japan) and used for cDNA synthesis with oligo $(dT)_{12\text{-}18}$ primer, deoxy nucleotide triphosphate mix, RT buffer and MultiScribe™ reverse transcriptase (Applied Biosystems, Foster City, CA). The individual cDNA species were amplified in a reaction mixture containing a cDNA aliquot, relevant sense and antisense primers as shown in supplementary table 1, and THUNDERBIRD SYBR qPCR Mix (TOYOBO, Osaka, Japan). An Mx3005P real-time PCR apparatus (Agilent Technologies, Santa

Clara, CA) was employed. PCR reactions were initiated by template denaturation at 95 °C for 15 min, followed by 40 cycles of amplification (denaturation at 95°C for 10 s, and primer annealing and extension at 60°C for 30 s). Data were analyzed by delta-delta-Ct method.

Data Analysis

The results were expressed as mean ± SEM. The statistical significance of differences was determined by means of Student's *t*-test or one-way ANOVA with the Bonferroni/Dunn test, and $p < 0.05$ was regarded as denoting a significant difference.

Results

Marked fibrosis and oxidative stress in liver of octn1$^{-/-}$ mice after treatment with DMN or ConA

Hepatic fibrosis models were constructed in both wild-type and *octn1*$^{-/-}$ mice by injection of DMN or ConA in order to clarify the possible role of Octn1 in liver fibrosis. The degree of fibrosis and oxidative stress were assessed in liver sections in

terms of Sirius red staining and 4HNE staining, respectively. At one week after DMN treatment, marked Sirius red staining was observed in *octn1*$^{-/-}$, while weaker staining was observed in wild-type mice (Fig. 1A, 1B). 4HNE staining was also marked in *octn1*$^{-/-}$, but weaker in wild-type mice (Fig. 1C, 1D). The effect of Octn1 on fibrosis and oxidative stress was also examined in another fibrosis model prepared with ConA. At two weeks after ConA treatment, both Sirius red and 4HNE staining was more marked in *octn1*$^{-/-}$ than in wild-type mice (Fig. 1E, 1F, 1G, 1H). In both experiments, vehicle controls showed minimal staining (Fig. 1). To confirm that oxidative stress is higher in *octn1*$^{-/-}$ mice, gene expression of oxidative stress markers, superoxide dismutase (SOD) 1 and NADPH oxidase (NOX) 4, was examined. Expression of both genes at 1 week after DMN treatment was higher in *octn1*$^{-/-}$ than in wild-type mice (Table 1).

To further characterize the difference in liver fibrosis between wild-type and *octn1*$^{-/-}$ mice, activated HSC and Kupffer cells were identified by staining with anti-α-SMA and F4/80 antibodies, respectively. At 1 week after DMN treatment, gene expression of α-SMA tended to be higher in *octn1*$^{-/-}$ than in wild-type mice, although this difference was not statistically significant (Table 1). On the other hand, at 3 weeks

after DMN treatment, both α-SMA and F4/80 were detected and were more strongly stained in liver of *octn1*$^{-/-}$ than wild-type mice (Fig. 2B, 2C). At 3 weeks after DMN treatment, Sirius red staining was more intense in *octn1*$^{-/-}$ than in wild-type mice (Fig. 2A), though the difference was not large. Sirius red staining was marked in both wild-type and *octn1*$^{-/-}$ mice at 4 and 5 weeks after DMN treatment, and at 4 weeks after ConA treatment, but the difference between the two strains was minimal at these time points (data not shown). Oxidative stress, assessed as MDA, tended to be increased in the liver of *octn1*$^{-/-}$ mice (Fig. 2D).

Up-regulation of OCTN1 in activated HSC

Since Octn1 accepts ERGO as a substrate, the higher oxidative stress in *octn1*$^{-/-}$ may be compatible with the lower ERGO concentration in these mice. Food-derived ERGO in the liver was measured; it was detected in wild-type mice, but was under the detection limit in *octn1*$^{-/-}$ (Fig. 3D). Interestingly, ERGO concentration in the liver, but not kidney, after DMN treatment of wild-type mice was significantly higher than that in the wild-type control (Fig. 3D). Therefore, immunostaining with

Octn1 antiserum [9] was performed using sequential liver slices at three weeks after DMN treatment. Octn1 staining was increased after DMN treatment, compared with the control (Fig. 3A). The Octn1 staining in control mice was minimally detected in the present immunostaining condition (Fig. 3A). The signal pattern of Octn1 in DMN-treated mice resembled that of α-SMA, rather than F4/80 (Fig. 3B). Nonspecific binding of the Octn1 antiserum was checked by comparing signals in the liver sections of wild-type and *octn1*$^{-/-}$ mice. Although weak nonspecific signal was observed in *octn1*$^{-/-}$ mice, the signal was much more obvious in wild-type mice. To examine the localization of Octn1 in activated HSC, we performed flow-cytometric analysis using anti-OCTN1 antibody. The population of NPCs positive for both Octn1 and α-SMA after DMN treatment was higher than that in the control (50.2 vs 30.7%, Fig. 3C), suggesting up-regulation of OCTN1 in activated HSCs.

OCTN1 is functionally expressed in human hepatic stellate cell lines LI90 and LX-2

To further support the localization of OCTN1 in activated HSCs, functional expression of OCTN1 in LI90 was assessed in terms of uptake of [^{3}H]ERGO. The

uptake was time-dependent and reduced in the presence of an excess of unlabeled ERGO (Fig. 4A). Next, we investigated the effect of knockdown of OCTN1 on the expression and function of OCTN1 in LI90. After OCTN1 siRNA (siOCTN1) treatment, gene expression of OCTN1 was almost completely blocked with a concomitant reduction in [^{3}H]ERGO uptake, compared with the situation after treatment with control siRNA (siControl, Fig. 4B). Immunocytochemical analysis confirmed the expression of α-SMA (Fig. 4C, bottom) and OCTN1 (Fig. 4C, top) in LI90. HSCs are known to be activated by fibrosis-related cytokines such as tumor necrosis factor (TNF)-α and transforming growth factor (TGF)-β. Thus, we examined the effect of these cytokines on the expression and function of OCTN1 in LI90 cells. TNF-α significantly increased gene expression of *SLC22A4* in LI90 cells whereas TGF-β minimally showed such effect (Fig. 4D). Uptake of [^{3}H]ERGO in LI90 cells was also increased by treatment with TNF-α, but not TGF-β (Fig. 4E). To further investigate whether OCTN1 is functionally expressed in HSCs, another human HSC line LX-2 was used. ERGO-d9 was taken up into LX-2 cells in time-dependent manner (Fig. 4F, top). Concentration dependent uptake of ERGO was also examined, yielding Km and V_{max} values of 194

μM and 90.7 pmol/mg protein/min, respectively (Fig. 4F, bottom). Expression of OCTN1 gene product was also confirmed in LX-2 cells according to immunocytochemical analysis (Fig. 4G). Direct effect of ERGO on oxidative stress markers was also evaluated in LI90 cells. Gene expression of NOX4 in LI90 was significantly reduced by treatment with ERGO whereas that of α-SMA tended to be reduced with ERGO (Fig. 4H).

Oral intake of ERGO-rich feed suppressed oxidative stress and liver fibrosis

The up-regulation of Octn1 during liver fibrosis (Figs. 3) would be consistent with an ameliorative effect of orally administered antioxidant ERGO, which would be delivered to activated HSCs via Octn1. To investigate the effect of ERGO *in vivo*, mice were given an ERGO-rich diet. Liver fibrosis was induced by DMN at 7 weeks after the start of this diet, and then Sirius red staining and immunostaining of 4HNE were examined. Ingestion of the ERGO-rich diet decreased Sirius red staining, compared with normal diet (Fig. 5A). Quantification revealed significantly weaker Sirius red staining after the ERGO-rich diet than after the control diet (Fig. 5B). 4HNE staining

was also reduced, compared with that in mice given the control diet (Fig. 5C, 5D). To support the pharmacological effect of ERGO, its concentrations in blood and liver were measured in the same experiments. ERGO concentration in blood of mice fed the ERGO-rich diet gradually increased, and at the end of the experiment, ERGO concentration in blood and liver was more than 50-fold higher than in the control group (Fig. 5E).

Up-regulation of Octn1 in the liver of NASH model mice

To further understand the pathological relevance of Octn1, we constructed HFD-induced NASH model mice. Sirius red staining was markedly elevated in the liver at 14, 24 and 36 weeks after the start of HFD treatment, compared with the control (Fig. 6A). Immunostaining of Octn1 in the liver was also obvious (Fig. 6B).

Discussion

Here, we proposed a protective role of xenobiotic transporter Octn1 in two independent hepatic fibrosis models in mice: Sirius red staining in the liver of *octn1*$^{-/-}$

was greater than that in wild-type mice at 1 and 2 weeks after treatment with DMN (Fig. 1A, 1B) and ConA (Fig. 1E, 1F), respectively, indicating a higher degree of hepatic fibrosis in *octn1*$^{-/-}$. The protective role of Octn1 against liver fibrosis was further supported by the stronger staining of α-SMA (Fig. 2B) and F4/80 (Fig. 2C), which represent activated HSC and Kupffer cells, respectively, in liver of *octn1*$^{-/-}$, compared with wild-type mice, at 3 weeks after DMN treatment. At this time, hepatic fibrosis was severe in *octn1*$^{-/-}$, but was also present in wild-type mice (Fig. 2A). Thus, OCTN1 may influence the early stage (~1 week after DMN treatment) of liver fibrosis. Association of Octn1 with hepatic fibrosis was further confirmed by the up-regulation of this transporter in the liver after DMN treatment (Fig. 3A, 3C). The increase in Octn1 gene product with increase in fibrosis stage was also observed in HFD-induced NASH model mice (Fig. 5A, 5B). Up-regulation of Octn1 is consistent with the increase in hepatic concentration of food-derived Octn1 substrate ERGO in the liver after DMN treatment in wild-type mice (Fig. 3D).

Hepatic fibrosis is known to primarily occur via activation of HSCs, which produce ECMs such as collagen. Therefore, we next examined possible localization of

OCTN1 in HSCs. Immunostaining of Octn1 resembled that of α-SMA, but not F4/80, in liver sections (Fig. 3B). Colocalization of Octn1 with α-SMA was confirmed in flow-cytometric analysis of hepatic NPCs isolated from the liver of DMN-treated mice (Fig. 3C). OCTN1 was also colocalized with α-SMA in two different activated human HSC lines, LI90 and LX-2 (Fig.4C, 4G). Functional expression of OCTN1 in HSCs was supported by the observation of time-dependent uptake of [^{3}H]ERGO and ERGO-d9 in LI90 and LX-2 cells, respectively (Fig. 4A, 4F), and its decrease in response to siOCTN1 treatment (Fig. 4B). Thus, OCTN1 is possibly localized in HSCs, and the increase in the population of Octn1/α-SMA double-positive cells after DMN treatment (Fig. 3C) indicates that Octn1 is up-regulated in activated HSCs. These results support the protective role of OCTN1 in experimental fibrosis (Figs. 1 and 2) possibly linked to the functional expression and up-regulation of this transporter in activated HSCs.

The progression of liver fibrosis is promoted by oxidative stress [12, 13], although this is likely to be just one of multiple processes involved. In the present study, oxidative stress in the liver of *octn1*$^{-/-}$ was much greater than that in wild-type mice at 1 and 2 weeks after treatment with DMN (Fig. 1C, 1D) and ConA (Fig. 1G, 1H),

respectively, supporting the association of oxidative stress with progression of fibrosis. The increased level of oxidative stress in *octn1*$^{-/-}$ may suggest that OCTN1 plays a protective role against oxidative stress. OCTN1 has been characterized as a unique transporter expressed in NPCs [9] and transporting the food-derived antioxidant ERGO [7, 8]. The major source of ERGO is fungi and mycobacteria, and endogenous biosynthesis is minimal in mammals [16, 17]. Hepatic extraction of ERGO is almost complete, meaning that essentially all orally absorbed ERGO is taken up by the liver [9]. Therefore, OCTN1-mediated uptake of ERGO in hepatic NPCs may explain the protective role of OCTN1 in HSCs against oxidative stress. Actually, food-derived ERGO was detected in the liver of wild-type mice, but not in *octn1*$^{-/-}$ mice (Fig. 3D), which exhibited more severe 4HNE staining (Fig. 1). Additionally, orally administered ERGO-rich feed exerted anti-fibrotic effect in DMN-treated mice (Fig. 5). OCTN1 is multispecific transporter which accepts various types of organic cations as substrates. Therefore, OCTN1-mediated ERGO uptake and anti-fibrotic effect may imply that OCTN1 is a novel target to deliver therapeutic agents into HSCs, thereby protecting hepatic fibrosis. Hepatic fibrosis is a progressive disease, leading to cirrhosis and liver

cancer. Elevation of hepatic enzyme activities in serum is not obvious in relatively mild chronic liver injury, and therefore, establishment of techniques for early diagnosis of liver fibrosis is also required. Measurements of OCTN1 and/or its substrates may be available for early diagnosis of liver fibrosis. In addition, since ERGO-rich diet ameliorated liver fibrosis (Fig. 5), ERGO may have value as a protective agent against liver fibrosis.

It should be noted that ERGO is included in normal diets and is therefore usually present at a substantial concentration in the liver under the normal diet condition (Fig. 3D). This may hinder direct demonstration of the amelioration of liver fibrosis by ERGO *in vivo*. In order to examine the anti-fibrosis effect of exogenously administered ERGO, therefore, we screened several brands of mouse chaw to find an ERGO-free diet in the present study. Indeed, mice in the control group given this ERGO-free diet showed a decrease of ERGO concentration in blood from 13.7±0.5 at the start of the experiment to 1.34±0.14 μg/mL at 8 weeks on the ERGO-free diet. At this time point, mice on the ERGO-rich diet showed a blood ERGO concentration of 183±3 μg/mL (Fig. 5E). This large difference in exposure to ERGO supports the idea that the

anti-fibrosis and oxidative stress effects (Fig. 5A-5D) resulted at least partially from ingestion of ERGO, although we cannot rule out possible effects of other components included in the ERGO-rich diet due to the supplementation with mushroom extract. It has been reported that mushroom extract suppresses hepatic fibrosis [28, 29], but little is known about the mechanism. Considering that ERGO is present at high levels in fungi and mushrooms [16, 17], the beneficial effect of mushroom extract could be largely due to ERGO.

Gene expression of *SLC22A4* is induced by treatment with TNF-α, but not other inflammatory cytokines such as interleukin (IL)-1β, 6 and interferon-γ in human intestinal epithelial cells Caco-2 [30], whereas both TNF-α and IL-1β induce *SLC22A4* gene expression in human fibroblast-like synoviocyte cell line MH7A [31]. In the present study using LI90, both OCTN1 mRNA and [^{3}H]ERGO uptake were significantly increased by TNF-α, but not TGF-β (Fig. 4D, 4E). TNF-α is a key mediator in hepatic fibrosis [33] and, therefore, may serve as a trigger to induce *SLC22A4* gene expression during liver fibrosis, leading to up-regulation of *SLC22A4* gene product in activated HSCs. On the other hand, TGF-ß is also an important cytokine

for inducing collagen production in hepatic fibrosis [33]. Therefore, TGF-ß may not be directly associated with the up-regulation of OCTN1, but may play a distinct role in liver fibrosis.

ERGO exerts its antioxidant activity at sub-mM to mM concentrations [34-36], whereas the hepatic ERGO concentration in control and DMN-treated mice was at most 100~200 μM (Fig. 3D). However, considering that OCTN1 is almost exclusively expressed in HSCs (Fig. 3B) and HSCs comprise only a minor population among total hepatic cells, it is plausible that the ERGO concentration in HSCs could be at mM level. Nevertheless, it is not clear whether loss of the antioxidant effect of ERGO alone can explain the higher liver fibrosis and higher oxidative stress found in *octn1*$^{-/-}$ mice. We should consider the possibility of other protective effect(s) exerted by ERGO on liver fibrosis. For example, NADPH oxidase (NOX) is an important source of ROS, which could be involved in progression of liver fibrosis [37-39]. NOX4, an isoform of NOX, is highly expressed in hepatocytes and HSCs [40]. In the present study, mRNA for NOX4 in the liver of *octn1*$^{-/-}$ was significantly higher than in wild-type mice after DMN treatment (Table 1). In addition, gene expression of NOX4 in LI90 was significantly

reduced by treatment with ERGO (Fig. 4H). These results may mean that one of the candidate target genes for ERGO is NOX4, and down-regulation of this gene may be involved in the reduction of oxidative stress and suppression of liver fibrosis.

In conclusion, OCTN1 plays a protective role in liver fibrosis models. This probably results from up-regulation in activated HSCs and increase in uptake of ERGO at an early stage of liver fibrosis. OCTN1 may be thus a novel target for the delivery of therapeutic agents into activated HSCs.

Acknowledgement

We thank Ms Lica Ishida for technical assistance and Ms Shiori Kawabata for performing cell biology studies.

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Figure legends

Fig. 1. Fibrosis and oxidative stress in liver of wild-type and *octn1*$^{-/-}$ mice treated with DMN and ConA

Liver sections were obtained 1 and 2 weeks after treatment with DMN (A-D) or ConA (E-H), respectively, and stained with Sirius red (A, B, E, F) and anti-4HNE antibody (C, D, G, H). The control represents treatment with vehicle alone. Representative results from at least three individual mice in each group are shown (A, C, E, G). The histochemical staining was subjected to image analysis, and the area occupied by the signal was quantified and shown (B, D, F, H, mean±SEM, n=3). *Significantly different from wild-type mice treated with DMN (p<0.05).

Fig. 2 Activation of hepatic stellate and Kupffer cells in the liver of wild-type and *octn1*$^{-/-}$ mice after DMN treatment

Liver tissues were obtained from wild-type and *octn1*$^{-/-}$ mice 3 weeks after treatment with DMN. The liver sections were then stained with Sirius red (A), and anti-α-SMA (B) and anti-F4/80 (C) antibodies. Representative results from at least three individual

mice in each group are shown. (D) Concentration of MDA in liver was determined (mean±SEM, n=4-8). The control represents treatment with vehicle alone.

Fig. 3 Localization of OCTN1 in activated stellate cells and its up-regulation after DMN treatment.

(A) Liver sections were obtained from wild-type mice at 3 weeks after treatment with DMN or vehicle alone (Control), and stained with anti-OCTN1 antibody. Representative results from seven DMN-treated and five control mice are shown. (B) Serial slice sections were obtained from wild-type mice at 3 weeks after DMN treatment and immunostained with anti-OCTN1, α-SMA and F4/80 antibodies (OCTN1-positive areas are surrounded by circles). (C) Isolated hepatic NPCs were incubated with anti-OCTN1 and α-SMA antibodies, and analyzed by flow cytometry to quantify the population of OCTN1 and α-SMA double-positive cells to total NPCs (%). The whole experiment was repeated three times, and typical data are shown. (D) Concentration of ERGO in liver and kidney was determined (mean±SEM, n=4-8). *Significantly different from the control (p<0.05). N.D., under the detection limit (< 1.5 μg/g tissue).

Fig. 4 Functional expression of OCTN1 in LI90 (A-E, H) and LX-2 (F, G).

(A) Uptake of [^{3}H]ERGO was measured in LI90 cells in the absence (○) or presence (●) of 200 μM unlabeled ERGO. The experiment was repeated three times, and typical data representing mean±SEM of three wells are shown. (B) Gene expression of OCTN1 and uptake of [^{3}H]ERGO were measured after transfection of siRNA for OCTN1. Data represent mean±SEM of three wells. (C) Immunocytochemical analysis was performed for both OCTN1 and α-SMA. The experiment was repeated three times, and typical results are shown. (D, E) Gene expression of *SLC22A4* (D) and uptake of [^{3}H]ERGO (E) were measured after treatment with TNF-α (10 ng/mL) or TGF-ß (1 ng/mL) for 48 hr. Data represent mean±SEM of three wells. *Significantly different from control (p < 0.05). (F) Time- and concentration-dependent uptake of ERGO was measured in LX-2 cells. The experiment was repeated twice, and the data represent mean±SEM of three wells. (G) LX-2 cells were activated with TGF-β (10ng/mL) for 24 hr, and both OCTN1 and α-SMA were immunostained. The experiment was repeated three times, and typical results are shown. (H) Gene expression of NOX4 and α-SMA was examined at 48 hours

after treatment with 0 or 200 μM ERGO in LI90 cells. Data represent mean±SEM of three wells.

Fig. 5 Protective effect of ERGO-rich feed against liver fibrosis and oxidative stress.

Wild-type mice were fed ERGO-free (ERGO(-)) or ERGO-rich (ERGO(+)) diet for 8 weeks, and treated with DMN or vehicle (control) for the last one week. Liver sections were stained with Sirius red (A) and anti-4HNE antibody (C), and quantitative values are shown in panels B and D, respectively. Each value represents the mean ± SEM of 4 to 6 mice in a single experiment. *Significantly different from control mice fed ERGO-free diet. #Significantly different from DMN-treated mice fed ERGO-free diet. Panel E shows the ERGO concentration in blood and liver (mean±SEM, n=10~12).

Fig. 6 Histochemical staining of liver sections obtained from NASH model mice.

Liver sections were obtained from mice fed either a normal diet (control) or HFD, and stained with Sirius red (A) or anti-OCTN1 antibody (B). 3 mice for each group were

used in the experiment.