Research Article

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Sustained repression and translocation of Ntcp and expression of Mrp4 for cholestasis after rat 90% partial hepatectomy

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Background & Aims: To clarify the mechanism of persistent cholestasis after massive hepatectomy, the relationship between such cholestasis and the expression and localization of organic anion transporters for bile acids was examined in a rat model.

Methods: Male Sprague–Dawley rats were subjected to 90% hepatectomy, and tissues were harvested at 0, 1, 3, and 7 days for microarray analysis, quantitative real-time polymerase chain reaction (RT-PCR), Western blotting, and immunohistochemistry to examine the expression of multidrug resistance protein 4 (Mrp4), bile salt export pump (Bsep), and sodium-dependent taurocholate cotransporting polypeptide (Ntcp).

Results: Persistently elevated levels of serum bile acids were observed at days 3 and 7. RT-PCR and Western blotting indicated that the expression of Mrp4, a bile acid export pump located in the basolateral membrane, was increased at day 3. The expression of Ntcp, a transporter used to uptake bile acids from the sinusoids, was significantly decreased throughout the period. The levels of Bsep, an export pump localized to the canalicular membrane, were unchanged. Immunohistochemistry revealed the localization of Mrp4 and Bsep in the basolateral and canalicular membranes, respectively. On the other hand, at days 3 and 7, Ntcp was localized in the cytoplasm and was hardly detected in the basolateral membrane.

Conclusions: These results suggested that the sustained repression and translocation of Ntcp and the expression of Mrp4 at the basolateral membrane seem to be responsible for the high blood bile acids levels after massive hepatectomy.

Keywords: Ntcp; Mrp; Bsep; Translocation; Bile acids; Cholestasis; Liver regeneration; Rat hepatectomy.

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Abbreviations: Mrp, multidrug resistance protein; Bsep, bile salt export pump; Ntcp, sodium-dependent taurocholate cotransporting polypeptide; Oatp1, sodium-independent organic anion transporting polypeptides; Ost, organic solute transporter; RT-PCR, real-time polymerase chain reaction; Cyp, cytochrome P450; Pcna, proliferating cell nuclear antigen.

Introduction

In the modern setting of hepatobiliary surgery and adult living donor liver transplantation, a small remnant liver volume is of major concern because this is associated with cholestasis and mortality [1,2]. Cholestasis refers to the suppression of bile secretion, which causes biliary constituents to be retained within hepatocytes and the systemic circulation. In particular, bile acids, the major components of bile, can damage cell membranes because of their detergent properties [3]. It has been suggested that when the intracellular concentration of bile acids exceeds the cytosolic binding capacity, the acids induce apoptosis and necrosis by damaging mitochondria [4]. Therefore, it is crucial to clarify the regulation of the bile acid transport system in small remnant livers.

Hepatocytes efficiently extract bile acids from sinusoidal blood plasma and excrete them into bile canaliculi using organic anion transporters. The hepatic uptake of bile acids from the sinusoids is mainly mediated by the sodium-dependent taurocholate cotransporting polypeptide (Ntcp, gene symbol *Slc10a1*) [5] and the sodium-independent organic anion transporting polypeptide (Oatp1, gene *Slc21a1*) [6], which are located at the basolateral membrane [7,8]. The extracted bile acids are excreted into the bile canaliculi by adenosine triphosphate (ATP) -dependent canalicular transporters, such as the bile salt export pump (Bsep, gene *Abcb11*) [9] and the multidrug resistance protein 2 (Mrp2, gene *Abcc2*) [10]. In addition to these basolateral uptake and canalicular export systems, the basolateral membrane also contains efflux pumps, such as the multidrug resistance protein 3 (Mrp3, gene *Abcc3*), the multidrug resistance protein 4 (Mrp4, gene *Abcc4*), and the organic solute transporter (Ostα–Ostβ, gene *Ostalpha–Ostbeta*), which are normally expressed at very low levels and whose expression levels are upregulated during obstructive cholestasis [11–13].

After partial hepatectomy, the remnant liver is exposed to high bile acid flux, which may aggravate liver injuries. Liver transporters are suggested to play a role in protecting the regenerating liver [14,15] because they are determinants of the intracellular bile acid concentration [16]. However, little is known about how these transporters behave in proliferating hepatocytes and are involved in persistent cholestasis during the recovery phase after massive hepatectomy. We previously reported that

hyperbilirubinemia after 90% hepatectomy was associated with Mrp2 repression and Mrp3 expression [17]. Therefore, our aim in this study was to investigate the regulation and tissue localization of the main transporters for bile acids such as Ntcp, Bsep, and Mrp4, in order to elucidate the association between these transporters and persistent cholestasis. We provide evidence for the sustained repression and translocation of Ntcp and the expression of Mrp4 after massive hepatectomy, which may be linked to the high serum bile acid levels.

Materials and methods

Animals and animal treatment

Male Sprague–Dawley rats aged 6 to 7-weeks purchased from Charles River Japan, Inc. (Tsukuba, Japan) underwent 70% or 90% hepatectomy as described previously [18,19], with 100% survival rates. The rats were sacrificed at days 0, 1, 3, or 7, and blood and tissues were obtained for analysis. We selected the day 0 rats; i.e., those representing the status just before hepatectomy, as controls. Serum total bile acids were measured using an enzymatic assay with Enzabile Auto kits (Sekisui Medical Co., Ltd., Tokyo, Japan). This study was carried out in accordance with the *Guidelines for Animal Experimentation* of Hirosaki University.

Microarray analysis

Total RNA was extracted from frozen liver samples at days 0, 1, 3, and 7 after 90% hepatectomy with TRIzol reagent (Invitrogen, Carlsbad, CA). Equal amounts of RNA from three individual livers were combined and ten micrograms of RNA were used for biotin-labeled complementary RNA (cRNA). The labeled and fragmented cRNA was subsequently hybridized to the GeneChip Rat Genome 230 2.0 Array (Affymetrix, Santa Clara, CA). Labeling, hybridization, image scanning, and data analysis were performed at Takara Bio Inc. (Shiga, Japan).

Quantitative real-time polymerase chain reaction (RT-PCR)

Complementary DNA (cDNA) was reverse-transcribed from one microgram of total RNA using the Omniscript RT kit (Qiagen, Tokyo, Japan). A MiniOpticon Detection System (Bio-Rad Laboratories, Hercules, CA) and SYBR Green Supermix (Bio-Rad Laboratories) were used for the quantitation of specific messenger RNA (mRNA). The amplification of *ubiquitin C* cDNA was performed to standardize the levels of the target cDNA. Gene-specific primers were designed according to known rat sequences using the Primer3Plus software (Supplementary Table 1). No non-specific PCR products, as detected by melting temperature curves, were found in any case. After normalizing the expression of the target gene to *ubiquitin C* expression, the level of the expressed mRNA in each sample was expressed relative to the control values.

Western blot analysis

Crude liver membranes were prepared according to the method of Gant *et al.* [20] and preparations (100 μg protein each) were dissolved in sample buffer and loaded onto a 7.5% or 10% sodium dodecyl sulfate polyacrylamide electrophoresis gel with a 4.4% stacking gel. After electrophoresis, the proteins were transferred to polyvinylidene fluoride membranes (Hybond-P; GE Healthcare, Buckinghamshire, UK). After being blocked, the membranes were incubated overnight at 4 °C with the following concentrations of the primary antibodies: anti-Mrp4 antibody (1:500, a gift from Dr. John D Schuetz), anti-Bsep antibody (1:500, Abcam, Cambridge, MA), anti-Ntcp antibody (1:10,000, Santa Cruz Biotechnology, Santa Cruz, CA), and anti-Ostα antibody (1:10,000, Santa Cruz Biotechnology). Immune complexes were detected using a species-appropriate horseradish peroxidase-conjugated secondary antibody and an enhanced chemiluminescent kit (ECL Plus; GE Healthcare). Immunoreactive bands were quantified by densitometry (ImageJ software). After normalizing the individual protein bands to a membrane protein band as an internal reference, the protein levels were expressed relative to the control values.

Immunofluorescence microscopy

Before excising the liver specimens, the rats were perfused with physiologic saline followed by 4% paraformaldehyde via the left ventricle. The perfused liver specimens were fixed in 4% paraformaldehyde for two nights and then embedded in paraffin. These paraffin blocks were sliced into 4 μm thick sections for hematoxylin-eosin staining and Mrp4, Ntcp, and Pcna staining. For immunohistochemical staining, the tissue sections were treated with citrate buffer (Target Retrieval Solution; Dako, Kyoto, Japan) at 121 °C for 3 min. For Bsep staining, small cubes of fresh liver tissue were embedded in OCT compound (Sakura, Tokyo, Japan), and 7 μm frozen sections were fixed with 4% paraformaldehyde for 5 min. The slides were blocked and then incubated overnight at 4 °C with the primary antibodies against Mrp4, Ntcp, and Pcna (diluted 1:200), and against Bsep (diluted 1:50). After the washings, the slides were incubated with species-appropriate Alexa 488 IgG or/and Alexa 546 IgG (Invitrogen). Images were captured with an

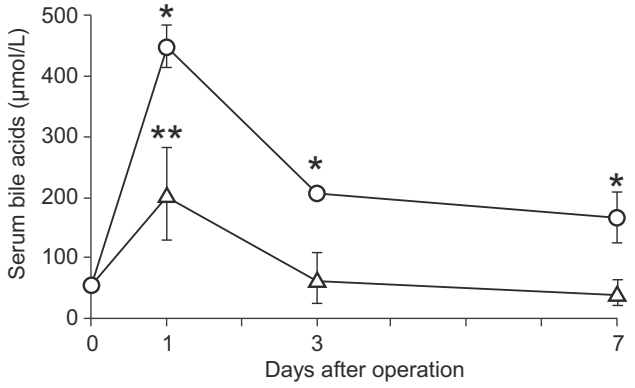

Fig. 2. Levels of serum total bile acids at days 0, 1, 3, and 7 after 70% (triangles) or 90% (circles) hepatectomy. The serum total bile acid level was quantified with a commercial kit. Data are the mean ± SD from three rats. $^{*}p$ <0.05 *vs.* the value in the 70% hepatectomy group; $^{**}p$ <0.05 *vs.* the day 0 value.

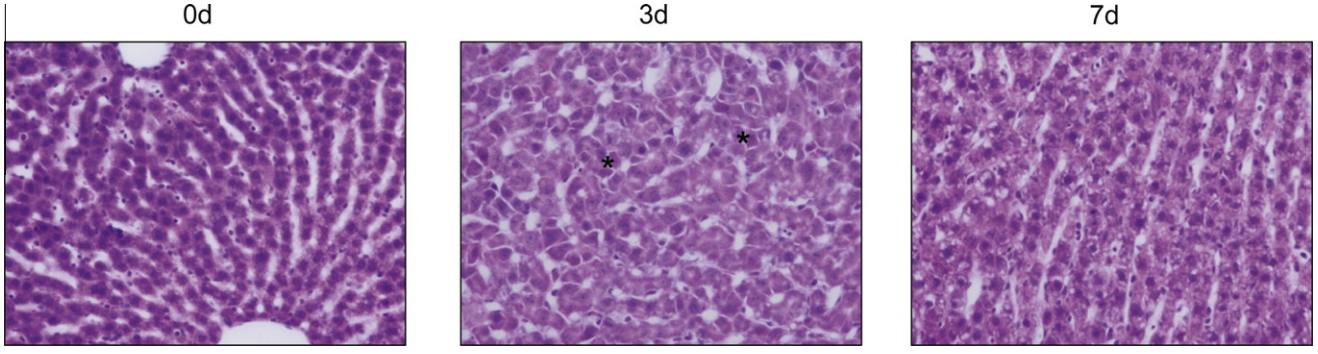

Fig. 1. Hematoxylin-eosin staining of liver sections at days 0 (0d), 3 (3d), and 7 (7d) after 90% hepatectomy. Paraformaldehyde-fixed liver sections were stained with hematoxylin-eosin as described in Section 2. The asterisks on the day 3 panel show clusters of hepatocytes devoid of sinusoidal architecture.