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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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Abstract

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 on 0, 1, 3, and 7 days for microarray analysis, the 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 serum bile acids were observed on 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 on day 3. Ntcp, a transporter used to uptake bile acids from the sinusoids, was significantly decreased throughout the period. Bsep, an export pump localized to the canalicular membrane, was unchanged. Immunohistochemistry revealed the localization of Mrp4 and Bsep in the basolateral and canalicular membranes, respectively. On the other hand, Ntcp was localized in the cytoplasm on days 3 and 7 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 seemed to be responsible for the high blood bile acids levels after massive hepatectomy.

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Key words: Ntcp; Translocation; Bile acids; Cholestasis; Liver regeneration

1. 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

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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) [5] and the sodium-independent organic anion transporting polypeptide (Oatp1) [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) [9] and multidrug resistance protein 2 (Mrp2) [10]. In addition to these basolateral uptake and canalicular export systems, the basolateral membrane also contains efflux pumps, such as multidrug resistance protein 3 (Mrp3) and multidrug resistance protein 4 (Mrp4), which are normally expressed at very low levels and whose expression levels are upregulated during obstructive cholestasis [11, 12].

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[13, 14] because they are determinants of the intracellular bile acid concentration[15]. 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. 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 and this may link to the high serum bile acid levels.

2. Materials and Methods

2.1. Animals and animal treatment

Male Sprague-Dawley rats aged 6 to 7-weeks underwent 70% or 90% hepatectomy as described previously[16, 17], with 100% survival rates. The rats were sacrificed on 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.

2.2. Microarray analysis

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. Labeling, hybridization, image scanning and data analysis were performed at Takara Bio Inc.

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

A MiniOpticon Detection System and SYBR Green Supermix were used for the quantification of specific messenger RNA (mRNA). The amplification of *ubiquitin* C cDNA was performed to standardize the levels of the target cDNA. 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.

2.4. Western blot analysis

Crude liver membranes were prepared according to the method of Gant et al[18] and preparations (100 μg protein each) were dissolved in sample buffer. Immunoreactive bands were quantified with densitometry. 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.

2.5. Immunofluorescence microscopy

Paraffin blocks were sliced into 4 μm thick for hematoxylin-eosin staining and Mrp4, Ntcp and Pcna staining. For Bsep staining, small cubes of fresh liver tissue were embedded in OCT compound, and 7 μm frozen sections were fixed with 4% paraformaldehyde. Images were captured with an Olympus IX71 fluorescent microscope or a Zeiss LSM710 confocal scanning microscope.

2.6. Statistical analysis

Data are presented as means ± SD. Differences between experimental groups were assessed for significance using the two-tailed unpaired Student's *t*-test. *P* values <0.05 were considered to be statistically significant.

3. Results

3.1. Restoration of liver weight and lobule structure

On days 1 and 3, the liver weight of the 90% hepatectomy rats was significantly lower than that of the 70% hepatectomy rats. On day 3, the weight of the remnant liver after 90% hepatectomy was only 26% of that at day 0. On day 7, liver weight had reached 55% of the value on day 0 in the 90% hepatectomy group and 58% in the 70% hepatectomy group, and the difference between 70% and 90% hepatectomy was not significant. On day 3, clusters of hepatocytes were seen sporadically, suggesting that hepatocyte proliferation continued and that the sinusoidal architecture had not completely formed. On day 7, the structure of the sinusoids had almost returned to normal; i.e., they passed between the plates of hepatocytes, with individual cells being exposed to blood on two sides.

3.2. Persistently elevated serum bile acids

Serum bile acid levels initially increased on day 1 in both hepatectomy groups, but the level was higher in 90% hepatectomy. On day 3, it had returned to the level seen on day 0 in 70% hepatectomy, whereas it remained high in 90% hepatectomy and the higher value was maintained on day 7.

3.3. Gene expression profiles during regeneration after 90% hepatectomy

At first, in order to understand the relationship between cell proliferation and liver specific functions, including bile acid metabolism during regeneration after 90% hepatectomy, gene expression profiles were examined by microarray analysis. Data are presented as signal values and values above the two-fold threshold, as compared with the value on day 0, were defined as indicating significant and reproducible alterations in gene regulation[19].

Following hepatectomy, the remaining liver showed upregulated expression of the genes involved in DNA replication and cell proliferation in accordance with the results for liver weight on days 1 and 3. Genes for fatty acid and cholesterol synthesis were also upregulated on days 3 and 7. *Ntcp* was downregulated on days 1 and 3, and *Oatp1* and *Mrp2* were also repressed on day 1. *Bsep* was unchanged during the regeneration period. Contrary to the upregulation of the cholesterol synthesis genes, *cytochrome P450 8b1* was downregulated on days 3 and 7, consistent with the findings of a previous study[20]. Proteasome subunit genes *Psmb8* and *Psmb9* were upregulated on days 3 and 7.

3.4. Changes in organic anion transporter mRNA levels after 90% hepatectomy

Next, to confirm the microarray results, the mRNA levels of the organic anion transporters for bile acids in the samples of 90% hepatectomy were quantified by RT-PCR. *Ntcp* and *Oatp1* mRNA were significantly decreased to 10.0 ± 5.7 and 31.2 ± 12.4, 35.4 ± 6.5 and 36.5 ± 9.4, and 56.9 ± 9.5 and 49.5 ± 15.2% of the controls on days 1, 3, and 7, respectively. *Mrp2* mRNA was also decreased to 55.1 ± 8.7 and 60.6 ± 11.1% of the control on days 1 and 3, respectively. *Mrp4* mRNA was increased to 272

± 105 and 317 ± 150% of the control on days 1 and 3, and *Mrp3* mRNA increased to 774 ± 677 and 548 ± 350% on days 3 and 7, respectively. No alterations in the *Bsep* mRNA level were observed. The mRNA level of cytochrome P450 7a1 (*Cyp7a1*), the rate-limiting enzyme of the bile acid synthesis pathway, was decreased to 45.8% ± 35.9% on day 1 but increased to 630% ± 347% on day 3.

3.5. Increased Mrp4 protein and decreased Ntcp after 90% hepatectomy in Western blotting

To examine whether the changes in *Bsep*, *Mrp4* and Ntcp mRNA expression were associated with changes in the levels of the respective proteins, Western blotting was performed using membrane fractions from the 90% hepatectomy samples. Ntcp protein levels were significantly decreased to 54.5 ± 8.2 and 45.3 ± 17.0% of the control on days 1 and 3, respectively. The Mrp4 level was increased to 221 ± 59% of the control on day 3. Bsep protein did not change throughout the regeneration period. These protein changes were almost in accord with the changes in mRNA expression.

3.6. Translocation of Ntcp into the cytoplasm after 90% hepatectomy

The tissue distributions of Bsep, Mrp4 and Ntcp in 90% hepatectomy samples were examined by immunofluorescence microscopy. As shown in Fig. 1, Bsep was localized to the canalicular membranes of hepatocytes throughout the period. Nuclear staining seemed to be nonspecific, because a similar staining pattern was seen with nonimmune γ-globulin (Bsep insert, Fig. 1). Mrp4 staining was increased during the period but was restricted

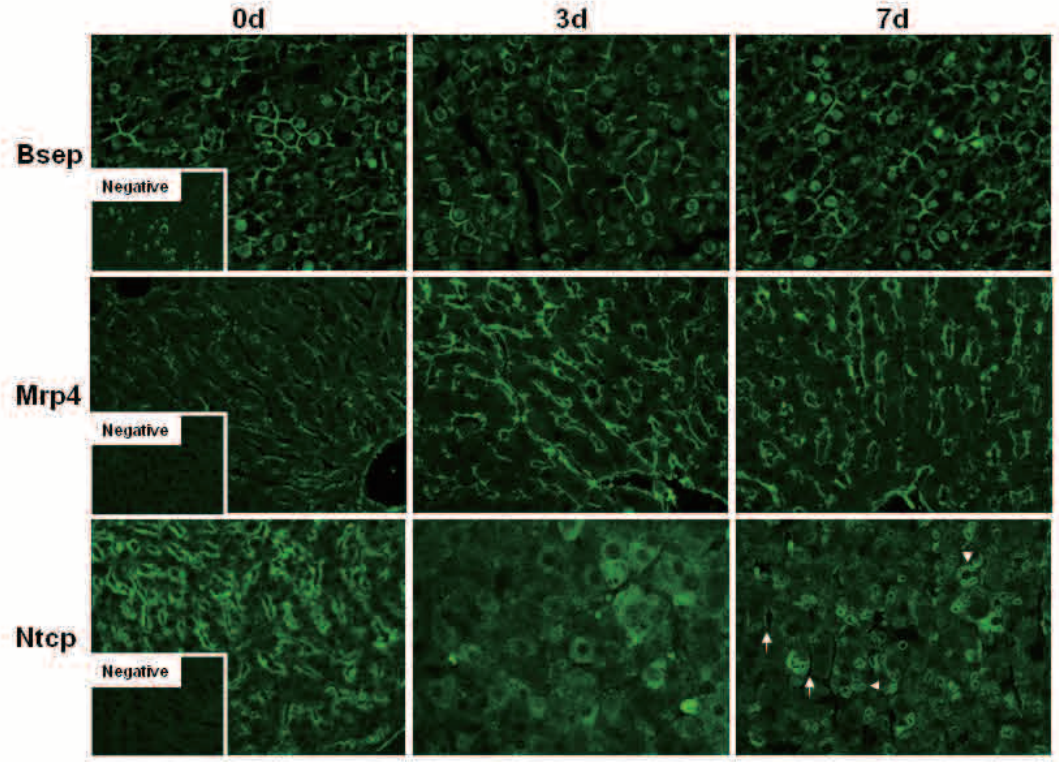

Figure 1. Immunofluorescent localization of Bsep, Mrp4, and Ntcp on days 0 (0d), 3 (3d), and 7 (7d) after 90% hepatectomy.

Immunofluorescence microscopy was performed as described in Materials and Methods. The respective primary antibody and Alexa 488-labeled secondary antibody (green) were used.No immune γ-globulin was used instead of the primary antibodies for the negative controls (inserts in 0d). The arrows and arrowheads indicate the staining of the basolateral membrane and cytoplasm, respectively. Original magnification: 200X.

to the basolateral membrane. On the other hand, although Ntcp was localized to the basolateral membrane on day 0, it was diffusely distributed in the cytoplasm of many hepatocytes on day 3. On day 7, Ntcp was stained as large granules scattered throughout the cytoplasm (Fig. 1, arrowheads in the Ntcp panel on day 7) and in part of the basolateral membrane (arrows in the same panel). To further examine the alterations in Ntcp localization during the regeneration process, its expression was compared with Mrp4 by two-color analysis. This analysis clearly demonstrated the different localizations of Ntcp and Mrp4 on day 3, Ntcp in the cytoplasm and Mrp4 in the basolateral membrane and no overlapping between them (Fig. 2A). On day 7, most Ntcp and Mrp4 also demonstrated different localizations, but the merged image revealed some overlapping in part of the basolateral membrane (Fig. 2A, yellow). Thus, Ntcp exhibited translocation into the cytoplasm and a delay in the recovery of its expression in the basolateral membrane. To examine whether changes in Ntcp localization occurred in proliferating hepatocytes, Ntcp expression was examined in cells expressing proliferating cell nuclear antigen (Pcna) (Fig. 2B). On day 3, many cells were positive for Pcna, and Ntcp was distributed as fine spots in the cytoplasm of the Pcna-positive cells. Pcna staining was negative

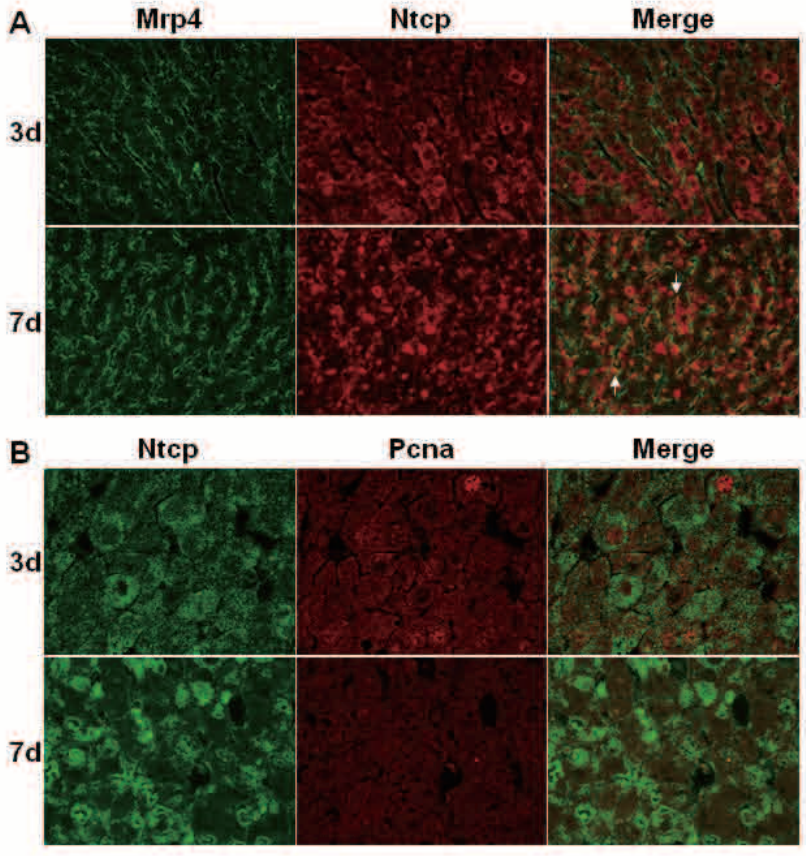

Figure 2. Immunofluorescence staining of Ntcp and Mrp4, and Ntcp and Pcna on days 3 (3d) and 7 (7d) after 90% hepatectomy.

(**A**) Cytoplasmic localization of Ntcp on days 3 and 7. The Mrp4-Alexa 488 (green), Ntcp-Alexa 546 (red), and merged signals are shown. On day 7, some overlapping between Ntcp and Mrp4 was observed in the basolateral membrane (arrows). Original magnification: 200X. (**B**) Cytoplasmic localization of Ntcp in proliferating hepatocytes. The Ntcp-Alexa 488 (green), Pcna-Alexa 546 (red), and the merged image are shown. Original magnification: 400X.

on day 7, and Ntcp was concentrated as large granules in the cytoplasm near to the plasma membrane.

4. Discussion

To clarify the mechanism of persistent cholestasis after massive hepatectomy, alterations in factors involved in bile acid metabolism during the regeneration period were examined using microarray, RT-PCR, Western blotting, and immunofluorescence microscopy. Expression array analysis revealed maximal DNA replication on day 1 and enhanced cell proliferation on days 1 and 3, which were in accordance with the findings of hematoxylin-eosin staining.

The present study showed significant decreases in the *Ntcp* mRNA level on days 1, 3, and 7 after 90% hepatectomy, in accordance with previous studies reporting the downregulation of *Ntcp* mRNA and protein levels during the early phase of rat liver regeneration after 70% partial hepatectomy[13]. However, in contrast to 70% hepatectomy, the levels of serum bile acids on day 7 after 90% hepatectomy remained higher than those on day 0, despite the restoration of liver weight and lobular structure. Besides the downregulation of Ntcp protein in 90% hepatectomy, immunofluorescence microscopy revealed that most Ntcp was localized in the cytoplasm, even on day 7, and only a small fraction in the basolateral membrane. These results suggest that the Ntcp present in the cytoplasm is not functional as a transporter for bile acids and that poor uptake of these compounds from the basolateral membrane may be responsible for the higher serum bile acid levels. In spite of the marked downregulation of *Ntcp* mRNA on days 1 and 3, its protein was demonstrated as fine spots in the cytoplasm. As the amount of Ntcp protein was very high on day 0, as shown by Western blotting and immunofluorescence microscopy, the cytoplasmic localization of the protein suggested a possible degradation process rather than synthesis. In this context, in microarray analysis, some genes encoding proteasome subunits were upregulated on day 3. It was also reported that wild-type rat Ntcp is degraded by the ubiquitin-proteasome system[21]. Moreover, similar endocytotic translocation of Ntcp was reported after treatment with a mitogen, phorbol myristate acetate[22]. On day 7, Ntcp was found as large granules in the cytoplasm near the plasma membrane. The relative recovery of the *Ntcp* mRNA level and positive Western blotting findings raised the possibility that the Ntcp-positive granules might represent exocytotic vesicles containing newly synthesized Ntcp to be transported to the basolateral membrane. These results indicated a delay in the recovery of Ntcp-mediated bile acid uptake after massive hepatectomy. The downregulation of Ntcp expression may be favorable for hepatocyte regeneration and the subsequent reorganization of lobule structure as it helps to avoid the toxic effects of bile acids[4, 22].

Mrp4 mRNA and protein were upregulated after 90% hepatectomy, and the protein was localized to the basolateral membrane throughout the regeneration period. This finding was in clear contrast to the cytoplasmic localization of Ntcp. Although Mrp3 is present in the basolateral membrane, a study employing *Mrp3*-null mice showed that it was not a significant determinant of the serum bile acid level[23]. On the other hand, as Mrp4 is an ATP-dependent pump that exports bile acids from hepatocytes into the blood[24], the upregulation and localization of Mrp4 to the basolateral membrane, in addition to the downregulation of Ntcp function, seem to be responsible for the high blood bile acid levels during the regeneration period. Although Ntcp, the main transporter for bile acid uptake, was downregulated, Mrp4 induction raised the possibility of enhanced intracellular bile acid

concentrations. As the expression and localization of Bsep were not changed, the canalicular export system seemed to be maintained during the regeneration period. The induction of Cyp7a1 suggested increased synthesis of bile acids in hepatocytes. Thus, this may be involved in a possible high intracellular bile acid level. However, the disproportion between the export and uptake of bile acids may also determine the levels inside hepatocytes. Further studies are needed to clarify the contribution of synthesis and transport to the intracellular bile acid level during the regeneration process.

Considering these alterations in the transport of bile acids in hepatocytes during the regeneration period, in order to compensate for the downregulation of Ntcp activity, the activation of alternative excretory routes may prevent the systemic dysfunction caused by high blood bile acid levels[25]. The kidney Mrp2 protein is a possible candidate for such an alternative pathway because it has been reported to function as an efflux pump into urine[26].

In summary, the persistent elevation of serum bile acids after massive hepatectomy was suggested to be due to sustained low uptake from the sinusoids by repressed and translocated Ntcp and increased export from hepatocytes into the blood by Mrp4 in the basolateral membrane.

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