Abnormal Activation of OPN Inflammation Pathway in Livers of Children with Biliary Atresia and Relationship to Hepatic Fibrosis

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Key words
- biliary atresia
- fibrosis
- osteopontin
- nuclear factor-κB
- transforming growth factor-β1

Abstract

Objective: Aim of the study was to investigate the expression of OPN (osteopontin) and its upper-downstream regulating factors in the biliary atretic liver and explore the relationship to progressive intrahepatic fibro-inflammation.

Method: OPN expression in the livers of 18 children with biliary atresia (BA), 15 children with congenital biliary dilatation (CBD) and 8 normal controls were examined by immunostaining. Masson's trichrome stain was used to evaluate the level of hepatic fibrosis in each group. Western blotting and RT-polymerase chain reaction were respectively used to semiquantitatively analyze the NF-κB (nuclear factor-κB) and the TGF-β1mRNA (transforming growth factor-β1) expression in each group.

Results: OPN expression was found in the epithelial cells of the intrahepatic bile duct in the BA group, and its intensity was 0.33 ± 0.10, while there was only little expression of OPN in the epithelial cells of the intrahepatic bile ducts in the CBD group and normal controls. There was a positive correlation between the intensity of OPN and the level of hepatic fibrosis in BA livers (r = 0.97). The intensity of NF-κB expression in BA livers (0.76 ± 0.07) was much higher than that in CBD livers (0.25 ± 0.04) or the livers of normal controls (0.22 ± 0.02). A positive correlation was detected between the intensity of NF-κB and OPN in BA livers (r = 0.94). The expression of TGF-β1mRNA in BA livers (1.46 ± 0.17) was much higher than that in CBD livers (0.68 ± 0.11). Little expression of TGF-β1mRNA was detected in the livers of normal controls. A positive correlation was detected between the expression of TGF-β1mRNA and the intensity of OPN in BA livers (r = 0.88).

Conclusion: The abnormal activation of the OPN inflammation pathway might play a key role in the generation of intrahepatic fibrosis in BA. This progressive fibro-inflammation might be controlled by OPN and its upper-downstream regulating factors NF-κB and TGF-β1.

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Introduction

BA (biliary atresia) is the most common cause of neonatal progressive jaundice, and can lead to cirrhosis, end-stage liver disease and the need for liver transplantation. A small number (10%) of cases associated with other developmental anomalies are thought to be congenital and are referred to as the embryonic or fetal form. The majority (90%) of cases are in the perinatal or acquired form [12,19]. These abnormal infants are presumably born with a patent biliary system that undergoes progressive inflammation and fibro-obliteration initiated by a perinatal insult. At the time of diagnosis, portal areas are expanded by fibrosis and exhibit marked proliferation of intrahepatic bile ductules, surrounded by a mixed cellular infiltration. The extrahepatic bile ducts are partially or entirely obliterated by fibrosis in association with inflammation within the fibrous remnant [9,20].

Recently, a gene chip array analysis showed that OPN (osteopontin) was the most highly overexpressed gene in biliary atresia relative to cholestatic control livers [1]. At the same time, it was found to be an important Th1 proinflammatory factor to mediate the fibro-inflammatory process [5]. These findings suggest a potential importance for OPN in the Th1 mediated fibro-inflammatory process that characterizes BA. In this study, we sought to investigate the expression of OPN and its upper-downstream regulators in biliary atresia using immunohistochemistry and molecular biology.

Patients and Methods

Clinical samples

We obtained and collected surgical biopsies of 18 infants diagnosed with typical type 3 biliary atresia when they were undergoing portoenterostomy in our hospital. None of these patients had other developmental anomalies and they were considered to have the acquired form of this disease. Surgical biopsies of 15 infants with congenital biliary dilatation were also collected during the operations. Finally, we examined livers from a group of "normal" children comprised of 5 children with extrahepatic portal vein thrombosis and 3 with rupture of the liver, which were proved to have a completely normal hepatic histopathology. All surgical biopsy samples were cut from the right anterior segments of livers and wrapped in tinfoil, immediately snap-frozen in liquid nitrogen and stored at – 80 °C until analysis. Specimens for histology and immunostaining were directly fixed in formalin and imbedded in paraffin. All parents of these infants gave their informed consent and the approval of the local ethics committee to perform this study has been obtained.

H&E and Masson's trichrome stain

Formalin-fixed liver tissues were processed into 5 µm thick paraffin sections and stained with H&E and Masson's trichrome stain for histologic analysis. H&E staining was performed according to common procedure and Masson's trichrome was done in accordance with the manufacturer's protocols. After the Masson's trichrome stain, collagen fibers appeared blue. Under light microscopy using a HMIAS-2000 image analysis system, 5 visual fields of every slide (4 circumferences and the center) were chosen and analyzed independently by two authors. Results were given using of a double-blind method and the extent of positive areas in collagen fibers was used to evaluate the hepatic fibrosis level (each data was analyzed twice and the mean was taken).

Immunohistochemistry

Osteopontin immunostaining was performed using an ABC method. Briefly, sections were deparaffinized, washed, and preincubated, followed by incubation with a monoclonal antihuman OPN antibody (ZhongShan Biological Engineer Company, Wuhan, China; 1 : 50 dilution). Sections were then incubated with biotinylated secondary antibody, washed, covered with DAB, and counterstained with hematoxylin. Sections treated with PBS were used as negative controls.

Western blotting

NF-κB (nuclear factor-κB) protein expression in liver samples was assessed by Western blotting using a kit procedure (WuHan ZhongShan Biological Engineer Company, China). Liver samples were homogenized in a lysis buffer and then the tissue protein concentration was quantitated. Liver homogenates were mixed with 5× reducing electrophoresis sample buffer and heated for 5 min at 95 °C. Samples containing about 20 µg protein were resolved by 12 % SDS-PAGE and then transferred overnight onto nitrocellulose membranes by electrophoresis. NF-κB protein was detected using a monoclonal anti-NF-κB antibody (1 : 200 dilution) with overnight incubation at 4 °C. The bound primary antibodies were detected with a horseradish peroxidase conjugated secondary antibody (1 : 1000 dilution; ZhongShan Biological Engineer Company, China) and visualized with an enhanced chemiluminescence method. Quantitation of protein levels was performed by densitometric analysis using an Eagle Eye II video system. A β-actin test was performed as an internal reference. We used this imaging system to scan the density of strips and calculate data for NF-κB/β-actin.

RT-polymerase chain reaction

Total RNA was extracted using the Trizol method (RNA PCR Kit, TaKaRa Company, Dalian, China, for 150 frequency use). The primers were designed and synthesized by the Shanghai biochemistry institution of Academia Sinica. Primer sequences used were TGF-β1 forward sequence 5′-CTGCGGATCTCTGTGTCATT-3′ and reverse sequence 5′-GTGGTATCGTTGTAGACTC-3′, the amplification fragments were 247bp. Sequences of the housekeeping gene GAPDH were GAPDH forward 5′-ACCACAGTCCATGCCATCAC-3′ and reverse 5′-TCCACCACCCGTTT GCTGTA-3′, the amplification fragments were 425bp. For the first step, the following components were added to the denatured RNA to obtain the specified concentrations in a final 20 µL reaction volume: total RNA 1 µl, 2 × buffer 10 µl, dNTP mixture 1 µl, RNase inhibitor 0.5 µl, $MgSO_4$ 4 µl, RNase free DH_2O 1.5 µl, downstream primers 1 µl, polymerase 1 µl. The reaction was performed at 65 °C for 1 min, 30 °C for 5 min, 47 °C for 5 min, 65 °C for 1 hour, 98 °C for 5 min, and 5 °C for 5 min. In the second step, these productions were mixed with $MgSO_4$ 6 µl, 5 × buffer 16 µl, RNase free DH_2O 55.5 µl, Taq enzyme 0.5 µl, upstream primers 1 µl and downstream primers 1 µl, then overlaid with 50 µL of mineral oil. Subsequently, the cDNA synthesis reaction was performed as follows: denaturation at 94 °C for 30 sec, annealing at 61 °C for 40 sec, and elongation at 72 °C for 4 min, total of 32 cycles of PCR amplification. The PCR products were separated by electrophoresis using 2.0% agarose gels and visualized by ethidium bromide staining and ultraviolet illumination. We used the Kodak science gel image analysis system to scan the density of strips and calculate the data for TGF-β1/GAPDH.

Statistical analysis

The SPSS 13.0 package (SPSS Inc., Chicago, IL, USA) was used to analysis our data. Comparisons between groups were performed using the *t*-test for unpaired samples. A p value of < 0.05 was considered statistically significant.

Results

Assessment of fibrosis in hepatic tissues in the BA group and controls

The percentage of positive areas for collagen fibers of BA group was 39.6 ± 3.1 %, which was much higher than that of the CBD group (23.9 ± 2.6 %) or of normal controls (4.5 ± 0.9 %), $p < 0.01$ (**Figs. 1 – 3** and **9**).

Detection of OPN in the BA group and controls

OPN positive staining was visible in the cellular membrane and cytoplasm, which presented a yellow brown color. Both groups of "normal" children showed a slight expression of OPN in hepatocytes, but none in the interlobular bile ducts. Children with biliary atresia showed marked OPN staining in the portal areas, predominantly in the biliary epithelium, but OPN expression in hepatocytes was obviously weak. The results were analyzed using a medical color image analysis system and indicated by average intensity of light. The OPN expression intensity of biliary epitheliums in the portal areas in the BA group was as high as

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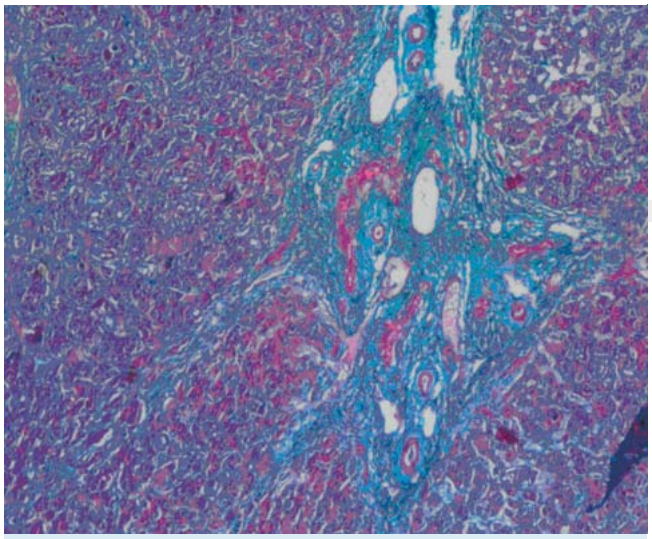

Fig. 1 Masson's trichrome stain of liver tissues in BA infants (40 × 10).

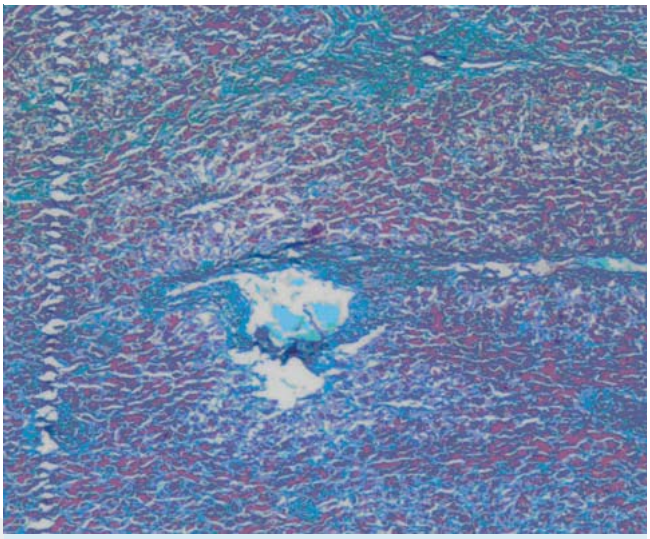

Fig. 2 Masson's trichrome stain of liver tissues in CBD infants (40 × 10).

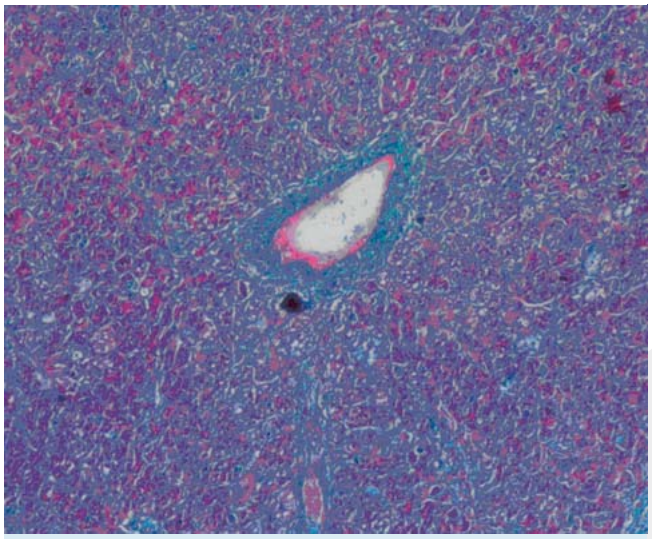

Fig. 3 Masson's trichrome stain of liver tissues in normal controls (40 × 10).

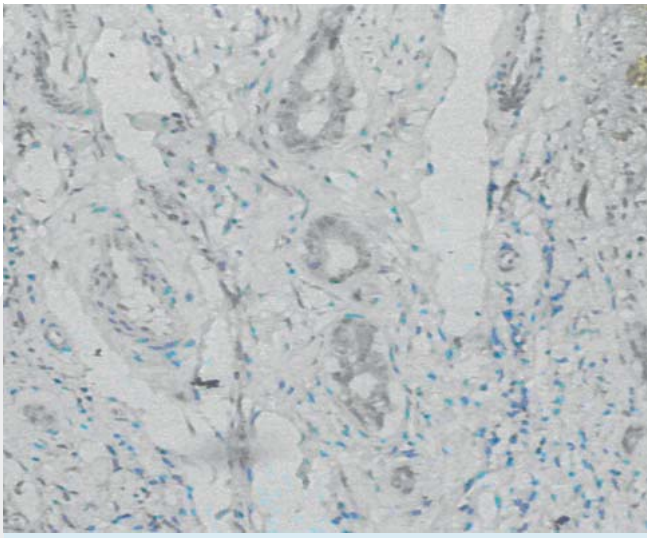

Fig. 4 Osteopontin immunostaining of liver tissues in BA infants (40 × 10).

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0.33 ± 0.10, but the intensities in the CBD and normal control groups were all zero. The intensity of OPN expression in hepatocytes of the BA group, CBD group and normal controls was 0.03 ± 0.02, 0.04 ± 0.02, 0.03 ± 0.01, respectively, and there was no statistical difference between these three groups ($p < 0.01$). Correlation analysis confirmed that there was a strong positive correlation between OPN expression in the biliary epithelium of the BA group and the hepatic fibrosis level ($r = 0.97$, $p < 0.01$) (**Figs. 4 – 6**).

Semiquantitative analysis of the expression of NF-κB protein in the BA group and controls

The expression of NF-κB protein in liver tissues of BA infants (0.76 ± 0.07) was obviously much higher than that of CBD infants (0.25 ± 0.04) and normal controls (0.22 ± 0.02), $p < 0.01$. The intensity of NF-κB protein expression in CBD infants seemed higher than that of normal controls, but there was no statistical difference between these two groups ($p > 0.05$). Correlation analysis confirmed that NF-κB protein expression in liver tissues of the BA group and OPN expression in biliary epitheliums of these infants showed a strong positive correlation ($r = 0.94$, $p < 0.01$) (**Figs. 7** and **10**).

Semiquantitative analysis of the expression of TGF-β1mRNA in the BA group and controls

The expression of TGF-β1mRNA in the liver tissue of BA infants (1.46 ± 0.17) was much higher than that of CBD infants (0.68 ± 0.11), $p < 0.01$. Little expression of TGF-β1mRNA was detected in the livers of normal controls. Correlation analysis showed that the expression of TGF-β1mRNA in the liver tissue of BA infants was positively correlated not only to the level of hepatic fibrosis ($r = 0.96$, $p < 0.01$) but also to the intensity of OPN expression in biliary epitheliums ($r = 0.88$, $p < 0.01$) (**Figs. 8** and **11**).

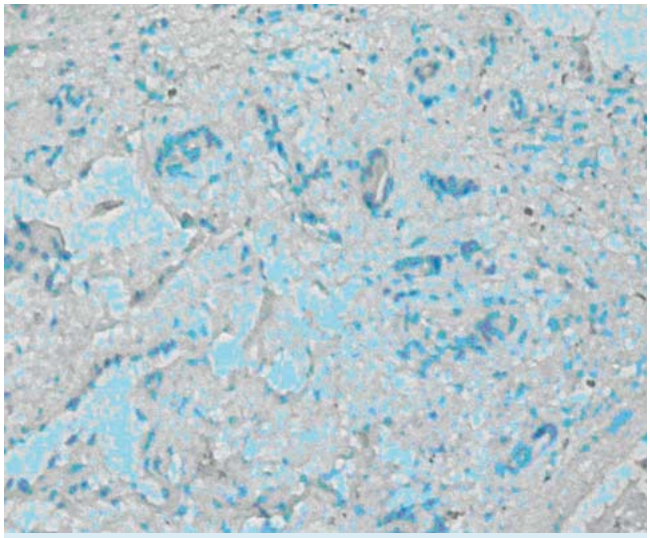

Fig. 5 Osteopontin immunostaining of liver tissues in CBD infants (40 × 10).

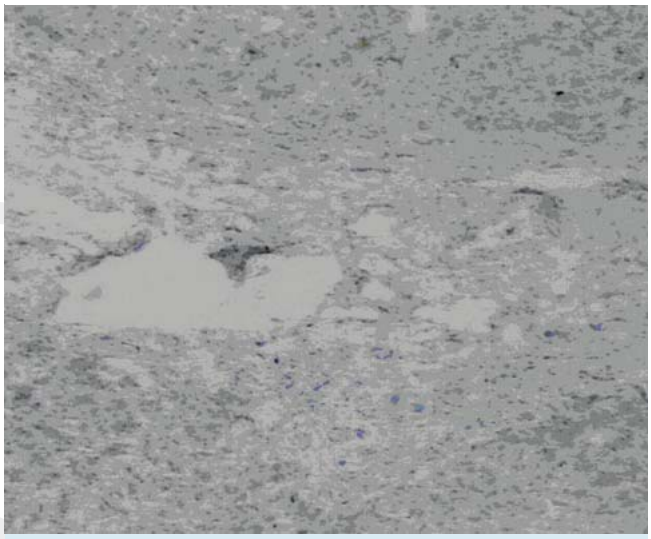

Fig. 6 Osteopontin immunostaining of liver tissues in normal controls (40 × 10).

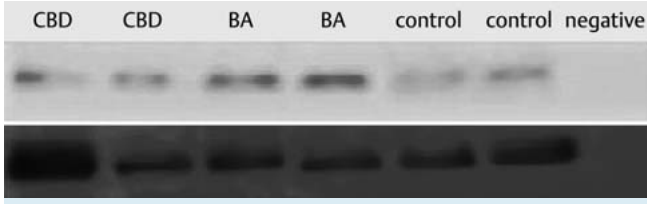

Fig. 7 NF-κB protein expression in liver tissue in the BA group and controls (Western blot).

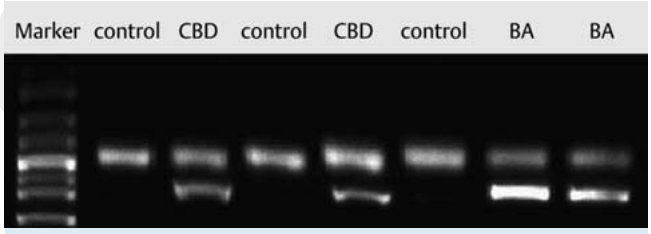

Fig. 8 TGF-β1mRNA expression in liver tissue in the BA group and controls (RT-PCR).

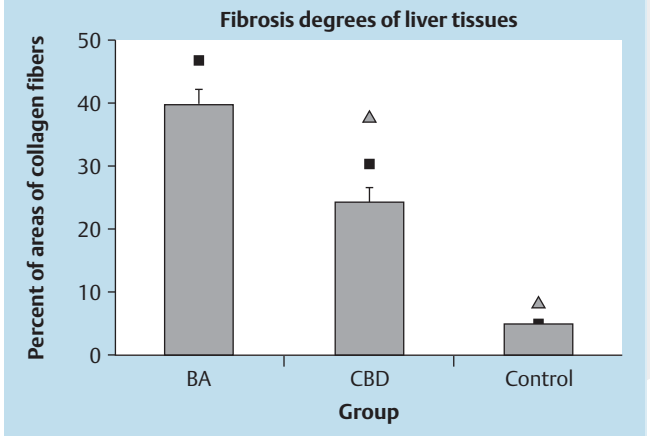

Fig. 9 Comparison of the degree of fibrosis in liver tissue in the BA group and controls. ■ $p < 0.01$, ▲ $p < 0.01$.

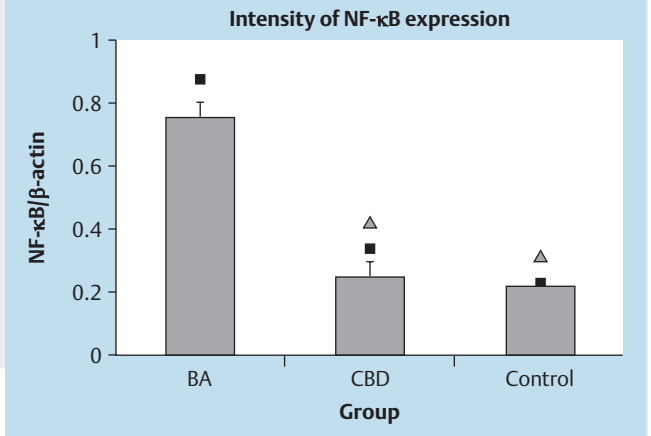

Fig. 10 Semiquantitative comparison of NF-κB expression in liver tissue in the BA group and controls. ■ $p < 0.01$, ▲ $p > 0.05$.

Discussion

Osteopontin (OPN) is a hyperphosphorylated secreting glycoprotein which can be synthesized and secreted by a variety of cell types. It plays a key role in immune cell recruitment during inflammation, acts as a mitogenic factor for epithelial cells, and is associated with extracellular matrix synthesis and fibrosis [2, 3, 6]. The human OPN gene is located at 4q13 and is composed of 7 extrons and 6 introns [14]. In an experimental mice model of hepatic fibrosis, increased OPN expression preceded the development of fibrosis in portal areas. OPN-null mice exposed to the same experimental conditions had a marked reduction in serum ALT compared with wild-type littermates and had not developed hepatic fibrosis [17]. An *ex vivo* cell experiment confirmed that

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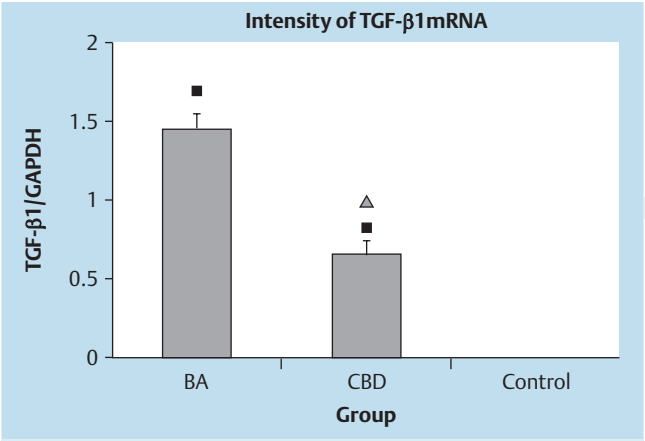

Fig. 11 Semiquantitative comparision of TGF-β1 mRNA expression in liver tissue in the BA group and controls. ■ $p < 0.01$.

OPN could stimulate the proliferation and expression transforming growth factor-β1 (TGF-β1) of many inflammation cells [14]. OPN can work as a Th1 cytokine, which plays an important role in recruiting macrophages to the parenchymal liver, and assists these accumulative Kupffer cells and macrophages to induce hepatic satellite cell activation as a waterfall; this specific procedure may lead to progressive hepatic fibrosis [11].

Through immunohistochemistry staining, we demonstrated a high expression of OPN in the portal areas of the livers of BA infants, located in the proliferative bile duct epitheliums with retention of bile. At the same time, we found that many inflammatory cells infiltrated and surrounded the proliferative interlobular bile ducts. There was no OPN expression in the intrahepatic epitheliums of the CBD group and normal controls. On the one hand, this phenomenon demonstrated that the proliferative intrahepatic epitheliums were a main source of high OPN expression in BA livers; on the other hand, it might indicate that the high expression of OPN in the epithelium surrounding the bile ducts might induce migration of inflammatory cells and local infiltration. In the normal control group, only hepatocytes weakly expressed OPN, indicating that the intrahepatic epithelium is not a normal location for OPN secretion. Compared with the abnormally high expression of OPN in BA livers, we considered that these changes in the location and intensity of expression could be induced by two reasons: primary bile epithelium injury or secondary cholestasis caused by bile duct obstruction. To investigate this question further, we used a typical cholestatic liver injury disease – congenital biliary dilatation – as a comparison. The immunohistochemistry demonstrated that there was no OPN expression in the intrahepatic epitheliums of CBD infants; this proved that cholestasis itself was not the reason for the abnormally high expression of OPN in the intrahepatic epitheliums of BA infants. This change in OPN expression in the interlobular bile duct structures of BA could only potentially result from a specific primary injury to the bile epithelium.

NF-κB is an important multifunctional nucleus transcription factor which can stimulate target gene expression [16,23,24]. With extensive biological activity, NF-κB is closely connected to immunological reaction, inflammation, cell regeneration and apoptosis, etc. [13]. Many stimulations can induce the expression of NF-κB [18,25]. Ophascharoensuk et al. reported that OPN had been shown to be critically highly expressed in the epitheliums of renal tubules in a fibrosis mice model with renal tubulo-interstitial disease. When they inhibited the activity of NF-κB by using pyrolidine dithiocarbamate (PDTC), the transcription level of OPN was obviously depressed [15]. This experiment confirmed that the transcription level of OPN could be regulated by NF-κB. In the current study, we detected the NF-κB protein expression using Western blotting; samples from the liver tissues of BA infants showed a high expression of NF-κB protein, which was much higher than that in the CBD group and normal controls. Correlation analysis confirmed a strong positive correlation between the NF-κB protein expression in liver tissue and the OPN expression in biliary epitheliums of these BA infants; our findings were consistent with the published reports by Ophascharoensuk et al. Our research supports the supposition that some special foreign injury factors induced an increased NF-κB activity in the intrahepatic epitheliums of BA children, which directly resulted in an alteration of OPN expression in the intrahepatic epitheliums. NF-κB might be the upstream regulator of the progressive hepatic fibrosis inflammation path in BA liver. Professor Feng in our department created a neonatal mice model of BA induced by an infection of rotavirus MMU18006; he found that when these mice were given PDTC (NF-κB activity inhibitor) prior to virus infection, the inflammation and fibrosis level were obviously relieved [4]. His research confirmed that NF-κB is an important upstream regulator of the progressive fibro-inflammatory process in BA.

TGF-β1 is the most important cytokine that can induce hepatic fibrosis; this cytokine is able to activate the HSCs (hepatic satellite cells) directly through the TGF-β1/Smad signal conduction pathway and lead to hepatic fibrosis [7,21,22]. Carbon tetrachloride intoxication in rats has been shown to increase OPN expression that was localized mainly to bile epithelium cells and this change directly induced the upregulation of TGF-β1mRNA. This experiment also found that human recombinant OPN had a positive effect on the hepatic macrophage migration *in vitro*; they presumed that OPN was the reason for the recruitment of inflammatory cells to the parenchymal liver [8]. We detected TGF-β1mRNA expression with a semiquantitive PCR method; marked increases of TGF-β1mRNA expression in the BA group were observed when compared with the CBD group and normal controls. Correlation analysis showed that the expression of TGF-β1mRNA in the liver tissue of BA infants was positively correlated not only with the level of hepatic fibrosis, but also with the intensity of OPN. Our research confirmed that the overexpression of OPN positively regulated the level of its downstream regulator TGF-β1; this change stimulated the proliferation of HSCs through the TGF-β1/Smad signal conduction path and then induced hepatic fibrosis. Previous studies had shown that increased expression of TGF-β1 was associated with increases in the collagen synthesis of HSC, and that the activated HSC could secrete greater amounts of TGF-β1, too [10]. This positive feedback might induce a progressive development of hepatic fibrosis in BA. It is worth noting that, although we could detect obvious hepatic fibrosis in the CBD group, our study found that OPN and its upper-downstream regulators were all not activated; these findings could indicate that CBD takes a different path to generate hepatic fibrosis compared with BA.

Our study confirmed that the OPN inflammatory pathway plays a key role in mediating progressive hepatic fibrosis inflammation in BA livers. Initially, foreign bile ducts damage factors activate the expression of NF-κB in the BA liver; subsequently, NF-κB induces the upregulation of OPN expression in bile epithelium;

finally, OPN stimulates the synthesis of TGF-β1mRNA and activates HSC through the TGF-β1/Smad signal conduction path. By drawing on the positive feedback effect of TGF-β1 and HSC, this hepatic fibrosis procedure becomes progressive. Trying to approach the molecular pathway that how OPN is activated intra-epithelium cells and explore the target sites of blocking the signal transduction of OPN and its upper-downstream regulators will be our future research directions.

Conflict of Interest: None

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