Cyclooxygenase-2 Overexpression is Associated with Clinical Outcome in Biliary Atresia

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Key words
- biliary atresia
- clinical outcome
- cyclooxygenase-2
- jaundice

Abstract

Objective: The purpose of this study was to investigate the association between cyclooxygenase-2 (COX-2) expression and clinical outcome in biliary atresia (BA) patients.

Methods: Six months after surgery, twenty-eight BA patients were divided into three groups according to their liver function tests: group A with satisfactory liver function (n=11), group B with moderate liver dysfunction (n=8), and group C with severe liver dysfunction (n=9). COX-2 expression was determined by immunohistochemistry. Choledochal cysts (n=5) and normal liver samples (n=4) served as controls.

Results: Our data have shown that the intrahepatic biliary epithelium in BA specimens expressed COX-2. The mean immunoreactive score of COX-2 in BA patients was significantly higher than that in choledochal cyst and normal liver (4.0±0.6, 0.9±0.3, and 0.7±0.3, respectively, $p<0.002$). Strong expression of COX-2 was observed in BA patients with severe liver dysfunction. Subgroup analysis showed that the mean COX-2 immunoreactive scores of patients in group A, B, and C were 2.1±0.6, 3.6±1.1, and 5.9±0.9, respectively. The COX-2 immunoreactive score in BA patients with severe liver dysfunction was higher than in patients with satisfactory liver function ($p<0.005$).

Conclusion: Increased COX-2 expression of biliary epithelial cells at the time of Kasai operation was associated with an adverse therapeutic outcome in BA, suggesting that COX-2 could play a plausible role in the liver pathology of BA.

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Introduction

Biliary atresia (BA) is a neonatal disorder characterized by progressive cholangiopathy affecting both extrahepatic and intrahepatic bile ducts. It may lead to obliteration or discontinuity of the biliary tract at any point between the porta hepatis and the duodenum. When untreated, BA patients develop severe liver injury, biliary cirrhosis, and ultimately die by the age of two years [2]. The standard surgical therapy of BA is early Kasai portoenterostomy. Despite early diagnosis and successful Kasai operation, more than half of BA patients still develop hepatic fibrosis, portal hypertension, and end-stage liver disease [6]. Alternatively, liver transplantation is an effective treatment modality if the Kasai portoenterostomy fails and serious complications occur such as recurrent cholangitis, persistent jaundice, cirrhosis, progressive ascites, and bleeding esophageal varices [7]. Although several etiologies of BA have been postulated, including neonatal viral infections, genetic disorders, and abnormalities in immune response, the precise pathogenesis of BA remains unknown [1].

Cyclooxygenase (COX) is a membrane-bound enzyme that catalyzes the conversion of arachidonic acid to prostaglandin H_2, the precursor of various compounds including prostaglandins, prostacyclins, and thromboxanes [23]. Two isozymes of this enzyme, COX-1 and COX-2, have been characterized and shown to share more than 60% homology at the amino acid level. COX-1 is constitutively expressed in various tissues and is involved in normal physiological functions including maintenance of the gastric mucosa, regulation of renal blood flow, and the control of platelet aggregation. In contrast, COX-2 is a highly inducible isoform usually undetectable in most tissues but upregulated in response to a number of stimuli including pro-inflammatory and mitogenic signals [8]. There is growing evidence that COX-2 plays a critical role in liver injury and hepatic fibrosis [13]. Recent studies have shown

overexpression of COX-2 in the livers of patients with chronic viral hepatitis [4], cirrhosis [18], and hepatocellular carcinoma [19,15]. In bile duct ligation animal models of liver injury, COX-2 expression was significantly upregulated with COX-2 mRNA and protein localized to proliferating bile duct epithelial cells [14,24]. However, the possibility that COX-2 could contribute to the pathogenesis and clinical outcome of BA has not been investigated.

To the best of our knowledge, there have been no published studies on COX-2 expression in liver specimens of patients with BA. We postulated that increased COX-2 expression would be associated with severity of the liver injury in BA and the expression pattern of COX-2 would be primarily in biliary epithelial cells in BA. Therefore, the aims of this study were to investigate the pattern of COX-2 expression in liver specimens from children with BA and to determine the potential association between the COX-2 expression and clinical outcome of BA patients. In the present study, COX-2 expression was analyzed in the intrahepatic biliary epithelium in BA patients using an immunohistochemical technique.

Patients and Methods

This study was approved by the Ethics Committee on Human Research of the Faculty of Medicine, Chulalongkorn University. All parents of children with BA and controls were informed of the study's purpose. Written informed consent was obtained for all patients who participated in this study.

Patients

Liver samples were obtained from 28 BA patients (11 boys and 17 girls; age range 1–5 months; mean 86 days) while undergoing Kasai portoenterostomy with a Roux-en-Y (original Kasai) operation at the Department of Surgery, Chulalongkorn University, between 2001 and 2006. Specimens from choledochal cysts (n=5) from patients with ages ranging from 1 month to 10 months (mean 4.5 months) and normal liver (n=4) specimens from patients with an age range from 10 months to 98 months (mean 34.4 months) served as controls. Liver specimens were routinely evaluated histologically with hematoxylin and eosin staining. Formalin-fixed and paraffin-embedded specimens for immunostaining were obtained from our pathology archives.

The duration of follow-up after the Kasai operation ranged from 6 months to 72 months (mean 34 months). Serum samples were collected from each patient at the time of their follow-up and stored at −80 °C. Liver function tests, including total bilirubin (TB), direct bilirubin (DB), aspartate aminotransferase (AST), and alanine aminotransferase (ALT) were performed by routine laboratory methods using an automated machine (Hitachi 912, Roche Diagnostics, Basel, Switzerland). In order to associate COX-2 expression with clinical outcome at 6 months post-Kasai in BA children, the children were categorized into 3 groups according to their levels of serum total bilirubin and liver enzymes. Group A consisted of 11 patients with satisfactory liver function (TB < 2 mg/dl, AST < 40 IU/l, ALT < 40 IU/l). Group B consisted of 8 patients with moderate liver dysfunction (TB < 2 mg/dl, AST ≥ 40 IU/l, ALT ≥ 40 IU/l). Group C consisted of 9 patients with severe liver dysfunction (TB ≥ 2 mg/dl, AST ≥ 40 IU/l, ALT ≥ 40 IU/l), who subsequently underwent liver transplantation.

Immunohistochemistry of the COX-2 stained liver specimens

Tissue specimens were fixed in 10% buffered formalin and embedded in paraffin. Serial sections (5 µm) of paraffin-embedded tissue were cut and processed for COX-2 staining. Sections were deparaffinized and rehydrated in Tris-buffered saline. Endogenous peroxidase activity was blocked with 0.3% H_2O_2 for 10 min. For antigen retrieval, tissue sections were microwave heated in 10 mmol/L citrate buffer for 5 min. Nonspecific binding was blocked for 10 min with 5% goat serum (DAKO, Glostrup, Denmark), followed by incubation with antibody to COX-2 (1:100; Diagnostic Biosystems, Pleasanton, CA, USA) in Tris-buffered saline containing 2% rabbit serum and 1% bovine serum albumin for 2 hours. Tissues were incubated with the same buffer without the antibody to serve as negative controls. Sections were subsequently stained with biotinylated goat antirabbit immunoglobulins (1:400; DAKO) and streptavidin/horseradish peroxidase complex (1:400; DAKO) and incubated at room temperature for 45 min. Reaction products were visualized using diaminobenzidine (Sigma, St. Louis, MO, USA) as the chromogen. The sections were subsequently counterstained with Mayer's hematoxylin and mounted onto microscope slides using a permanent medium.

COX-2 immunohistochemistry staining score

All stained specimens were assessed blindly without knowledge of the patient subgroups, clinical and histology parameters. Scores were assigned on the basis of intensity and extensiveness as follows: (a) maximum intensity (the maximum level among all positive cells); (b) dominant intensity (the level observed in the majority of positive cells); and (c) extensiveness (by percentage population) of positively stained cells. Both maximum intensity and dominant intensity were scored on a scale from 0 to 3, with 0 = negative staining, 1 = weak positive staining, 2 = moderate positive staining, and 3 = strong positive staining. Extensiveness was scored on a scale from 0 to 4, with 0 = negative, 1 = positive staining in 1–25% of cells, 2 = positive staining in 26–50% of cells, 3 = positive staining in 51–75% of cells, and 4 = positive staining in 76–100% of cells. Each specimen's score represented the sum of these three parameters.

Statistical analysis

Comparisons of clinicopathological parameters between groups were performed using Student's *t*-test and Mann–Whitney *U*-test. Statistical significance was considered achieved if the p-value was less than 0.05. Data are expressed as mean ± SEM. All analyses were conducted using the Statistical Package for Social Sciences (SPSS) for Windows (version 16) computer program.

Results

COX-2 expression was barely detectable as a faint cytoplasm staining in the control liver specimens. However, the expression of COX-2 in the BA specimens was upregulated in inflammatory cells, vascular endothelial cells, the epithelial cells lining the intrahepatic bile ducts, and cells of the surrounding connective tissue (**Fig. 1**). COX-2 was expressed in the cytoplasm of the positive cells. More intense expression was observed in BA subjects compared with controls for COX-2 expression on intrahepatic bile ducts. As illustrated in **Table 1**, the mean immunoreactive score of COX-2 in the BA patients was signifi-

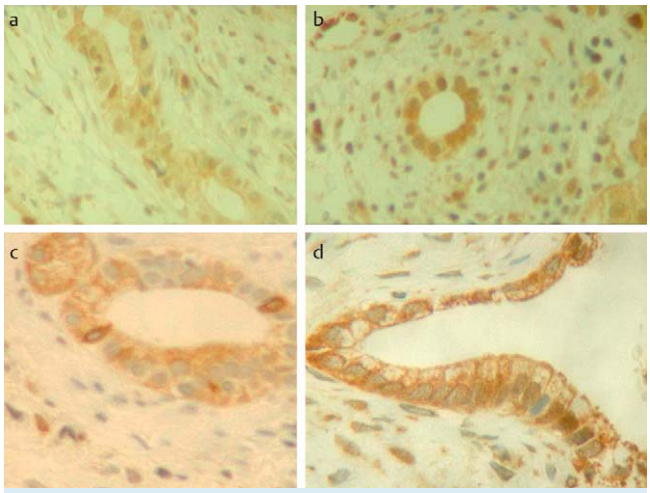

Fig. 1 Histological staining intensity findings after COX-2 immunostaining of liver specimens in controls and BA patients. Score 0 (**a**), score 1 (**b**), score 2 (**c**), score 3 (**d**).

Table 1 COX-2 immunoreactive scores of liver specimens of BA patients and controls.

Tissue type	COX-2 immunoreactive score (mean ± SEM)
biliary atresia (n = 28)	4.0 ± 0.6*
choledochal cyst (n = 5)	0.9 ± 0.3
normal liver (n = 4)	0.7 ± 0.3

* $p < 0.002$ when compared with choledochal cyst and normal liver

cantly higher than in choledochal cysts and normal liver (4.0 ± 0.6, 0.9 ± 0.3, and 0.7 ± 0.3, respectively; $p < 0.002$).

The COX-2 immunoreactive scores of the distinctive groups of BA patients are shown in **Table 2**. Liver biopsy specimens from group C subjects revealed that COX-2 was expressed strongly on biliary epithelial cells and some inflammatory cells but not on the hepatocytes. In contrast, liver biopsy specimens from group A subjects showed slight COX-2 reactivity on the epithelial lining of intrahepatic bile ducts (**Fig. 1**). As demonstrated in **Table 2**, the total COX-2 immunoreactive scores of BA patients in groups A, B, and C were 2.1 ± 0.6, 3.6 ± 1.1, and 5.9 ± 0.9, respectively. The average immunoreactive score for COX-2 expression was significantly increased in group C subjects compared with that in group A subjects ($p < 0.005$, **Fig. 2**). However, there was no significant difference in the COX-2 immunoreactive score of group B compared with other groups.

Discussion

Prostaglandins (PG) are essential mediators of inflammation, with their synthesis initiated by the release of arachidonic acid from cell membranes. COX stimulates the conversion of arachidonic acid to PGH_2 and is a rate-limiting enzyme in the biosynthesis of prostanoid [20,10]. COX-1 is generally present in almost all normal tissues and performs housekeeping functions such as gastric cytoprotective action and homeostasis. COX-2 accounts for the increased production of PG in many cell types induced by various factors, such as mitogens, hormones, growth factors, and cytokines. COX-2 is also actively involved in inflammation, fibrogenesis, and wound healing [16]. Previous studies have shown that COX-2 is not expressed in normal biliary epithelium whereas COX-2 overexpression is correlated with the degree of hepatic inflammation and the development of hepatic fibrosis [17]. However, the impact of COX-2 expression on the clinical course of BA has not been characterized.

Biliary atresia (BA) remains one of the most common causes of pathologic jaundice in neonates and presents with the triad of jaundice, acholic stools and hepatosplenomegaly. The etiology and pathogenesis of BA largely remain unknown, and therapeutic options are unsatisfactory. Irrespective of the initiating disorder, all children with BA share a unique progressive inflammatory and fibrotic obstruction of the biliary system [2,6,1]. Macroscopically, the livers of children with BA are firm with dark green discoloration and nodular degeneration of cirrhosis. During the early stages, microscopic findings of hepatic histology typically show bile pigment in hepatocytes and canaliculi, portal tract widening, and bile duct proliferation [7]. Disease progression leads to liver fibrosis and cirrhosis with concomitant portal hypertension and hepatic failure. Although there have been several studies focusing on the role of growth factors in BA patients including serum levels of growth factors [11,21] and cytokines [3,12,22], the determination of COX-2 expression in biliary atresia patients has received little attention.

To the best of our knowledge, this is the first study that demonstrates the relationship between COX-2 expression in liver specimens and the clinical outcome of BA. The current investigation has demonstrated that COX-2 expression in the intrahepatic biliary epithelium of BA patients is significantly higher than that of controls. Moreover, we have shown that COX-2 expression is associated with serum levels of total bilirubin, aspartate aminotransferase, and alanine aminotransferase, although we cannot rule out that these associations might be attributable to advanced hepatic fibrosis in the BA children. COX-2 overexpression has been documented in a number of liver diseases including chronic hepatitis [4], liver cirrhosis [5,18], and hepatocellular carcinoma [9,15,19]. In agreement with our findings, Pazirandeh et al. have demonstrated that COX-2 expression is significantly increased in patients with liver cirrhosis and is associated with liver inflammation and fibrosis stage [19]. Cheng et al. have also shown upregulation of hepatic COX-2 expression along with progression of liver fibrosis in chronic liver diseases [5]. These findings suggest that upregulated COX-2 expression is associated with hepatic damage and hence, reflects hepatic fibrosis.

Our immunohistochemistry data demonstrate positive cytoplasmic COX-2 expression in inflammatory cells and biliary epithelial cells of bile ductules. Subsequent analysis has shown that the total COX-2 immunoreactive score is significantly higher in BA patients with severe liver dysfunction compared to those with satisfactory liver function. COX-2 overexpression in the bile ductule epithelium of liver specimens at the initial operation might be a poor prognostic factor in BA and appears to be associated with the severity of liver injury, suggesting its significant involvement in the pathophysiology of BA. Although these findings do not prove a direct cause and effect relationship, they provide further evidence that liver inflammation and hepatic dysfunction could be COX-2 dependent processes. More studies using animal models of liver injury such as bile duct ligation, and transgenic mice with liver-specific overexpression of COX-2 will be required to elucidate the role of COX-2 in hepatic injury and fibrosis of BA.

Table 2 Variables for total scores of COX-2 immunostaining of liver specimens in BA patients. Group A, satisfactory liver function; group B, moderate liver dysfunction; group C, severe liver dysfunction.

Group	Age (years)	Maximum intensity (I)	Dominant intensity (II)	Extensiveness (III)	Total (I+II+III)
A (n=11)	3.0±0.5	0.7±0.2*	0.6±0.3*	0.8±0.2*	2.1±0.6$
B (n=8)	3.0±0.4	1.3±0.3	1.0±0.4	1.3±0.4	3.6±1.1
C (n=9)	3.8±0.5	1.8±0.3	2.2±0.3	1.9±0.3	5.9±0.9

* $p<0.02$ when compared with group C; $ $p<0.005$ when compared with group C. Mann-Whitney *U*-test was used for statistical analysis of non-parametric data of COX-2 immunoreactive scores. Values are expressed as mean±SEM

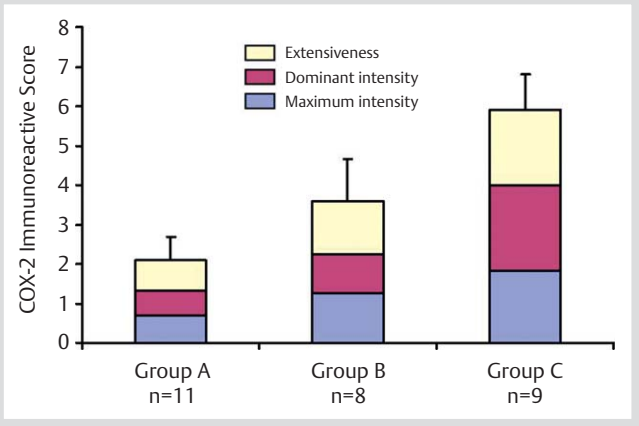

Fig. 2 COX-2 immunoreactive scores for different groups of BA patients. Data are expressed as mean and SEM. The mean COX-2 immunoreactive score of group C was significantly increased when compared with that of group A ($p<0.005$). Group A, satisfactory liver function; group B, moderate liver dysfunction; group C, severe liver dysfunction.

In this study, we are aware of some limitations. Firstly, most data were gathered retrospectively, making its quality variable. Secondly, the sample size of BA patients was not large enough to draw strong conclusions. The small number of patients makes it difficult to show statistical differences between patient groups even though the data appear to show differences. However, with the scarcity of biliary atresia, the results of a study based on liver specimens of 28 BA patients cannot be disregarded. Lastly, upregulation of hepatic COX-2 expression might be just the nonspecific findings of cholestasis. Additional investigations of non-BA children with cholestasis will clarify this point.

To summarize, the present study has demonstrated the overexpression of COX-2 in the liver specimens of BA patients compared to those of controls. The COX-2 immunoreactive score in BA patients with poor outcomes was significantly higher than that of patients with good outcomes and controls. Our study has shown that increased COX-2 expression in intrahepatic biliary epithelium at the time of Kasai operation was associated with an adverse therapeutic outcome and could play a possible role in the liver pathology of BA. Clinical application of COX-2 modulation may contribute to the development of therapeutic agents for BA patients. Further studies examining the impact of selective COX-2 inhibitors on inflammation and progression of liver fibrosis in BA are required. The use of selective COX-2 inhibitors in BA patients may help attenuate hepatic inflammation and potentially help prevent or provide treatment for hepatic fibrosis.

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Conflict of Interest: None

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