รายงานวิจัยฉบับสมบูรณ์

โครงการ บทบาทของเฮปปาโทไซด์โกรทแฟกเตอร์ อิพิเดอร์มัลโกรทแฟกเตอร์ และตัวรับสเตียรอยด์ในโรคทางเดินน้ำดีตีบตัน

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สัญญาเลขที่ RMU4980017

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โครงการ บทบาทของเฮปปาโทไซด์โกรทแฟกเตอร์ อิพิเดอร์มัลโกรทแฟกเตอร์ และตัวรับสเตียรอยด์ในโรคทางเดินน้ำดีตีบตัน

หัวหน้าโครงการวิจัย
รองศาสตราจารย์ ดร. ไพศาล เวชชพิพัฒน์ คณะแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย

สนับสนุนโดยสำนักงานคณะกรรมการการอุดมศึกษา
และสำนักงานกองทุนสนับสนุนการวิจัย

Executive Summary

1. Elevated serum soluble E-selectin is associated with poor outcome and correlated with serum ALT in biliary atresia

Serum levels of soluble E-selectin were determined from 53 post-operative Biliary atresia (BA) patients and 10 healthy children. **Results:** Serum E-selectin of BA patients was higher than the controls (p=0.01). Further analysis showed that there was an increase in serum E-selectin of BA patients with jaundice compared to those without jaundice (p=0.035). Serum E-selectin was positively correlated with serum ALT. **Conclusion:** Serum E-selectin was elevated in BA patients. The elevated serum E-selectin was also associated with poor outcome. Additionally, there was a positive correlation between serum E-selectin and serum ALT. These suggest that E-selectin plays a role in the pathophysiology of liver injury in post-operative BA.

2. High-dose steroids do not improve early clinical outcome in biliary atresia

BA patients between 2001 and 2005 were reviewed. The use of steroids for 1 to 3 months has been implemented since 2003. **Results:** At 6 months post-op, 30 patients (56.6%) were jaundice-free and 24 patients (45.3%) experienced cholangitis at least once. Of the 53 patients, there were 33 patients in steroid group and 20 patients in non-steroid group. The proportion of jaundice-free patients in steroid group was higher than that in non-steroid group, and the proportion of patients with cholangitis in steroid group was lower than that in non-steroid group. However, these discrepancies did not reach a statistically significant difference **Conclusions:** Although the use of steroids seems to have benefits, it did not statistically improve early outcome in BA patients.

3. Serum transforming growth factor-β1 and epidermal growth factor in biliary atresia

Serum levels of TGF-β1 and EGF were determined from 67 BA patients and 10 healthy children. **Results:** Serum TGF-β1 levels of BA patients were higher than the controls (P=0.036). However, there was no difference in serum EGF between BA patients and the controls (P=0.74). Further analysis showed that patients with good outcome had their serum TGF-β1 and serum EGF levels higher than those of patients with poor outcome. In addition, serum TGF-β1 was positively correlated with serum EGF. **Conclusions:** The elevated serum TGF-β1 and serum EGF levels were associated with good outcome in BA. These suggest that the resultant actions of TGF-β1 and EGF pathways possibly involve in the pathophysiologic process in post-operative BA.

4. Overexpression of inducible nitric oxide synthase in biliary atresia

Hepatic iNOS expression was determined from liver biopsies of 24 BA patients, and 16 non-BA patients. The iNOS expression was evaluated based on its intensity using ImageJ software. **Results:** Hepatic iNOS expression of BA was significantly stronger than that of non-BA (P<0.0001). The largest area of hepatic iNOS expression was the area of hepatocytes. Subgroup analysis of BA patients at 6 months post-op revealed that there was no difference in iNOS expression between the patients with good outcome and those with poor outcome (P=0.732). **Conclusions:** Overexpression of hepatic iNOS in BA was demonstrated. Hepatocytes were the major source of hepatic iNOS production. These suggest that iNOS plays a role in the liver pathology of BA but its expression cannot be used as a predictor for therapeutic outcome.

5. Non-correctable Biliary Atresia with Large Extrahepatic Cyst: a Report of Two Cases

We report 2 unusual cases of biliary atresia type III with cystic structure that is possibly mistaken as a correctable biliary atresia or choledochal cyst.

6. Hepatic Expression of hepatocyte growth factor and its receptor in biliary atresia

Hepatic HGF and C-met expression were studied from liver biopsies of 41 BA patients at the time of Kasai operation, and 17 non-cholestatic pediatric patients. The HGF and C-met expression of hepatocyte areas was scored as per its intensity and percentage of stained area. **Results:** Hepatic HGF and C-met staining scores of BA patients were higher than those of non-cholestatic patients (P<0.0001). Analysis of BA patients at 6 months post-Kasai revealed that there was no difference in either hepatic HGF or C-met expression at the time of surgery between the patients with good outcome and those with poor outcome. **Conclusions:** Strong expression of hepatic HGF and its receptor in BA patients was demonstrated. However, the expression was not associated with the early therapeutic outcome. These suggest that resultant actions of HGF involve in the liver pathology of BA but its expression cannot be used as a predictor for therapeutic outcome.

รายงานสรุปย่อ

๑. ระดับซีรั่ม E-selectin ที่สูงขึ้นมีความสัมพันธ์กับผลการรักษาที่ไม่ดีและแปรผันกับระดับซีรั่ม ALT ในโรคทางเดินน้ำดีตับตัน

เป็นการศึกษาระดับซีรั่มของ E-selectin โดยใช้หลักการ ELISA ในผู้ป่วยโรคทางเดินน้ำดีตีบตัน 53 ราย โดยเปรียบเทียบกับกลุ่มควบคุม 10 คน ผลการศึกษา พบว่า ระดับของซีรั่ม E-selectin ในผู้ป่วยทางเดินน้ำดีตีบตันสูงกว่ากลุ่มควบคุม การวิเคราะห์เพิ่มเติมพบว่า ระดับซีรั่ม E-selectin ในผู้ป่วยทางเดินน้ำดีตีบตันที่มีผลการรักษาที่ไม่ดีจะสูงกว่า ผู้ป่วยทางเดินน้ำดีตีบตันที่มีผลการรักษาที่ดี นอกจากนั้นระดับซีรั่ม E-selectin ยังแปรผันตรงกับระดับซีรั่ม ALT สรุป ระดับซีรั่ม E-selectin ที่สูงในผู้ป่วยทางเดินน้ำดีตีบตันมีความสัมพันธ์กับผลการรักษาที่ไม่ดี ผลการศึกษานี้แสดงให้เห็นว่า E-selectin น่าจะมีบทบาทในพยาธิสรีรวิทยาของโรคทางเดินน้ำดีตีบตัน

๒. การใช้ยาเสตียรอยด์ระดับสูงไม่ทำให้ผลการรักษาโรคทางเดินน้ำดีตีบตันดีขึ้น

เป็นการศึกษาผู้ป่วยทางเดินน้ำดีตีบตันจำนวน 53 คน มีผู้ป่วยที่ได้รับยาเสตียรอยด์ระดับสูง 33 คน และไม่ได้รับยาเสตียรอยด์ 20 คน ผลการศึกษาพบว่า สัดส่วนผู้ป่วยที่มีผลการรักษาดีในกลุ่มที่ได้รับยาเสตียรอยด์ (60%) มีมากกว่ากลุ่มที่ไม่ได้รับยาเสตียรอยด์ (50%) แต่สัดส่วนความแตกต่างนี้ไม่มีนัยสำคัญทางสถิติ ($p>0.05$) สรุป การใช้ยาเสตียรอยด์ระดับสูงไม่ทำให้ผลการรักษาโรคทางเดินน้ำดีตีบตันดีขึ้นอย่างมีนัยสำคัญทางสถิติ

๓. ระดับซีรั่ม transforming growth factor-beta1 และ epidermal growth factor ในโรคทางเดินน้ำดีตีบตัน

เป็นการศึกษาวัดระดับของ TGF-b1 และ EGF ในซีรั่มในผู้ป่วยทางเดินน้ำดีตีบตันจำนวน 67 รายและเด็กปกติทั่วไปจำนวน 10 ราย ผลการศึกษาพบว่าระดับของซีรั่ม TGF-beta1 ในผู้ป่วยทางเดินน้ำดีตีบตันมีค่าสูงกว่ากลุ่มควบคุม ($p = 0.0362$) อย่างไรก็ดี ไม่มีความแตกต่างกันของระดับของซีรั่ม EGF ระหว่างผู้ป่วยทางเดินน้ำดีตีบตันและกลุ่มควบคุม ($p = 0.744$) นอกจากนี้ผู้ป่วยทางเดินน้ำดีตีบตันที่มีผลการรักษาดีจะมีระดับของซีรั่ม TGF-b1 และ EGF สูงกว่าผู้ป่วยทางเดินน้ำดีตีบตันที่มีผลการรักษาไม่ดีอย่างมีนัยสำคัญทางสถิติ และพบว่ามีพหุสัมพันธ์ระหว่างระดับของ TGF-b1 และ EGF ในซีรั่ม (Pearson's $r = 0.3418$, $p = 0.0046$) สรุป ระดับของ TGF-b1 และ EGF ในซีรั่มของผู้ป่วยทางเดินน้ำดีตีบตันที่สูงขึ้น มีความสัมพันธ์กับผลการรักษาที่ดี ผลการศึกษาในครั้งนี้บ่งว่าผลของ TGF-b1 และ EGF น่าจะเกี่ยวข้องกับ พยาธิสรีรวิทยาของโรคทางเดินน้ำดีตีบตัน

๔. การแสดงออกของเอนไซม์ inducible nitric oxide synthase (iNOS) ของตับในโรคทางเดินน้ำดีตีบตัน

ศึกษาการแสดงออกของเอนไซม์ iNOS ของตับอาศัยเทคนิค Immunohistochemistry โดยศึกษาชิ้นเนื้อตับของผู้ป่วยทางเดินน้ำดี 24 คน และกลุ่มควบคุม 16 คน ผลการศึกษา พบว่าระดับการแสดงออกของเอนไซม์ inducible nitric oxide synthase ของตับในผู้ป่วยทางเดินน้ำดีตีบตันสูงกว่าในกลุ่มควบคุมอย่างมีนัยสำคัญทางสถิติ ($p<0.0001$) การวิเคราะห์เพิ่มเติมพบว่าระดับการแสดงออกของเอนไซม์ iNOS ของตับไม่มีความสัมพันธ์กับผลการรักษาในผู้ป่วยโรคทางเดินน้ำดีตีบตันเมื่อ 6 เดือนหลังผ่าตัด ($p=0.732$) สรุป ระดับการแสดงออกของเอนไซม์ inducible nitric oxide synthase ของตับในผู้ป่วยโรคทางเดินน้ำดีตีบตันสูงกว่ากลุ่มควบคุมแต่ไม่สัมพันธ์กับผลการรักษา แสดงให้เห็นว่า iNOS มีบทบาทต่อพยาธิสภาพของตับในผู้ป่วยโรคทางเดินน้ำดีตีบตัน แต่ระดับการแสดงออกของเอนไซม์ iNOS ของตับไม่สามารถนำมาใช้เพื่อการพยากรณ์โรคได้

๕. การรายงานผู้ป่วย 2 รายที่เป็นโรคทางเดินน้ำดีตีบตันแบบที่มี ถุงน้ำขนาดใหญ่นอกตับ

ผู้วิจัยได้ทำการรักษาและรายงานผู้ป่วยโรคทางเดินน้ำดีตีบตันชนิดที่พบยากคือ ชนิดที่มีถุงน้ำขนาดใหญ่นอกตับร่วมด้วย ซึ่งอาจจะทำให้แพทย์ผู้ทำการรักษา เข้าใจผิดว่าเป็นโรคทางเดินน้ำดีโป่งพองแต่กำเนิดซึ่งมีการรักษาที่ต่างกันได้

๖. การแสดงออกของ เฮปปาโทไซท์โกรทแฟกเตอร์และตัวรับ C-met ในตับโรคทางเดินน้ำดีตีบตัน

เป็นการศึกษาการแสดงออกของ เฮปปาโทไซท์โกรทแฟกเตอร์และตัวรับ C-met ของตับอาศัยเทคนิค Immunohistochemistry โดยศึกษาชิ้นเนื้อตับของผู้ป่วยทางเดินน้ำดี 41 คน และกลุ่มควบคุม 17 คน โดยผลการศึกษา พบว่าระดับการแสดงออกของ เฮปปาโทไซท์โกรทแฟกเตอร์และตัวรับ C-met ของตับในผู้ป่วยทางเดินน้ำดีตีบตันสูงกว่าในกลุ่มควบคุมอย่างมีนัยสำคัญทางสถิติ ($p<0.0001$) การวิเคราะห์เพิ่มเติมพบว่าการแสดงออกของ เฮปปาโทไซท์โกรทแฟกเตอร์และตัวรับ C-met ของตับไม่มีความสัมพันธ์กับผลการรักษาในผู้ป่วยโรคทางเดินน้ำดีตีบตันเมื่อ 6 เดือนหลังผ่าตัด โดยสรุป ระดับการแสดงออกของ เฮปปาโทไซท์โกรทแฟกเตอร์และตัวรับ C-met ของตับในผู้ป่วยโรคทางเดินน้ำดีตีบตันสูงกว่ากลุ่มควบคุมแต่ไม่สัมพันธ์กับผลการรักษา

Study 1: Elevated serum soluble E-selectin is associated with poor outcome and correlated with serum ALT in biliary atresia

Introduction

Biliary atresia (BA) is known as a disease characterized by chronic progressive inflammation and obliteration of extra- and intrahepatic bile ducts. If the BA patients are left without surgery either by Kasai operation or liver transplantation, the majority of them will die by 2 years of life from complications of portal hypertension [20]. Although there have been a number of studies regarding its pathophysiology, the plausible explanation remains unclear. These hypotheses include the involvement in the expression of serum levels of various cytokines, serum growth factors and the apoptosis of the bile duct cells [12, 14, 17, 27, 29]. Recently, various adhesion molecules seems to be an important part of the pathophysiology of BA [2, 14, 28].

Adhesion molecules such as E-selectin are found to shed from cell surfaces into the circulation. This soluble form (sE-selectin) mediates slow rolling and stable arrest of leukocytes on endothelium during inflammation [15, 16, 22]. They can be detected by enzyme-linked immunosorbent assays (ELISA) and their expression may reflect on activated or injured endothelial cells. Soluble E-selectin can be found in the circulation, probably arising from proteolytic cleavage of the surface-expressed molecule. Elevated levels of sE-selectin in serum have been reported in conditions characterized by systemic inflammatory processes [9, 10, 21, 23, 25]. However, the study of serum sE-selectin levels in post-operative BA receives little attention. Hence, the aim of this study was to investigate possible roles of serum sE-selectin in BA.

Methods

The ethical approval was obtained from the Institutional Review Board of the institution (No. 131/2006). All parents of the children were explained and informed of the purpose of the study. The written informed consents were obtained.

The study group consisted of 53 post-operative BA patients (25 males and 28 females with the mean age of 103.36+51.76 months during annual follow-up. The specimens of peripheral venous blood were drawn and transferred to a centrifuge tube, and then performed at 4 degrees Celsius. The serum was stored at -70 C until they can be assayed. The serum levels of sE-selectin were measured using ELISA method according to manufacturer's instructions (Quantikine, R&D Systems, USA; catalogue number BBE2B) and then compared with 10 age-comparable healthy children. The serum sE-selectin levels were expressed as nanogram per milliliter (ng/mL). Also, liver function tests (LFT) including serum total bilirubin, direct bilirubin, alkaline phosphatase (AP), aspartate aminotransferase (AST), alanine aminotransferase (ALT), and gamma-glutamyl transpeptidase (GGT) were determined using an automated chemical analyzer (Hitachi 911) at the central laboratory of our hospital.

In addition, the patients were categorized into two groups according to their jaundice status; jaundice-free patients (total bilirubin <2.0 mg%) and jaundice patients (total bilirubin >2.0 mg%). The cut-point of 2.0 mg% was selected based on the normal value of our central laboratory and based on published articles [12, 14]. The comparisons of demographic data and serum sE-selectin between jaundice-free patients and jaundice patients were performed. Correlation analysis of serum ALT and serum GGT was carried out. Data are expressed as mean and SD. SPSS software version 10.0 (SPSS Inc., Chicago, IL) was used for all statistical analyses.

Results

Comparisons between 53 BA patients and 10 healthy children

There was no significant difference in the terms of age (103.3+51.7 vs. 115.5+49.8 months, P=0.498) and gender (25:28 vs. 7:3, P= 0.302) between the BA patients and normal children. However, serum sE-selectin of BA patients was higher than the normal healthy children (114.1+44.0 vs. 88.7+22.2 ng/mL, P=0.01). The liver function tests of all children in control group were within normal limits as follows: serum albumin (4.0+0.2 g/dL), total bilirubin (0.5+0.3 mg%), direct bilirubin (0.1+0.07 mg%), AST (22.7+11.3 IU/L), and ALT (15.6+10.2 IU/L).

Comparisons within the group of BA patients

The demographic data and liver function test of BA patients based on the status of jaundice were shown in Table 1. Subgroup analysis showed that there was an increase in serum sE-selectin levels of BA patients with jaundice (n=21) compared to those without jaundice (n=32); (129.7+48.6 vs. 103.9+38.1 ng/mL, P=0.035). In addition, serum sE-selectin was positively correlated with serum ALT, a marker for liver injury (pearson r =0.355, P=0.009), but not with serum GGT (pearson r =0.223, P=0.12), as shown in Figure 1.

Discussion

Since the introduction of hepatic portoenterostomy by Kasai, the prognosis of BA has been improved and the pathophysiology has been widely investigated [13, 20]. Nevertheless, its real pathophysiology of the disease remains to be solved. One possible hypothesis is that immune-mediated process is responsible for the pathology of BA particularly in those with genetic susceptibility to toxic or infectious insult [3,

24]. Several investigators suggested that there are associations between serum levels of inflammatory markers (interleukins, intercellular adhesion molecule-1 or ICAM-1, nitric oxide metabolites, etc.) and BA patients [11, 12, 18, 26, 28]. In addition, the immunoglobulin superfamily of adhesion molecules, including ICAM-1 and vascular cell adhesion molecule-1 (VCAM-1), has been detected on hepatocytes, epithelial cells in all intrahepatic bile duct structures, and vascular endothelial cells in all patients with BA compared with negative staining in controls [4-6]. Thus, the role of adhesion molecules in the chronic progressive inflammation of liver is likely to be critical.

E-selectin (CD62E), formerly referred to as endothelial leukocyte adhesion molecule or ELAM, is a cell adhesion molecule expressed mostly on endothelial cells activated by cytokines. It plays an important part in inflammation for involving in the process of leukocyte rolling on vascular endothelium under blood flow. This is essential for blood leukocyte extravasations into the inflammatory or infectious sites [16]. The overlap of Endothelial (E-), Platelet (P-), and Leukocyte (L-) types of selectin function is also seen at the level of leukocyte rolling. However, E-selectin mediates leukocyte rolling at a significantly lower velocity than the other selectins [15, 16]. Therefore, the presence of E-selectin partly contributes to the transition from rolling to firm adhesion under the localized inflammatory circumstance. A soluble form of E-selectin (sE-selectin) was reported to be released into the circulation during the course of inflammatory response [19].

Although there have been some reports regarding the association between E-selectin and chronic liver diseases [1, 7, 8], the study of possible roles of serum sE-selectin levels in BA, however, receives little attention. Therefore, our objectives of

this study were to investigate whether serum levels of sE-selectin is associated with BA and to determine its relationship to the disease activity.

The present study clearly demonstrated that serum sE-selectin levels were significantly elevated in post-operative BA patients compared to the age-comparable healthy children. High levels of serum sE-selectin are probably associated with systemic inflammatory response. Hence, the process is likely to be related to the ongoing liver inflammation in BA. Interestingly, according to a study by Davenport et al [2], levels of sE-selectin in pre-operative BA patients (age of 1-4 months) were not different from those in controls. Since the mean age of the BA patients in our study was approximately 8-9 years old, therefore, it is possible that the elevation of serum sE-selectin levels found in this study is a secondary effect caused by long-standing chronic progressive inflammation, not directly associated with BA during infancy period. In addition, we found that the elevated serum sE-selectin levels were associated with clinical outcome (jaundice-free or persistent jaundice) in post-operative BA patients. The elevated serum sE-selectin was also positively correlated with the serum ALT, a marker for liver injury, but not with serum GGT, a marker for the degree of biliary obstruction. These findings suggest that serum levels of sE-selectin can be used as another prognostic marker for ongoing liver injury in post-operative BA patients.

One limitation of this study is that the serum levels of sE-selectin did not necessarily reflect the activity of selectin signaling pathways. However, with the supported evidence from other studies regarding the real association between serum sE-selectin levels and the degree of systemic inflammatory response [9, 10, 21, 23, 25], it is likely that the elevated serum sE-selectin levels found in post-operative BA patients play a role in long-term progressive liver inflammatory pathology.

In conclusion, serum sE-selectin was significantly elevated in postoperative BA patients. There was also an association between serum sE-selectin and clinical outcome in BA. A positive correlation of serum sE-selectin with serum ALT in BA patients suggests that E-selectin plays a role in the pathophysiology of liver injury in postoperative BA.

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Table and Figure Legends

	Jaundice-free (n=32)	Persistent jaundice (n=21)	P-value
Age (months)	101.9+41.9	105.6+65.6	0.81
Gender (M:F)	17:15	8:13	0.40
Total bilirubin (mg/dl)	0.79+0.40	10.72+8.38	<0.001
Direct bilirubin (mg/dl)	0.22+0.22	8.78+7.18	<0.001
AST (IU/L)	87.75+60.95	217.38+86.73	<0.001
ALT (IU/L)	97.22+73.53	180.90+99.26	0.001
Alkaline phosphatase (IU/L)	373.28+220.96	630.80+242.52	<0.001
GGT (IU/L)	178.17+189.82	389.00+267.78	0.002
sE-selectin (ng/mL)	103.92+38.10	129.74+48.60	0.035

Table 1: Comparisons between BA patients without jaundice and those with persistent jaundice

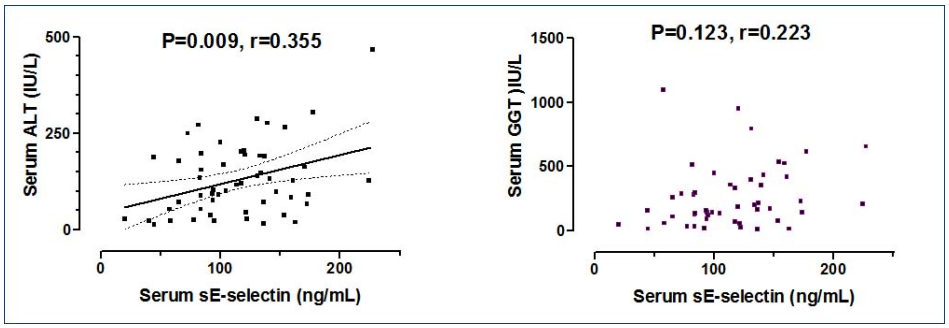

Figure 1: The correlation plot of serum sE-selectin with serum ALT (left) and serum GGT (right). The trend line on the left is the regression line +/- 95% confidence interval and r is pearson correlation coefficient.

Study 2: High-dose steroids did not improve early outcome in biliary atresia

Introduction

Hepatic portoenterostomy or Kasai operation has been accepted as a standard treatment to drain the biliary system in children with biliary atresia (BA) for many years [1]. Although a successful Kasai operation can restore bile drainage, long-term success of the Kasai procedure has been achieved in a minority of patients. Recent reports estimate that 70-80% of the patients will eventually require liver transplantation or die secondary to the progression of liver impairment [2, 3].

Several previous attempts to improve outcome of the Kasai procedure have concentrated on technical details such as the extent of resection of the fibrous mass at porta hepatis and variations in the reconstruction of the Roux-en-Y limb. Additionally, to make an impact on the outcome of infants with BA, several studies investigating its pathophysiology have been widely conducted. The results lead to the refinement of post-operative treatment [1, 4]. Even so, the fundamental problem of BA is that we do not fully understand the underlying disease process. Theoretically, viral infection, genetic predisposition, abnormal bile acid metabolism, and ductal plate malformations have been suggested as possible inciting events [3, 5-7]. Immunologic, inflammatory, infectious and obstructive pathways of progressive intrahepatic biliary epithelial destruction and portal fibrosis all have been hypothesized [8].

With the realization that immune-mediated inflammatory pathways of tissue injury attack both biliary caniliculi and liver parenchyma and serve as the fundamental pathophysiology of BA, anti-inflammatory drugs may come as an additional therapy [9-11]. Therefore, adjuvant post-operative medical therapy using short-term high-dose steroids aimed at enhancing native liver function after has been

practiced by some surgeons [12-15]. Since there are no randomized controlled trials on the effects of steroids, at present, due to the rarity of the disease, the benefits of high-dose steroids in BA have always been questioned.

In this study, the effects of post-operative high-dose steroids on early outcome of infants with BA, in terms of jaundice-free status and cholangitis within 6 months following the operation, were investigated.

Materials and Methods

The study was approved by the Institutional Review Board of Faculty of Medicine, Chulalongkorn University (No. 131/2006). A retrospective review of patients treated for BA, from Jan 2001 to Dec 2005, was performed at the Pediatric Liver Center. Over the studied period, only one team of pediatric surgeons (PV and SC) operated these patients using original Kasai operation with similar surgical techniques.

Before May 2003, all BA patients treated at our hospital received a routine protocol of post-operative treatment as follows; intravenous antibiotics for 10 days followed by oral co-trimoxazole for at least one year, urso-deoxycholic acid (10-15 mg/kg/d) for at least one month, and Vitamin A, D, E, K for at least one year, without any steroid therapy. BA patients treated during this period served as historical controls. From May 2003 onward, the use of high-dose steroids (prednisolone 4 mg/kg/d for 3-4 consecutive days began at 7^{th} day post-op, then at alternate day for 1-3 months) were added to the protocol. The duration of steroid therapy depends on the jaundice-free status. If the patients were jaundice-free very soon after the operation, steroid dosage would be tapered and stopped (but not before one month of steroid therapy after surgery). Generally, two weeks were used for the tapering of steroids.

However, if the patients were still jaundice after 3 months following the operation, steroid therapy would also be tapered and stopped. The protocol of post-operative medical treatment in this study was modified by our medical team from the information of related articles [11, 14, 16-18].

Categorization of the BA patients

In order to compare various aspects among BA patients, they were divided into 2 groups according to steroid treatment; steroid group and non-steroid group (historical control). Further subgroup analysis of early clinical outcome based on the jaundice-free status (serum total bilirubin or TB <2.0 mg/dl) at 6 months post-op and the occurrence of ascending cholangitis within 6 months after surgery were carried out. Ascending cholangitis, in this study, was defined as a combination of fever more than 38.5 C, changes of yellow stool to acholic stool, and leukocytosis (wbc >12,000 cells/mm^3) with PMN predominance.

Statistical analyses

Demographic and clinical data between steroid and non-steroid groups were compared by Fisher's exact tests and unpaired t-tests where appropriate. The mean and standard deviation were calculated for each variable. Jaundice-free status at 6 months post-op and the occurrence of cholangitis within 6 months post-op between patients receiving steroids and those not receiving steroids were compared using Fisher's exact test. Significance is set at the 95% confidence interval, $P < 0.05$.

Results

A total of 53 BA patients (M:F=27:26) undergoing Kasai operation were studied. Mean age at surgery was 89.85+31.33 days. Pre-operative serum TB was 10.81+2.70 mg/dl and serum direct bilirubin (DB) was 8.20+2.15 mg/dl. At 6 months post-op, 30 patients (56.6%) were jaundice-free and 24 patients (45.3%) experienced cholangitis at least once.

Of the 53 patients, there were 33 patients in steroid group and 20 patients in non-steroid group. The 2 groups were comparable in terms of age at surgery, gender, and pre-op serum TB (Table 1). The proportion of jaundice-free patients in steroid group was higher than that in non-steroid group (60.6% vs. 50%), and the proportion of patients with cholangitis in steroid group was lower than that in non-steroid group (39.4% vs. 55%). However, by using Fisher exact's tests, these discrepancies did not reach a statistically significant difference (jaundice-free status: steroid vs. non-steroid = 20/33 vs. 10/20, P=0.57 and cholangitis at 6 months post-op: 13/33 vs. 11/20, P=0.39) as shown in Table 1.

In spite of the high dosage of steroids used in this study, we did not identify any specific complications due to steroid treatment other than fluid retention and increased appetite. Surgical complications in the steroid group included one wound infection, and one gut obstruction from adhesion band requiring re-operation at 5^{th} day post-op. Complications from surgery in the non-steroid group included one child with wound infection and two children with gut obstruction responded to non-operative management.

Discussion

Biliary atresia (BA) is characterized by atresia of both extra- and intrabiliary system with a progressive sclerosing and inflammatory process. It has been

hypothesized, based on a liver histology, that the inflammatory process is associated with immune-mediated reaction [6, 7, 19]. Liver pathology illustrates bile ductular proliferation, canalicular stasis, vacuolization of cholangiocytes, portal edema and fibrosis, and monocytic and lymphocytic cell infiltration of the portal tracts [6, 20]. In addition, a number of cellular inflammatory markers have been studied including CD14-positive macrophages which secrete a number of inflammatory cytokines into the periductular tissue when activated by endotoxin [21]. Molecules involving in inflammatory pathways including ICAM-1 [7], nitric oxide metabolites [22], interleukin-8 [23] and monocyte chemoattractant protein-1 [24] have been proposed to be involved in the pathophysiology of progressive liver fibrosis in BA.

Steroids have been used as an adjunct for post-operative BA patients for many years, in spite of without a single randomized controlled trial at present. The beneficial effects of steroids for BA are believed to be choleretic action, immuno-supression, and anti-inflammation. In the past, the use of steroids has been reported to have synergistic effects with antibiotic treatment for intractable postoperative cholangitis in BA [9, 25]. Later, high-dose steroids have been subsequently proposed to have potential choleretic and anti-inflammatory properties that might benefit post-operative BA patients [11, 12, 14, 15, 26-28]. Steroids increase canalicular electrolyte transport and stimulate bile flow independent of the bile salt concentration. Furthermore, high-dose steroids have significant anti-inflammatory and immuno-suppressive effects decreasing tissue edema and collagen deposition, inhibiting fibrogenesis and migration of infiltrating monocytes and lymphocytes [5, 8, 10].

In an attempt to ameliorate progressive liver fibrosis following Kasai operation in BA patients, several retrospective studies demonstrated that high-dose steroids may improve the clinical outcome in BA after surgery via the mechanisms

mentioned above [12-15]. However, proof that the improved outcome is caused primarily by the steroids remains elusive, because none of these studies have been randomized or controlled and none of the studies are of sufficient size to properly answer the research questions.

Although the use of high-dose steroids, in the present study, showed some benefits, we cannot demonstrate the benefits of high-dose steroid towards early clinical outcome by means of statistically significant differences. Several reasons may be responsible for our results which show that there was no difference in the clearance of jaundice at 6 months and the occurrence of cholangitis within 6 months. Firstly, the dose of 4 mg/kg/d at alternate day might be too low. However, based on similar dosage reported elsewhere [14, 27] showing the benefits from steroids, it is likely that the dose of steroids used in this study was adequate. Secondly, the mean age at surgery (89.8 days) was quite late compared to other reports. Once the degree of cholestasis or liver fibrosis is significant, steroids may not maximally exert their effects. Thirdly, the sample size is probably too small due to the unexpected good results in non-steroid group. Our results showed that the effects of steroids increased the jaundice-free status by only 10% (from 50% to 60.6%), compared to non-steroid group. Statistically, this 10% difference needs at least 350 patients in each arm (calculated from StatMate version 1.01, GraphPad Software, Inc., CA, USA) to make the discrepancy statistically significant (at the power of 80%). Previous studies from other groups that show benefits of steroids by statistical differences have a significant gap of the percentage of jaundice-free status between steroid group and non-steroid group (79% vs. 21% [11] and 76% vs 37% [12]). Finally, the effects of surgical techniques may affect the outcome. However, all of the operations in this study were performed by one team of surgeons with similar details. Therefore, differences in

surgical technique are unlikely although the confounding factor of historical controls cannot be excluded. Another methodological aspect needed to be emphasized is that this study was carried out in a retrospective fashion together with a major drawback of likely Beta error due to relatively small patient number. Therefore, as mentioned above, the data of this study do not conclusively demonstrate that high-dose steroids did not improve early outcome in BA.

We do not know whether the beneficial effects of the adjuvant steroid therapy are caused by the choleretic, immunosuppressive, anti-inflammatory effects or the combination of these effects. At present, with the consistently promising observations of the adjuvant steroid studies published and the negative results reported in this study, a multi-center, randomized, controlled clinical trial is mandatory. Since the published articles usually proposed potential benefits of steroids in BA, this question may never be answered if the sample size in each arm of the patients in a randomized controlled trial is too small.

In conclusion, high-dose steroids can be used safely in post-operative BA. Although the use of steroids in post-operative BA patients seems to have some benefits, it did not statistically improve early outcome in BA patients based on jaundice-free status and cholangitis at 6 months post-op.

Acknowledgements

We are grateful to the Thailand Research Fund for generous support (Grant No RMU-4980017). Also, we would like to thank Rachadaphisek Somphot Endowment, Chulalongkorn University for supporting the research work.

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Study 3: Serum Transforming Growth Factor-β1 and Epidermal Growth Factor in Biliary Atresia

Introduction

Biliary atresia (BA) is a serious disease characterized by chronic progressive inflammation and obliteration of extra- and intra-hepatic biliary ducts with unknown etiology. If the BA patients do not received surgical treatment either by Kasai operation or liver transplantation, the majority of them will die inevitably within a few years due to portal hypertension [20]. Although there have been several investigations regarding its pathophysiology, the reasonable explanation remains unclear. Various plausible mechanisms for its etiology were proposed. Some of them include the involvement in the expression of various inflammatory cytokines, growth factors and the apoptosis of the biliary epithelial cells [5, 10, 14, 25, 27]. Recently, it has been suggested that various growth factors such as hepatocyte growth factor, insulin-like growth factor, and connective tissue growth factor play critical roles in the pathophysiology of liver fibrosis in BA [11, 15, 25, 27]. However, the information of possible roles of some growth factors including transforming growth factor-β1 (TGF-β1) and epidermal growth factor (EGF) in BA still receive little attention.

Transforming growth factor-β1 (TGF-β1) is a polypeptide member of the transforming growth factor superfamily of cytokines. It is a secreted protein that performs various cellular functions, including the regulation of cell growth, cell proliferation, cellular differentiation and apoptosis [15, 21]. For the liver, TGF-β1 is an important mediator of liver cell proliferation and replication that is implicated in liver fibrosis. In addition, hepatic stellate cells are activated by TGF-β1. Hepatic stellate cells are the main precursor cells involved in liver fibrogenesis [17].

Epidermal growth factor or EGF is a growth factor that plays an important role in the controls of cell growth, proliferation, and differentiation. Human EGF is a 6,045-Da protein with 53 amino acid residues and three intra-molecular disulfide bonds. EGF induces the increase in hepatocytes proliferation [26]. EGF given to animals receiving a hepatotoxic substance (carbon tetrachloride) can decrease organ damage and morbidity as compared to the control group without EGF [1]. It has been demonstrated that expression of EGF transcription was highly elevated in cirrhotic liver (regenerative nodules and bile duct epithelial cells) as compared to low expression in normal liver [13].

Both TGF-β1 and EGF seem to play important roles in pathological processes of the liver, especially progressive liver fibrosis. However, little information of serum TGF-β1 and EGF levels in post-operative BA, surprisingly, has been explored. Thus, the aim of this study was to investigate possible roles of serum TGF-β1 and EGF regarding their association with therapeutic outcome in BA.

Methods

The ethical approval was obtained from the Institutional Review Board of the Faculty of Medicine (No. 131/2006). All parents of the children were explained and informed of the purpose of the study.

The study group consisted of 67 post-operative BA patients (36 males and 31 females) with the mean age of 8.32 years during annual follow-up. None of the patients received liver transplantation. The specimens of peripheral venous blood were drawn and transferred to a centrifuge tube, and then performed at 4 C. The serum was stored at -70 C until they can be assayed. The serum levels of TGF-β1 and EGF were measured using ELISA method based on manufacturer's instructions

(Quantikine, R&D Systems, USA; catalogue number DB100B and DEG00, respectively) and then compared with 10 age-matched healthy children. The serum levels were expressed as ng/ml for TGF-β1 and pg/ml for EGF. Also, serum levels of total bilirubin (TB), and alanine aminotransferase (ALT) were determined.

For subgroup analysis, the patients were categorized into two groups as per their therapeutic outcome; patients with good outcome (serum TB <2.0 mg%) and poor outcome (serum TB >2.0 mg%). The comparisons of demographic data, serum TGF-β1 and serum EGF levels between the two groups of BA patients were performed. Correlation analysis of serum TGF-β1 and serum EGF was carried out. Data are expressed as mean and SD. SPSS software version 13.0 (SPSS Inc., Chicago, IL) was used for all statistical analyses.

Results

Comparisons between 67 BA patients and 10 healthy children

There was no significant difference in the terms of age and gender between the BA patients and normal controls. Serum TGF-β1 levels of BA patients were higher than the controls (86.6+15.7 vs. 75.7+8.8 ng/ml, P=0.0362). However, there was no difference in serum EGF between BA patients and the controls (133.1+66.6 vs. 125.4+88.9 pg/ml, P=0.744).

Comparisons among the 2 groups of BA patients with different therapeutic outcome

The demographic and clinical data of BA patients based on their therapeutic outcome were shown in Table 1. Further subgroup analysis among BA patients showed that patients with good outcome (n=40) had their serum TGF-β1 and serum EGF levels higher than those of patients with poor outcome (TGF-β1; 91.2+16.5 vs.

79.6+11.7 ng/ml, P=0.002, and EGF; 148.5+65.0 vs. 110.3+63.4 pg/ml, P=0.02). Moreover, serum TGF-β1 was positively correlated with serum EGF (pearson r =0.3418, P=0.0046), as shown in Figure 1. However, both serum TGF-β1 and serum EGF were not correlated with serum ALT, a marker for liver injury.

Discussion

Owing to the introduction of Kasai operation, the prognosis of BA has been greatly changed and the pathophysiology has been widely investigated [7, 19, 20]. Nevertheless, its real pathophysiology of the disease remains to be solved and its therapeutic outcome is yet to be improved. Several investigators suggested that there are associations between serum levels of various growth factors (hepatocyte growth factor, insulin-like growth factor, connective tissue growth factor, and TGF-β1) and BA patients [11, 12, 23, 25]. Therefore, the role of growth factors in the progressive liver fibrosis is likely to be critical. Although there have been several reports regarding the association between growth factors and BA as mentioned above, the study of possible roles of serum levels of TGF-β1 and EGF in BA receives not much attention. Therefore, the objective of this study was to explore the possible roles of serum TGF-β1 and EGF levels in post-operative BA patients who are long-term survivals.

TGF-β1 is a secreted protein that performs many cellular functions, including cell growth, cell proliferation, cell differentiation and apoptosis [15, 21]. TGF-β1 is an important mediator of liver cell proliferation and replication that is implicated in liver fibrosis. It is a potent cytokine for promoting hepatic fibrogenesis by stimulating the activation of HSCs. In addition, hepatic stellate cells are the main precursor cells involved in liver fibrogenesis [17].

The present study showed that serum levels of TGF-β1 were significantly elevated in post-operative BA patients compared to healthy children. The results are consistent with a previous study demonstrating that serum TGF-β1 levels were higher in adult cirrhotic patients than those of healthy adults [8]. Since TGF-β1 is a primary factor promoting fibroblast proliferation and inducing matrix protein synthesis (hepatic fibrogenesis), elevated serum TGF-β1 levels found in patients with BA may have a role in the pathophysiology of progressive liver fibrosis in BA. Interestingly, based on its therapeutic outcome, BA patients with good outcome had higher levels of TGF-β1 than those of the patients with poor outcome. Our results confirm the findings illustrated by Kobayashi et al. [12]. They showed that post-operative BA patients without jaundice had elevated serum TGF-β1. Therefore, according to the action of TGF-β1 alone, it is possible that BA patients with good outcome still had progressive liver fibrosis. The study of hepatic TGF-β1 expression and activated stellate cells in BA patients with good outcome are needed to prove this hypothesis.

We also demonstrated that there was no difference between serum levels of EGF between BA patients and controls. However, when looking at the comparisons between BA patients with different outcomes, the patients with good outcome had their serum EGF levels higher than those with poor outcome. Because there were two groups of BA patients with two groups of serum EGF levels due to therapeutic outcome, these may explain why there was no difference in serum EGF levels between BA patients and normal controls. Hence, it is not always straightforward when interpreting the comparisons of serum levels of substances factors between diseased patients and controls.

EGF is a growth factor that also plays an important role in the regulation of cell growth, proliferation, and differentiation [26]. It is an initiator of hepatic

regenerative response as well as hepatocyte DNA synthesis [22]. EGF when given to hepatotoxic animals can decrease organ damage and morbidity as compared to those without EGF [1]. It has also been demonstrated that EGF mRNA expression is low in the cirrhotic liver [2]. This probably resulted in the loss of liver parenchyma in cirrhosis. Therefore, the action of EGF is likely to plays advantageous roles in BA. These are in concordant with low serum EGF levels in BA patients with poor outcome found in this study. Additionally, EGF accumulation within biliary epithelial cells has been demonstrated [2]. This may be an evidence for the involvement of EGF in the proliferation of bile ducts. More studies on EGF expression of biliary ductular cells are needed to further understand the roles of EGF in BA.

The elevated serum levels of TGF-β1 and EGF were associated with good outcome (jaundice-free) in post-operative BA patients. Serum levels of TGF-β1 were also positively correlated with the serum levels of EGF in BA patients. Since the actions of TGF-β1 and EGF are in the opposite directions. TGF-β1 inhibits hepatocyte regeneration, promotes fibroblast proliferation, and induces fibrogenesis via matrix protein synthesis [3, 15] whereas EGF initiates hepatocyte regeneration, and stimulates hepatocyte DNA synthesis [4]. The positive correlation between serum TGF-β1 and serum EGF are probably caused by the effects of healing process of the liver against progressive liver fibrosis. Nevertheless, our findings suggest that serum levels of TGF-β1 and EGF can be used as prognostic markers for long-term clinical outcome in post-operative BA patients. In addition, we found that serum ALT (a marker for liver injury) was not correlated to either serum TGF-β1 or serum EGF. These suggested that actions of TGF-β1 and EGF involve in the process of liver fibrogenesis and regeneration but not liver injury.

This study inevitably does have limitations. Firstly, the serum levels of TGF-β1 and EGF did not necessarily reflect the action of their signaling pathways. However, with the supported evidence from other studies regarding the real association between their serum levels and the process of liver fibrosis, it is likely that the elevated serum levels of TGF-β1 and EGF found in BA play roles in the liver pathology. Secondly, there are other growth factors that are involved in the pathophysiology of liver fibrosis such as fibroblast growth factor [18], interleukin-6 [6], and hepatocyte growth factor [9, 16]. In our previous studies, it has been demonstrated that the elevated serum levels of hepatocyte growth factor were associated with poor outcome in post-operative BA [25]. However, the results from the present study showed that elevated serum levels of TGF-β1 and EGF were associated with good outcome. Although the resultant actions of EGF and HGF towards the liver seem to be similar [24], their changes in serum levels, as per their outcome, are interestingly in the opposite directions. Therefore, there are other influential factors outside the experimental boundaries needed to be taken into account. What we demonstrated here is just a small jigsaw from the big picture. More research work on other growth factors and cytokines related to growth factors involving in liver fibrosis is mandatory in order to better understand this complex process.

In conclusion, the elevated serum TGF-β1 and serum EGF levels were associated with good outcome in BA. The positive correlation between serum TGF-β1 and serum EGF was demonstrated. Serum levels of TGF-β1 and EGF may be used as prognostic markers in long-term post-operative BA. Our results also suggest that the resultant actions of TGF-β1 and EGF pathways possibly involve in the pathophysiology in post-operative BA.

Acknowledgements

We are grateful to the Thailand Research Fund for the support (Grant No RMU-4980017). Also, we would like to thank Faculty of Medicine, Chulalongkorn University for the permission to conduct the research work.

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	Good outcome (n=40)	Poor outcome (n=27)	P-value
Age (years)	8.28+4.95	8.37+5.72	0.957
Gender (M:F)	23: 17	13: 14	0.467
Serum albumin (g/dl)	4.48+0.34	3.35+0.60	<0.001
Serum Total bilirubin (mg/dl)	0.72+0.37	12.94+12.13	<0.001
Serum ALT (IU/l)	110.42+104.70	159.48+130.27	0.093
Serum TGF-β1 (ng/ml)	91.24+16.48	79.63+11.75	0.002
Serum EGF (pg/ml)	148.53+65.02	110.30+63.41	0.020

Table 1: Comparisons between BA patients without jaundice and those with persistent jaundice

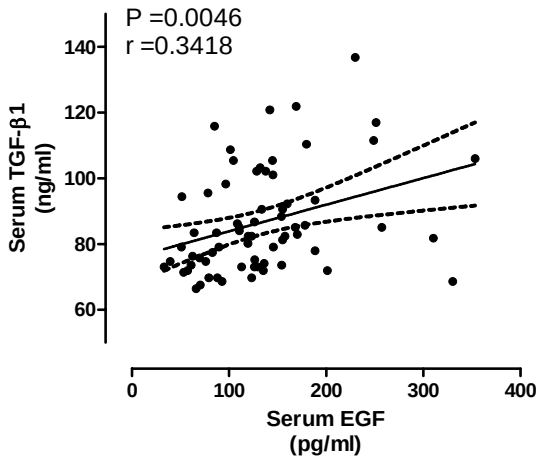

Figure 1: The correlation plot between serum TGF-β1 levels and serum EGF levels of 67 BA patients. The trend line is the regression line +/- 95% confidence interval and r is pearson correlation coefficient.

Study 4: Overexpression of inducible nitric oxide synthase in biliary atresia

Introduction

Biliary atresia (BA) is a rare and devastating cholestatic disease. Its clinical presentation is the development of obstructive jaundice, indicated by direct hyperbilirubinemia and acholic stools. Progressive liver fibrosis will lead to death within 2 years if left untreated [1, 2]. The patients who are left untreated will die from hepatic decompensation, esophageal variceal bleeding or infection [3]. It is accepted that hepatic portoenterostomy or Kasai operation at the early age is indispensable to the successful management of infants with BA. However, despite this procedure, BA remains the commonest indication for pediatric liver transplantation.

For decades, there is a hope that research on liver fibrosis in BA may finally lead to the improvement of long-term outcome. Several investigations regarding its pathophysiology have been explored. Although there have been a number of studies on pathophysiology of BA, including serum levels of various inflammatory markers [4-8], serum growth factors [9, 10], and the apoptosis of bile duct cells [11], the exact mechanism is still unclear.

In recent years, it has been illustrated that nitric oxide (NO) produced through inducible nitric oxide synthase (iNOS) plays an important role in primary biliary cirrhosis [12, 13] and liver diseases characterized by chronic inflammation [14, 15]. Although we do not believe that the roles of iNOS are identical between BA and primary biliary cirrhosis, they may share some resultant effects of NO. In addition, our previous results showed that there was an elevation in systemic NO production (serum nitrate and nitrite levels) in BA patients [16]. Interestingly, the elevated NO production was associated with serum alanine transaminase (ALT) levels, a marker for liver injury [16].

However, there is little information available regarding the role of iNOS enzyme in BA. Since progressive liver fibrosis is an important development in BA together with the possible role of NO in biliary cirrhosis and liver inflammation, it is of our interest to study the links between the expression of hepatic iNOS and BA.

Therefore, the aims of this study were to investigate the expression of hepatic iNOS in BA and to associate the iNOS expression with their early therapeutic outcome using immunohistochemistry (IHC) technique.

Methods

The study was approved by the Ethical Review Board of the Department.

Liver tissues and patients

Liver samples of BA patients undergoing Kasai operation and non-BA patients undergoing liver biopsies between July 2005 and July 2007 were retrospectively investigated. All patients were operated by one team of surgeons (PV and SC). The non-BA patients, who had no clinical jaundice, were served as controls. All non-BA patients underwent exploratory laparotomy as the therapeutic means for their diseases. Liver biopsies in this group of patients were an additional procedure and were indicated for medical reasons.

Immunohistochemistry of liver tissues for iNOS

Liver samples were fixed in formalin for 24 hours and kept in paraffin embedded blocks using standard procedure. New sections of 5 μm thickness were cut from the blocks and mounted on glass slides coated by amino-propyl-triethoxysilane (APES; Sigma Chemical Co., St Louis, MO, USA). Sections were deparaffinized and rehydrated. Endogenous peroxidase activity was blocked with 10-min incubation in

3% H_2O_2. Antigen retrieval was done by immersing the sections in 0.4% pepsin (Sigma Chemical Co) in 0.01 M HCL at 37 C for one hour. After washing with 0.1% Tween 20 (MERCK-Schuchardt, Hohanbrunn, Germany) in phosphate buffered saline (PBS), the sections were treated with 2% bovine serum albumin (BSA; Sigma Chemical Co) in PBS for 30 minutes and then treated with primary antibodies for two hours at room temperature. The primary antibodies used in this study were against iNOS (monoclonal anti-human iNOS antibody, Cat. Number MAB9502, R & D Systems, USA) diluted at 1:200. The primary antibodies were diluted in PBS. The immunoreactive complexes were detected using Envision System (Dako Corporation, Carpinteria, CA, USA) and visualized with the peroxidase substrate. Color was developed in freshly made diaminobenzidine (Sigma Chemical Co). Sections were washed briefly in running tap water and lightly stained with Mayer's hematoxylin. Negative controls were done by omission of the relevant primary antibody. Sections of salivary gland tissue were stained at the same run as positive controls for comparison. The ducts of salivary gland have been shown stain intensely for iNOS [17].

Evaluation of hepatic iNOS expression

Immunostained sections were evaluated independently by 2 investigators who were unaware of the diagnosis or clinical outcome of the patients. Two quantitative methods were used, based on the extent and intensity of the antibody stain. Areas of hepatic lobules excluding portal triads and central veins were evaluated using visual scoring method and computerized scoring method. The portal triads were excluded for 2 reasons. Firstly, the hepatocytes areas are likely to be the major source of iNOS expression within the liver of BA patients. Therefore, we would like to specifically investigate the iNOS expression of the hepatocytes areas. Secondly, portal triad areas

consisted of various components i.e. vascular endothelium, bile duct cells, smooth muscle cells, and fibrous connective tissue around these cells. The majority of the portal triad area including fibrous connective tissue was not stained by iNOS. We would like to make the selected areas as homogeneous as possible. Otherwise, significant areas of fibrous connective tissue would possibly affect the mean score of iNOS expression and mislead the results.

Visual scores for iNOS expression were assessed based on its intensity and percentage of staining areas. Expression of iNOS intensity was categorized into 3 levels as weak (intensity score =1), moderate (intensity score =2), and strong (intensity score =3). In addition, the percentage of staining areas of hepatocytes was graded into 4 levels (Grade 1; 0-25% of hepatocyte areas was stained; Grade 2: 26-50% of hepatocyte areas was stained; Grade 3: 51-75% of hepatocyte areas was stained; and Grade 4: 76-100% of hepatocyte areas was stained). Any differences in scores were resolved by a conference. Total scores of hepatic iNOS expression, evaluated by visual scoring method, were calculated as intensity score times staining grade.

In addition to the visual scoring method mentioned above, another computerized scoring method which is probably more objective to evaluate hepatic iNOS expression was used based on Ruifrok and Johnston's method [18, 19]. Five randomly selected areas of hepatocytes (under x10 magnification) excluding portal triads and central veins were pictured and saved as JPEG format. The parameters of the camera were similarly set in all cases. The intensity of the iNOS expression was evaluated using the Histogram mode from ImageJ software version 1.38x [20] with color deconvolution plugin [21]. By using this option, the blue color of hematoxylin and brown color of diaminobenzidine (DAB) can be separated. With this computerized

method, the intensity in DAB channel of a pixel in the selected area is scored into 256 arbitrary levels (0 to 255; bright to dark). Five selected areas of hepatic lobules from each patient were evaluated and then averaged. The equation to quantify the iNOS expression into the arbitrary levels is as follows: iNOS expression = Mean intensity in DAB channel of selected hepatocyte areas – Mean intensity in DAB channel of background areas.

Categorization of the BA patients

In order to associate hepatic iNOS expression with clinical outcome at 6 months post-op among BA patients, they were divided into 2 groups according to the status of jaundice (TB <2 mg/dL; good outcome vs. TB ≥2 mg/dL; poor outcome). Subgroup analysis of hepatic iNOS expression based on early clinical outcome in BA patients was carried out.

Statistical analyses

Demographic and clinical data between groups were compared by chi-square tests and unpaired t-tests where appropriate. The mean and standard deviation were calculated for each variable. The comparisons of iNOS expression among the two groups (BA and non-BA patients) were performed using Mann-Whitney U tests (visual score) and unpaired t-tests (computerized method). Spearman correlation analysis of visual scoring method with computerized scoring method was performed. Association between outcome of BA patients at 6 months post-op and hepatic iNOS expression was analyzed using univariate analysis.

Significant differences were established at $P<0.05$. For all statistical analyses, either GraphPad Prism version 3.02 (GraphPad Software Inc., California, USA) or

SPSS software version 10.0 (SPSS Inc., Chicago, IL) was used. Data are expressed as mean and SD.

Results

Twenty-four BA patients and 16 non-BA patients were studied. No BA patients were excluded from the study during the studied period. All non-BA patients had no clinical jaundice. The diagnosis of non-BA patients were as follows: 6 choledochal cysts, 4 thalassemias, 3 neuroblastomas, 2 portal vein thromboses, and 1 hepatoblastoma. The demographic and clinical data are demonstrated in Table 1. Descriptively, the staining intensity was stronger and the percentage of staining area of hepatic iNOS was higher than those of controls. Hepatic iNOS expression score of BA patients was significantly higher than that of non-BA patients ($P=0.0015$ using visual scoring method and $P<0.0001$ using computerized method), as shown in Figure 1. There was a positive correlation between hepatic iNOS expression evaluated by visual scoring method and by computerized scoring method ($P<0.0001$, spearman $r = 0.7387$). The representative histological sections of BA patients and non-BA patients are illustrated in Figure 2. For Figure 3, higher magnification (x40) of histological section of a hepatocytes area in a BA patient is demonstrated in order to prove that iNOS expression was exclusively stained in hepatocytes. In addition, iNOS expression was detected in nuclei of biliary epithelial cells at portal triad areas from BA patients (Figure 4).

Regarding the clinical outcome at 6 months post-Kasai among BA patients, there were 13 patients with good outcome (serum TB < 2.0 mg/dL) and 11 patients with poor outcome (serum TB >2.0 mg/dL). The demographic and clinical data are shown in Table 2. Subgroup analysis showed that there was no association between

hepatic iNOS expression and early clinical outcome in BA patients (P=0.995 by visual scoring method and P=0.732 by computerized method).

Discussion

Biliary atresia (BA) is a rare disease characterized by progressive sclerosing fibrous obliteration of the intra- and extra-hepatic biliary systems. Without hepatic portoenterostomy or Kasai operation, the resultant cholestasis leads to hepatic fibrosis and death within a few years [1, 22]. However, the procedure is not the final answer because, even after a successful Kasai operation, a significant number of patients still progress to liver fibrosis requiring liver transplantation later in their lives [23]. Although there have been several studies concentrating on the pathophysiology of progressive liver fibrosis in post-Kasai BA patients [5, 8, 10, 24], its mechanism is still unclear.

In recent years, one of the molecules being extensively studied regarding liver injury of various conditions is nitric oxide (NO). It is a short-living biological mediator generated from L-arginine by nitric oxide synthase (NOS). The NOS family of enzymes includes endothelial NOS (eNOS or type 3 NOS), neuronal NOS (nNOS or type 1 NOS), and inducible NOS (iNOS or type 2 NOS). NO exerts a broad spectrum of physiological functions, including regulation of vascular reactivity, platelet and leukocyte activation, neurotransmission, regulation of cellular proliferation, and nonspecific immunity reactions [25]. Inappropriate release of NO have been associated with diverse vascular, ischemic, thrombotic, and inflammatory pathologies [25-27]. For the liver, NO is generated by eNOS and iNOS, and NO can mediate a number of physiological and disease reactions involving this organ [27]. With a broad range of molecular targets, NO acts as an inhibitor or an agonist of cell signaling events. Constitutively generated NO maintains the hepatic microcirculation

and endothelial integrity, while inducible NO synthase (iNOS)-governed NO production can be either beneficial or hepatotoxic [26]. When the conditions are right, NO (via formation of peroxynitrite) can damage cellular components by its strong oxidizing effect. There is substantial evidence that NO or peroxynitrite can act on the mitochondria to inhibit cellular respiration [28]. Perhaps the significant direct cellular effect of NO with respect to hepatic injury concerns apoptosis [29]. Our previous studies also demonstrated that there is an elevation of systemic NO production (serum nitrites and nitrates) in post-operative BA patients [16]. Therefore, possible implications of hepatic iNOS expression in the pathophysiology of liver injury in BA are obvious.

In this study, immunohistochemistry (IHC) was used to investigate the hepatic iNOS expression. The increased use of IHC in both clinical and basic research settings has led to the development of various computerized techniques for acquiring quantitative information from immunostaining levels. Quantitative IHC techniques have often yielded important information regarding patient diagnosis, prognosis, or both. In fact, a correlation between IHC staining and protein levels, using other conventional methods including Western blotting analysis [30] and enzyme immunoassays [31], has been demonstrated. By comparing immunostaining quantification obtained by a digital computer-assisted method (ImageJ software with color deconvolution plugin) with a standard semi-quantitative analysis in this study, it is clear that results of both methods are concordant. However, digital measurement is more objective and could resolve either researcher's bias or disagreement between two observers.

Our results clearly demonstrated that there is an overexpression of hepatic iNOS in BA patients compared to other non-BA patients. Overexpression of hepatic iNOS found in BA patients suggested that NO production by the liver in BA is

increased. This is consistent with our previous studies in post-operative BA patients illustrating that there is an elevation of serum NO metabolites compared to normal children [16]. In addition, this study demonstrated that most of the iNOS stained within the liver tissues was from the area of hepatocytes. It is therefore likely that hepatocytes, not cholangiocytes or endothelial cells or inflammatory cells, are the major source of hepatic NO production in BA patients.

Interestingly, the iNOS expression found in the nuclei of biliary ductular cells possibly contributes to the injury of bile ducts leading to fibrosis and perhaps atresia of biliary system. High levels of hepatic NO production could also damage both hepatocytes and biliary ductules through oxidative stress possibly resulting in biliary obstruction from progressive liver fibrosis. Additionally, subsequent analysis among BA patients revealed that hepatic iNOS expression at the time of Kasai operation was not associated with early clinical outcome in BA at 6 months post-Kasai. Therefore, the levels of hepatic iNOS expression cannot be used as a prognostic marker for predicting the early outcome at 6 months post-op.

We are also aware of some limitations in this study. Firstly, we do not know whether the overexpression of hepatic iNOS found in BA patients is a cause or is an effect to the disease pathology caused by biliary obstruction. If it is an effect, hepatic iNOS expression will decrease in BA patients with good outcome. The study of hepatic iNOS expression in post-Kasai patients with different clinical outcomes will be able to answer this research question. Secondly, overexpression of hepatic iNOS may be just the non-specific findings of cholestasis. More studies on non-BA infants with cholestasis will solve this concern. Thirdly, the sample size of BA patients was probably too small in terms of statistical analysis. However, with the rarity of this disease, the results of a study of liver tissues from 24 BA patients cannot be

overlooked. Finally, the mean age of non-BA patients is higher than that of BA patients. This is because of the rarity of infants whose open liver biopsies were mandatory for their therapeutic goals. Liver biopsies in normal infants will be unethical. However, there were 4 non-BA patients with their age below 6 months in this study. All of hepatic iNOS expression in these 4 non-BA patients showed low levels of iNOS expression. In addition, it has been reported that hepatic iNOS expression is low in non-cholestatic livers [25, 26, 32, 33]. Therefore, it is likely that hepatic iNOS expression is low in non-BA patients without jaundice.

A recent report illustrated that iNOS inhibitor reduces liver fibrosis by the inhibition of transforming growth factor-beta1 and retards the development of cirrhosis in rats [34]. Together with the findings from this study that hepatic iNOS in BA patients is overexpressed, and with several evidences that NO production via iNOS enzyme induces liver injury [35-38], inhibition of NO production may have therapeutic roles in the amelioration of progressive liver fibrosis in BA. More studies are needed to prove this hypothesis.

In conclusion, overexpression of hepatic iNOS in BA was demonstrated. Hepatocytes were the major source of iNOS expression within the liver in BA. However, hepatic iNOS expression was not associated with the early therapeutic outcome in BA patients. These suggest that iNOS plays a role in the liver pathology of BA but its expression cannot be used as a predictor for therapeutic outcome. More studies on this aspect are needed to better understand the exact role of iNOS in BA.

Acknowledgments

We are grateful to the Thailand Research Fund for the support (Grant No. RMU-4980017). Also, we would like to thank Faculty of Medicine, Chulalongkorn

University for the permission to conduct the research work. The authors also thank Ms. Meesakul O for her technical assistance.

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Table and Figure legends

Table 1: The demographic data and hepatic iNOS expression of BA patients and non-BA patients (data are expressed as mean+SD). N/A = not available because liver function tests were not performed in half of the non-BA patients.

Table 2: BA patients are categorized based on clinical outcome at 6 months post-Kasai (data are expressed as mean+SD)

Figure 1: Hepatic iNOS expression between BA patients and non-BA patients based on visual scoring method and computerized scoring method.

Figure 2: Representative immunohistochemical features of the livers from a patient with neuroblastoma (A&B) and a BA patient (C&D). Note the strong expression of iNOS stained on hepatocytes in BA patients (x5 and x10 magnification)

Figure 3: Representative immunohistochemical features of iNOS at hepatocytes in BA patients at high magnification (x40). Note the expression of iNOS exclusively stained within the hepatocytes.

Figure 4: Representative immunohistochemical features of iNOS at portal triad area of a BA patient. Note the iNOS expression of the nuclei of proliferative biliary dutular cells (x20 magnification).

	BA Patients (n=24)	Non-BA Patients (n=16)	P-value
Age at operation (days)	89.42+30.05	1881.5+1773.05	<0.0001
Gender (male: female)	8: 16	8: 8	0.339
Albumin (g/dL)	4.05+0.35	N/A	-
Total bilirubin (mg%)	12.11+3.20	N/A	-
ALT (IU/L)	179.25+113.17	N/A	-
Hepatic iNOS expression (computerized method)	89.39+20.38	46.01+29.60	<0.0001

Table 1: The demographic data and hepatic iNOS expression of BA patients and non-BA patients (data are expressed as mean+SD). N/A = not available because liver function tests were not performed in half of the non-BA patients.

	BA patients with good outcome (n=13)	BA patients with poor outcome (n=11)	P-value
Age at operation (days)	79.23+13.43	101.45+39.57	0.07
Gender (male: female)	6: 7	2: 9	0.211
Total bilirubin (mg%)	0.71+0.56	9.15+6.36	<0.0001
Albumin (g/dL)	4.05+0.45	3.33+0.67	0.006
ALT (IU/L)	91.61+56.36	134.72+9.81	0.201
Hepatic iNOS expression (computerized method)	90.75+20.46	87.80+21.16	0.732

Table 2: BA patients are categorized based on clinical outcome at 6 months post-Kasai (data are expressed as mean+SD)

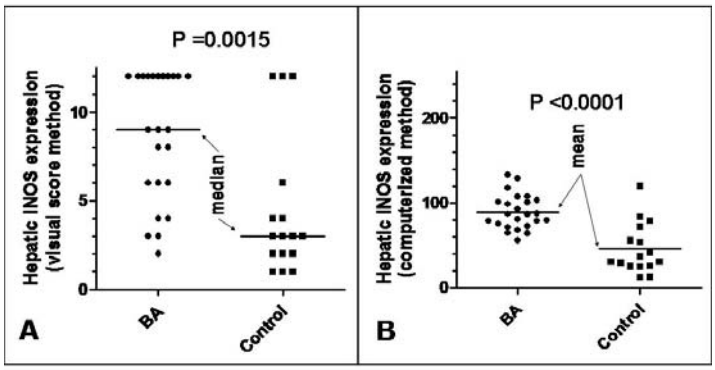

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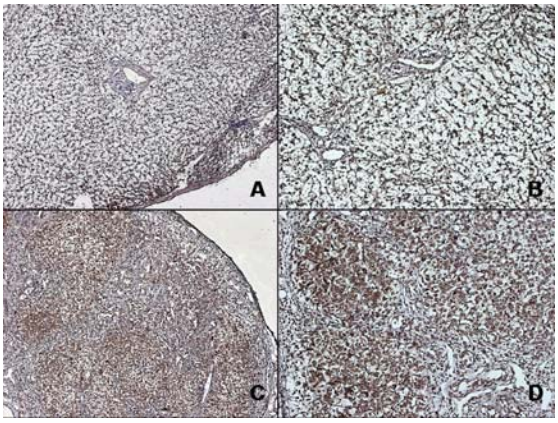
Figure 2: 

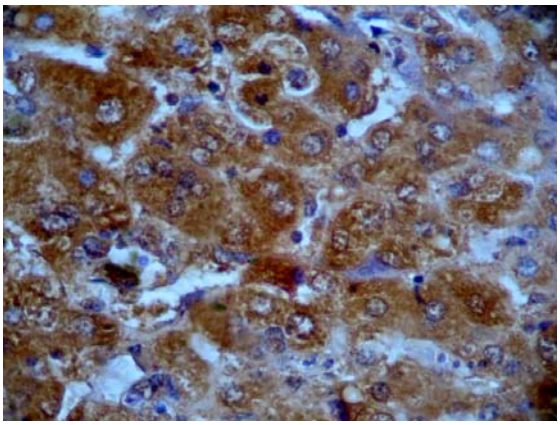
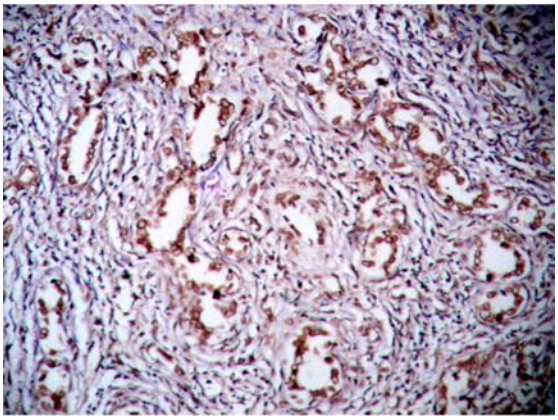
Figure 3: 

Figure 4: 

Study 5: Non-correctable Biliary Atresia with Large Extrahepatic Cyst: a Report of Two Cases

Introduction

Generally and conventionally, biliary atresia can be classified by using macroscopic appearance and cholangiography findings based on three main categories: main types, subtypes according to the pattern of distal bile ducts, and subgroups according to the pattern of hepatic hilus [10, 11]. We report two cases of an unusual variant of biliary atresia which had bile duct atresia at porta hepatis, together with a true cystic structure at common hepatic or common bile duct without distal connection to the duodenum.

Case 1

The baby was a 70-day-old girl who had persistent jaundice. Her prenatal history was unremarkable. Clinical jaundice and acholic stool were recognized after the first month of life. She was then referred to the pediatric surgical service. Laboratory findings revealed a total bilirubin of 12.04 mg/dL, direct bilirubin of 8.59 mg/dL, AST (aspartate aminotransferase) of 172 U/L (normal; 0-38 U/L), ALT (alanine aminotransferase) of 109 U/L (normal; 0-38 U/L), and alkaline phosphatase of 541 U/L (normal; 39-117 U/L).

Pre-operative ultrasonography revealed a 2.5x2 cm of cystic mass at extrahepatic bile duct without intrahepatic bile duct dilatation. Hepatobiliary scintigraphy using Tc-99m DISIDA scan showed homogeneous radiotracer accumulation of the liver. However, gall bladder and small intestinal tracer activity was not visualized up to 24 hours after radiotracer injection. In addition, no accumulation of radiotracer at so-called choledochal cyst was identified. Therefore,

our differential diagnosis was either correctable biliary atresia (type I or II) or choledochal cyst.

On exploratory laparotomy, a small gall bladder containing clear fluid together with a cystic mass at hepatoduodenal ligament was demonstrated. Intra-operative cholangiography was carried out. During contrast injection, a significant resistant pressure was felt. The radiographic image revealed a 2.5-cm cystic collection of the contrast material with no connection to intrahepatic bile duct or intestinal lumen (Figure 1). Further upward dissection illustrated that there was a fibrous mass at the bifurcation of portal vein. The mass of fibrous remnant together with the cyst was then completely resected. Finally, hepatic portojejunostomy with Roux-en-Y construction or Kasai operation was performed. Dissection of the cyst on the bench demonstrated that there was no any cystic connection other than gall bladder via cystic duct. The histological findings of the fibrous tissue showed fibrosis of the remnant with surrounding ductular proliferation. Section from the cystic structure showed focally eroded mucosal epithelium with diffuse submucosal fibrosis. Post-operative course was uneventful. The patient's bile excretion improved gradually after surgery and she became jaundice-free approximately 2 months postoperatively.

Case 2

A 65-day-old male infant was born at term gestational age (birth weight, 3,140 g). Jaundice with acholic stool was noted after a few weeks of life. Pertinent physical findings included jaundice and enlarged liver. Laboratory studies revealed a total bilirubin of 13.8 mg/dL, direct bilirubin of 10.08 mg/dL, AST of 338 U/L, ALT of 286 U/L, and alkaline phosphatase of 535 U/L. An abdominal ultrasound was not performed due to the long waiting list. Hepatobiliary scintigraphy failed to identify

the isotope in the gastrointestinal tract by 24 h. Therefore, biliary atresia cannot be excluded and the surgery was scheduled.

On exploratory laparotomy, a normal-sized gall bladder containing clear fluid was identified together with a partially collapsed cystic structure at hepatoduodenal ligament. The cyst extended downward behind the pancreas. Intraoperative cholangiography revealed a 3-cm contrast-filled cystic structure with no communication to the liver or the intestine, as shown in Figure 2. Further dissection from the cyst towards porta-hepatis demonstrated that there was no left or right hepatic duct. Only a fibrous mass at the bifurcation of the portal vein was identified. The fibrous remnant of the bile duct together with the cystic mucosa and gallbladder was resected. Original Kasai operation was then performed. Subsequent microscopic evaluation of the liver specimens revealed moderate cholestasis and ductular proliferation. The cystic structure showed mucosal epithelium of biliary origin. Well-formed bile ducts were absent at tissue obtained from porta hepatis. Postoperatively, the baby did well with the presence of bile-stained stools. She was discharged home on the 10^{th} days after surgery. The infant became jaundice-free at three months following surgery.

Discussion

It is well established that Kasai operation is still the most appropriate treatment of choice for biliary atresia [6-8, 10, 11]. The morphologic classification of biliary atresia proposed by the Japanese Biliary Atresia Registry (JBAR) is currently used in most centers [3, 9]. Generally, biliary atresia type I and type II, by which hepatico-jejunostomy with Roux-en-Y is the surgery of choice, has a better prognosis than biliary atresia type III, if treated early [1]. Unfortunately, biliary atresia type III

(atresia of the porta hepatis) is the most common main type seen (approximately 88%) at the time of surgery [12]. Approximately 10% of the patients present with biliary atresia type I. Cystic dilatation at the distal end of patent duct might be encountered in some cases with this type. Only minority of the cases are biliary atresia type II.

Biliary atresia type III requires additional technique after completion of dissection of the fibrous remnants of the extrahepatic bile ducts. Schweizer in 1980 [14, 15] was first who has mentioned that biliary atresia type III requires an extended exploration of the porta hepatis including the regions beneath and around the branches of the portal vein in order to incorporate all potentially remnants of the bile ducts. This technique can be called 'extended hepatoportoenterostomy'. When this type is demonstrated, the technique of extensive dissection is imperative [2, 13].

With respect to our cases, had we mistaken the intraoperative radiologic findings as correctable biliary atresia or choledochal cyst, the patients' surgical correction would have been cystojejunostomy with Roux-en-Y reconstruction, instead of Kasai operation. Postoperatively, they both recovered uneventfully and were jaundice-free. Occasionally, extrahepatic biliary cyst in cholestatic jaundice is seen in infants with choledochal cyst or in some cases of correctable biliary atresia [1, 5].

Although non-correctable cystic biliary atresia has also been diagnosed prenatally [4], the rarity and the difficulties in intra-operative surgical decision of this entity deserves more attention. Here, we report an unusual variant of non-correctable biliary atresia (type III), with a true cystic structure, without distal connection to the duodenum. Infants with this unusual morphologic finding should be treated with proper surgical approach to ensure that ones do not miss this condition. Clear fluid in gall bladder or in the cyst in jaundice infants is an important finding of this type of biliary atresia. This can be easily confirmed by intra-operative cholangiography. In

addition, we speculate that the prognosis of these two infants is similar to those of biliary atresia type III. Although our patients were jaundice-free following Kasai operation, the number of the patients is too small and the follow-up time is too short to make any strong comments regarding post-operative outcome.

In conclusion, an unusual variant based on morphologic classification of biliary atresia has been demonstrated. Clear fluid in gall bladder or in extrahepatic cyst is an important finding. Awareness of this type of biliary atresia, bile duct atresia of porta-hepatis with cystic structure of common hepatic or bile duct, may facilitate the surgeons during dissection of hepatoduodenal ligament in neonates with cholestasis.

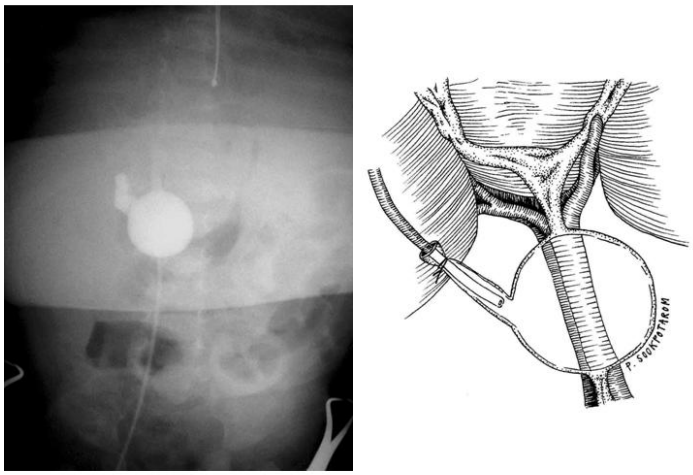


Figure 1: Intra-operative cholangiography via gall bladder of Case 1 and the diagrammatic picture of operative findings showing a smooth cystic structure without connection to either intrahepatic bile duct or distal common bile duct.

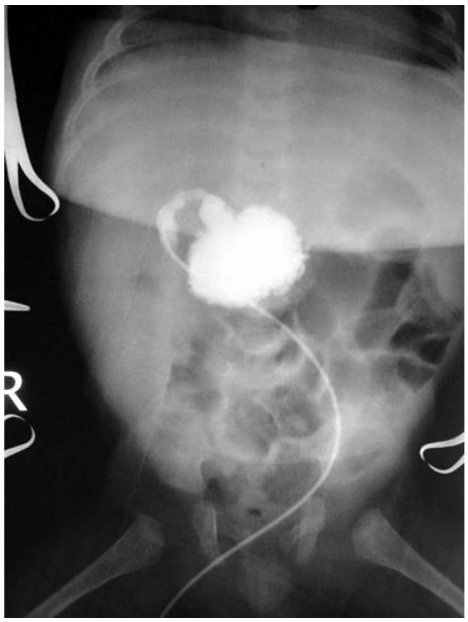


Figure 2: Intra-operative cholangiography via gall bladder of Case 2 demonstrating a large cystic mass without connection to either intrahepatic bile duct or distal common bile duct.

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Study 6: Hepatic Expression of hepatocyte growth factor and its receptor in biliary atresia

Introduction

Biliary atresia (BA) is a devastating cholestatic disease in pediatric patients. Progressive liver fibrosis will lead to death within 2 years if left untreated [1, 2]. The patients who are left untreated will die from hepatic decompensation, esophageal variceal bleeding or infection [3]. Hepatic portoenterostomy or Kasai operation at the early age is indispensable to the successful management of infants with BA.

For years, extensive research on liver fibrosis in BA has been advocated in order to find the way to the improvement of long-term outcome. Several investigations regarding its pathophysiology have been explored. Although there have been a number of studies on pathophysiology of BA, including serum levels of various inflammatory markers [4-8], serum growth factors [9, 10], and the apoptosis of bile duct cells [11], the exact mechanism is still unclear.

Hepatocyte growth factor (HGF) has been shown to induce hepatocyte proliferation. HGF is a paracrine cellular growth factor. It is secreted by mesenchymal cells and targets and acts primarily upon epithelial cells and endothelial cells, but also acts on haemopoietic progenitor cells. It has been shown to have a major role in embryonic organ development, in adult organ regeneration and in wound healing [12]. HGF regulates cell growth, cell motility, and morphogenesis by activating a tyrosine kinase signaling cascade after binding to the C-Met receptor. Its ability to stimulate mitogenesis, cell motility, and matrix invasion gives it a central role in angiogenesis, tumorogenesis, and tissue regeneration [13].

In addition, our previous results showed that there was an elevation in serum HGF in BA patients was associated with the poor therapeutic outcome [10]. However, there is little information available regarding the role of HGF/C-met in BA. Since progressive liver fibrosis is an important development in BA together with the possible role of HGF/C-met pathway in liver reparative process, it is of our interest to study the links between the expression of hepatic HGF/C-met and BA.

Therefore, the aims of this study were to investigate the expression of hepatic HGF and its receptor, C-met, in BA and to associate HGF and C-met expression with early therapeutic outcome using immunohistochemistry (IHC) technique.

Methods

Liver tissues and patients

Liver samples of BA patients undergoing Kasai operation and non-BA patients undergoing liver biopsies between July 2005 and July 2008 were retrospectively investigated. All patients were operated by one team of surgeons (PV and SC). The non-BA patients, who had no clinical jaundice, were served as controls. All non-BA patients underwent exploratory laparotomy as the therapeutic means for their diseases. Liver biopsies in this group of patients were an additional procedure and were indicated for medical reasons.

Immunohistochemistry of liver tissues for HGF

Liver samples were fixed in formalin for 24 hours and kept in paraffin embedded blocks using standard procedure. New sections of 4 μm thickness were cut from the formalin-fixed paraffin embedded blocks and mounted on glass slides coated by aminopropyltriethoxysilane (APES; Sigma Chemical Co., St Louise, MO, USA).

Sections were deparaffinized and rehydrated. Endogenous peroxidase activity was blocked with 10-minute incubation in 3% H_2O_2. Antigen retrieval was done by immersing the sections in 0.4% pepsin (Sigma Chemical Co., St Louise, MO, USA) in 0.01 M HCL at 37^{O}C, one hour. After washing with 0.1% Tween 20 (MERCK-Schuchardt, Hohanbrunn, Germany) in phosphate buffered saline (PBS), the sections were treated with 5% bovine serum albumin (BSA; Sigma Chemical Co., St Louise, MO, USA) in PBS for 30 minutes and then treated with primary antibodies for two hours at room temperature. The primary antibodies used in this study were against HGF (Santa Cruz, USA) diluted at 1:20. The primary antibodies were diluted in PBS. After thorough washing in 0.1% Tween 20 in PBS, labeled polymer (Dako Envision System, Dako Corporation, Carpinteria, CA, USA) was applied to the sections for 30 minutes and followed by three washes of 0.1% Tween 20 in PBS. Color was developed in freshly made diaminobenzidine (Sigma Chemical Co). Sections were washed briefly in running tap water and lightly stained with Mayer's hematoxylin.

A negative control omitting the primary antibody was included for each specimen. In addition, negative controls of some sections were incubated with non-immune rabbit serum. Furthermore, sections of liver cancer, known to have strong cytoplasmic staining were stained at the same run as positive controls.

Immunohistochemistry of liver tissues for C-met

Sections were deparaffinized and rehydrated. Endogenous peroxidase activity was blocked with 10-minute incubation in 3% H_2O_2. Antigen retrieval was done by immersing the sections in 0.4% pepsin (Sigma Chemical Co., St Louise, MO, USA) in 0.01 M HCL at 37^{O}C, one hour. After washing with 0.1% Tween 20 (MERCK-Schuchardt, Hohanbrunn, Germany) in phosphate buffered saline (PBS), the sections were treated with 5% bovine serum albumin (BSA; Sigma Chemical Co., St Louise,

MO, USA) in PBS for 30 minutes and then treated with primary antibodies for two hours at room temperature. The primary antibodies used in this study were against C-met (Santa Cruz, USA) diluted at 1:100. The primary antibodies were diluted in PBS. After thorough washing in 0.1% Tween 20 in PBS, labeled polymer (Dako Envision System, Dako Corporation, Carpinteria, CA, USA) was applied to the sections for 30 minutes and followed by three washes of 0.1% Tween 20 in PBS. Color was developed in freshly made diaminobenzidine (Sigma Chemical Co). Sections were washed briefly in running tap water and lightly stained with Mayer's hematoxylin.

A negative control omitting the primary antibody was included for each specimen. In addition, negative controls of some sections were incubated with non-immune rabbit serum. Furthermore, sections of normal intestine were stained at the same run as positive controls.

Evaluation of hepatic HGF and C-met expression

Immunostained sections were evaluated independently by 2 investigators who were unaware of the diagnosis or clinical outcome of the patients. Two quantitative methods were used, based on the extent and intensity of the antibody stain. Areas of hepatic lobules excluding portal triads and central veins were evaluated using visual scoring method.

Visual scores for HGF and C-met expression were assessed based on its intensity and percentage of staining areas. Expression of HGF and C-met intensity was categorized into 3 levels as weak (intensity score =1), moderate (intensity score =2), and strong (intensity score =3). In addition, the percentage of staining areas of hepatocytes was graded into 4 levels (Grade 1; 0-25% of hepatocyte areas was stained; Grade 2: 26-50% of hepatocyte areas was stained; Grade 3: 51-75% of hepatocyte

areas was stained; and Grade 4: 76-100% of hepatocyte areas was stained). Any differences in scores were resolved by a conference. Total scores of hepatic HGF and C-met expression, were calculated as intensity score times staining grade.

Categorization of the BA patients

In order to associate hepatic HGF and C-met expression with clinical outcome at 6 months post-op among BA patients, they were divided into 2 groups according to the status of jaundice (TB <2 mg/dL; good outcome vs. TB ≥2 mg/dL; poor outcome). Subgroup analysis of hepatic HGF/C-met expression based on early clinical outcome in BA patients was carried out.

Statistical analyses

The comparisons of HGF and C-met expression among the two groups (BA and non-BA patients) were performed using Mann-Whitney U tests (visual score). Spearman correlation analysis of HGF with C-met was performed. Association between outcome of BA patients at 6 months post-op and hepatic HGF and C-met expression was analyzed using univariate analysis.

Significant differences were established at $P<0.05$. For all statistical analyses, either GraphPad Prism version 3.02 (GraphPad Software Inc., California, USA) or SPSS software version 10.0 (SPSS Inc., Chicago, IL) was used. Data are expressed as median and range.

Results

Hepatic HGF and C-met expression was determined using immunohistochemistry from liver biopsies of 41 BA patients at the time of Kasai

operation, and 17 non-cholestatic pediatric patients whose liver tissues were needed in the treatment process. All non-BA patients had no clinical jaundice. The diagnosis of non-BA patients were as follows: 6 choledochal cysts, 5 thalassemias, 3 neuroblastomas, 2 portal vein thromboses, and 1 hepatoblastoma.

Hepatic HGF and C-met staining scores of BA patients were significantly higher than those of non-cholestatic patients (HGF; 9 [2, 12] versus 2 [1, 12], C-met; 8 [1, 12] versus 1 [1, 6], both $P<0.0001$), as shown in Figure 1. There was a correlation between HGF and C-met (spearman $r = 0.77$, $P<0.0001$).

Regarding the clinical outcome at 6 months post-Kasai among BA patients, there were 24 patients with good outcome (serum TB < 2.0 mg/dL) and 17 patients with poor outcome (serum TB >2.0 mg/dL). Subgroup analysis showed that there was no association between either hepatic HGF or C-met expression and early clinical outcome in BA patients.

Discussion

Biliary atresia (BA) is a rare disease characterized by progressive sclerosing fibrous obliteration of the intra- and extra-hepatic biliary systems. Without hepatic portoenterostomy or Kasai operation, the resultant cholestasis leads to hepatic fibrosis and death within a few years [1, 14].

Our results clearly demonstrated that there is an overexpression of hepatic HGF and C-met in BA patients compared to other non-BA patients. Overexpression of hepatic HGF and C-met found in BA patients suggested that HGF and C-met production by the liver in BA is increased. This is consistent with our previous studies in post-operative BA patients illustrating that there is an elevation of serum HGF compared to normal children. In addition, this study demonstrated that most of the

HGF stained within the liver tissues was from the area of hepatocytes. It is therefore likely that hepatocytes, not cholangiocytes or endothelial cells or inflammatory cells, are the major source of hepatic HGF production in BA patients. Strong expression HGF and C-met in BA patients reflected the high activity of HGF/C-met pathway.

Subsequent analysis among BA patients revealed that either hepatic HGF or C-met expression at the time of Kasai operation was not associated with early clinical outcome in BA at 6 months post-Kasai. Therefore, the levels of hepatic HGF/C-met expression cannot be used as a prognostic marker for predicting the early outcome at 6 months post-op.

We are also aware of some limitations in this study. Firstly, we do not know whether the overexpression of HGF/C-met found in BA patients is a cause or is an effect to the disease pathology caused by cholestasis. If it is an effect, their expression will decrease in BA patients with good outcome. The study of hepatic HGF/C-met expression in post-Kasai patients with different clinical outcomes will be able to answer this research question. Secondly, overexpression of hepatic HGF/C-met may be just the non-specific findings of cholestasis. More studies on non-BA infants with cholestasis will solve this concern. Thirdly, the sample size of BA patients was probably too small in terms of statistical analysis. However, with the rarity of this disease, the results of a study of liver tissues from 41 BA patients cannot be underestimated.

In conclusion, strong expression of hepatic HGF and C-met receptor in BA patients was demonstrated. However, the expression was not associated with the early therapeutic outcome. These suggest that resultant actions of HGF involve in the liver pathology of BA but its expression cannot be used as a predictor for therapeutic outcome.

Acknowledgments

We are grateful to the Thailand Research Fund for the support (Grant No. RMU-4980017). Also, we would like to thank Faculty of Medicine, Chulalongkorn University for the permission to conduct the research work. The authors also thank Ms. Meesakul O for her technical assistance.

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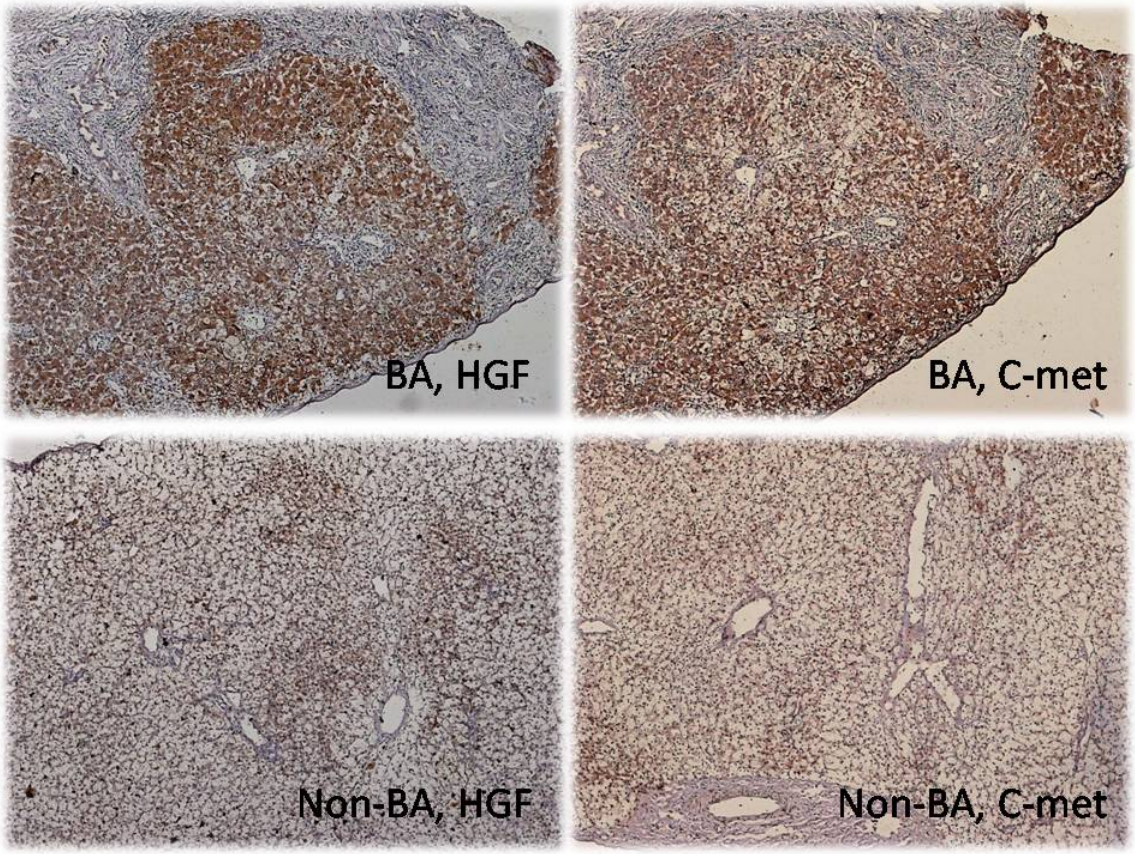

Figure 1: Representative immunohistochemical features of the livers from a BA patient and a non-BA patient. Note the strong expression of HGF and C-met stained on hepatocytes in BA patients

ผลงานที่ได้จากโครงการ

ผลงานวิจัยที่ตีพิมพ์ในวารสารวิชาการระดับนานาชาติ

ได้รับการตีพิมพ์ในวารสารวิชาการระดับนานาชาติ ที่มี Impact factor โดยมีหัวหน้าโครงการเป็นผู้วิจัยหลัก 5 เรื่องดังนี้

1. **Vejchapipat P**, Sookpotarom P, Theamboonlers A, Chittmittrapap S, Poovorawan Y. Elevated serum soluble E-selectin is associated with poor outcome and correlated with serum ALT in biliary atresia. Eur J Pediatr Surg 2008; 18:254-7, impact factor = 0.768
2. **Vejchapipat P**, Passakonnirin R, Sookpotarom P, Chittmittrapap S, Poovorawan Y. High-dose steroids do not improve early outcome in biliary atresia. J Pediatr Surg 2007; 42:2102-5, impact factor =1.557
3. **Vejchapipat P**, Theamboonlers A, Poomsawat S, Chittmittrapap S, Poovorawan Y. Serum transforming growth factor-beta1 and epidermal growth factor in biliary atresia. Eur J Pediatr Surg 2008; 18:415-8, impact factor = 0.768
4. **Vejchapipat P**, Poomsawat S, Imvised T, Chongsrisawat V, Chittmittrapap S, Poovorawan Y. Overexpression of hepatic inducible nitric oxide synthase in biliary atresia. Hepatol Res 2008; 38:1018-25, impact factor = 1.562
5. Sookpotarom P, **Vejchapipat P**. Non-correctable biliary atresia with large extrahepatic cyst: a report of two cases. Eur J Pediatr Surg 2007; 17:295-7, impact factor = 0.768

นอกจากนี้ ผู้รับทุนยังเป็นผู้วิจัยร่วม ในงานวิจัยที่ได้รับการตีพิมพ์ในวารสารวิชาการระดับนานาชาติที่มี Impact factor ที่เกี่ยวข้องกับโรคทางเดินน้ำดีตีบตัน อีก 6 เรื่อง ดังนี้

1. Honsawek S, Chongsrisawat V, **Vejchapipat P**, Thawornsuk N, Tangkijvanich P, Poovorawan Y. Elevation of serum stem-cell factor in postoperative biliary atresia. Pediatr Int 2007; 49:888-93, impact factor = 0.677

2. Honsawek S, Chaiwatanarat T, Chongsrisawat V, Thawornsuk N, **Vejchapipat P**, Poovorawan Y. Circulating leptin levels and bone mineral density in children with biliary atresia. Acta Paediatr 2008; 97:206-11, impact factor = 1.517
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6. Chayanupatkul M, Honsawek S, **Vejchapipat P**, Chongsrisawat V, Poovorawan Y. Elevated serum bone morphogenetic protein 7 levels and clinical outcome in children with biliary atresia. Eur J Pediatr Surg 2009 Apr 22. [Epub ahead of print], impact factor = 0.768

กิจกรรมอื่นๆที่เกี่ยวข้อง

การไปเสนอผลงาน

ผู้รับทุนได้เดินทางไปเสนอผลงานวิจัยเกี่ยวกับเรื่องโรคทางเดินน้ำดีตีบตันระดับนานาชาติ แบบ Oral presentation 4 ครั้ง ดังนี้

1. การประชุม 40th Annual Meeting of Pacific Association of Pediatric Surgeons ประเทศนิวซีแลนด์ วันที่ 15 เมษายน 2550 ถึง 19 เมษายน 2550
    a. Vejchapipat P, Passakonnirin R, Sookpotarom P, Chittmittrapap S, Poovorawan Y. High-dose steroids did not improve early outcome in

biliary atresia. Proceedings of 40th Annual Meeting of Pacific Association of Pediatric Surgeons: page 73 (abstract)

2. การประชุม 44th Annual Meeting of Japanese Society of Pediatric Surgeons ประเทศญี่ปุ่น ระหว่างวันที่ 31 พฤษภาคม 2550 ถึง 2 มิถุนายน 2550
    a. Vejchapipat P, Sookpotarom P, Theamboonlers A, Chittmittrapap S, Poovorawan Y. Elevated serum soluble E-selectin is associated with poor outcome and correlated with serum ALT in biliary atresia. Journal of the Japanese Society of Pediatric Surgeons 2007; 43: 404 (abstract)
3. การประชุม 9th European Congress of Pediatric Surgery ประเทศสาธารณรัฐตุรกี ระหว่างวันที่ 18 มิถุนายน 2551 ถึง 21 มิถุนายน 2551
    a. Vejchapipat P, Theamboonlers A, Poomsawat S, Chittmittrapap S, Poovorawan Y. Serum transforming growth factor-beta1 and epidermal growth factor in biliary atresia.
4. การประชุม 10th European Congress of Pediatric Surgery joint with 56th Congress of British Association of Paediatric Surgeons ประเทศออสเตรีย ระหว่างวันที่ 17 มิถุนายน 2551 ถึง 20 มิถุนายน 2552
    a. Vejchapipat P, Poomsawat S, Chittmittrapap S, Poovorawan Y. Hepatic expression of hepatocyte growth factor and its receptor in biliary atresia.

การได้รับรางวัลที่เกี่ยวข้องกับการทำวิจัยโรคทางเดินน้ำดีตีบตัน

ผู้รับทุนได้รับรางวัล ดังนี้

1. International Guest Scholarship 2009 ของ American College of Surgeons จากงานวิจัยที่เกี่ยวข้องกับโรคทางเดินน้ำดีตีบตันในประเทศไทย โดยจะเดินทางไปรับรางวัล ในการประชุม 95th Clinical Congress of American College of Surgeons และเสนอผลงานวิจัยเรื่อง Biliary atresia: Thailand's experience ณ เมือง ชิคาโก ประเทศสหรัฐอเมริกา วันที่ 11-15 ตุลาคม พ.ศ. 2552
2. ได้รับเชิญให้เข้าร่วมประชุมในฐานะ honorary guest ในการประชุมวิชาการ Fundamentals of surgical research course และ Career development course ของ

Association for Academic Surgery วันที่ 9-10 ตุลาคม พ.ศ. 2552 ณ เมือง ชิคาโก ประเทศสหรัฐอเมริกา

การเชื่อมโยงทางวิชาการกับนักวิชาการอื่นๆ

ผู้รับทุนได้สร้างเครือข่ายในการส่งต่อผู้ป่วยโรคทางเดินน้ำดีตีบตันเพื่อการรักษาและการวิจัยกับกุมารศัลยแพทย์ที่อยู่ตามโรงพยาบาลศูนย์และโรงพยาบาลจังหวัดดังนี้

๑. นายแพทย์ ไพบูลย์ สุขโพธารมย์ โรงพยาบาลชลประทาน จังหวัดนนทบุรี
๒. แพทย์หญิง รพีพรรณ ชาวคอนไชย โรงพยาบาลกาญจนบุรี จังหวัดกาญจนบุรี
๓. แพทย์หญิง จำเรียง คุ้มจันอัด โรงพยาบาลบุรีรัมย์ จังหวัดบุรีรัมย์
๔. แพทย์หญิง เสาวลักษณ์ คล่องธนาพันธ์ โรงพยาบาลสรรพสิทธิ์ประสงค์ จังหวัดอุบลราชธานี
๕. แพทย์หญิง กตเวที เดชอรัญ วชิระพยาบาล จังหวัดกรุงเทพฯ
๖. แพทย์หญิง รจนา ภาสกรนิรินทร์ โรงพยาบาลศูนย์ราชบุรี จังหวัดราชบุรี
๗. นายแพทย์ นิติวุฒิ วงศ์เสงี่ยม โรงพยาบาลศูนย์จังหวัดชลบุรี
๘. แพทย์หญิง ตะวัน อิ่มวิเศษ โรงพยาบาลธรรมศาสตร์ ศูนย์รังสิต

ภาคผนวก

๑. สำเนาบทความทางวิชาการ 5 เรื่องโดยมีผู้รับทุนเป็นผู้วิจัยหลัก ที่ได้รับการตีพิมพ์ในวารสารระดับนานาชาติและมี impact factor และมี กิตติกรรมประกาศขอบคุณ Thailand Research Fund

๒. สำเนาเอกสารการไปเสนอผลงานวิจัยในการประชุมวิชาการระดับนานาชาติในต่างประเทศ จำนวน 4 ครั้ง

๓. สำเนาจดหมาย ได้รับรางวัล International Guest Scholarship 2009 ของ American College of Surgeons

๔. สำเนาจดหมาย ได้รับเชิญ ไปร่วมประชุม Fundamentals of surgical research course และ Career development course ของ Association for Academic Surgery ณ เมืองชิคาโก ประเทศสหรัฐอเมริกา

Journal of Pediatric Surgery (2007) 42, 2102–2105

Journal of Pediatric Surgery

www.elsevier.com/locate/jpedsurg

High-dose steroids do not improve early outcome in biliary atresia

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Received 8 August 2007; accepted 8 August 2007

Index words:
Biliary atresia;
Steroids;
Jaundice

Abstract

Purpose: Our objective was to evaluate the effects of steroids on early outcome in biliary atresia (BA).

Methods: Patients with BA between 2001 and 2005 were reviewed. The use of steroids (4 mg/kg per day at alternate days for 1 to 3 months) has been routinely implemented since 2003. Jaundice-free status and the occurrence of cholangitis at 6 months postoperatively between patients receiving steroids and those not receiving steroids were compared.

Results: Fifty-three patients with BA were studied. At 6 months postoperation, 30 patients (56.6%) were jaundice free and 24 patients (45.3%) experienced cholangitis at least once. Of the 53 patients, there were 33 patients in the steroid group and 20 patients in the nonsteroid group. The proportion of jaundice-free patients in the steroid group was higher than that in the nonsteroid group, and the proportion of patients with cholangitis in the steroid group was lower than that in the nonsteroid group. However, these discrepancies did not reach a statistically significant difference (jaundice-free status [steroid vs nonsteroid]: 20/33 [60.6%] vs 10/20 [50%], $P = .57$; cholangitis: 13/33 [39.4%] vs 11/20 [55%], $P = .39$).

Conclusions: Although the use of steroids seems to have benefits, it did not statistically improve early outcome in patients with BA.

Hepatic portoenterostomy or Kasai operation has been accepted as a standard treatment to drain the biliary system in children with biliary atresia (BA) for many years [1]. Although a successful Kasai operation can restore bile drainage, long-term success of the Kasai procedure has been achieved in a minority of patients. Recent reports estimate that 70% to 80% of the patients will eventually require liver transplantation or die secondary to the progression of liver impairment [2,3].

Several previous attempts to improve the outcome of the Kasai procedure have concentrated on technical details such as the extent of resection of the fibrous mass at porta hepatis

Presented at the 40th annual meeting of the Pacific Association of Pediatric Surgeons, Queenstown, New Zealand, April 15–19, 2007.

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0022-3468/$ – see front matter © 2007 Published by Elsevier Inc.
doi:10.1016/j.jpedsurg.2007.08.038

and variations in the reconstruction of the Roux-en-Y limb. In addition, to make an impact on the outcome of infants with BA, several studies investigating its pathophysiology have been widely conducted. The results lead to the refinement of postoperative treatment [1,4]. Even so, the fundamental problem of BA is that we do not fully understand the underlying disease process. Theoretically, viral infection, genetic predisposition, abnormal bile acid metabolism, and ductal plate malformations have been suggested as possible inciting events [3,5-7]. Immunologic, inflammatory, infectious, and obstructive pathways of progressive intrahepatic biliary epithelial destruction and portal fibrosis have all been hypothesized [8].

With the realization that immune-mediated inflammatory pathways of tissue injury attack both biliary caniliculi and liver parenchyma and serve as the fundamental pathophysiology of BA, anti-inflammatory drugs may come as an additional therapy [9-11]. Therefore, adjuvant postoperative medical therapy using short-term high-dose steroids aimed at enhancing native liver function after surgery has been practiced by some surgeons [12-15]. As there are no randomized controlled trials on the effects of steroids at present, owing to the rarity of the disease, the benefits of high-dose steroids in BA have always been questioned.

In this study, the effects of postoperative high-dose steroids on early outcome of infants with BA, in terms of jaundice-free status and cholangitis within 6 months after the operation, were investigated.

1. Materials and methods

The study was approved by the Institutional Review Board of the Faculty of Medicine of Chulalongkorn University (No. 131/2006). A retrospective review of patients treated for BA, from January 2001 to December 2005, was performed at the Pediatric Liver Center. Over the studied period, only 1 team of pediatric surgeons (PV and SC) operated on these patients using the original Kasai operation with similar surgical techniques.

Before May 2003, all patients with BA treated at our hospital received a routine protocol of postoperative treatment as follows: intravenous antibiotics for 10 days followed by oral cotrimoxazole for at least 1 year; ursodeoxycholic acid (10-15 mg/kg per day) for at least 1 month; and vitamins A, D, E, and K for at least 1 year, without any steroid therapy. Patients with BA treated during this period served as historical controls. From May 2003 onward, the use of high-dose steroids (prednisolone 4 mg/kg per day for 3-4 consecutive days began on the 7th day postoperatively, then at alternate days for 1-3 months) was added to the protocol. The duration of steroid therapy depends on the jaundice-free status. If the patients were jaundice free very soon after the operation, steroid dosage would be tapered and stopped (but not before 1 month of steroid therapy after surgery). Generally, 2 weeks were used for the tapering of steroids. However, if the patients were still jaundice free 3 months after the operation, steroid therapy would also be tapered and stopped. The protocol of postoperative medical treatment in this study was modified by our medical team from the information of related articles [11,14,16-18].

1.1. Categorization of the patients with BA

To compare various aspects among patients with BA, they were divided into 2 groups according to steroid treatment: the steroid group and the nonsteroid group (historical control). Further subgroup analysis of early clinical outcome based on the jaundice-free status (serum total bilirubin [TB] <2.0 mg/dL) at 6 months postoperatively and the occurrence of ascending cholangitis within 6 months after surgery was carried out. Ascending cholangitis, in this study, was defined as a combination of fever higher than 38.5°C, change of yellow stool to acholic stool, and leukocytosis (white blood cell count >12,000 cells per cubic millimeter) with polymorphonuclear leukocyte predominance.

1.2. Statistical analyses

Demographic and clinical data between steroid and nonsteroid groups were compared by Fisher exact tests and unpaired *t* tests where appropriate. The mean and SD were calculated for each variable. Jaundice-free status at 6 months postoperatively and the occurrence of cholangitis within 6 months postoperatively between patients receiving steroids and those not receiving steroids were compared using Fisher exact test. Significance is set at the 95% confidence interval, $P < .05$.

2. Results

A total of 53 patients with BA (male/female ratio, 27:26) undergoing Kasai operation were studied. Mean age at surgery was 89.85 ± 31.33 days. Preoperative serum TB was 10.81 ± 2.70 mg/dL and serum direct bilirubin was 8.20 ± 2.15 mg/dL. At 6 months postoperation, 30 patients (56.6%) were jaundice free and 24 patients (45.3%) experienced cholangitis at least once.

Of the 53 patients, there were 33 patients in the steroid group and 20 patients in the nonsteroid group. The 2 groups were comparable in terms of age at surgery, sex, and preoperative serum TB (Table 1). The proportion of jaundice-free patients in the steroid group was higher than that in the nonsteroid group (60.6% vs 50%), and the proportion of patients with cholangitis in the steroid group was lower than that in the nonsteroid group (39.4% vs 55%). However, by using Fisher exact tests, these discrepancies did not reach a statistically significant difference (jaundice-free status [steroid vs nonsteroid]: 20/33 vs 10/20, $P = .57$;

Table 1 Comparisons between the steroid group and nonsteroid group among 53 patients with BA

	Steroid (n = 33)	Nonsteroid (n = 20)	*P* value
Age at surgery (d)	84.7 ± 25.7	98.3 ± 38.0	.125
Sex (male/female)	15:18	12:8	.39
Preoperative TB (mg/dL)	11.1 ± 2.6	10.3 ± 2.9	.302
Proportion of patients jaundice free at 6 mo	20/33 (60.6%)	10/20 (50%)	.57
Proportion of the occurrence of cholangitis within 6 mo	13/33 (39.4%)	11/20 (55%)	.39

cholangitis at 6 months postoperatively: 13/33 vs 11/20, $P = 0.39$) as shown in Table 1.

Despite the high dosage of steroids used in this study, we did not identify any specific complications caused by steroid treatment other than fluid retention and increased appetite. Surgical complications in the steroid group included one wound infection and one gut obstruction from adhesion band requiring reoperation on the fifth day postoperatively. Complications from surgery in the nonsteroid group included one child with wound infection and 2 children with gut obstruction who responded to nonoperative management.

2.1. Discussion

Biliary atresia is characterized by atresia of both the extra- and intrabiliary system with a progressive sclerosing and inflammatory process. It has been hypothesized, based on a liver histology, that the inflammatory process is associated with immune-mediated reaction [6,7,19]. Liver pathology illustrates bile ductular proliferation, canalicular stasis, vacuolization of cholangiocytes, portal edema and fibrosis, and monocytic and lymphocytic cell infiltration of the portal tracts [6,20]. In addition, a number of cellular inflammatory markers have been studied including CD14-positive macrophages which secrete a number of inflammatory cytokines into the periductular tissue when activated by endotoxin [21]. Molecules involved in inflammatory pathways including ICAM-1 [7], nitric oxide metabolites [22], interleukin-8 [23], and monocyte chemoattractant protein-1 [24] have been proposed to be involved in the pathophysiology of progressive liver fibrosis in BA.

Steroids have been used as an adjunct for postoperative patients with BA for many years, despite the current lack of a single randomized controlled trial. The beneficial effects of steroids for BA are believed to be choleretic action, immunosuppression, and anti-inflammation. In the past, the use of steroids has been reported to have synergistic effects with antibiotic treatment for intractable postoperative cholangitis in BA [9,25]. Later, high-dose steroids have been subsequently proposed to have potential choleretic and anti-inflammatory properties that might benefit postoperative patients with BA [11,12,14,15,26-28]. Steroids increase canalicular electrolyte transport and stimulate bile flow independent of the bile salt concentration. Furthermore, high-dose steroids have significant anti-inflammatory and immunosuppressive effects such as decreasing tissue edema and collagen deposition, and inhibiting fibrogenesis and migration of infiltrating monocytes and lymphocytes [5,8,10].

In an attempt to ameliorate progressive liver fibrosis after Kasai operation in patients with BA, several retrospective studies demonstrated that high-dose steroids may improve the clinical outcome in BA after surgery via the mechanisms mentioned above [12-15]. However, proof that the improved outcome is caused primarily by the steroids remains elusive, because none of these studies have been randomized or controlled and none of the studies are of sufficient size to properly answer the research questions.

Although the use of high-dose steroids in the present study showed some benefits, we cannot demonstrate the benefits of high-dose steroid toward early clinical outcome by means of statistically significant differences. Several reasons may be responsible for our results which show that there was no difference in the clearance of jaundice at 6 months and the occurrence of cholangitis within 6 months. First, the dosage of 4 mg/kg per day at alternate days might be too low. However, based on similar dosage reported elsewhere [14,27] showing the benefits from steroids, it is likely that the dose of steroids used in this study was adequate. Second, the mean age at surgery (89.8 days) was quite late compared to other reports. Once the degree of cholestasis or liver fibrosis is significant, steroids may not maximally exert their effects. Third, the sample size is probably too small owing to the unexpected good results in the nonsteroid group. Our results showed that the effects of steroids increased the jaundice-free status by only 10% (from 50% to 60.6%), compared to the nonsteroid group. Statistically, this 10% difference needs at least 350 patients in each arm (calculated from StatMate version 1.01, GraphPad Software, Inc, San Diego, Calif) to make the discrepancy statistically significant (at the power of 80%). Previous studies from other groups that show benefits of steroids by statistical differences have a significant gap in the percentage of jaundice-free status between the steroid group and the nonsteroid group (79% vs 21% [11] and 76% vs 37% [12]). Finally, the effects of surgical techniques may affect the outcome. However, all of the operations in this study were performed by one team of surgeons with similar details. Therefore, differences in surgical technique are unlikely although the confounding factor of historical controls cannot be excluded. Another methodological aspect needed to be emphasized is that this study was carried out in a retrospective fashion together with a major drawback of likely β error owing to the relatively small patient number. Therefore, as mentioned above, the data of this study do not conclusively demonstrate that high-dose steroids did not improve early outcome in BA.

We do not know whether the beneficial effects of the adjuvant steroid therapy are caused by the choleretic, immunosuppressive, anti-inflammatory effects or the combination of these effects. At present, with the consistently promising observations of the adjuvant steroid studies published and the negative results reported in this study, a multi-center, randomized, controlled clinical trial is mandatory. As the published articles usually proposed potential benefits of steroids in BA, this question may never be answered if the sample size in each arm of the patients in a randomized controlled trial is too small.

High-dose steroids can be used safely in postoperative BA. Although the use of steroids in postoperative patients with BA seems to have some benefits, it did not statistically improve early outcome in patients with BA based on jaundice-free status and cholangitis at 6 months postoperatively.

Acknowledgments

We are grateful to the Thailand Research Fund for generous support (Grant No RMU-4980017). Also, we would like to thank Rachadaphisek Somphot Endowment, Chulalongkorn University for supporting the research work.

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Hepatology Research 2008; **38**: 1018–1025 doi: 10.1111/j.1872-034X.2008.00385.x

Original Article

Overexpression of hepatic inducible nitric oxide synthase in biliary atresia

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Aims: Biliary atresia (BA) is a rare and serious liver disease in infants characterized by progressive inflammatory cholangiopathy. The aims of this study were to investigate hepatic expression of inducible nitric oxide synthase (iNOS) in BA and to associate the iNOS expression with their early therapeutic outcome.

Methods: Hepatic iNOS expression was determined using immunohistochemistry from liver biopsies of 24 BA patients, and 16 non-BA patients whose liver tissues were needed in the treatment process. Six months after surgery, the BA patients were categorized into two groups;good and poor outcome. The iNOS expression of hepatocyte areas was evaluated based on its intensity using ImageJ software. Unpaired *t*-tests were used for the comparisons of iNOS expression between groups.

Results: Hepatic iNOS expression of BA patients was significantly stronger than that of non-BA patients ($P < 0.0001$). The largest area of hepatic iNOS expression was the area of hepatocytes. Subgroup analysis of BA patients at 6 months post-op revealed that there was no difference in iNOS expression between the patients with good outcome and those with poor outcome ($P = 0.732$).

Conclusions: Overexpression of hepatic iNOS in BA patients was demonstrated. Within liver tissues, hepatocytes were the major source of hepatic iNOS production. However, the expression was not associated with the early therapeutic outcome. These results suggest that iNOS plays a role in the liver pathology of BA but its expression cannot be used as a predictor for therapeutic outcome.

Key words: Biliary atresia; iNOS; immunohistochemistry; nitric oxide

INTRODUCTION

BILIARY ATRESIA (BA) is a rare and devastating cholestatic disease. Its clinical presentation is the development of obstructive jaundice, indicated by direct hyperbilirubinemia and acholic stools. Progressive liver fibrosis will lead to death within 2 years if left untreated.[1,2] The patients who are left untreated will die from hepatic decompensation, esophageal variceal bleeding or infection.[3] It is accepted that hepatic portoenterostomy or the Kasai operation at the early age is indispensable to the successful management of infants with BA. Despite surgical intervention, BA remains the most common cause of pediatric liver transplantation.

Research on liver fibrosis has striven to improve the long-term outcome for BA patients. Several investigations regarding its pathophysiology have been undertaken. Although there have been a number of studies on pathophysiology of BA, including serum levels of various inflammatory markers,[4–8] serum growth factors,[9,10] and the apoptosis of bile duct cells,[11] the exact mechanism is still unclear.

In recent years, it has been illustrated that nitric oxide (NO) produced through inducible nitric oxide synthase (iNOS) plays an important role in primary biliary cirrhosis[12,13] and liver diseases characterized by chronic inflammation.[14,15] Although we do not believe that the roles of iNOS are identical in BA and primary biliary cirrhosis, they may share some resultant NO effects. In addition, our previous results show that there is an

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Received 23 February 2008; revision 25 March 2008; accepted 8 April 2008.

elevation in systemic NO production (serum nitrate and nitrite levels) in BA patients.[16] Interestingly, the elevated NO production is associated with serum alanine transaminase (ALT) levels, a marker for liver injury.[16] However, there is little information available regarding the role of iNOS enzyme in BA. Since progressive liver fibrosis is an important development in BA together with the possible role of NO in biliary cirrhosis and liver inflammation, it is important to study the links between the expression of hepatic iNOS and BA.

The aims of this study were to investigate the expression of hepatic iNOS in BA and to associate the iNOS expression with their early therapeutic outcome using immunohistochemistry (IHC) technique.

METHODS

THIS STUDY WAS approved by the Ethical Review Board of the Department of Surgery, Chulalongkorn Hospital, Thailand.

Liver tissues and patients

Liver samples of BA patients undergoing the Kasai operation and non-BA patients undergoing liver biopsies between July 2005 and July 2007 were retrospectively investigated. All patients were operated on by one team of surgeons (PV and SC). The non-BA patients, who had no clinical jaundice, served as controls. All non-BA patients underwent exploratory laparotomy as the therapeutic treatment for their diseases. Liver biopsies in this group of patients were an additional procedure and were required for medical reasons.

Immunohistochemical analysis of liver tissues for iNOS

Liver samples were fixed in formalin for 24 h and kept in paraffin embedded blocks using standard procedure. New sections of 5 μm thickness were cut from the blocks and mounted on glass slides coated by aminopropyl-triethoxysilane (Sigma Chemical, MO, USA). Sections were deparaffinized and rehydrated. Endogenous peroxidase activity was blocked by 10-min incubation in 3% H_2O_2. Antigen retrieval was done by immersing the sections in 0.4% pepsin (Sigma Chemical) in 0.01 M HCL at 37 C for one hour. After washing with 0.1% Tween 20 (MERCK-Schuchardt, Germany) in phosphate buffered saline (PBS), the sections were treated with 2% bovine serum albumin (Sigma Chemical) in PBS for 30 min and then treated with primary antibodies for 2 h at room temperature. The primary antibodies used in this study were against iNOS (monoclonal anti-human iNOS antibody, Cat. Number MAB9502, R & D Systems, MN, USA) diluted at 1:200. The primary antibodies were diluted in PBS. The immunoreactive complexes were detected using Envision System (Dako Corporation, CA, USA) and visualized with the peroxidase substrate. Color was developed in freshly made diaminobenzidine (Sigma Chemical). Sections were washed briefly in running tap water and lightly stained with Mayer's hematoxylin. Negative controls were done by omission of the relevant primary antibody. Sections of salivary gland tissue were stained at the same run as positive controls for comparison. The ducts of the salivary gland have been shown to stain intensely for iNOS.[17]

Evaluation of hepatic iNOS expression

Immunostained sections were evaluated independently by two investigators who were unaware of the diagnosis or clinical outcome of the patients. Two quantitative methods were used, based on the extent and intensity of the antibody stain. Areas of hepatic lobules excluding portal triads and central veins were evaluated using a visual scoring method and a computerized scoring method. The portal triads were excluded for two reasons. First, the hepatocyte areas are likely to be the major source of iNOS expression within the liver of BA patients and therefore we specifically investigated the iNOS expression of the hepatocyte areas. Second, we selected only areas of hepatocytes and excluded portal triad areas so as to avoid significant areas of fibrous connective tissue that could possibly affect the mean score of iNOS expression and skew the results.

Visual scores for iNOS expression were assessed based on the intensity and percentage of stained areas. Expression of iNOS intensity was categorized into three levels: weak (intensity score = 1); moderate (intensity score = 2); and strong (intensity score = 3). In addition, the percentage of stained areas of hepatocytes was graded into four levels: grade 1, 0–25% of hepatocyte areas stained; grade 2, 26–50% of hepatocyte areas stained; grade 3, 51–75% of hepatocyte areas stained; and grade 4, 76–100% of hepatocyte areas stained. Any differences in scores were resolved by a conference. Total scores of hepatic iNOS expression, evaluated by the visual scoring method, were calculated as intensity score times staining grade.

In addition to the visual scoring method mentioned above, a more objective computerized scoring method was used to evaluate hepatic iNOS expression based on Ruifrok and Johnston's method.[18,19] Five randomly selected areas of hepatocytes (under x10 magnification),

excluding portal triads and central veins, were photographed – the parameters of the camera were similarly set in all cases. The intensity of the iNOS expression was evaluated using the Histogram mode from ImageJ version 1.38x (http://rsbweb.nih.gov/ij) with color deconvolution plugin.[20] Using this program, the blue color of the hematoxylin and the brown color of the diaminobenzidine (DAB) were separated and the intensity of a DAB channel pixel in the selected area was scored according to 256 arbitrary levels (0–255; bright to dark). Five selected areas of hepatic lobules from each patient were evaluated and then averaged. The equation to quantify the iNOS expression into the arbitrary levels was as follows: iNOS expression = mean intensity in DAB channel of selected hepatocyte areas – mean intensity in DAB channel of background areas.

Categorization of the BA patients

In order to associate hepatic iNOS expression with clinical outcome at 6 months post-op among BA patients, they were divided into two groups according to the status of jaundice (TB < 2 mg/dL; good outcome vs TB ≥ 2 mg/dL; poor outcome). Subgroup analysis of hepatic iNOS expression based on earlier clinical outcome in BA patients was carried out.

Statistical analyses

Demographic and clinical data between groups were compared by χ^2 tests and unpaired *t*-tests where appropriate. The mean and standard deviation were calculated for each variable. The comparisons of iNOS expression among the two groups (BA and non-BA patients) were performed using Mann-Whitney *U*-tests (visual score) and unpaired *t*-tests (computerized method). Spearman correlation analysis of visual scoring method with computerized scoring method was performed. Association between outcome of BA patients at 6 months post-op and hepatic iNOS expression was analyzed using univariate analysis.

Significant differences were established at $P < 0.05$. For all statistical analyses, either GraphPad Prism version 3.02 (GraphPad Software, CA, USA) or SPSS version 10.0 (SPSS, IL, USA) were used. Data are expressed as mean and SD.

RESULTS

TWENTY-FOUR BA patients and 16 non-BA patients were studied. No BA patients were excluded from the study during the studied period. All non-BA patients had no clinical jaundice. The diagnosis of non-BA patients were as follows: 6 choledochal cysts, 4 thalassemias, 3 neuroblastomas, 2 portal vein thromboses, and 1 hepatoblastoma. The demographic and clinical data are demonstrated in Table 1. Descriptively, the staining intensity was stronger and the percentage of staining area of hepatic iNOS was higher than those of controls. Hepatic iNOS expression score of BA patients was significantly higher than that of non-BA patients ($P = 0.0015$ using visual scoring method and $P < 0.0001$ using computerized method), as shown in Figure 1. There was a positive correlation between hepatic iNOS expression evaluated by visual scoring method and by computerized scoring method ($P < 0.0001$, Spearman $r = 0.7387$). The representative histological sections of BA patients and non-BA patients are illustrated in Figure 2. For Figure 3, higher magnification of histological section of a hepatocytes area in a BA patient shows that iNOS expression was exclusively stained in hepatocytes. In addition, iNOS expression was detected in nuclei of biliary epithelial cells at portal triad areas from BA patients (Fig. 4).

Table 1 The demographic data and hepatic inducible nitric oxide synthase (iNOS) expression of biliary atresia (BA) and non-BA patients†

	BA patients ($n = 24$)	Non-BA patients ($n = 16$)	*P*-value
Age at operation (days)	89.42 + 30.05	1881.5 + 1773.05	<0.0001
Sex (male : female)	8:16	8:8	0.339
Albumin (g/dL)	4.05 + 0.35	NA	–
Total bilirubin (mg%)	12.11 + 3.20	NA	–
ALT (IU/L)	179.25 + 113.17	NA	–
Hepatic iNOS expression (computerized method)	89.39 + 20.38	46.01 + 29.60	<0.0001

†Data expressed as mean + SD. ALT, alanine transaminase; NA, data not available because liver function tests were not performed in half of the non-BA patients.

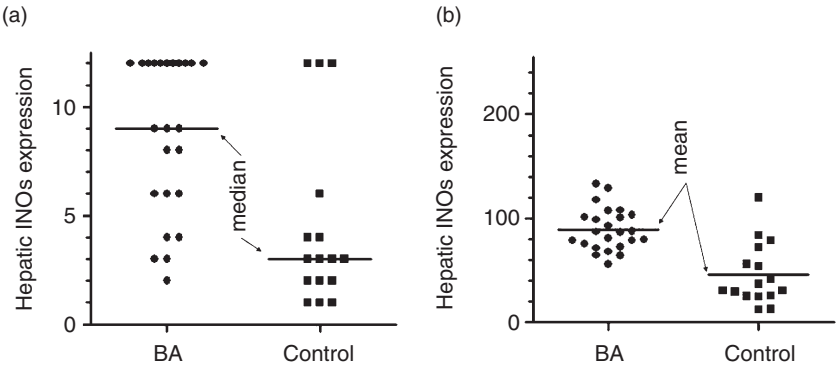

Figure 1 Hepatic inducible nitric oxide synthase expression between biliary atresia patients and non-biliary atresia patients based on (a) visual (P = 0.0015) and (b) computerized (P < 0.0001) scoring methods.

Regarding the clinical outcome at 6 months post-op among BA patients, there were 13 patients with a good outcome (serum TB < 2.0 mg/dL) and 11 patients with a poor outcome (serum TB > 2.0 mg/dL). The demographic and clinical data are shown in Table 2. Subgroup analysis showed that there was no association between hepatic iNOS expression and early clinical outcome in BA patients (P = 0.995 by visual scoring method and P = 0.732 by computerized method).

DISCUSSION

BILIARY ATRESIA (BA) is a rare disease characterized by progressive sclerosing fibrous obliteration of the intra- and extra-hepatic biliary systems. Without hepatic portoenterostomy or the Kasai operation, the resultant cholestasis leads to hepatic fibrosis and death within a few years.[1,21] However, the procedure is not the final answer as even after a successful Kasai operation, a significant number of patients still progress to liver fibrosis requiring liver transplantation later in life.[22] Although there have been several studies concentrating on the pathophysiology of progressive liver fibrosis in post-Kasai BA patients,[5,8,10,23] its mechanism is still unclear.

In recent years, one of the molecules being extensively studied regarding liver injury of various conditions is nitric oxide (NO). It is a short-living biological mediator generated from L-arginine by nitric oxide synthase (NOS). The NOS family of enzymes includes endothe-

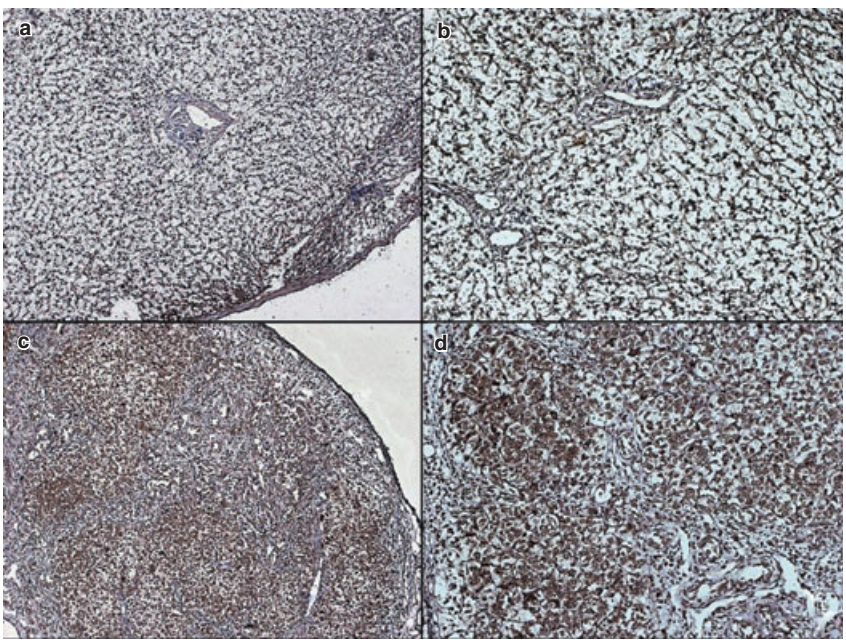

Figure 2 Representative immunohistochemical features of the livers from a patients with (a–b) neuroblastoma and (c–d) biliary atresia. Note the strong expression of inducible nitric oxide synthase in the stained hepatocytes of the biliary atresia patients. (× 5, × 10)

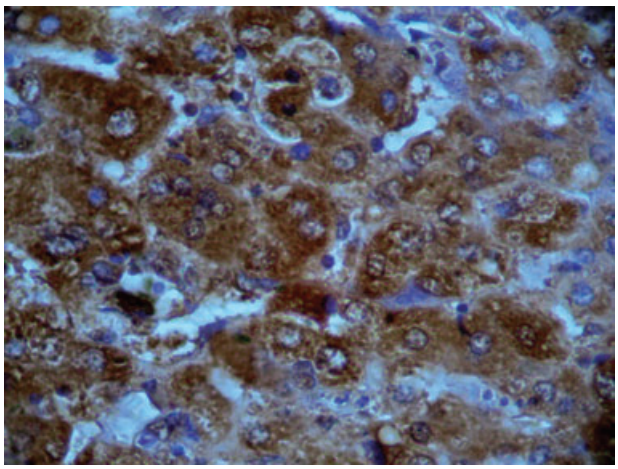

Figure 3 Representative immunohistochemical features of inducible nitric oxide synthase in hepatocytes in biliary atresia patients at high magnification. Note the expression of inducible nitric oxide synthase exclusively within the hepatocytes. (× 40)

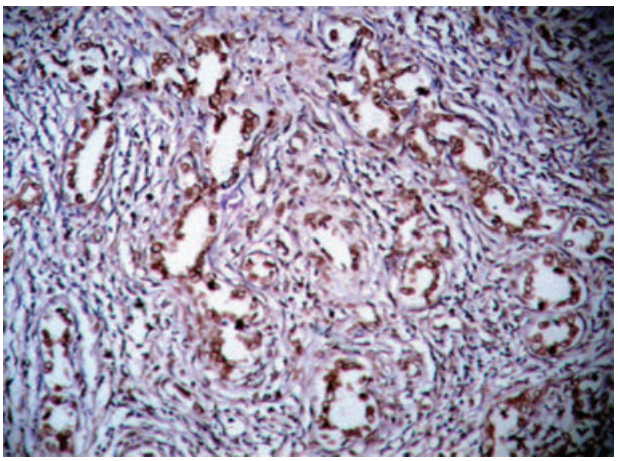

Figure 4 Representative immunohistochemical features of inducible nitric oxide synthase in the portal triad area of a biliary atresia patient. Note the inducible nitric oxide synthase expression in the nuclei of proliferative biliary ductular cells. (× 20)

lial NOS (eNOS or type 3 NOS), neuronal NOS (nNOS or type 1 NOS), and inducible NOS (iNOS or type 2 NOS). NO exerts a broad spectrum of physiological functions, including regulation of vascular reactivity, platelet and leukocyte activation, neurotransmission, regulation of cellular proliferation, and nonspecific immunity reactions.[24] Inappropriate release of NO have been associated with diverse vascular, ischemic, thrombotic, and inflammatory pathologies.[24–26] For the liver, NO is generated by eNOS and iNOS, and NO can mediate a number of physiological and disease reactions involving this organ.[26] With a broad range of molecular targets, NO acts as an inhibitor or an agonist of cell signaling events. Constitutively generated NO maintains the hepatic microcirculation and endothelial integrity, while inducible NO synthase (iNOS)-governed NO production can be either beneficial or hepatotoxic.[25] When the conditions are right, NO (via formation of peroxynitrite) can damage cellular components by its strong oxidizing effect. There is substantial evidence that NO or peroxynitrite can act on the mitochondria to inhibit cellular respiration.[27] Perhaps the significant direct cellular effect of NO with respect to hepatic injury concerns apoptosis.[28] Our previous studies also demonstrated that there is an elevation of systemic NO production (serum nitrites and nitrates) in post-operative BA patients.[16] Therefore, possible implications of hepatic iNOS expression in the pathophysiology of liver injury in BA are obvious.

In this study, immunohistochemistry was used to investigate the hepatic iNOS expression. The increased use of immunohistochemistry in both clinical and basic

Table 2 Biliary atresia (BA) patients are categorized based on clinical outcome at 6 months post-Kasai†

	BA patients with good outcome ($n = 13$)	BA patients with poor outcome ($n = 11$)	*P*-value
Age at operation (days)	79.23 + 13.43	101.45 + 39.57	0.07
Gender (male : female)	6:7	2:9	0.211
Total bilirubin (mg%)	0.71 + 0.56	9.15 + 6.36	<0.0001
Albumin (g/dL)	4.05 + 0.45	3.33 + 0.67	0.006
ALT (IU/L)	91.61 + 56.36	134.72 + 9.81	0.201
Hepatic iNOS expression (computerized method)	90.75 + 20.46	87.80 + 21.16	0.732

†Data expressed as mean + SD. ALT, alanine transaminase; iNOS, inducible nitric oxide synthase.

research settings has led to the development of various computerized techniques for acquiring quantitative information from immunostaining levels. Quantitative immunohistochemistry techniques have often yielded important information regarding patient diagnosis, prognosis, or both. In fact, a correlation between immunohistochemical staining and protein levels, using other conventional methods including Western blotting analysis[29] and enzyme immunoassays,[30] has been demonstrated. By comparing immunostaining quantification obtained by a digital computer-assisted method with a standard semi-quantitative analysis in this study, it is clear that results of both methods are concordant. However, digital measurement is more objective and could resolve either researcher's bias or disagreement between two observers.

Our results clearly demonstrated that there is an overexpression of hepatic iNOS in BA patients compared to other non-BA patients. Overexpression of hepatic iNOS found in BA patients suggested that NO production by the liver in BA is increased. This is consistent with our previous studies in post-operative BA patients illustrating that there is an elevation of serum NO metabolites compared to normal children.[16] In addition, this study demonstrated that most of the iNOS stained within the liver tissues was from the area of hepatocytes. It is therefore likely that hepatocytes, not cholangiocytes or endothelial cells or inflammatory cells, are the major source of hepatic NO production in BA patients.

Interestingly, the iNOS expression found in the nuclei of biliary ductular cells possibly contributes to the injury of bile ducts leading to fibrosis and perhaps atresia of biliary system. High levels of hepatic NO production could also damage both hepatocytes and biliary ductules through oxidative stress possibly resulting in biliary obstruction from progressive liver fibrosis. Additionally, subsequent analysis among BA patients revealed that hepatic iNOS expression at the time of Kasai operation was not associated with early clinical outcome in BA at 6 months post-Kasai. Therefore, the levels of hepatic iNOS expression cannot be used as a prognostic marker for predicting the early outcome at 6 months post-op.

We are also aware of some limitations in this study. Firstly, we do not know whether the overexpression of hepatic iNOS found in BA patients is a cause or is an effect to the disease pathology caused by biliary obstruction. If it is an effect, hepatic iNOS expression will decrease in BA patients with good outcome. The study of hepatic iNOS expression in post-Kasai patients with different clinical outcomes will be able to answer this research question. Secondly, overexpression of hepatic iNOS may be just the non-specific findings of cholestasis. More studies on non-BA infants with cholestasis will solve this concern. Thirdly, the sample size of BA patients was probably too small in terms of statistical analysis. However, with the rarity of this disease, the results of a study of liver tissues from 24 BA patients cannot be overlooked. Finally, the mean age of non-BA patients is higher than that of BA patients. This is because of the rarity of infants whose open liver biopsies were mandatory for their therapeutic goals. Liver biopsies in normal infants will be unethical. However, there were 4 non-BA patients with their age below 6 months in this study. All of hepatic iNOS expression in these 4 non-BA patients showed low levels of iNOS expression. In addition, it has been reported that hepatic iNOS expression is low in non-cholestatic livers.[24,25,31,32] Therefore, it is likely that hepatic iNOS expression is low in non-BA patients without jaundice.

A recent report illustrated that iNOS inhibitor reduces liver fibrosis by the inhibition of transforming growth factor-beta1 and retards the development of cirrhosis in rats.[33] Together with the findings from this study that hepatic iNOS in BA patients is overexpressed, and with several evidences that NO production via iNOS enzyme induces liver injury,[34–37] inhibition of NO production may have therapeutic roles in the amelioration of progressive liver fibrosis in BA. More studies are needed to prove this hypothesis.

In conclusion, overexpression of hepatic iNOS in BA was demonstrated. Hepatocytes were the major source of iNOS expression within the liver in BA. However, hepatic iNOS expression was not associated with the early therapeutic outcome in BA patients. These suggest that iNOS plays a role in the liver pathology of BA but its expression cannot be used as a predictor for therapeutic outcome. More studies on this aspect are needed to better understand the exact role of iNOS in BA.

ACKNOWLEDGMENTS

WE ARE GRATEFUL to the Thailand Research Fund for their support (grant no. RMU-4980017). We would like to thank the Faculty of Medicine, Chulalongkorn University for permission to conduct the research. The authors also thank Ms. Meesakul O for her technical assistance.

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