Correlation of Circulating Endoglin with Clinical Outcome in Biliary Atresia

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Key words
- biliary atresia
- clinical outcome
- endoglin
- jaundice

Abstract

Background and Aim: Biliary atresia (BA) is a chronic progressive inflammatory disorder of the extrahepatic and intrahepatic biliary system in children. The aim of the present study was to investigate circulating endoglin levels in BA patients compared with healthy controls and to determine the relationship between plasma endoglin levels and outcome parameters of BA patients after Kasai operation.

Methods: Fifty-five postoperative BA patients and 14 healthy controls were recruited. The patients were divided into two groups based on their serum total bilirubin levels (TB < 34.2, no jaundice vs. TB ≥ 34.2 μmol/L, persistent jaundice) and serum alanine aminotransferase (ALT < 45, normal ALT vs. ALT ≥ 45 IU/L, high ALT). Circulating endoglin levels were analyzed by enzyme-linked immunosorbert assay.

Results: Average levels of plasma endoglin were significantly higher in BA patients compared to healthy controls (7.8 ± 0.4 vs. 6.5 ± 0.4 ng/mL; p = 0.02). BA patients with persistent jaundice had higher plasma endoglin levels than those without jaundice (9.2 ± 0.8 vs. 6.9 ± 0.3 ng/mL; p = 0.006). Furthermore, the concentrations of plasma endoglin in BA patients with high ALT were significantly higher compared to those with normal ALT (8.5 ± 0.5 vs. 6.3 ± 0.5 ng/mL; p = 0.003). In addition, BA patients with portal hypertension had more elevated plasma endoglin levels than those without portal hypertension (8.8 ± 0.6 vs. 6.1 ± 0.3 ng/mL; p = 0.001). Plasma endoglin was positively correlated with serum ALT (r = 0.36, p = 0.007) and serum GGT (r = 0.44, p = 0.001).

Conclusion: High circulating endoglin correlated with a poor outcome for BA. Plasma endoglin can be utilized as a potential biomarker reflecting the severity of ongoing liver injury and biliary obstruction in BA patients after Kasai procedure.

Introduction

Biliary atresia (BA) is a neonatal cholestatic liver disease that predominantly manifests with progressive inflammatory cholangiopathy and portal fibrosis. To date, obstructive cholestasis can be surgically relieved by Kasai portoenterostomy to re-establish ductal continuity for adequate bile drainage [7]. Despite early Kasai operation, the great majority of patients generally develop biliary cirrhosis, portal hypertension and liver failure [20]. Such chronic liver damage subsequently leads to life-threatening complications including recurrent ascending cholangitis, progressive ascites and esophageal varices [19]. Eventually, liver transplantation is needed when the Kasai procedure has failed and severe end-stage liver disease develops. Although extensive clinical studies have proposed several possible causes including perinatal and neonatal viral infections, congenital malformations, vascular abnormalities and autoimmune defects, the etiopathogenesis of BA remains poorly understood [1].

Transforming growth factor-β1 (TGF-β1) is a polypeptide member of the transforming growth factor superfamily of cytokines that plays a key role in hepatic fibrogenesis [4]. It promotes the production, secretion and accumulation of various proteins in the extracellular matrix, for instance collagen, fibronectin and proteoglycan [14]. TGF-β1 functions by interacting with specific receptors, including endoglin, TGF-β receptor type I and type II [5]. Endoglin, also known as
CD105, a 180 kDa homodimeric transmembrane glycoprotein of the receptor complex for fibrogenic cytokines TGF-β1 and TGF-β3 [25]. Endoglin is highly expressed on vascular endothelial cells, monocytes, and erythroid precursors [13]. Several studies have implicated endoglin as playing a role in the development of fibrosis in systemic sclerosis, glomerulosclerosis and hepatic cirrhosis [6,8,9]. Although circulating levels of various growth factors and cytokines have been extensively studied in patients with BA, published data on serum endoglin levels from various clinical stages of BA are not currently available [15–18,23,24]. To our knowledge, this study is the first to demonstrate the important role of circulating endoglin in postoperative BA. We postulated that circulating endoglin would be more elevated in BA patients compared to healthy controls, and to prove this hypothesis, we examined circulating endoglin levels in BA patients and healthy controls. Thus, the aim of the current study was to investigate the circulating concentrations of endoglin collected from postoperative BA patients and determine the relationship between plasma endoglin and outcome parameters of BA patients after Kasai operation.

Patients and Methods

Study population
Fifty-five pediatric patients with BA (22 boys and 33 girls; mean age = 8.0 ± 0.5 years) who had undergone hepatic portojejunostomy with Roux-en-Y reconstruction (Kasai procedure) and never received a liver transplantation were enrolled in the study. The control group comprised 14 healthy children (6 boys and 8 girls; mean age = 8.1 ± 0.3 years) who attended the Well Baby Clinic at King Chulalongkorn Memorial Hospital for vaccination. All parents of children with persistent jaundice had evidence of portal hypertension (PH) was validated by the presence of ascites and/or esophageal varices as diagnosed by endoscopic screening. Fifteen of the 33 BA children without jaundice and 20 of 22 children with persistent jaundice had evidence of portal hypertension. The study protocol was approved by the Institutional Ethical Review Board of the Faculty of Medicine, Chulalongkorn University. The present study was conducted in accordance with the guidelines of the Declaration of Helsinki. All parents of children were informed of the purpose of this study and of any interventions involved in this study. Written informed consent was obtained prior to the children participating in this study.

Laboratory methods
Samples of peripheral venous blood were collected from each patient and healthy controls, centrifuged and then stored at −80°C until analysis. Plasma endoglin concentrations were measured using a commercially available enzyme-linked immunosorbent assay (ELISA) kit (Quantikine high sensitivity human endoglin/CD105 immunoassay, R&D Systems, Minneapolis, MN, USA) following the manufacturer’s recommendations. Briefly, standards of recombinant human endoglin and plasma samples were added to microtiter plates pre-coated with mouse monoclonal antibody against human endoglin and incubated for 2 h at room temperature. The wells were then washed four times with washing buffer and incubated for 2 h at room temperature with a horseradish peroxidase-conjugated monoclonal antibody against endoglin. After four washes, substrate solution was added to each well, and the plate was incubated in the dark for 30 min at room temperature. Finally, the reaction was stopped with the stop solution, and absorbance was then measured at 450 nm using an automated microplate reader. Recombinant human endoglin was used to generate a linear standard calibration curve (range 0–10 ng/mL). The manufacturer reported precision was 2.8–3.2% intra-assay, and 6.3–6.7% inter-assay. In addition, liver function tests including serum albumin, total bilirubin (TB), direct bilirubin (DB), aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP) and gamma-glutamyl transpeptidase (GGT) were done using an automated chemical analyzer (Hitachi 911) at the central laboratory of our hospital.

Statistical analysis
Statistical analysis was performed using the statistical package for social sciences (SPSS) software, version 16.0. All values are expressed as mean ± standard error of the mean (SEM) unless otherwise specified. Comparisons of demographic data and biochemical parameters between groups were analyzed by unpaired t-test. Correlations between numerical data were investigated using Pearson’s correlation coefficient (r). A p-value < 0.05 indicated statistical significance.

Results
Endoglin levels were analyzed in 55 plasma samples from BA patients and 14 from healthy controls. There were no statistically significant differences with respect to age (8.1 ± 0.3 vs. 8.0 ± 0.5 years) or gender (male/female, 6/8 vs. 22/33) between healthy controls and BA patients. The demographic data and biochemical parameters including liver function tests and plasma endoglin levels based on jaundice status are given in Table 1. BA patients with persistent jaundice had significantly lower albumin levels than those without jaundice, while serum levels of AST, ALT, and ALP were highly elevated in the BA patients with jaundice compared to those without jaundice. As shown in Fig. 1, circulating endoglin values measured in the healthy control group, total BA group, jaundice-free group, and jaundice group were 6.5 ± 0.4, 7.8 ± 0.4, 6.9 ± 0.3, and 9.2 ± 0.8 ng/mL, respectively. BA patients had significantly higher plasma endoglin concentrations than healthy controls (p = 0.02). In BA patients, plasma endoglin levels of patients with persistent jaundice were significantly elevated compared to those of patients without jaundice (p = 0.006). There was no significant difference in plasma endoglin levels between the jaundice-free group and controls (Fig. 1).

Subsequently, BA patients were re-evaluated with regard to serum ALT levels, as demonstrated in Table 2. A subgroup analysis of BA children revealed that patients with high ALT levels had more elevated plasma endoglin levels than patients with...
normal ALT levels and healthy controls (8.5 ± 0.5 vs. 6.3 ± 0.5 vs. 6.5 ± 0.4 ng/mL, respectively; *p* = 0.003) (Fig. 2). In addition, BA patients with portal hypertension (PH) had significantly higher levels of plasma endoglin compared to those without PH (8.8 ± 0.6 vs. 6.1 ± 0.3 ng/mL; *p* = 0.001), as displayed in Fig. 3. Particularly in BA patients without jaundice, plasma endoglin levels

### Table 1
Demographic data, liver function tests and plasma endoglin levels of biliary atresia patients categorized according to their jaundice status. Data are expressed as mean ± SEM.

<table>
<thead>
<tr>
<th></th>
<th>Total</th>
<th>Persistent jaundice</th>
<th>No jaundice</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>no. of patients</td>
<td>55</td>
<td>22</td>
<td>33</td>
<td></td>
</tr>
<tr>
<td>no. of patients with PH</td>
<td>35</td>
<td>20</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>gender (M/F)</td>
<td>22/33</td>
<td>9/13</td>
<td>13/20</td>
<td>NS</td>
</tr>
<tr>
<td>age (years)</td>
<td>8.0 ± 0.5</td>
<td>7.3 ± 0.8</td>
<td>8.6 ± 0.6</td>
<td>NS</td>
</tr>
<tr>
<td>albumin (g/L)</td>
<td>39.2 ± 1.0</td>
<td>33.5 ± 1.4</td>
<td>43.0 ± 0.8</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>total bilirubin (μmol/L)</td>
<td>73.5 ± 15.4</td>
<td>164.2 ± 32.5</td>
<td>15.4 ± 1.7</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>direct bilirubin (μmol/L)</td>
<td>47.9 ± 12.0</td>
<td>114.6 ± 25.7</td>
<td>5.1 ± 1.7</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>AST (IU/L)</td>
<td>150.4 ± 16.6</td>
<td>235.9 ± 28.5</td>
<td>93.4 ± 12.7</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>ALT (IU/L)</td>
<td>131.8 ± 15.1</td>
<td>174.6 ± 22.6</td>
<td>103.2 ± 18.9</td>
<td>0.02</td>
</tr>
<tr>
<td>ALP (IU/L)</td>
<td>476.9 ± 41.6</td>
<td>594.7 ± 54.4</td>
<td>398.4 ± 55.6</td>
<td>0.015</td>
</tr>
<tr>
<td>GGT (IU/L)</td>
<td>227.8 ± 29.0</td>
<td>271.0 ± 38.9</td>
<td>199.0 ± 40.5</td>
<td>NS</td>
</tr>
<tr>
<td>endoglin (ng/mL)</td>
<td>7.8 ± 0.4</td>
<td>9.2 ± 0.8</td>
<td>6.9 ± 0.3</td>
<td>0.006</td>
</tr>
</tbody>
</table>

NS: not significant

### Table 2
Demographic data, liver function tests and plasma endoglin levels of biliary atresia patients categorized according to serum ALT status. Data are expressed as mean ± SEM.

<table>
<thead>
<tr>
<th></th>
<th>Normal ALT</th>
<th>High ALT</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>no. of patients</td>
<td>17</td>
<td>38</td>
<td></td>
</tr>
<tr>
<td>gender (M/F)</td>
<td>6/11</td>
<td>16/22</td>
<td>NS</td>
</tr>
<tr>
<td>age (years)</td>
<td>10.1 ± 0.8</td>
<td>7.1 ± 0.5</td>
<td>0.006</td>
</tr>
<tr>
<td>albumin (g/L)</td>
<td>43.5 ± 1.2</td>
<td>36.7 ± 1.2</td>
<td>0.009</td>
</tr>
<tr>
<td>total bilirubin (μmol/L)</td>
<td>32.5 ± 22.2</td>
<td>94.1 ± 20.5</td>
<td>0.05</td>
</tr>
<tr>
<td>direct bilirubin (μmol/L)</td>
<td>15.4 ± 13.7</td>
<td>63.3 ± 17.1</td>
<td>0.03</td>
</tr>
<tr>
<td>AST (IU/L)</td>
<td>37.6 ± 3.5</td>
<td>200.8 ± 18.8</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>ALT (IU/L)</td>
<td>23.5 ± 1.5</td>
<td>180.2 ± 16.7</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>ALP (IU/L)</td>
<td>225.7 ± 19.2</td>
<td>589.3 ± 49.8</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>GGT (IU/L)</td>
<td>82.4 ± 39.6</td>
<td>292.8 ± 33.2</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>endoglin (ng/mL)</td>
<td>6.3 ± 0.5</td>
<td>8.5 ± 0.5</td>
<td>0.003</td>
</tr>
</tbody>
</table>

NS: not significant

**Fig. 1** Correlation of plasma endoglin levels in biliary atresia patients with total bilirubin levels and controls. Data are expressed as mean ± SEM.

**Fig. 2** Correlation of plasma endoglin levels in biliary atresia patients with serum ALT and controls. Data are expressed as mean ± SEM. ALT, alanine aminotransferase.

**Fig. 3** Correlation of plasma endoglin levels in biliary atresia patients with the presence of portal hypertension (PH) and in controls. Data are expressed as mean ± SEM. PH, portal hypertension.
were significantly higher in the patients with PH compared to those without PH (8.0±0.5 vs. 6.0±0.3 ng/mL; p=0.001). Moreover, plasma endoglin was positively correlated with serum ALT (r=0.36, p=0.007) and with serum GGT (r=0.44, p=0.001) (Figs. 4 and 5).

Discussion
Endoglin, an accessory component for TGF-β receptor system, functions as a modulator of cellular TGF-β responses [22]. This auxiliary receptor binds TGF-β1, TGF-β3, activin, bone morphogenetic protein-2 and bone morphogenetic protein-7 in the presence of the signaling receptor type I and II, and regulates the effect of TGF-β on extracellular matrix synthesis [3,5,21]. The presence of endoglin as a part of the TGF-β receptor complex could be important for determining the extent of extracellular matrix protein accumulation following the binding of TGF-β1. A number of studies have previously demonstrated the upregulation of endoglin expression on septal myofibroblasts and sinusoidal lining cells of patients with chronic liver diseases [2,11,12]. Furthermore, a previous investigation reported that elevated intrahepatic and circulating endoglin was associated with progressive hepatic fibrosis in chronic hepatitis C virus infection [6]. However, there have been no published studies on the role of endoglin in biliary atresia and on the relationship between endoglin and the clinical outcome of BA.

The current study revealed that circulating endoglin levels in BA patients were significantly higher than those in healthy controls. In the BA patients, plasma endoglin levels were more elevated in BA patients with persistent jaundice than in those without jaundice. Further analysis demonstrated that BA patients with high ALT levels had increased concentrations of plasma endoglin compared to those with normal ALT. Elevated plasma endoglin was positively correlated with serum ALT and serum GGT in postoperative BA patients. Serum ALT routinely serves as a specific biochemical parameter of liver dysfunction reflecting hepatocellular damage. In addition, serum GGT is likely to be an indicator for the severity of biliary obstruction. Therefore, these findings suggest that plasma endoglin can be utilized as a prognostic biomarker for ongoing liver injury and biliary obstruction in postoperative BA.

To our knowledge, the present study is the first to show that circulating endoglin is elevated in BA patients compared with healthy controls, and that endoglin concentration is associated with a therapeutic outcome (jaundice status, hepatic dysfunction, and portal hypertension) in BA. Recently, an increase of endoglin expression in circulation and/or liver tissue has been documented for a number of liver diseases, including chronic hepatitis, liver cirrhosis and hepatocellular carcinoma [6,26,27]. In accordance with the findings of this study, Clemente et al. demonstrated an elevation of circulating endoglin with advancing chronic hepatitis [6]. Furthermore, Yagmur et al. revealed that circulating endoglin increased significantly in patients with cirrhosis and hepatocellular carcinoma and was correlated with the severity of hepatic damage [26]. In addition, the expression of intrahepatic endoglin has been demonstrated in bile duct-ligated rats as described by Díaz-Gil et al. [10]. More prospective studies on hepatic endoglin expression are required to elucidate the mechanisms of endoglin in BA.

It is interesting to point out that the results of this study are consistent with our recent publication showing that serum tissue inhibitors of metalloproteinase-1, stem cell factor, basic fibroblast growth factor, and transforming growth factor-β1 were more pronounced in BA children than in healthy controls [16-18,24]. These cytokines are significantly associated with outcomes in postoperative BA and have been implicated in progressive hepatic fibrosis. Endoglin levels, like those of previous cytokines, were found to be correlated to the progression of liver dysfunction and portal hypertension. Future research on additional cytokines and growth factors may help identify more pieces of the inflammatory jigsaw of BA; nevertheless, the challenge remains to piece them together to create a logical solid hypothesis regarding their precise role.

In the light of these considerations, various possible mechanisms could be responsible for the elevation of circulating endoglin in BA patients. Firstly, production of endoglin in the damaged liver may result in high levels of plasma endoglin. Secondly, elevated endoglin concentrations could be ascribed to an imbalance between endoglin production and endoglin clearance. In advanced BA stages, reduced endoglin clearance may possibly contribute to increased circulating endoglin levels. Moreover, because other organs apart from the liver can synthesize and
secret endoglin, the major sources of elevated plasma endoglin in the present study could be extrahepatic organs. Additional investigation will be needed to clarify this observation.

It should be mentioned, however, that our study has some limitations. The sample size of patients enrolled in this study was small. A further study with a random sample from a larger population will be needed to draw a more definite conclusion. In addition, incomplete assessment of potential confounders such as age, gender, and medical comorbidities needs to be taken into account. As this study was designed as a cross-sectional study, a definite cause and effect relationship cannot be concluded. However, with the supporting evidence from other studies based on the role of endoglin in liver cirrhosis and hepatic fibrosis [6,10,26,27], it is likely that the elevated plasma endoglin levels found in postoperative BA patients may be involved in the pathophysiology of hepatocellular injury and the development of portal hypertension.

To summarize, the current study demonstrated that BA patients had significantly elevated circulating levels of endoglin compared with healthy controls. Plasma endoglin was more pronounced in BA children with persistent jaundice compared to those without jaundice. Additionally, plasma endoglin in BA patients with high ALT was significantly elevated compared to those with normal ALT. Further analysis showed that BA patients with PH had markedly higher plasma endoglin levels than those without PH. These findings suggest that high levels of circulating endoglin are associated with hepatic injury and hence reflect the magnitude of liver dysfunction in BA. There was a positive correlation between plasma endoglin, serum ALT, and serum GGT. Endoglin measurement may be used as a potential biomarker for progression to hepatic dysfunction and could be predictive of prognosis with respect to the development of PH during the follow-up of postoperative BA patients.

Acknowledgement

The present study was supported by National Research Council of Thailand, Thailand Research fund and the Commission on Higher Education. We are thankful to Ms. Nutchanart Thaworn-suk for technical assistance.

Conflict of Interest: None

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