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Are stable postoperative biliary atresia patients really stable?

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Abstract Transforming growth factor-beta 1 (TGF β-1) is an important mediator of liver-cell proliferation and replication that is implicated in hepatic fibrosis (HF). Hepatic stellate cells (HSC) are activated by TGF β-1 and are the main precursor cells involved in fibrogenesis. The correlation between serum TGF β-1, activated HSC in liver-biopsy specimens, and liver biochemistry was investigated to determine the value of TGF β-1 as an indicator of clinical status in postoperative biliary atresia (BA) patients. Thirty-two postoperative BA patients (mean age 11.2 ± 2.8 years) and 13 normal controls (mean age 10.3 ± 3.7 years) were studied. Based on average liver function test (LFT) results over a 3-month period immediately prior to this study, the BA patients were divided into group I (anicteric, normal LFT; n = 10); group II (anicteric, elevated liver transaminases; n = 12), and group III (jaundiced end-stage liver fibrosis awaiting liver transplantation; n = 10). Serum TGF β-1 was determined using ELISA. Liver-biopsy specimens were examined with antibody against TGF β-1 and α-smooth muscle actin (SMA) antibody for detection of activated HSC. Serum TGF β-1 was significantly higher in groups I (11.4 ± 3.7 ng/ml; P < 0.01) and II (23.3 ± 11.3 ng/ml; P < 0.001) than in group III (3.0 ± 1.5 ng/ml) and controls (4.5 ± 2.5 ng/ml) despite normal LFT in group I. The 3 subjects with the highest serum TGF β-1 in group II had bile lakes. Biopsies from groups I and II were strongly positive for TGF β-1 in hepatocytes and Kupffer cells and for activated HSC detected by SMA compared with group III and controls. Because serum TGF β-1 and activated HSC are only present during active fibrosis, we conclude that there is progressive fibrogenesis even in seemingly normal postoperative BA patients. In particular, bile lakes should be regarded as a key sign of progressive HF, the presence of which should be regarded with extreme caution. We suggest that serum TGF β-1 could be used as an accurate indicator of progressive fibrogenesis in postoperative BA patients.

Keywords Biliary atresia · Transforming growth factor-beta 1 · Prognosis · Hepatic stellate cells · α-Smooth muscle actin

Introduction

The initial surgical treatment of biliary atresia (BA) is the Kasai portoenterostomy. Unfortunately, the long-term success of the Kasai procedure is poor, with the incidence of jaundice-free patients ranging from 25% to 40%, and most patients develop progressive hepatic fibrosis (HF) [8].

Transforming growth factor-beta 1 (TGF β-1), a peptide widely distributed in tissues, is known to have diverse biological activities [3]. TGF β-1 is an important mediator of liver-cell proliferation and replication that is implicated in HF [1], and may also regulate the proliferation of epithelial and mesenchymal cells as well as immunocompetent cells [10]. Hepatic stellate cells (HSC) are activated by TGF β-1 and are the main precursor cells involved in fibrogenesis [4]. Activated HSC (lipoocytes, retinoid-storing cells, fat-storing cells, and Ito cells), localized in the space of Disse, are the most important source of connective tissue in liver injury and disease [2].

We examined long-term follow-up postoperative BA patients to determine the value of measuring serum TGF β-1 and assessing the extent of activated HSC in liver-biopsy specimens for evaluating clinical status.
Materials and methods

We classified 32 long-term outpatient follow-up postoperative BA patients (mean age 11.2 ± 2.8 years) and 13 normal controls (mean age 10.3 ± 3.7 years) into three groups according to their average liver function tests (LFT) over the 3 months prior to this study. Classification was based on total bilirubin (T-Bil), glutamic oxaloacetic transaminase (GOT), glutamic pyruvic transaminase (GPT), and γ-glutamyl transpeptidase (γ-GTP) levels [5].

Group I comprised 10 patients who were jaundice-free, with normal LFT (T-Bil < 1.5 mg/dl; GOT < 40 IU/l; GPT < 35 IU/l; γ-GTP < 55 IU/l) and no evidence of severe cholangitis or portal hypertension. Group II comprised 12 patients with moderate liver dysfunction (T-Bil < 1.5 mg/dl; GOT > 40 IU/l; GPT > 35 IU/l; γ-GTP > 55 IU/l). Group III, the “unfavorable prognosis group”, comprised 10 patients with severe liver dysfunction (T-Bil > 1.5 mg/dl; GOT > 40 IU/l; GPT > 35 IU/l; γ-GTP > 55 IU/l). Serum TGF-β-1 was determined using ELISA. All subjects were investigated after obtaining parental informed consent to participate in this study.

Four patients in group I, 4 in group II, and 6 in group III had liver needle biopsies. In addition, histologically normal liver biopsies were obtained from 3 children undergoing radical surgery for choleodochal cysts. Biopsy specimens were examined with antibody against TGF-β-1 and α-smooth muscle actin (SMA) antibody for detection of activated HSC [6].

Samples of peripheral venous blood were collected and immediately separated and stored at −80 °C until they could be assayed. All were fasting specimens taken after each subject had rested for at least 1 h. Serum TGF-β-1 levels were measured in duplicate using a Quantikine human immunoassay kit (R&D Systems, Japan) according to the manufacturer’s instructions. Results were expressed in ng/ml.

All biopsy specimens were fixed in Bouin’s solution and embedded in paraffin for immunohistochemistry for TGF-β-1 or α-SMA antigens, and a three-step indirect immunoperoxidase procedure was performed. Sections were flooded with 5% species-specific normal rabbit serum and incubated overnight at 4 °C with rabbit polyclonal antibody to TGF-β-1 (Santa Cruz Biotechnology, Japan) or primary monoclonal antibody for α-SMA (1:50 dilution, DAKO, Denmark). After washing in phosphate-buffered saline (PBS, pH 7.2), sections were treated for 30 min at room temperature with biotinylated anti-rabbit antibody (1:50 dilution, DAKO) or biotinylated anti-mouse immunoglobulin (1:50 dilution, DAKO), then washed thoroughly in three changes of PBS (pH 7.2) and incubated in avidin-biotin-horseradish peroxidase complex preparation (DAKO) for 30 min. Staining was visualized using 0.015% 3,3-diaminobenzidine (DAB, Sigma, UK) solution in 50 mmol/L Tris–HCl buffer (pH 7.6) containing 10 mmol/L hydrogen peroxide. Counterstaining was performed with 1% methyl green solution (pH 4.0). To provide a negative control in immunohistochemical studies, the primary antibody was omitted.

The mean, standard deviation, and standard error were calculated for each group, and Student’s t-test was performed to test differences between the results from each clinical group and the control group.

Results

Serum TGF-β-1 was significantly higher in groups I (11.4 ± 3.7 ng/ml; P < 0.01) and II (23.3 ± 11.3 ng/ml; P < 0.001) than in group III (3.0 ± 1.5 ng/ml) and controls (4.5 ± 2.5 ng/ml) despite normal LFT in group I (Fig. 1). Interestingly, the 3 subjects with the highest serum TGF-β-1 in group II had bile lakes.

Biopsies from groups I and II were strongly positive for TGF-β-1 in hepatocyte and Kupffer cells compared with group III and controls (Fig. 2a, b). Proliferating bile ducts in the portal tracts showed slightly stronger TGF-β-1 immunoreactivity in group II compared with minimal to no staining in group III and controls (Fig. 3a, b).

Biopsies from groups I and II were strongly positive for activated HSC detected by SMA compared with no staining in group III and controls (Fig. 4a, b).

Discussion

Irrespective of the success of surgical procedures for BA, long-term follow-up of postoperative BA patients indicates that progressive HF and portal hypertension develop even if good bile flow is achieved. Thus, BA must be considered a progressive disease. TGF-β-1 is a multifunctional cytokine that plays an important role in embryogenesis, collagenesis, hematopoiesis, and regulation of cell growth. Recently, overexpression of TGF-β-1 in end-stage BA has been reported [9], but little is known about the plasma levels of TGF-β-1 in BA.

Ramm et al. [6] reported that TGF-β-1 mRNA expression can be demonstrated in bile-duct epithelial cells, activated HSC, and hepatocytes in close proximity to fibrotic septa in cases of BA less than 4 years old. They concluded that activated HSC are responsible for increased collagen production in patients with BA, and therefore play a definitive role in the fibrogenetic process. Rosensweig et al. [7] examined the plasma levels of TGF-β-1 protein in patients with BA immediately after liver transplantation (LT) and found them decreased compared with values for healthy children. However, they found that the hepatic expression of TGF-β-1 can also increase in the presence of HF in BA [7]. The data reported in these two studies are very interesting, but both examined very young patients (less than 4 years old) who were likely to progress rapidly to