Proliferation of intrahepatic bile-duct epithelium in biliary atresia: A useful predictor of clinical outcome

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Abstract
Proliferating cell nuclear antigen (PCNA) and transforming growth factor α (TGF α) are considered as markers of cell proliferation. The expression of PCNA and TGF α was evaluated immunohistochemically using anti-PCNA antibody and TGF α in 31 patients with biliary atresia (HA) (15 jaundice-free and 16 with persistent jaundice) and 6 control infants. The labeling indices (LI) for PCNA and TGF α-positive bile-duct epithelium in HA were 14.1±14.0% and 51.4±33.7%, respectively, which was significantly higher than in the controls (P <0.01). In HA, the number of PCNA-immunoreactive cells was higher in the peripheral bile ductules than in the central bile ducts of the portal tract (P <0.01). 11 was not related to patient age at the time of hepatic portoenterostomy in two groups divided at the age of 60 days. Patients in the persistent jaundice group had greater expression of PCNA and TGF α (21.7±16.0% and 76.9±20.7%, respectively) compared to those in the jaundice-free group (6.0±1.2% and 24.3±20.9%, p <0.001). PCNA and TGF α expression in the bile-duct epithelium of the portal tract was closely related to prognosis in HA patients, and thus could be useful as a prognostic marker.

Introduction
Recent progress in immunology has made many new and useful methods of morphologic studies of various cellular proteins available. Proliferating cell nuclear antigen (PCNA) and transforming growth factor α (TGF α) are two such substances. PCNA/cyclin is an auxiliary protein of DNA-polymerase δ, and its synthesis is directly related to the proliferative state of the cell. It can be detected in dividing and newly formed cells. TGF α on the other hand, has been demonstrated to be a secreted polypeptide available in both normal and transformed cells. These two proliferating agents are widely used as markers in studying various types of neoplasms.

Kasai's hepatic portoenterostomy has resulted in long-term survival in approximately 25%-30% biliary atresia (BA) patients. Some less fortunate patients have developed portal hypertension and progressive hepatic insufficiency and eventually died. Although age at operation, histologic alterations of the hepatic parenchyma, and episodes of postoperative cholangitis were thought to be related to the clinical outcome, clinical experience has shown that none serve as reliable indicators of prognosis. Our previous studies indicated that intrahepatic bile ducts are one of the most important factors that determine the outcome of BA patients. To investigate the pathological changes of the bile ducts in more detail, we used monoclonal antibodies for PCNA and TGF α in immunohistochemical studies of biopsied liver specimens taken at the time of portoenterostomy. This is the first application of these proteins to study the intrahepatic bile ducts and ductules in BA, and expression of these proteins correlate well with the clinical outcome of BA patients.

Materials and methods
Hepatic tissue was obtained by surgical
biopsy from the right anterior hepatic segment of 31 EA patients at hepatic portoenterostomy. Six liver specimens from autopsied infants without hepatobiliary disease were used as histologically normal controls. Tissue sections of 3 µm were prepared from 10% formalin-fixed liver specimens. For immunohistochemical study, they were deparaffinized using xylene and graded ethanol and then incubated in methyl alcohol containing 1.5% hydrogen peroxide for 20 min to block the endogenous peroxidase activity. For PCNA immunostaining only, sections were then incubated in 2N HCl for 30 min at 20°C, followed by washing in two successive baths (5 min each) in 0.1 mol/L borax at pH 8.5, then incubated in 0.5% tween 20 in phosphate buffer solution (PES) for 10 min and washed in PES twice, for 10 min each.

The indirect peroxide-labeled antibody method was applied for immunohistochemical staining. The monoclonal antibodies for PCNA (DAKO, PC-10, Denmark) and TGF α (Oncogene Science, Uniondale, NY) were used as first antibody in 1:400 and 1:200 dilutions, respectively. Tissue sections were then allowed to react for 12 h at 40°C. After washing with PES, anti-mouse IgG was conjugated with horseradish peroxidase (MBL, Japan) and used as a second antibody in 1:400 dilutions and allowed to react for 3 h. PBS was used as a substitute for primary antibody for the negative controls, which confirmed the specificity of the procedure. Bound peroxidase activity was visualized with 0.25% 3,3-diaminobenzidine solution containing 10 mM hydrogen peroxide. Sections were allowed to react for 3 min, after which they were counterstained with 10% methyl green solution and mounted for microscopic studies.

For quantitative evaluation of the bile-duct epithelium of haematoxylin-eosin and immunostained sections, at least 4-10 portal areas were investigated. Centering the interlobular artery, we divided the bile ducts in the portal tract equally into three groups: central, mid, and peripheral, according to their locations. We excluded portal tracts where the interlobular artery was ill defined. Positive and negative immunoreactive cells were counted in the sections, regardless of the degree of reactivity with monoclonal antibodies for PCNA and TGF α; nuclei that were stained brown were considered positive. Sections were examined without any prior knowledge of the patient's prognosis. The labeling index (LI) was applied as previously described, corresponding to the number of nuclei positive for PCNA and TGF α among a total of 1,000 nuclei and expressed as percentage of positive cells.

Ten patients were operated upon before 60 days of age and the remaining 21 after 60 days. Fifteen patients with serum bilirubin levels less than 1 mg/dl were considered to have cleared jaundice and the remaining 16 were never completely anicteric during the postoperative period. Statistical analysis was performed using the unpaired student t-test. Differences were considered significant if p was less than 0.05. Results were expressed as mean ± SD.

Results

Histologic findings: Conventional histologic studies of the liver specimens showed findings characteristic of BA. There was widening of the portal tracts, cholestasis in the bile ductules, infiltration of mixed inflammatory cells, fibrosis, and proliferation of bile ductules with or without intraluminal accumulation of cellular debris or bile plugs. Nonspecific cholangitis with degeneration and necrosis was always observed in the epithelial lining of bile ducts although the degrees varied.

Immunohistochemical findings: PCNA and TGF α were found diffusely in the bile-duct epithelium of all 31 patients with BA. PCNA was detected in the bile duct epithelium of 1.32 ±0.35% of the normal controls compared to 14.1 ± 14.0% of BA patients (P<0.02), while TGF α was found in 0.32 ± 0.42% and 51.4 ± 33.7% (P<0.001), respectively. Although PCNA immunoreactive cells were observed in central, mid, and peripheral bile ducts in the portal tract (Table 1), their
numbers were less in the central than the peripheral ducts ($P < 0.02$). No remarkable difference in TGF-α expression was observed among the three groups of bile ducts (*Table 1*).

**Table 1.** Distribution of proliferating cell nuclear antigen (PCNA) and transforming growth factor α (TGF-α)-immunoreactive bile duct epithelium in the portal areas of 31 patients with biliary atresia (HA)

<table>
<thead>
<tr>
<th>Nos. of positive cells</th>
<th>Central duct</th>
<th>Mid-duct</th>
<th>Peripheral duct</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCNA</td>
<td>8.8 ± 9.1</td>
<td>14.3 ± 15.3</td>
<td>16.7 ± 16.7</td>
</tr>
<tr>
<td>TGF-α</td>
<td>52.1 ± 35.1</td>
<td>52.0 ± 35.1</td>
<td>53.7 ± 35.8</td>
</tr>
</tbody>
</table>

$P < 0.02$

**Relation to clinical outcome:** Patients who never became anicteric had greater expression of PCNA in the portal-tract bile-duct epithelium, (Fig. 1 A) compared to jaundice-free patients (Fig. 1 B). The LIs of PCNA expression in jaundice-persistent and jaundice-free groups were 21.7±16.0% and 6.0±2.7%, respectively ($P < 0.001$). Expression of TGF-α was also higher in jaundice-persistent than jaundice-free patients, (Fig. 2A and B). LI differed significantly between the two groups of patients, at 76.9±20.7% and 24.3±20.9%, respectively ($P < 0.001$). A highly significant association was observed between the expression of both PCNA and TGF-α and the clinical outcome: jaundice-persistent patients showed higher expression of PCNA and TGF-α than jaundice-free patients (Fig. 3).

**Fig. 1 A, B.** Microphotographs showing expression of proliferating cell nuclear antigen/cyclin in portal bile-duct epithelium from A a jaundice-persistent patient (labeling index [LI] = 78%) (arrowheads) and B a jaundice-free patient (LI = 4%) (arrows).

**Fig. 2A, B.** Specimens showing expression of transforming growth factor α in portal bile-duct epithelium from A jaundice-persistent patient (labeling index [LI] = 97% (arrows) and B a jaundice-free patient (LI = 3.55) (arrows).

The LIs of PCNA and TGF-α; were not related to patient age at the time of hepatic portoenterostomy in the two groups divided at the age of 60 days. In the patients operated upon before and after 60 days of age, the expression of PCNA was 13.1±11.6% and 14.4 ± 15.3% ($P < 0.8$) and that of TGF-α; 47.1 ± 31.9% and 53.5±35.1 %, respectively ($P < 0.6$).

**Fig. 3 A, B.** Expression of A proliferating cell nuclear antigen (PCNA) and B transforming growth factor alabeling indices (LI) in intrahepatic bile-duct epithelium of biliary atresia patients in relation of clinical outcome

**Discussion**

Achievements in modern immunohistochemistry have opened a new era in the identification of various cellular proteins in both neoplastic and non-neoplastic conditions.$^{3,6,17,18,20}$ PCNA and TGF-α are detected in proliferating cells because of their high concentrations during the proliferating state.$^{3,5,14,15}$ We have studied them in portal
bile-duct epithelium in patients with BA by applying monoclonal anti-PCNA and -TGF α are expressed at a high frequency in intrahepatic portal bile-duct epithelium in BA: they were expressed in 100% of the cases of BA examined. PCNA staining was restricted to the nucleus and TGF α to the cytoplasm and cell membrane of biliary epithelial cells. The study confirms that proliferation of portal bile-duct epithelium has a positive correlation with clinical outcome in BA patients.

Our previous studies of the intrahepatic bile ducts using light and electron microscopy disclosed that the bile ducts are pathological even in the early stages of BA8,9. Other histologic studies have also indicated that bile-duct injuries in the form of inflammation, degeneration, and obstruction are the usual features of BA2. A proliferate response of the biliary epithelium in BA would most likely take place as a regenerative process to repair injured bile ducts of any etiology. However, factors that determine the proliferative response of biliary epithelial cells are not yet clearly understood. The fact that there were variations in the expression of proliferate activity in individual patients can be explained by the concept that a proliferate response may depend partly on the severity of previous damage and the differing abilities of individual patients to repair damage. The present study confirms for the first time using immunohistochemical methods that the epithelial response of bile ducts and ductules is proliferate rather than degenerative in BA.

The pathology of the intrahepatic bile ducts is regarded as one of the most important factors determining the prognosis of BA8,9. The present study demonstrates that patients with persisting jaundice have significantly higher proliferating indices of PCNA and TGF α expression in: the bile-duct epithelium than patients who are jaundice-free during their postoperative course. The intimate relationship between the regenerating response of bile-duct epithelium and clinical outcome may indicate that less prominent proliferation of the biliary epithelial cells in jaundice-free patients is the result of a lesser degree of injury to the portal bile-duct epithelium. In contrast, the prominent regenerative response of the biliary epithelial cells in the group with persistent jaundice may be due to more severe bile-ducts injury from unknown aetiologic agents or to severe, long-standing obstruction. Our previous study of PCNA expression in the hepatocytes in HA also disclosed a similar finding: PCNA expression in the hepatocytes of jaundice-free patients was significantly lower than in those of jaundiced patients16.

This study also reveals that the expression of PCNA-immunoreactive epithelial cells is more prominent in peripheral than in central bile ducts in the portal area. It would be interesting to know why the structures near the hepatic lobules (peripheral portal tract) appeared more regenerative. The damaging effects of the unknown aetiologic agents seemed much more severe in the periphery adjacent to the hepatic lobules than in the centre of the portal tract. Nostrant et al found that rat liver-cell regeneration after selective zonal injury was directly related to the site of damage of a particular area in the liver, and concluded that the contribution of different acinar zones to regeneration after toxic liver injury depends upon the distribution of damage within the acinus21.

In conclusion, PCNA and TGF α expression in the epithelial bile ducts of the portal tract could be a good marker to assess the overall condition of the bile ducts, and thereby serve as a useful parameter to predict the clinical outcome of HA patients.

References


