Chemopreventive effect of a cyclooxygenase-2-specific inhibitor (etodolac) on chemically induced biliary carcinogenesis in hamsters

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The present study was designed to evaluate whether etodolac, a cyclooxygenase-2 (COX-2)-specific inhibitor, could prevent chemically induced biliary carcinogenesis in bileoenterostomized hamsters. Syrian golden hamsters were subjected to choledochojejunostomy and then received subcutaneous injections of \textit{N}-nitrosobis(2-oxopropyl)-amine (BOP) every 2 weeks at a dose of 10 mg/kg body wt. BOP administration was started 4 weeks after surgery, and continued for 18 weeks. The animals were simultaneously orally administered etodolac three times per week at a dose of 10 mg/kg body wt in 0.5% methylcellulose solution (etodolac group). The control hamsters were administered methylcellulose solution alone. The hamsters were killed 22 weeks after surgery, and the biliary carcinomas were evaluated histologically. The presence and degree of cholangitis and the cell kinetic status of the biliary epithelium were also evaluated with special reference to biliary carcinogenesis. Intrahepatic bile duct carcinomas developed in 15 of 17 (88%) hamsters in the control group, and in only 6 of 18 (33%) hamsters in the etodolac group (\textit{P} < 0.01). The incidence and number of developing biliary carcinomas were well correlated with the degree of cholangitis, and severe cholangitis was evident in the controls. The cell kinetic study demonstrated that the proliferating cell nuclear antigen-labeling index of the biliary epithelium was 9.67 and 5.14% in the control and etodolac groups, respectively (\textit{P} < 0.05). The mean levels of prostaglandin \textit{E}$_2$ (PGE$_2$) products in the liver tissue were 14.14 ± 3.31 pg/total protein (TP) mg in the control group, and 7.46 ± 2.34 pg/TP mg in the etodolac group (\textit{P} < 0.05). These findings indicated that etodolac reduced both the occurrence of severe cholangitis and the acceleration of biliary epithelial cell kinetics after bileoenterostomy, resulting in the prevention of BOP-induced biliary carcinogenesis in hamsters. In conclusion, COX-2-specific inhibitor (etodolac) may be a possible agent against not only reflux cholangitis, but also biliary carcinoma after bileoenterostomy.

Abbreviations: BOP, \textit{N}-nitrosobis (2-oxopropyl) amine; COX-2, cyclooxygenase-2; NSAIDs, non-steroidal anti-inflammatory drugs; PGE$_2$, prostaglandin \textit{E}$_2$; PCNA-LI, proliferating cell nuclear antigen labeling index.

Introduction

Bileoenterostomy is a common surgical technique that is widely used in the field of hepatobiliary pancreatic surgery. Reflux cholangitis (1–3), biliary stones (3,4) and liver abscess (2) are well-known complications after bileoenterostomy. Recently, clinical studies have revealed that biliary carcinomas occur as a delayed complication of bileoenterostomy for benign disease (5–7). We have demonstrated that persistent reflux cholangitis after bileoenterostomy accelerated biliary carcinogenesis through an activation of biliary epithelial cell kinetics in hamsters (8,9).

Non-steroidal anti-inflammatory drugs (NSAIDs) are known to have anticarcinogenic effects on chemically induced or genetic mutational carcinogenesis (10–15), and numerous epidemiological studies have indicated that NSAIDs reduced the incidence of colorectal cancer in humans (16–23). On the other hand, cyclooxygenase-2 (COX-2), one of the major target molecules of NSAIDs has recently been demonstrated to be over-expressed in the inflamed biliary epithelium and biliary neoplasms in humans (24,25).

In this study, we investigated whether etodolac, a COX-2-specific inhibitor, could prevent biliary carcinogenesis in bileoenterostomized hamsters. We used Syrian golden hamsters because the anatomical structure of their pancreaticobiliary ductal system and the bile acid composition and pancreatic juice components in this species are similar to those of humans (26–28), and concluded that etodolac is a possible agent for the prevention of biliary carcinogenesis in bileoenterostomized hamsters through the restraint of cholangitis. To the best of our knowledge, this is the first successful \textit{in vivo} study on chemoprevention of biliary carcinogenesis by means of a COX-2-specific inhibitor.

Materials and methods

Animals

Seven-week-old female Syrian golden hamsters (SLC, Shizuoka, Japan) were housed, one per plastic cage, on sawdust bedding. They were kept at 24 ± 2°C and 50 ± 20% humidity with a 12-h light/12-h dark cycle, fed a CE-2 pelleted diet (Clea Japan, Tokyo, Japan), and provided drinking water \textit{ad libitum}. The animals were checked daily and weighed weekly throughout the experiments. All experiments were conducted according to the Guidelines for Animal Experimentation of Nagasaki University.

Surgical techniques

Choledochojjunostomy using a Roux-en Y procedure was performed on all hamsters. The schemata of the completed surgical procedure of the choledochojejunostomy is illustrated in Figure 1. Following anesthesia with sodium pentobarbital (50 mg/kg of body wt), an upper abdominal midline incision was made, and the distal end of the common bile duct was double-ligated with 6-0 nylon and divided. Following ligation of the cystic duct, the gallbladder was removed. The jejunum was double-ligated with 6-0 nylon and cut 7 cm distal to the pyloric ring of the stomach. About 4 cm of the anal side of the jejunum was used for the Roux-en Y anastomosis, and an intestinal anastomosis was made in a side-to-side manner with 5-0 nylon. A 20-G needle was inserted into the elevated jejunal wall ~10 mm distal to the jejunal stump, and the tied common bile duct was then inserted into the hole that had been made by the

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Morphological and biochemical changes in the hepatobiliary system of hamsters after bilioenterostomy

c) Side-to-side intestinal anastomosis; 7-cm from the pyloric ring.
(b) The common bile duct was transected at the distal end. (a) The gallbladder was removed. (Fig. 1).

Operating scheme: choledochojejunostomy in the hamster using the Roux-en Y procedure. The jejunal limb was 4-cm long. In the control group, 20 hamsters were provided with the same dose of methylcellulose solution alone. At postoperative week 22, all hamsters were killed.

Morphological and biochemical analyses

At autopsy, the largest diameter of the extrahepatic bile duct was measured. Blood samples from the vena cava were collected in ice-chilled tubes containing heparin, centrifuged (3000 r.p.m) for 10 min, and then serum samples were collected in new ice-chilled tubes. The serum levels of total bilirubin (T-Bil), alkaline phosphatase (ALP), glutamic-oxaloacetic transaminase (GOT) and glutamic-pyruvic transaminase (GPT) were measured.

Histological studies

The liver, biliary system and pancreas were removed en bloc at autopsy. After fixation in 10% neutral formalin, the specimens were cut into five blocks so that four sections contained the liver and one section contained the hepatic duct, and embedded in paraffin. The histological sections were stained with hematoxylin and eosin (H&E), and then examined by a pathologist who was blinded to the treatment allocation of the sections. The number of histologically verified adenomas and carcinomas was counted. Carcinoma was diagnosed based on the basis of disruption of epithelial cell polarity and evidence of an invasive event.

Table I summarizes the morphological and biochemical changes in the hepatobiliary system of hamsters after bilioenterostomy

<table>
<thead>
<tr>
<th>Groups</th>
<th>No. of hamsters</th>
<th>Average diameter of the EBD (mm)</th>
<th>Serum levels¹</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>T.Bil (mg/l)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>17</td>
<td>2.8 ± 1.9</td>
<td>1.9 ± 1.8</td>
</tr>
<tr>
<td>Etdolac</td>
<td>18</td>
<td>1.9 ± 1.3</td>
<td>1.4 ± 2.8b</td>
</tr>
</tbody>
</table>

¹Mean ± SD.
²Significantly different from control group (P < 0.05).

Inflammatory changes

To evaluate the relationship between cholangitis and biliary carcinogenesis, the grade of cholangitis was scored in accordance with the infiltration of inflammatory cells and the fibrous change of Glisson as follows: grade 0, no cholangitis; grade 1, mild invasion of inflammatory cells around the bile duct without fibrous change of Glisson; grade 2, severe invasion of inflammatory cells around the bile duct and/or fibrous change of Glisson; grade 3, abscess formation in the liver (8).

Cell kinetic studies

Proliferating cell nuclear antigen (PCNA) was used as a marker of biliary epithelial cell kinetics. Tissue sections were cut at 4-μm, mounted on glass slides coated with 5-aminoprophyltriethoxy saline and deawxed in xylene. The sections were treated with microwave heating for 5 min in phosphate-buffered saline at 500 W. After the blocking of endogenous peroxidase, the sections were incubated with mouse monoclonal antibodies against PCNA (clone-PC 10; DAKO, Kyoto, Japan) at a dilution of 1:100. The cell nuclei were counter-stained with hematoxylin. The proportion of labeled nuclei (labeling index; LI) was determined by counting the labeled nuclei in ≥1000 non-neoplastic epithelial cells of the intrahepatic bile ducts (33).

Activity of cyclooxygenase

Prostagrandin E₂ (PGE₂) production in the liver tissue was used as a marker of inflammation and COXs activity. At autopsy, the right lateral lobe of the liver was removed, immediately frozen in liquid nitrogen, and stored at −80°C in a sterile 1.5 ml Eppendorf tube until analysis. The frozen tissue was homogenized in saline containing 10 mg/l indomethacin, and ethanol was added to achieve the final proportion of 20%. After centrifugation, the supernatant was removed and agitated in the octadeccysilyl silica (ODS) suspension to adsorb PGE₂. Protein and lipids were eliminated from the ODS carrier by washing with ethanol, hydrochloric acid and petroleum ether in turn. Then, PGE₂ was eluted from the ODS carrier by adding acetic ether, and measured by a radioimmunoassay (RIA) technique using a prostaglandin E₂ [125I]RIA kit (PerkinElmer Life Sciences, Boston, MA).

In order to evaluate normal levels of PGE₂ production in the liver tissue and PCNA-LI of the biliary epithelium, 12 29-week-old hamsters that had received no treatment were also investigated.

Adverse effects of etodolac on vital state

The side effects of etodolac, such as gastrointestinal mucosal injury, liver and renal dysfunction, and eosinophilic pneumonia, may have adverse effects on the vital state. Accordingly, the animals were checked daily and weighed weekly throughout the experiments.

Statistical analyses

The incidence of tumor development and grade of cholangitis were analyzed using the χ² exact test. The Mann–Whitney test was also used for statistical analyses of the diameter of the extrahepatic bile duct, number of tumors per animal, laboratory data on serum, and PCNA-LI and PGE₂ production. Differences of P < 0.05 were considered statistically significant.

Results

Morphological and biochemical changes

Table I summarizes the morphological and biochemical changes in the hepatobiliary system of the hamsters. The total number of hamsters examined was 17 and 18 in the control and etodolac groups, respectively. Three hamsters in the control group and two in the etodolac group died of liver abscess and/or obstructive jaundice before death. The average diameter of the extrahepatic bile duct was 2.8 ± 1.9 mm in the...
control and 1.9 ± 1.3 mm in the etodolac group, and there was no significant difference between the two groups. However, the serum levels of T.Bil, GOT and ALP were significantly higher in the control group than in the etodolac group (P < 0.05).

Occurrence of biliary tumors

Biliary adenomas and carcinomas were observed in the hamsters in both groups (Table II). The occurrence rates of adenoma were 88 and 61% of the hamsters in the control and etodolac groups, respectively, and there was no significant difference in the incidence of adenoma between the two groups. However, numerous biliary adenomas occurred in the control group, and the average number of adenomas per animal was much higher in the control group than in the etodolac group (P < 0.01).

In the control group, intrahepatic bile duct carcinoma developed in 15 of 17 (88%) hamsters, and the average number of carcinomas per animal was 11.4. In the etodolac group, only six of 18 (33%) hamsters developed intrahepatic bile duct carcinoma, and the average number of carcinomas per animal was 1.8. Both the incidence of carcinoma and the average number of carcinomas per animal were significantly lower in the etodolac group than in the control group (P < 0.01).

Cholangitis, biliary epithelial cell kinetics, PGE2 production and biliary carcinogenesis

Cholangitis was recognized in all hamsters in the control group and 89% of the hamsters in the etodolac group (Table III). However, severe cholangitis was frequently observed in the control hamsters, and the average cholangitis score was significantly higher in the control group (P < 0.05).

PCNA-LI of the biliary epithelium was 9.67 ± 5.90% in the control group, and this value was significantly higher than that of the etodolac group (P < 0.05). In addition, the PCNA-LI of the biliary epithelium in hamsters that received no treatment was 2.4 ± 1.5%.

The mean level of PGE2 products in the liver tissue was 14.14 ± 3.31 pg/TP mg in the control group, and this value was significantly higher than the value of 7.46 ± 2.34 pg/total protein (TP) mg for the etodolac group (P < 0.05). In hamsters that received no treatment, it was 1.5 ± 0.1 pg/TP mg, and this value was significantly different from the value of both the control and etodolac groups (P < 0.05).

Figure 2 shows the correlation between the cholangitis score and biliary carcinogenesis. Concerning the degree of cholangitis, highly scored cholangitis of grade 2 or 3 was observed in 59% of the hamsters in the control group, and 22% of the hamsters in the etodolac group. In the control group, biliary carcinomas were frequently observed in each grade of cholangitis. In the etodolac group, in contrast, the incidence of biliary carcinoma decreased along with the degree of cholangitis, and carcinomas were observed in only two of the 14 hamsters with cholangitis of grade 0 or 1.

Transition of body weight

Figure 3 shows the transition curves of the body weight of hamsters during the experiment. Until the 15th week of the study, the average body weight of the hamsters in both groups increased in a similar fashion. Thereafter, the body weight of the hamsters in the etodolac group increased continuously, while that in the control group gradually decreased.

Discussion

Intrahepatic and extrahepatic biliary carcinomas have a dismal outcome even if the tumor is resected, and tolerate with traditional cytotoxic chemo- and radio-therapeutic approaches.
Cancer chemoprevention is, thus, now expected to be a new approach in the management of biliary carcinoma. Recently, numerous studies have proved that NSAIDs reduce the incidence of several kinds of cancer (34–36). We obtained evidence that persistent cholangitis after biliointerostomy accelerated the development of biliary carcinoma in hamsters, and that more severe cholangitis was associated with a higher occurrence of biliary carcinoma through an increase in the proliferative activity of the biliary epithelium (8,9). Therefore, we expected that NSAIDs could reduce biliary carcinogenesis by inhibiting biliary inflammation.

The present study clearly demonstrated a preventative effect of etodolac on BOP-induced biliary carcinogenesis in hamsters undergoing biliointerostomy. Although etodolac failed to inhibit the occurrence of reflux cholangitis, a reduction in the degree of cholangitis was apparent in the etodolac group. Moreover, etodolac down-regulated the cell kinetic activity of the biliary epithelium. These findings indicated that reflux cholangitis was inevitably induced after choledochojejunostomy, but the aggravation of cholangitis was attenuated by etodolac. The inhibitory effects of etodolac on inflammation and inflammation-activated biliary epithelial cell kinetics may participate in the tumor-preventing mechanisms in our hamster model.

It is well known that COXs mediate the rate-limiting step of prostaglandin, including PGE2, biosynthesis in the arachidonic acid cascade, and PGE2 is considered to be a reliable biomarker of COXs activity (38). On the other hand, PGE2 is well known to be up-regulated in inflammatory sites through up-regulated COXs activity. In addition, recent in vitro studies have demonstrated that COX-2 and COX-2-mediated prostanooids, including PGE2, are strongly related to cancer development and progression through their anti-apoptotic effects (38), enhancement of angiogenesis or suppression of cell-to-cell adhesive activity (39–41). In the present study, PGE2 products in the liver tissue were significantly decreased in the etodolac group. Although the tissue of interest is the epithelium of the intrahepatic bile duct, it is probable that the changes seen in the liver will be similar to those in the bile duct epithelium. Considering our findings and the evidence reported above, the inhibition of COXs activity, especially COX-2, is considered to be another possible mechanism of the chemopreventive effect of etodolac on biliary carcinogenesis in biliointerostomized hamsters.

The toxicity and efficacy of NSAIDs are mediated through the inhibition of COX-mediated prostaglandin synthesis (42). Due to the non-selective inhibitory effects of conventional NSAIDs on COX-1 and COX-2, which results in many untoward side effects, including gastrointestinal bleeding, their clinical use is limited. However, etodolac mainly inhibits COX-2, and its adverse effects on the gastrointestinal tract can occur less frequently (43). In our study, the body weight of hamsters in the etodolac group was well maintained throughout the experiment, in contrast to the hamsters in the control group. These facts suggested that etodolac had no critical adverse effects. Thus, long-term administration of etodolac might be feasible and also convenient for cancer prevention.

In conclusion, etodolac inhibited BOP-induced biliary carcinogenesis in hamsters undergoing choledochojejunostomy. Suppression of the proliferative activity of the biliary epithelial cells and reduction of PGE2 products in the liver in association with the attenuation of persistent cholangitis by etodolac were considered possible mechanisms of cancer prevention in this hamster model. Information that supports the benefits of etodolac in the management of reflux cholangitis was also obtained in this study. Clinical trials will be necessary to assess the utility of specific COX-2 inhibitors, not only in the prevention of biliary carcinogenesis, but also in the treatment of reflux cholangitis in patients undergoing biliointerostomy.

### Table III. The occurrence of cholangitis and changes in biliary epithelial cell kinetics and PGE2 products in hamsters after biliointerostomy

<table>
<thead>
<tr>
<th>Groups</th>
<th>No. of hamsters</th>
<th>No. (%) of hamsters with cholangitis</th>
<th>Average of Cholangitis scorea</th>
<th>PCNA-LI (%)a</th>
<th>PGE2 products (pg/TP mg)a</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>17</td>
<td>17 (100)</td>
<td>2.08 ± 0.97</td>
<td>9.67 ± 5.90</td>
<td>14.14 ± 3.31</td>
</tr>
<tr>
<td>Etodolac</td>
<td>18</td>
<td>16 (89)</td>
<td>1.28 ± 0.89b</td>
<td>5.14 ± 4.55b</td>
<td>7.46 ± 2.34b</td>
</tr>
</tbody>
</table>

PCNA-LI, proliferating cell nuclear antigen labeling index; PGE2, prostaglandin E2.

aMean ± SD.
bSignificantly different from control group (P < 0.05).

### References


