Chemopreventive effect of an inducible nitric oxide synthase inhibitor, ONO-1714, on inflammation-associated biliary carcinogenesis in hamsters

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The present study was designed to investigate whether an inducible nitric oxide synthase (iNOS)-specific inhibitor, ONO-1714 [(1S, 5S, 6R, 7R)-7-chloro-3-imino-5-methyl-2-azabicyclo[4.1.0]heptane], could prevent inflammation-associated biliary carcinogenesis in bilioenterostomized hamsters. Syrian golden hamsters underwent choledochojejunostomy and then received subcutaneous injections of the chemical carcinogen N-nitrosobis(2-oxopropyl)amine every 2 weeks at a dose of 10 mg/kg body wt, starting 4 weeks after surgery and continuing for 18 weeks. The hamsters were divided into two groups according to their oral intake of either a standard pelleted diet containing ONO-1714 at 100 p.p.m. for 18 weeks (ONO group, n = 15) or an ordinary diet alone (control group, n = 15). The animals were killed 22 weeks after surgery, and the development of biliary tumors was examined histologically. The presence and degree of cholangitis, cell kinetic status of the biliary epithelium and iNOS expression were evaluated. Intrahepatic biliary adenomas developed in all control animals, whereas they developed in only seven (47%) hamsters treated with ONO-1714 (P < 0.05). Intrahepatic biliary carcinomas were present in 13 (87%) hamsters in the control group and in only 6 (40%) hamsters in the ONO groups (P < 0.05). Histological and immunohistochemical examinations demonstrated a significant decrease in the degree of cholangitis, cell kinetic status of the biliary epithelium and iNOS expression in the biliary epithelium in the ONO group in comparison with the control (P < 0.05). These results indicate that ONO-1714 represses N-nitrosobis(2-oxopropyl)amine-induced biliary carcinogenesis in bilioenterostomized hamsters and inhibits iNOS expression in the biliary epithelium. ONO-1714 may therefore be a promising agent for the prevention of biliary carcinoma in various inflammation-associated biliary disorders.

Materials and methods

Animals
Seven-week-old female Syrian golden hamsters (SLC, Shizuoka, Japan) were used. They were housed one per plastic cage on sawdust bedding, kept at 24 ± 2°C and 50 ± 20% humidity with a 12 h light–dark cycle, fed a CE-2 pelleted diet (Clea Japan, Tokyo, Japan) and provided drinking water 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
Choledochojejunostomy using a Roux-en Y procedure was performed on all hamsters. A schematic drawing of the completed choledochojejunostomy surgical procedure is illustrated in Figure 1. Briefly, following anesthesia with sodium pentobarbital (50 mg/kg body wt), an upper abdominal midline incision was made and the distal end of the common bile duct was doubly ligated with 6-0 nylon and divided. The gallbladder was then removed following the ligation of the cystic duct. The jejunum was doubly 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 7-0 nylon. The animals were checked and weighed weekly throughout the experiments. All experiments were conducted according to the Guidelines for Animal Experimentation of Nagasaki University.

Chemoprevention protocol
All hamsters were given subcutaneous injections of the chemical carcinogen N-nitrosobis(2-oxopropyl)amine (Nakarai Tesque, Kyoto, Japan) every 2 weeks at a dose of 10 mg/kg body wt in 0.9% saline. N-nitrosobis(2-oxopropyl)amine administration was started 4 weeks after surgery and continued thereafter for 18 weeks. The animals were randomly divided into two groups according to the

Abbreviations: iNOS, inducible nitric oxide synthase; NO, nitric oxide; NOS, nitric oxide synthase; ONO-1714, (1S, 5S, 6R, 7R)-7-chloro-3-imino-5-methyl-2-azabicyclo[4.1.0]heptane; PCNA, proliferating cell nuclear antigen.

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different regimens, i.e. 15 hamsters were provided with a standard pelleted diet containing ONO-1714 (donated by Ono Pharmaceutical Co., Ltd, Osaka, Japan) at 100 p.p.m., starting 4 weeks after surgery and continuing for 18 weeks (ONO group), whereas 15 hamsters were fed the ordinary diet alone (control group). Twenty-two weeks after the operation, all hamsters were killed in order to perform histological examinations.

**Histological studies**

The liver and the extrahepatic bile duct were removed at autopsy. After fixation in 10% neutral formalin, the specimens were cut into five blocks, so that four sections contained the liver and one contained the hepatic duct. The specimens were subsequently embedded in paraffin. The histological sections were stained with hematoxylin and eosin and examined by a pathologist who was blinded to the treatment allocation of the sections. The number of histologically verified biliary adenomas and carcinomas was counted in the five tissue sections in each animal. Adenoma and carcinoma were defined as lesions that showed signs of expansive growth, in accordance with the WHO classification of tumors in hamsters (30,31). In contrast to adenomas, carcinomas displayed signs of malignancy, such as nuclear atypia, mitotic activities, a disruption of epithelial cell polarity and invasion.

**Inflammatory changes**

In order to evaluate the relationship between cholangitis and biliary carcinogenesis, the grade of cholangitis was scored according to 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, moderate invasion of inflammatory cells around the bile duct and/or partial fibrous change of Glisson and grade 3, severe invasion of inflammatory cells around the bile duct and/or diffuse fibrous change of Glisson (29).

**Immunohistochemical staining for iNOS**

Immunohistochemical staining for iNOS was performed using the streptavidin-biotin-peroxidase method (32) on formalin-fixed, paraffin-embedded tissue sections. The sections were cut at 4 μm, deparaffinized and dehydrated through xylene and graded alcohols. After antigen retrieval, endogenous peroxidase, 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 counterstained with hematoxylin. The proportion of labeled nuclei (labeling index) was determined by counting the labeled nuclei in >1000 non-neoplastic epithelial cells of the intrahepatic bile ducts.

**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-aminophenyltriethoxy saline and dewaxed 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 avidin-biotin-horseradish peroxidase conjugate (VECTASTAIN® Elite ABC Reagent; Vector Laboratories, CA) for 30 min, according to the Vectastain protocol. iNOS was visualized with the chromogen 3,3'-diaminobenzidine. The slides were counterstained with hematoxylin, dehydrated in a graded alcohol series, cleared in xylene and coverslipped. The expression grade of iNOS was scored using the percentage of positively stained biliary epithelial cells as follows: class 0, <30%; class 1, 30–70% and class 2, >70% (33).

**Statistical analysis**

The incidence of tumor development and the grade of cholangitis were analyzed using the chi-square exact test. The Mann–Whitney U-test was also used for statistical analyses to compare the number of tumors per animal, PCNA-labeling index and the iNOS expression between groups. A logistic regression analysis was used to clarify the correlation between the grade of cholangitis and the occurrence of biliary tumors. A P value of <0.05 was regarded as statistically significant.

**Results**

**Occurrence of biliary tumors**

Intrahepatic biliary adenomas (Figure 2) and carcinomas (Figure 3) were observed in hamsters from both groups (Table I). The occurrence rates of adenoma were 100 and 47% in the hamsters from the control and ONO groups, respectively. The difference in the incidence of adenoma between the two groups was significant (P < 0.05). Adenomas displayed a multicentric occurrence, and the average number of adenomas per animal was 15.0 and 5.0 in the control and ONO groups, respectively (P < 0.05).

Intrahepatic biliary carcinomas developed in 13 (87%) hamsters in the control group, and the average number of carcinomas per animal was 3.3. In the ONO group, only 6 (40%) hamsters developed intrahepatic biliary carcinoma, and the average number of carcinomas per animal was 1.1. In addition, statistically significant differences were observed in both the incidence of carcinoma and the average number of carcinomas per animal between the two groups (P < 0.05).

**Cholangitis, biliary epithelial cell kinetics and biliary carcinogenesis**

Cholangitis was observed in most hamsters of both groups (Table II), however, the severity of cholangitis was significantly different; namely, the average cholangitis score was 1.7 in the control group and 1.1 in the ONO group (P < 0.05). There was a tendency for the grade of cholangitis to be more severe in the large bile ducts in comparison with the small ducts or ductules in both groups of hamsters.

The PCNA-labeling index of the biliary epithelium in the control group was 27.0%, which was significantly higher than that in the ONO group (19.3%, P < 0.05).

Figure 4 shows the relationship between the cholangitis score and biliary tumorigenesis. All hamsters with severe cholangitis of grades 2 or 3 developed biliary adenoma and carcinoma. In contrast, hamsters with grade 0 or 1 cholangitis, especially hamsters in the ONO group, rarely developed biliary carcinoma. The occurrence of adenoma and carcinoma was thus correlated with the severity of cholangitis in the ONO group (P < 0.05).
**iNOS expression**

Upon immunohistochemical staining, iNOS expression was identified in the cytoplasm of the biliary epithelial cells (Figure 5) where the biliary mucosa showed various degrees of inflammatory changes. Although iNOS expression was seen in hamsters of both groups, it was weaker in the ONO group (Table II). The average score of iNOS expression was 0.7 in the ONO group and 1.4 in the control group \((P < 0.05)\). In the ONO group, a significant correlation between the iNOS expression score and the occurrence of biliary carcinoma was evident \((P < 0.05)\).

**Adverse effects of ONO-1714**

Figure 6 shows the transition curves of the body weight of hamsters in each group during the experiment. Throughout the course of the study, the average body weight of hamsters in both groups increased in a similar fashion and food consumption did not differ between the two groups. No gross or histological changes in the lungs, heart, kidney and digestive tracts of either group were observed.

**Discussion**

The present study successfully demonstrated the preventative effect of iNOS inhibitor on \(N\)-nitroso(2-oxopropyl)amine-induced biliary carcinogenesis in hamsters undergoing choledochojejunostomy. To the best of our knowledge, this is the first successful *in vivo* study on the chemoprevention of biliary carcinogenesis by means of an iNOS-specific inhibitor.
In this study, both histological and immunohistochemical examinations revealed the degree of cholangitis, expression of iNOS and cell kinetic activity of the biliary epithelium to be significantly suppressed with the use of an iNOS inhibitor, ONO-1714, in bilioenterostomized hamsters. ONO-1714 reduces not only iNOS activity but also cyclooxygenase-2 activity that is involved in K-ras-activating mutations (22). Moreover, K-ras mutations enhance the iNOS expression mediated by some cytokines (22). These factors may be involved in the anticancer mechanisms of ONO-1714 in our hamster model because it is reasonable to assume that the reduced iNOS expression should result in a decrease in NO production and subsequent NO-mediated genotoxicity. In addition, the reduced biliary epithelial cell kinetics should decrease the susceptibility of biliary epithelial cells to carcinoma because cells in the DNA synthesis phase are more susceptible to the tumorigenic effects of chemical carcinogens (34,35).

iNOS inhibitors may have adverse effects by causing vasoconstriction and thrombosis in various organs such as the liver, kidney, lung and heart (36). In this study, the body weight and food consumption of the hamsters in both the ONO group and the control group were almost equal throughout the experiment. In addition, both gross and histological evaluations revealed no remarkable findings suspicious of any adverse effects of iNOS inhibitor on the lungs, heart, kidney or digestive tracts. Therefore, the long-term administration of an iNOS inhibitor may be feasible for cancer prevention in clinical practice.

In conclusion, the suppression of the iNOS expression and proliferative activity of biliary epithelial cells, along with the restraint of cholangitis, are possible mechanisms of cancer prevention in this hamster model. Further studies should be conducted to investigate the iNOS activity and the expression of iNOS messenger RNA in our hamster model using molecular biological technology (such as reverse transcription–polymerase chain reaction and Southern blotting assays) as well as to elucidate the role of the iNOS/NO pathway in human carcinogenesis. Although additional studies are needed, iNOS inhibitors appear to be potentially promising agents for the prevention of biliary carcinogenesis in several inflammatory

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**Table II.** The occurrence of cholangitis and changes in iNOS expression and biliary epithelial cell kinetics in hamsters after bilioenterostomy

<table>
<thead>
<tr>
<th>Groups</th>
<th>No. of hamsters examined</th>
<th>No. (%)* of hamsters with cholangitis</th>
<th>Cholangitis score*</th>
<th>iNOS score*</th>
<th>PCNA-LI*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>15</td>
<td>14 (93)</td>
<td>1.7 ± 0.9</td>
<td>1.4 ± 0.7</td>
<td>27.0 ± 11.3</td>
</tr>
<tr>
<td>ONO</td>
<td>15</td>
<td>10 (67)</td>
<td>1.1 ± 0.9*</td>
<td>0.7 ± 0.7*</td>
<td>19.3 ± 9.8*</td>
</tr>
</tbody>
</table>

PCNA-LI, proliferating cell nuclear antigen-labeling index.
*Mean ± SD.
*Significantly different from the control group (P < 0.05).

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**Fig. 4.** Correlation between the cholangitis score and biliary carcinogenesis in hamsters undergoing bilioenterostomy. Filled circle, a hamster with tumor. Open circle, a hamster without tumor.

**Fig. 5.** Immunohistochemistry for iNOS. The cytoplasm of the biliary epithelial cells was positively stained according to the degree of iNOS expression. (A) A class 2 expression of iNOS seen in a hamster undergoing choledochojejunostomy without ONO-1714 treatment. The majority of the cytoplasm in the biliary epithelial cells is positively stained with iNOS (×100). (B) A class 0 expression of iNOS seen in a hamster with ONO-1714 treatment. The iNOS expression is seen in a few biliary epithelial cells (×100).

**Fig. 6.** Transition curves of the average body weight of the hamsters in each group during the experiment. Solid line, ONO group and dashed line, control group. The bars demonstrate standard errors.
cholangiopathies, such as primary sclerosing cholangitis, hepatolithiasis or recurrent cholangitis complicating biliary-enteric anastomosis.

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Conflict of Interest Statement: None declared.

References
