Inhibitory effect of meloxicam, a cyclooxygenase-2 inhibitor, on \(N\)-nitrosobis(2-oxopropyl) amine induced biliary carcinogenesis in Syrian hamsters

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Pancreaticobiliary maljunction (PBM) is a high risk factor in biliary tract carcinoma. The chemopreventive action of a cyclooxygenase (COX)-2 inhibitor (meloxicam) on \(N\)-nitrosobis (2-oxopropyl) amine (BOP)-induced gallbladder cancer in hamster PBM models was investigated. In 7-week-old female Syrian golden hamsters, the extrahepatic bile duct at the distal end of the common duct was ligated and cholecystoduodenostomy was performed (group I). In group II, the same surgery was performed and from week 4 after surgery, 10 mg/kg/day of BOP was injected subcutaneously once a week with a 1-week interval. In group III, in addition to the measures employed in group II, 5 mg/kg/day of meloxicam was administered once a day, every weekday. Pathological findings in the gallbladder in week 20 after surgery were as follows. In group I, proper epithelium (PE) was predominant and there was no cancer. In group II, PE was predominant, but there was also hyperplasia and atypical epithelium (AE) recognized in 8 of 11 cases (72.7%); the area of AE was more extensive compared with group II, the incidence of AE decreased to 27.3% and no cancerous lesion was observed. In week 20 after surgery, the proliferative cell nuclear antigen labeling index in group III was statistically significantly lower than in group II \((P = 0.045)\). No statistically significant differences were noted among the groups in terms of apoptosis labeling index in week 20 after surgery. In conclusion, it was confirmed that meloxicam suppresses carcinogenesis in hamster PBM models and its mechanism may be based on the suppression of cell growth.

Introduction

Pancreatobiliary maljunction (PBM), namely anomalous pancreaticobiliary ductal junction, is a congenital disorder and is a high risk factor for biliary tract cancer. In PBM cases, since the common bile duct and the main pancreatic duct join outside the duodenal wall, the sphincter of Oddi has no influence on the long common duct, and pancreatic juice and bile regurgitate alternately (1,2). When bacterial infection or the action of enterokinase is added to this, active pancreatic enzymes, secondary bile acid and mutagenic substances are easily produced, and these cause a process of repeated damage and repair of the biliary tract mucosa (3,4). As a result, various histological changes or genetic mutations are produced and biliary tract cancer occurs at a high rate (5–8). Pathologically, the carcinogenesis of PBM is based on the hyperplasia–dysplasia–carcinoma sequence. We were the first to clarify that from the early phase of this sequence, cyclooxygenase (COX)-2 appears at a high rate in PBM gallbladder epithelium and that there is a strong correlation between angiogenesis and tumorigenesis (9). COX is a rate-limiting enzyme in prostaglandin (PG) synthesis and exists in two isoforms. COX-1 appears constitutively in many organs, and COX-2 is induced by the stimulation from various cytokines or growth factors (10,11). Recent studies have shown that in many samples of human colon cancer tissue, COX-2 is remarkably expressed and plays a vital role in tumorigenesis, cancer growth or progression (12). In animal experiments, it was shown that non-steroidal anti-inflammatory drugs (NSAIDs), which are COX inhibitors, suppress carcinogenesis (13). It was also found, epidemiologically, that in patients who have used aspirin for a long period, the mortality rate from colon cancer is ~40% lower than in natural controls (14). Moreover, at the cellular level, it has been demonstrated that COX-2 has a strong influence on cell growth, carcinogenesis, invasion and metastasis (15,16). The same results are also reported in various tumors apart from colorectal lesions (17–19). These findings suggest that COX-2 might be useful for chemoprevention or treatment of various cancers.

Recently, Tajima et al. (20) established PBM models using Syrian golden hamster with a high incidence of adenocarcinoma development in the biliary tract induced by \(N\)-nitrosobis (2-oxopropyl) amine (BOP). There are several reports on the chemoprevention of BOP-induced pancreatic cancer using hamsters (21–26), but only a few reports cover chemoprevention of biliary tract cancer in PBM models (27–29). In addition, to the best of our knowledge, no studies have been performed on chemoprevention by COX-2 inhibitor in this model. In the present study, we investigated the chemopreventive efficacy of meloxicam, a selective COX-2 inhibitor, on BOP-induced biliary carcinogenesis in PBM models using Syrian golden hamsters.

Materials and methods

Animals

A total of 64 seven-week-old female Syrian golden hamsters (Shizuoka Laboratory Animal Center, Shizuoka, Japan) were housed one per cage and...
maintained under standard laboratory conditions in the Laboratory Animal Center, Tokyo Medical University. The hamsters were provided with a standard pellet diet and water ad libitum. All experiments were conducted in accordance with the Guidelines for Animal Experimentation of Tokyo Medical University.

Surgical procedure
Hamsters were anesthetized using ether, and a midline incision was made in the upper abdomen, followed by intraperitoneal anesthesia with nembutal (50 mg/kg body wt). In order to have pancreatic juice regurgitate into the biliary tract, the extrahepatic bile duct was doubly ligated at the distal end of the long common duct. Then, a 1 mm long incision was made in both the gallbladder fundus and duodenum ~10 mm from the pyloric ring of the stomach, and cholecystostroduodenostomy (CD) was performed with 9-0 nylon sutures.

Experimental protocol
Hamsters were divided into the following three groups. Group I: only CD surgery was performed as a control. Group II: from week 4 after CD surgery, 10 mg/kg of BOP (Nakarai Chemical, Kyoto, Japan) was subcutaneously injected once a week with a 1-week interval. Group III: from week 4 after CD surgery, 10 mg/kg of BOP was injected subcutaneously once a week with a 1-week interval, and 5 mg/kg/day of meloxicam (Daiichi Pharmaceutical, Tokyo, Japan; Boehringer Ingelheim, Ingelheim, Germany), a COX-2 inhibitor, was dissolved in water and administered orally once a day, every weekday. The interval, and 5 mg/kg/day of meloxicam (Daiichi Pharmaceutical, Tokyo, Japan) was subcutaneously injected once a week with a 1-week interval. Group III: from week 4 after CD surgery, 10 mg/kg of BOP was injected subcutaneously once a week with a 1-week interval. Group III: from week 4 after CD surgery, 10 mg/kg of BOP was injected subcutaneously once a week with a 1-week interval. Group III: from week 4 after CD surgery, 10 mg/kg of BOP was injected subcutaneously once a week with a 1-week interval. Group III: from week 4 after CD surgery, 10 mg/kg of BOP was injected subcutaneously once a week with a 1-week interval. Group III: from week 4 after CD surgery, 10 mg/kg of BOP was injected subcutaneously once a week with a 1-week interval.

Pathological examination
Formalin-fixed specimens of gallbladder resected from each group were prepared in five sections, embedded in paraffin and stained with hematoxylin and eosin. Gallbladder mucosa of the five sections was microscopically observed and the incidences of proper epithelium (PE), hyperplastic epithelium (HE), metaplastic epithelium (ME), atypical epithelium (AE) and carcinoma in situ (CIS) were evaluated. Histological findings were assessed according to the AFIP classification (30) and World Health Organization Classification (31). In brief, AE differs from CIS in consisting of a heterogeneous cell population in which columnar mucus-secreting cells, low cuboidal cells, atrophic-appearing epithelium and pencil-like cells are present. In cases where the cells have all the cytological features of malignancy with frequent mitotic figures, nuclear crowding and prominent pseudostratification, the term CIS may be used. Assessment criteria were as follows: not recognized at all, grade 0; recognized in up to half of five specimens, grade 1; recognized in more than half of five specimens, grade 2. All slides were assessed independently by two investigators. Normal gallbladder without CD surgery was taken as a control.

Cell kinetic study
Immunohistochemical staining for proliferative cell nuclear antigen (PCNA) was performed. Tissue was deparaffinized with xylene and dehydrated with a graded alcohol series. Slides were placed in a container and covered with 0.01 M sodium citrate buffer (pH 6.0) and heated in a microwave oven (500 W) for 20 min. Endogenous peroxidase was blocked with 0.3% hydrogen peroxide for 30 min. Following non-specific staining by incubation with 10% normal pig serum for 30 min at room temperature, the sections were incubated with diluted primary antibody (monoclonal mouse anti-PCNA clone PC10: DAKO, Glostrup, Denmark) overnight at 4°C. After washing with phosphate-buffer saline (PBS), the sections were treated with biotinylated rabbit anti-mouse IgG and peroxidase-conjugated streptavidin for 30 min at room temperature. They were then reacted with 20 mg of 3,3'-diaminobenzidine, tetrahydrochloride and 5 ml of 30% hydrogen peroxide for 5 min at room temperature, counter-stained with hematoxylin for 1 min, dehydrated, cleared and mounted. For PCNA labeling index (PCNA LI), 500 cells were selected primarily in areas having the most positive cells. Cells having brown-stained nuclei were taken as positive and the positive count (%) was calculated.

Apoptosis study
Immunohistochemical staining for apoptosis was performed by the TUNEL method. Tissue was deparaffinized with xylene and dehydrated with a graded alcohol series. Endogenous peroxidase was blocked with 0.3% hydrogen peroxide for 30 min. The sections were treated with 20 mg/ml proteinase K for 5–15 min at room temperature. After washing with PBS, they were incubated with reaction reagent (terminal transferase 3 µl, Biotin-16-DUTP 8 µl, TUNEL dilution buffer 1000 µl) for 60 min at 37°C and with diluted peroxidase-conjugated streptavidin for 30 min at room temperature. They were then reacted with 20 mg of 3,3'-diaminobenzidine, tetrahydrochloride and 5 µl of 30% hydrogen peroxide for 5 min at room temperature, counter-stained with hematoxylin for 30 min, dehydrated, cleared and mounted. For the apoptosis labeling index (ApO LI), 100 cells were selected primarily in areas having the most positive cells. Cells having brown-stained apoptotic bodies were taken as positive and the positive count (%) was calculated.

Statistical analysis
Differences in the incidence of gallbladder lesions were analyzed by the χ²-test or Fisher’s probability test. Data on PCNA LI and Apo LI were expressed as mean ± SD and examined using Student’s t-test. A P-value of <0.05 was considered to indicate the statistically significant difference.

Results
Pathological examination
Normal gallbladder had only PE without HE, ME or AE (Figure 2A). In group I, from week 8 after surgery, HE and AE were recognized at grade 1, but no cancerous lesion was recognized up to week 20. In group II, AE was recognized at grade 1 from week 8 after surgery and CIS was found at grade 1 from week 12 to week 20. In group III, AE was seen at grade 1 from week 8 but no CIS was observed up to week 20.

Pathological findings by hematoxylin and eosin staining in each group at week 20 after surgery are presented in Table I. In the gallbladder mucosa of group I, PE was predominant, and HE and ME were partly recognized at grade 1 (Figure 2B). AE was noted in 8 of 11 cases (72.7%), but no cancerous lesion was observed. In group II, HE as well as PE was predominant and ME was partly recognized in only one case. AE was recognized in 8 of 11 cases (72.7%) and the area of AE in group II was more extensive than that in group I. Moreover, CIS was recognized in 4 of 11 cases (36.4%) at grade 1.
Figure 2C. Group III showed almost the same pathological findings as group I (Figure 2D), whereas the incidence of AE was lower than in group II by 27.3% (3 of 11 cases) and no cancerous lesion was observed. No statistically significant differences were noted among any of the groups.

**Cell kinetic study**

The PCNA LI of each group in week 20 after surgery was 48.36 ± 14.28% in group I, 60.0 ± 21.88% in group II and 45.27 ± 16.43% in group III (Figure 3), respectively. No significant difference was recognized between groups I and II, whereas the PCNA LI of group III was significantly lower than in group II (P = 0.045).

**Apoptosis study**

The Apo LI of each group on week 20 after surgery was 2.64 ± 2.87% in group I, 3.91 ± 1.38% in group II and 3.0 ± 2.41% in group III (Figure 4). No statistically significant differences were noted between any of the groups.

**Discussion**

The main purpose of the present study is to clarify the possible inhibitory effect of COX-2 inhibitor on biliary carcinogenesis in Syrian hamster PBM carcinogenesis models. In human PBM, HE is frequently present, which can be observed only in very small amounts during the carcinogenesis of regular gallbladder cancer, from birth or early childhood. ME and AE appear with age, and finally, cancer develops (32). The rate of biliary tract cancer in PBM is extremely high, being
5–35 times higher than that of regular biliary tract cancer (33). A nationwide survey by the Japanese Study Group on PBM showed that biliary tract cancer was present in ~20% of PBM cases (34). In comparison with regular biliary tract cancer without PBM, the following unique characteristics were noted:

(i) In PBM, the age of cancer onset is 10 or more years younger;
(ii) complications with choledocholithiasis are rare; and
(iii) pathologically, HE is the main mucosal lesion.

The process of carcinogenesis in PBM is thus clearly different from that in regular gallbladder cancer. In general, the PBM carcinogenesis is based on a hyperplasia–dysplasia–carcinoma sequence. In the present study, HE was recognized in 30% of group I, and a high incidence of AE was also found.

In order to clarify PBM carcinogenesis, various animal models were used, including dogs, goats, rabbits and rats. We used the hamster PBM model established by Tajima et al. (20), the reason being that such hamsters resemble humans in terms of the anatomical structure of bile duct and pancreatic duct and the composition of bile and pancreatic juices. Normally, when BOP, a carcinogenic substance, is subcutaneously administered in hamsters without any surgery, pancreatic cancer and intrahepatic cholangiocarcinoma occur at high rates, but cancer onset is at a low rate in the gallbladder or extrahepatic bile duct. However in the models of Tajima et al. (20) because the common duct is ligated and CD is performed, pancreatic juice flows into the bile duct at the same time that intestinal juice regurgitates into it. When BOP was administered to these models once a week, gallbladder cancer developed in 80% and extrahepatic biliary tract cancer in 40% of animals (20). In our findings, there was a higher incidence of HE over a wider area in the BOP-administered group (group II) than in group I. It was interesting that not only was a high incidence of wide-area AE recognized, but also CIS was seen in 36% of animals. These results suggest that the BOP-induced biliary tract cancer models used in the present study, in which various epithelial changes were observed similar to those in humans, are well suited for PBM research.

In the original model of Tajima et al. (20) the carcinogenic substance BOP was injected once a week and invasive cancer developed at a high rate. In the present study, BOP was injected once a week with a 1-week interval in order to slowly establish biliary tract cancer and observe the action of COX-2 inhibitor. Thus, invasive cancer was not produced in the present study, probably because of the small amount of BOP used or because of the short observation period. In the group given BOP and COX-2 inhibitor (group III), the incidence of AE was less by about one-third than in the BOP-administered group (group II), and no cancerous lesion was recognized. These results directly suggest that COX-2 inhibitor has a chemopreventive effect. Interestingly, the incidence of AE was lower in group III than in group I, in which BOP was not administered. In this PBM model, there is not only a mixture of bile and pancreatic juice, but also of intestinal juice; thus secondary bile acid and activated pancreatic enzymes cause chronic inflammation with injury to biliary mucosa, resulting in the high incidence of AE. These results suggest that COX-2 inhibitor might suppress such inflammation connected with carcinogenesis.

In recent years, with advances in molecular biological research, it is reported that there are not only genetic anomalies including K-ras, p53 or microsatellite instability in PBM, as in other cancers, but also anomalies in cell-cycle regulating factors and increased expression of COX-2 (32). It is also reported that in the initial event of carcinogenesis in colon cancer, first the stabilization of beta-catenin induces an increase of Wnt
signal, leading to cell atypia. Subsequently, however, if there is no COX-2 expression and production of prostaglandin E₂ (PGE₂), tumor formation or growth will not occur (35). In addition, it is reported that K-ras might induce expression of COX-2 in colon adenoma (36). We clarified immunohistochemically that in the early stage of PBM carcinogenesis, COX-2 is highly expressed in HE with atypia (9). Furthermore, it is reported that K-ras gene mutation occurs at a high rate in HE irrespective of the presence or absence of atypia (7). From these results, we speculate that the formation of HE may have a strong association between K-ras gene mutation and the expression of COX-2. The present study showed that the appearance of diffuse HE was not recognized in group III, in which COX-2 inhibitor was used, as compared with group II. Strictly speaking, COX-2 expression should be examined, but these data might verify indirectly that in group III, COX-2 expression was suppressed by the administration of COX-2 inhibitor.

NSAIDs exhibit an antitumor effect by suppression of tumor growth, induction of apoptosis or inhibition of angiogenesis, but both COX- or PG-dependent and COX- or PG-independent mechanisms are involved. The COX-2-dependent cell growth mechanism has undergone various analyses primarily in cell culture experiments. It is reported that in research using colon cancer cells, PGE₂ produced by COX-2 activates epithelial cell growth factor receptor, either directly or indirectly, and this induces phosphorylation of Akt via PI3-kinase, leading to cell proliferation (37,38). It has also been shown that PGE₂ induces expression of insulin-like growth factor-I receptor via PI3/Akt signaling, and promotes cell proliferation (39). Another report suggests that PGE₂ might be related to cell proliferation via MAP kinase (40). Moreover, COX-2 and PG act not only on tumor cells, but also on stromal cells, contributing to cell proliferation. When PGE₁ or PGE₂ is made to act on cultured gastric mucosa fibroblasts, secretion of hepatocyte growth factor in high concentrations in the culture fluid is induced, promoting cell proliferation (41). On the contrary, Han et al. (42) report that in experiments on human cholangiocarcinoma, celecoxib, a COX-2 inhibitor, induces p21waf/cip1, p27kip1 and cell-cycle arrest. They also report that this is a mechanism independent of COX-2. In this study, we compared the results between groups II and III to evaluate the direct action of COX-2 inhibitor for the suppression of carcinogenesis. And we set out group I as a control to compare the degree of carcinogenic suppression for COX-2 inhibitor against group III. Since Kitajima et al. (43) reported that spontaneous biliary carcinogenesis in hamsters occurred 40–120 weeks after CD surgery, distinct histological changes may not be obtained within 20 weeks in group I. However, group I with meloxicam treatment needed to be established to elucidate the COX-2 specific versus independent effects of meloxicam at the molecular level. Notwithstanding, these results were obtained using in vitro experiments with cultured cells and the proliferation mechanism of tumor cells has not been fully investigated in vivo. In general, cells with an accelerated cell cycle are readily susceptible to genetic mutations and carcinogenesis can easily occur. In our study, the PCNA LI was significantly lower in group III than in group II, showing that cell growth was suppressed by COX-2 inhibitor. Accordingly, no cancerous lesion could be observed in group III. In addition, there is no significant difference in PCNA LI between groups I and III, whereas the incidence of AE in group III was less than in group I. This result also suggests that COX-2 inhibitor could make it more difficult for carcinogenesis to arise, even with the same cell kinetics.

With respect to COX-2-dependent apoptosis induction, Wu et al. (44) reported that celecoxib induced apoptosis by suppressing PGE₂ production using human cholangiocarcinoma cell lines. Similarly, using human cholangiocarcinoma cell lines, Nzeako et al. (45) showed that death receptor-mediated apoptosis is induced through suppression of PGE₂ production by NS-398, a COX-2 inhibitor. Regarding COX-2-independent apoptosis induction, however, Jendrossek et al. (46) reported that in using Jurkat and BJAB cells, celecoxib induces apoptosis by the activation of a mitochondrial signaling pathway, not a death receptor pathway. In the present study, in the observations of pathological specimens or in the search of apoptosis by the TUNEL method, a low Apo LI at <4% was manifested in each group. Moreover, no statistical difference was noted between any of the groups. These results suggest that meloxicam has less influence on apoptosis induction in this model and further investigation will be required.

Clinically, it is important to predict the COX selectivity of NSAIDs. Blain et al. (47) performed whole blood assay of COX isoenzymes from healthy male volunteers taking certain kinds of NSAIDs, and their in vitro study showed strong potencies of selective COX-2 inhibition by meloxicam. However, after oral intake, meloxicam inhibited COX-1 from 30 to 55% and COX-2 from 63 to 83% depending on the repetition of the dose and increase in plasma concentration. A similar dissociation between circulating drug levels and COX inhibition was reported after repeated intake of meloxicam (48). From a pharmacokinetic point of view, inhibition of COX isoenzymes by NSAIDs may differ between in vitro and in vivo conditions because drugs accumulating in the target cells can be degraded for varying degrees in different individuals or act through metabolites. There may be limitations to the use of meloxicam as a selective COX-2 inhibitor, because a different mechanism of inhibition of COX isoenzymes by meloxicam cannot be excluded (49). Further investigations are required for clinical use.

In conclusion, it has been confirmed that meloxicam, a COX-2 inhibitor, suppresses carcinogenesis in hamster PBM models. The mechanism may not be based on apoptosis induction, but on suppression of cell proliferation. In research using in vitro cell lines, analysis of cell signals related to cell proliferation or apoptosis is easily undertaken. A COX-2 inhibitor has diverse actions including suppression of angiogenesis, suppression of growth factors and enhancement of tumor immunity apart from suppression of cell proliferation or induction of apoptosis. In vivo, these actions mutually affect each other in complex ways and cause antitumor effects. Moreover, since there is mutual action against stromal cells as well as tumor cells, further investigation is required.

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References


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