

Inhibitory effects of the cyclooxygenase-2 inhibitor, etodolac, on colitis-associated tumorigenesis in p53-deficient mice treated with dextran sulfate sodium

KENICHIROH MUKAWA^{1,2}, SHIGEHIKO FUJII^{1,3}, KEIICHI TOMINAGA^{1,2}, NAOTO YOSHITAKE^{1,2}, AKIHITO ABE¹, TOKUYUKI KONO¹, AKIRA SEKIKAWA¹, HIROKAZU FUKUI¹, KAZUHITO ICHIKAWA¹, SHIGEKI TOMITA¹, JOHJI IMURA¹, YUKO ONO¹, MOTO SHINODA⁴, HIDEYUKI HIRASHI² and TAKAHIRO FUJIMORI¹

Departments of ¹Surgical and Molecular Pathology and ²Gastroenterology, Dokkyo Medical University School of Medicine, Tochigi; ³Department of Gastroenterology and Hepatology, Kyoto University Graduate School of Medicine, Kyoto; ⁴Laboratory Animal Research Center, Dokkyo Medical University, Tochigi, Japan

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Abstract. Non-steroidal anti-inflammatory drugs (NSAIDs) and cyclooxygenase-2 (COX-2) inhibitors are representative agents for the chemoprevention of sporadic colorectal neoplasia. However, few reports have described the chemopreventive effects of such agents on colitis-associated tumorigenesis. To clarify whether treatment with the COX-2 inhibitor may reduce the risk of colitis-associated neoplasia, we investigated the effect of one such agent, etodolac, on tumorigenesis in the colitis-associated neoplasia model using p53-deficient mice treated with dextran sulfate sodium (DSS). The p53^{-/-} mice were divided into four groups: i) treatment with DSS + etodolac, then after two cycles of DSS, the mice were given distilled water for 84 days. In addition, etodolac was administered three times a week at a dose of 10 mg/kg body weight throughout the experiment. ii) Treatment with two cycles of DSS only, followed by distilled water for 84 days. iii) Treatment with etodolac alone. iv) Distilled water alone was administered to the control group. The incidence of mice with neoplasia was 82.4% in the DSS + etodolac group and

100% in the DSS-alone group. No neoplasia was observed in the etodolac-alone and control groups. The mean (\pm SEM) number of total neoplasias per mouse was 1.29 ± 0.2 in the DSS + etodolac group and 3.0 ± 0.52 in the DSS-alone group, the inter-group difference being significant ($p < 0.01$). There was no significant difference in the inflammation score between these two groups. These results showed that treatment with etodolac significantly reduced the occurrence of neoplasia, suggesting that this COX-2 inhibitor has chemopreventive activity against colitis-associated tumorigenesis.

Introduction

Patients with ulcerative colitis (UC) show an increased incidence of colorectal neoplasia and UC-associated colorectal neoplasia represents a major cause of increased mortality in such patients (1). In order to improve the prognosis of patients with UC-associated neoplasia, diagnosis at an early or precancerous stage is crucial. A predisposition to colorectal neoplasia in patients with UC is generally considered to depend on two risk factors: the presence of long-standing disease and extensive colitis (2,3). Therefore, surveillance colonoscopy with multi-step biopsy has been widely recommended for patients with long-standing and/or extensive UC (4-6). However, studies on the efficacy of surveillance in UC have produced conflicting results and have suggested that surveillance allows for the detection of early-stage neoplasia in only a minority of patients and cannot guarantee cancer detection at a curable stage (7-10).

Non-steroidal anti-inflammatory drugs (NSAIDs) and cyclooxygenase-2 (COX-2) inhibitors are representative agents for the chemoprevention of sporadic colorectal neoplasia. Numerous experimental and clinical studies have indicated that such agents significantly inhibit tumorigenesis in humans with sporadic colorectal neoplasia and in the rodent model of colorectal tumorigenesis (11-14). However, few reports have described the chemopreventive effect of

Correspondence to: Dr Shigehiko Fujii, Department of Surgical and Molecular Pathology, Dokkyo Medical University School of Medicine, 880 Kitakobayashi, Mibu, Shimotsuga, Tochigi 321-0293, Japan
E-mail: s-fujii@dokkyomed.ac.jp

Abbreviations: UC, ulcerative colitis; NSAIDs, non-steroidal anti-inflammatory drugs; COX-2, cyclooxygenase-2; DSS, dextran sulfate sodium; AOM, azoxymethane; DMH, 1,2-dimethylhydrazine; IBD, inflammatory bowel disease; TNB, trinitrobenzene sulfonic acid

Key words: colitis-associated tumorigenesis, cyclooxygenase-2 inhibitor, chemoprevention

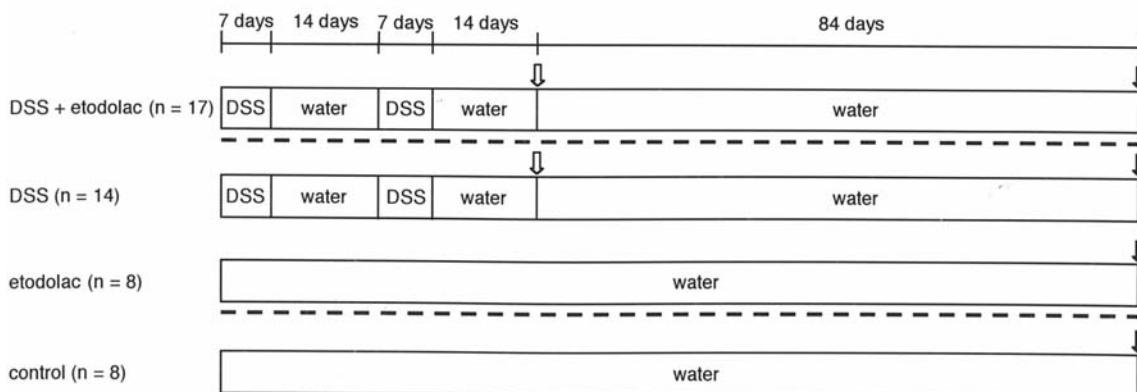


Figure 1. Experimental design. Broken line, oral administration of etodolac (10 mg/kg), 3 times/week; black arrows, sacrifice at 126 days; white arrows, sacrifice at 42 days.

NSAIDs and COX-2 inhibitors on colitis-associated tumorigenesis (15-18). Therefore, it is still unclear whether treatment with these agents provides as much protection against colorectal neoplasia in patients with UC as in patients without UC.

We recently developed a new model of colitis-associated neoplasia in p53-deficient mice by treatment with dextran sulfate sodium (DSS) and found that the macroscopic morphology and genetic alterations in the resulting neoplasia are similar to those of human UC-associated neoplasia (19). In the present study, to clarify whether treatment with the COX-2 inhibitor may reduce the risk of colitis-associated neoplasia, we investigated the effect of one such agent, etodolac, on tumorigenesis in the colitis-associated neoplasia model using p53-deficient mice.

Materials and methods

Animals. Specific pathogen-free mice with a homozygous p53 deficiency ($p53^{-/-}$ mice) were used for the study. The $p53^{-/-}$ mice with a C57BL/6 and CBA background were produced by Tsukada and colleagues (20) and kindly provided by Dr Norio Ishida (Clock Cell Biology Group, Institute for Biological Resources and Functions, National Institute of Advanced Industrial Science and Technology, Tsukuba, Japan).

Experimental procedure. Fig. 1 shows the experimental design. All of the mice were eight weeks old at the beginning of the experiment. The colitis was induced by feeding 3% DSS (ICN Biomedicals Inc., Aorano, OH, USA), molecular weight 36,000-50,000, dissolved in drinking water which was given *ad libitum*. One cycle was defined as seven days of DSS followed by 14 days of distilled water. The COX-2 inhibitor, etodolac (kindly provided by Nippon Shinyaku Co., Ltd., Kyoto, Japan), was suspended in 0.5% methylcellulose (Shin-Etsu Chemical Co., Ltd., Tokyo, Japan) to give a concentration of 1 mg/ml. The mice were divided into the following 4 groups: i) treatment with DSS + etodolac, then after two cycles of DSS, the mice were given distilled water for 84 days. In addition, etodolac was administered three times a week by oral gavage at a dose of 10 mg/kg body weight throughout

the experimental period. ii) Treatment with two cycles of DSS only, followed by distilled water for 84 days. In addition, distilled water was administered three times a week by oral gavage in a comparable volume (10 ml/kg body weight) throughout the experiment. iii) Treatment with etodolac-alone in the same manner as that in the DSS + etodolac group. iv) Distilled water alone was administered to the control group, in the same manner as that in the DSS-alone group. To evaluate the incidence of neoplasia and the severity of chronic colitis in the late phase, the mice in the four groups were sacrificed by cervical dislocation at 126 days. In addition, to evaluate the severity of acute colitis in the early phase, six mice in the DSS + etodolac group and five in the DSS-alone group were sacrificed at 42 days.

The colons of the mice were removed, cut open along the longitudinal median axis, rinsed with saline and fixed in neutral aqueous phosphate-buffered 4% formaldehyde for 8 h. Then the entire colon was stained with alcian blue (pH 2.5) and the surface microstructure was observed using a stereomicroscope (Olympus, Tokyo, Japan) and examined for the presence of neoplasia. All gross lesions suspected to be neoplasia were sectioned under stereomicroscopic observation. The remaining tissues were sectioned at intervals of 3 mm, embedded in paraffin and stained with hematoxylin and eosin. The whole extracted colon was divided equally into three portions starting from the proximal colon, followed by the middle and distal colon.

This experiment was carried out under the control of the Animal Care and Use Committee, Dokkyo Medical University, in accordance with the University Guidelines for the Care and Use of Laboratory Animals.

Histological assessment of neoplasia. Macroscopically, neoplasias were classified into two types: superficial and protruding. The superficial type was defined as a lesion with a height not greater than twice the thickness of the adjacent non-neoplastic epithelium. The protruding type was defined as a lesion that clearly projected above the surface of the adjacent non-neoplastic epithelium.

Histologically, each sample was examined by three experienced pathologists in a blinded manner and neoplasias were classified according to the Vienna classification of

body and liver weights and colon length in each group.

Group	n	Body weight (g)	Liver weight (g)	Colon length (cm)
DSS + etodolac	17	26.5±1.63	1.18±0.06	5.98±0.20
DSS	14	26.1±1.13	1.27±0.1	6.29±0.24
Etodolac	8	25.3±1.5	1.11±0.05	6.98±0.31
Control	8	22.9±1.73	1.19±0.15	6.73±0.38

Values are mean ± SEM.

Table II. Number of neoplasias and incidences of neoplasia in the mice of each group.

Group	n	Incidence	Total	No. of neoplasias		
				Category 3	Category 4	Category 5
DSS + etodolac	17	14/17	22	14	4	4
DSS	14	14/14	42	29	10	3
Etodolac	8	0/8	0	0	0	0
Control	8	0/8	0	0	0	0

gastrointestinal epithelial neoplasia (21). This classification is practical and has been recommended in order to resolve the large discrepancies that exist between Western and Japanese pathologists in the diagnosis of gastrointestinal epithelial neoplasias and as an aid to a better understanding of research data in the field of gastroenterology. This classification has five categories: category 1, negative for neoplasia; category 2, indefinite for neoplasia; category 3, non-invasive low-grade neoplasia; category 4, non-invasive high-grade neoplasia and category 5, invasive neoplasia. The number of neoplasias and their macroscopical and microscopical features were recorded.

Histological assessment of the severity of colitis (ulcer score). The severity of colitis was evaluated as an ulcer score according to the modified criteria of Cooper *et al* (22). Ulcer scores were determined as the number of ulcers for every piece of tissue on each slide and the total score was the sum for all pieces.

Statistical analysis. The number of neoplasias in each group were expressed as mean ± SEM and the differences between the groups were analyzed using the non-parametric Mann-Whitney U test. Differences at $p<0.05$ were considered significant. Welch's t-test was used to compare body and liver weights, colon lengths and ulcer scores among the groups at a significance level of $p<0.05$.

Results

General observations. Table I shows body and liver weights and the colon length in each group. There were no significant differences in body or liver weight, or colon length among the groups at the end of the experiment.

Number and incidence of neoplasias. The number of neoplasias and incidences of neoplasia in the mice of each group are summarized in Table II. Twenty-two neoplasias were found in the DSS + etodolac group of which 14 were low-grade neoplasia, four were high-grade neoplasia and four were carcinoma. Forty-two neoplasias were found in the DSS-alone group of which 29 were low-grade neoplasia, 10 were high-grade neoplasia and three were carcinoma. No neoplasia was observed in the etodolac-alone and control groups. The incidences of neoplasia in the mice were 82.4% in the DSS + etodolac group and 100% in the DSS-alone group.

The mean number of neoplasias per mouse (± SEM) is presented in Fig. 2. There were 1.29 ± 0.2 neoplasias (category 3-5) per mouse in the DSS + etodolac, 3.0 ± 0.52 in the DSS-alone, 0 ± 0 in the etodolac-alone and 0 ± 0 in the control group. The total number of neoplasias per mouse in the DSS + etodolac group was significantly lower than that in the DSS-alone group ($p<0.01$). With regard to the grade of neoplasia, the mean (± SEM) number of low-grade neoplasias (category 3) per mouse was 0.82 ± 0.13 in the DSS + etodolac group and 2.07 ± 0.42 in the DSS-alone group while the corresponding mean (± SEM) number of high-grade neoplasias (category 4) per mouse was 0.24 ± 0.09 and 0.71 ± 0.14 , respectively, and that of invasive neoplasias (category 5) per mouse was 0.24 ± 0.09 and 0.21 ± 0.1 , respectively. The number of low-grade and high-grade neoplasias per mouse in the DSS + etodolac group were significantly lower than those in the DSS-alone group ($p<0.05$).

Pathological findings. Macroscopically, in the DSS + etodolac group, 20 out of the 22 neoplasias (90.9%) were of the superficial type and 2 (9.1%) were of the protruding type. In the DSS-alone group, 41 out of the 42 neoplasias (97.6%)

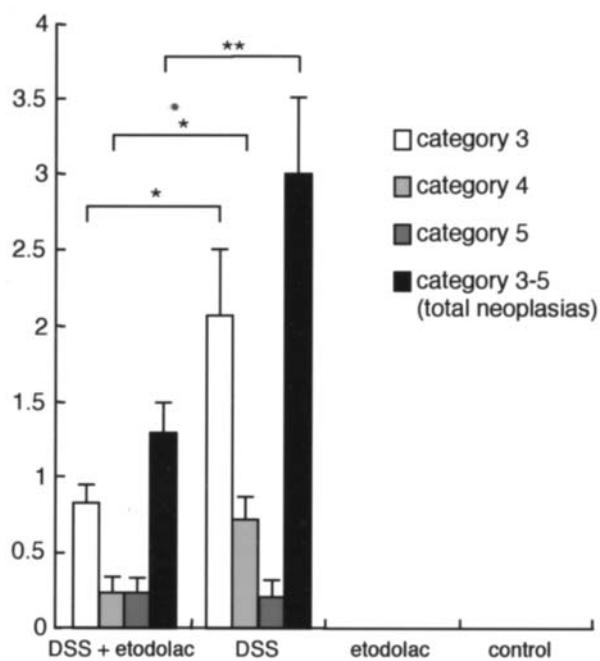


Figure 2. The mean number of neoplasias per mouse. The number of low- and high-grade neoplasias per mouse in the DSS + etodolac group was significantly lower than that in the DSS-alone group. The number of total neoplasias per mouse in the DSS + etodolac group was significantly lower than that in the DSS-alone group; * $p<0.05$, ** $p<0.01$.

were of the superficial type and 1 (2.4%) was of the protruding type. In these groups, 46 out of the 61 superficial-type lesions (75.4%) were flat lesions with the same height as the adjacent non-neoplastic epithelium (Fig. 3A). As with our study (19), most of the neoplasias that developed in this DSS colitis-associated neoplasia model using *p53*^{-/-} deficient mice were of the flat type.

Histologically, seven invasive neoplasias (category 5) were observed. Five of them were well-differentiated adenocarcinomas (Fig. 3B) and two were moderately-differentiated adenocarcinomas. Two of these seven neoplasias invaded the submucosal layer (Fig. 3C).

Severity of colitis (ulcer score). Table III shows the ulcer scores for all groups. With regard to acute colitis in the early phase, there was no significant difference in the ulcer score between the DSS + etodolac group and the DSS-alone group examined on day 42 (8.17 ± 1.01 vs. 11.2 ± 2.4 , $p=0.293$). In the chronic late phase, the ulcer score of the DSS + etodolac group had a tendency to be lower than that in the DSS-alone group examined on day 126 (3.94 ± 0.69 vs. 6.43 ± 1.03 , $p=0.086$). No ulcer was observed in either the etodolac-alone or control groups.

Discussion

The present study demonstrated that colonic tumorigenesis was effectively suppressed by the COX-2 inhibitor, etodolac, in *p53*^{-/-} mice with DSS colitis-associated neoplasia and that this COX-2 inhibitor did not exacerbate experimental colitis. These findings indicate that the inhibition of the COX-2

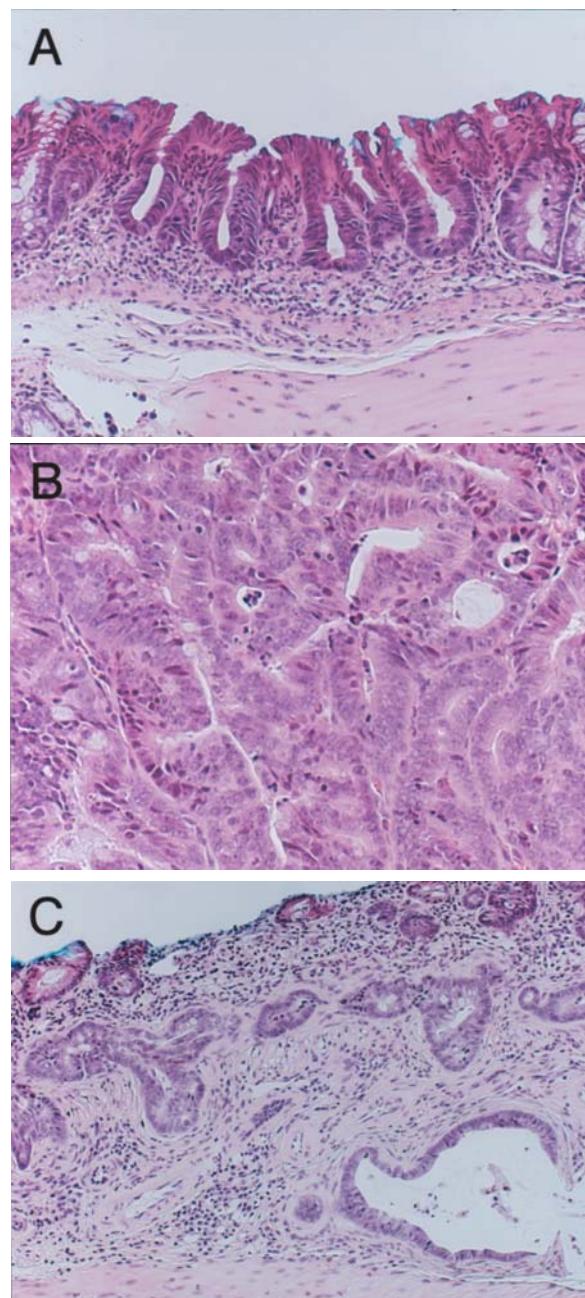


Figure 3. (A) Neoplasm in the DSS-alone group, classified as category 4 (high-grade neoplasia). This neoplasm is a flat type with the same height as the adjacent non-neoplastic epithelium. (B) Neoplasm in the DSS + etodolac group, classified as category 5 (invasive neoplasia). This neoplasm is a well-differentiated adenocarcinoma histologically. (C) Neoplasm in the DSS-alone group, classified as category 5. This neoplasm has invaded through the muscularis mucosae into the submucosa.

activity may be a novel and safe approach for the suppression of colitis-associated tumorigenesis and that the COX-2 inhibitor would have potential as a chemopreventive agent against colitis-associated neoplasia.

COX-2 is one of the key regulatory molecules in tumor development (23-25). It supports tumor angiogenesis, inhibits tumor cell apoptosis, promotes metastatic potential and is associated with the prognosis of cancer patients (24,25). Therefore, the COX-2 expression has been investigated in various kinds of tumors. In colorectal neoplasia, many reports confirmed the COX-2 expression in

Ulcer scores in the mice of each group.

Group	Proximal	Middle	Distal	Total
DSS + etodolac (126 days) ^a	0.71±0.16	0.47±0.17	2.76±0.51	3.94±0.69
DSS (126 days) ^a	0.86±0.22	0.86±0.41	4.71±0.7	6.43±1.03
DSS + etodolac (42 days) ^b	0.5±0.29	2.5±0.72	5.17±0.7	8.17±1.01
DSS (42 days) ^b	1.4±0.82	3.4±0.53	6.4±0.87	11.2±2.4
Etodolac (126 days)	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
Control (126 days)	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0

Values are mean ± SEM. ^aUlcer scores of the DSS + etodolac group versus the DSS group on day 126, p=0.086. ^bUlcer scores of the DSS + etodolac group versus the DSS group on day 42, p=0.293.

tumor tissues and demonstrated the association between the COX-2 expression level and tumor size (26-29) and that NSAIDs and COX-2 inhibitors substantially decrease the risk of sporadic colorectal neoplasia and the development of adenoma in patients with familial adenomatous polyposis (11,12,30,31). Experimentally, these agents have been shown to effectively prevent colorectal neoplasia induced by carcinogenic agents such as azoxymethane (AOM) or 1,2-dimethylhydrazine (DMH) in rodents (32,33) and spontaneous colorectal neoplasia in the APC min mouse model (34).

Agoff *et al* reported that the COX-2 expression is induced at an early stage of tumorigenesis in the UC-associated neoplasia, suggesting that the inhibition of COX-2 may be a useful chemopreventive approach (35). However, scarce data are available on the use of NSAIDs or COX-2 inhibitors for preventing UC-associated neoplasia development and progression. A case-control study by Bansal *et al* examined whether patients with inflammatory bowel disease (IBD) with comorbidity and requiring long-term therapy with NSAIDs had a reduced number of colorectal cancers (15). Although the difference did not reach statistical significance, patients with IBD who were also given long-term NSAID therapy to control other inflammatory diseases (e.g., rheumatoid arthritis and osteoarthritis) had a lower rate of colorectal neoplasia than those without such therapy. They also found a statistically significant protective influence against mortality in patients who were given long-term NSAID therapy compared to those without such therapy. Similarly, Eaden *et al* conducted a case-control study comparing 102 patients with colorectal carcinoma in UC with matched controls (16). They reported that aspirin use had a minor protective effect, although it was not statistically significant because of the small sample size. These findings may lend support to a protective role for NSAIDs in IBD-associated neoplasia, although currently the data are insufficient.

Although several experimental colitis-induced neoplasia models have been reported as candidate models of human UC-associated neoplasia, few studies exist on the protective effect of COX-2 inhibitors against colitis-associated tumorigenesis. In our previous study, we prepared a tumorigenesis rat model using trinitrobenzene sulfonic acid (TNB) and DMH and reported that treatment with the COX-2 inhibitor suppressed the occurrence of neoplasia in the model

(18). This result suggested that COX-2 inhibitors may serve as protective agents against UC-associated neoplasia.

However, these animal models, including the one used previously, would be inadequate as models of human UC-associated neoplasia for several reasons. In a model of colitis induced by an agent such as DSS-alone, the incidence of neoplasia is not high enough to test the effects of chemopreventive agents or analyze the molecular events participating in colitis-associated neoplasia (22,36). Although the main macroscopic characteristic of UC-associated neoplasia at the early stage, dysplasia or early cancer, is a flat configuration, the neoplasia developing in colitis models has predominantly been the protruding type (22). Furthermore, the incidence of the p53-gene alteration, which is high in human UC-associated neoplasia, has been reported to be rare in animal models (22). When carcinogen is added to colitis-inducing agents, the incidence of neoplasia increases (37). However, in colonic neoplasia induced by carcinogens, several studies have shown that K-ras gene mutation, which is considered infrequent in human UC-associated neoplasia (38,39), is a frequent feature (40,41). Thus, the neoplasias that develop in established experimental tumorigenesis models have properties that are different from those of human UC-associated neoplasia with respect to both morphology and genetic alterations.

Therefore, in our recent study, we developed a new colitis-associated neoplasia model in p53-deficient mice using DSS and succeeded in inducing a high rate of colonic neoplasia in a background of colitis (19). In this model, most of the neoplasias that developed were flat, multiple and synchronous. In these neoplasias, K-ras mutation was not detected but the translocation of β-catenin was frequently observed. Thus, this animal model would be suitable for the study of human UC-associated neoplasia because the morphological features and molecular genetics are similar to those of human UC-associated neoplasia. In the present study, using this model, we investigated the effect of the COX-2 inhibitor, etodolac, on colitis-associated tumorigenesis. Our results showed that treatment with etodolac significantly reduced the occurrence of neoplasia, suggesting that the COX-2 inhibitor may have a chemopreventive activity in colitis-associated tumorigenesis.

With regard to the chemopreventive effect of COX-2 inhibitors on colitis-associated tumorigenesis, a critical issue

has been whether COX-2 inhibitors are safe. A number of published reports have indicated a link between the use of NSAIDs or COX-2 inhibitors and inflammatory activity in UC (42-45). Although the use of these agents has been associated with the exacerbation of IBD in individual cases and small case series, there are still conflicting data regarding the effect of NSAIDs or COX-2 inhibitors on disease activity in patients with UC. Recently, Miedany *et al* conducted a double-blind, placebo-control study to assess the safety of the COX-2 inhibitor, etoricoxib and its effect on disease activity in patients with IBD (45). They indicated that etoricoxib did not cause the flare-up of IBD and that most of the patients were able to continue the drug therapy for 3 months. They also showed that there was no significant difference between the disease activity score before and after 3 months of etoricoxib therapy. They concluded that etoricoxib was safe and beneficial for most patients with IBD.

Similarly, in experimental colitis animal models, several studies investigating the influence of COX-2 inhibitors on colitis severity have yielded inconsistent results (46,47). Martin *et al* evaluated the efficacy of the COX-2 inhibitor for the prevention of experimental colitis and demonstrated that the COX-2 inhibitor ameliorated DSS-induced colitis in mice (48). In the present study, the use of the COX-2 inhibitor, etodolac, was not associated with DSS-induced colitis severity in p53-deficient mice at the early acute phase. Furthermore, although the difference did not reach statistical significance, the ulcer score of the DSS + etodolac group had a tendency to be lower than that in the DSS-alone group at the chronic late phase. These results demonstrated that the COX-2 inhibitor ameliorates rather than exacerbates experimental colitis, suggesting that colonic tumorigenesis may be suppressed by the COX-2 inhibitor as a result of the amelioration of colitis activity in the present study. Further studies addressing the influence of COX-2 inhibitors on colitis severity in rodent models and human UC are needed to determine whether these agents are safe, or have a deleterious effect on inflammatory activity.

COX-2 inhibitors have been used clinically as anti-inflammatory drugs that exert milder ulcerogenic effects in the gastrointestinal tract than classical NSAIDs. However, the long-term use of COX-2 inhibitors, especially those of the coxib family, is known to be associated with an increased risk of cardiovascular events. Motsko *et al* reported that the long-term use of etodolac, belonging to the pyrano acetic acid class, was not associated with a cardio-negative or cardio-protective effect in comparison with the long-term ibuprofen (49), suggesting that etodolac may be a good candidate for trials of chemoprevention against colorectal cancer. However, it would be unrealistic to give the COX-2 inhibitor to increased UC patients with long-standing and extensive colitis. Our previous study showed that individuals with estrogen-receptor gene methylation in non-neoplastic colorectal epithelium have a higher risk for the development of colorectal neoplasia among patients with long-standing and extensive UC (50,51). Although further studies are needed to confirm that the COX-2 inhibitor has a chemopreventive effect on colitis-associated neoplasia, treatment with the COX-2 inhibitor for those individuals may bring on more effective management in the reduction of UC-associated neoplasia risk.

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