

DA-6034, a Derivative of Flavonoid, Prevents and Ameliorates Dextran Sulfate Sodium–Induced Colitis and Inhibits Colon Carcinogenesis

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Previously, we have shown that DA-6034, a synthetic derivative of flavonoid eupatilin, inhibited NF- κ B activation in colon epithelial cells and prevented trinitrobenzene sulfonic acid–induced rat colitis. The aim of this study was to investigate the preventive and therapeutic effect of DA-6034 on dextran sulfate sodium (DSS)–induced colitis and on inflammation-related cancer. C57BL/6 mice were given 4% DSS for 5 days with and without DA-6034 in the acute preventive model. In the acute therapeutic model, mice were given 4% DSS for 5 days followed by rectal administration of DA-6034. Colitis was quantified by body weight, disease activity index (DAI), colon length, and histology. In the inflammation-related cancer model, mice were given a single intraperitoneal injection of azoxymethane, then three cycles of 2% DSS for 5 days, then 2 weeks of free water consumption. Apoptosis was determined by *in situ* terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick-end labeling assay, and the expression of Ki-67, phospho- κ B kinase α (IKK α), and COX-2 were evaluated by immunohistochemistry. In both the acute preventive and acute therapeutic models, DA-6034 significantly attenuated DSS-induced weight loss, an increase in DAI, and a shortening of colon length. DA-6034-treated mice maintained crypt architecture and revealed a scanty infiltration of inflammatory cells in

both the preventive and therapeutic models. In the inflammation-related cancer model, DA-6034 reduced the number of colon tumors and ameliorated weight loss and shortening of colon length. DA-6034 strongly enhanced apoptosis and inhibited the expression of COX-2 and phospho-IKK α in inflammation-related colon cancer models. Our results suggest that DA-6034 prevents acute murine colitis and inhibits inflammation-related colon carcinogenesis. DA-6034 could be a potential therapeutic agent for inflammatory bowel disease. *Exp Biol Med* 233:180–191, 2008

Key words: DA-6034; inflammatory bowel disease; colon cancer

Introduction

Inflammatory bowel diseases (IBDs), such as ulcerative colitis (UC) and Crohn's disease (CD), are each a chronic and relapsing intestinal inflammatory status caused by the immune dysregulation of the gut. Despite the significant advances in therapeutic strategy, including corticosteroids, 5-aminosalicylates, immunomodulators, and anti-tumor necrosis factor (TNF)- α monoclonal antibody, there are no medical agents or surgical methods of treating IBD that result in a nonrelapsing cure. In addition, only a few nontoxic therapeutic options are currently able to ameliorate intestinal inflammation.

Patients with UC have a significantly higher risk of developing colitis-associated colon cancer and precancerous dysplastic epithelial change (1). A recent meta-analysis of 116 studies showed that the prevalence of colorectal cancer (CRC) in patients with UC was about 3.7% (2). The 5-aminosalicylic acids (5-ASAs), including sulfasalazine and mesalazine, are the most commonly prescribed anti-inflammatory drugs for IBD. Regular 5-ASA intake may reduce the risk of CRC (3–6). Two case-control studies (7, 8) and one large observational study (4) have found lower

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risks of CRC in patients on regular 5-ASA therapy. Furthermore, two prospective studies have reported positive effects of 5-ASA therapy on surrogate markers of CRC, such as rectal cell proliferation and apoptotic index (9, 10). However, two recent case-control studies have found no reduction in CRC risk in regular 5-ASA users (11, 12). Therefore, it is still challenging to develop new agents for the chemoprevention of colitic cancer and the control the intestinal inflammation of IBD.

Cell proliferation plays an important role in multistep carcinogenesis (13). Suppressing apoptosis is one of the hallmarks of cancer, (14) and the induction of apoptosis during tumor promotion represents a critical step for chemoprevention. We assessed the proliferation and apoptotic indices in the colon section by means of immunohistochemistry using Ki-67 antibody and *in vivo* terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) assay, respectively. The modulation of NF- κ B signaling pathways could be the main target for the treatment of IBD (15, 16). In addition, NF- κ B activates the transcription of numerous genes that are capable of suppressing apoptosis, (17) and I κ B kinase (IKK) could be related to colon carcinogenesis in murine models of colitis-associated cancer (18). Therefore, selective IKK-dependent NF- κ B activation may play a major role in oncogenesis. We assessed the I κ B kinase alpha (IKK α) activation in the colon section by means of immunohistochemistry that used an anti-phospho-IKK α antibody to elucidate the target of action of DA-6034 more specifically *in vivo*. Colon cancer was decreased in cyclooxygenase-2 (COX-2) knockout mice, (19) and a COX-2 inhibitor inhibited both colon carcinogenesis and colitis in a murine colitis model (20, 21) and lead to a significant reduction in the number of colorectal polyps in patients with familial adenomatous polyposis (22). The COX-2 level was increased in IBD, (23) in dysplasia, and in colon cancer (24). Therefore, COX-2 may be an important mediator of inflammation and colon carcinogenesis. We assessed the COX-2 expression in the colon tissue by using immunohistochemistry to elucidate the effect of DA-6034 more specifically *in vivo*.

Flavonoids are known to engage in potentially useful anti-inflammatory and anticancer activities (25, 26). Eupatilin (5,7-dihydroxy-3',4',6-trimethoxyflavone), a derivative of flavone, is a main component of the extract of the *Artemisiae* species. (*Artemisiae* has been used as a folk medicine in Korea for the treatment of chronic diarrhea.) A recent study showed that eupatilin-induced apoptosis in human gastric cancer cells by means of the inhibition of antiapoptotic *bcl-2* gene expression (27). Oral eupatilin (Stillen) is currently marketed as a gastritis treatment in Korea. Previously, we have shown that DA-6034 (7-carboxymethoxy-3',4',5-trimethoxyflavone), a synthetic derivative of eupatilin, inhibited NF- κ B activation in colon epithelial cells and prevented trinitrobenzene sulfonic acid (TNBS)-induced rat colitis, which was comparable to

corticosteroid and sulfasalazine (28, 29). DA-6034 also reduced the histologic damage in the HLAB27 transgenic rat colitis (28). The therapeutic effect of oral DA-6034 could be a result of the local effect on the intestinal mucosa caused by the low bioavailability of DA-6034, which mainly is due to the intestinal first-pass effect (30).

Because TNBS-induced colitis would mimic the gross finding of CD, DA-6034 should be tested to see whether it could ameliorate dextran sulfate sodium (DSS)-induced colitis that shows UC-like features. In addition, the inhibitory effect of DA-6034 on the development of colitic cancer should be evaluated to test whether it could be a potential therapeutic agent for IBD. Therefore, the aim of this study was to investigate the preventive and therapeutic effects of DA-6034 on DSS-induced acute colitis and inflammation-related colon cancer.

Materials and Methods

Materials. DSS (36,000–50,000 Daltons; MP Bio-medicals, Aurora, OH) was dissolved in distilled water at a concentration of 2% or 4% (w/v). A total of 60 mg of DA-6034 (provided by Dong-A Pharmacy, Seoul, Korea) was dissolved with 10 ml water and adjusted to a pH of 11–13 with 1N NaOH. The suspension turned into a lucent solution and then adjusted to pH 7 with 0.1N HCl. A colonic carcinogen, azoxymethane (AOM; Sigma Chemical Co., St. Louis, MO) was obtained.

Animals. Eight- to nine-week-old male C57BL/6 mice (Orient, Seongnam, Korea) were purchased. Animals were maintained on a 12:12-hr light:dark cycle under specific pathogen-free conditions. The mice had *ad libitum* access to a standard diet and water until they reached the desired age (10 weeks) and/or weight (18–21 g). All procedures that used animals were reviewed and approved by the Institutional Animal Care and Use Committee of Seoul National University Hospital.

Acute Colitis Model. In the previous study, an acute preventive model was developed and established (31). Briefly, C57BL6 mice were given 4% DSS in drinking water for 5 days. DA-6034 (30 mg/kg) was administered twice daily by oral gavage until Day 7, beginning on the days of DSS exposure in the acute preventive model with DA-6034. DSS-treated groups received 0.9% normal saline in a comparable volume by the same route. Normal control mice received the filtered water by itself. Mice were sacrificed on Day 7, and clinical parameters and pathology were evaluated (Fig. 1A).

In the acute therapeutic model, mice were fed 4% DSS in their drinking water for 5 days; then DA-6034 (10 mg/kg) was administered once daily through a rectal enema. In the preliminary study, we administered 4% DSS for 5 days and then treated mice with DA-6034 *via* the rectum for 1, 2, 3, and 4 days ($n = 10$ for each group). We found that treatment of DA-6034 for 3 days after DSS was effective for acute

colitis. Therefore, mice were sacrificed on Day 8, and clinical parameters and survival were evaluated (Fig. 1B).

Colitis Associated Colon Cancer Model. Experimental mice were given a single intraperitoneal injection of AOM (10 mg/kg initial body weight) on Day 0. DA-6034 (30 mg/kg) or 0.9% normal saline (0.1 ml) were administered twice daily by oral gavage from Day 7 to the end of this study. Experimental mice were given three cycles of 2% DSS for 5 days followed by 2 weeks plus 2 days of consumption of free water. Mice were sacrificed 10 days after the final 2% DSS administration (Fig. 1C).

Assessment of Clinical Parameters in DSS-Induced Colitis. Animals were observed daily for fluid intake, weight changes, and symptoms such as loose stools, mucous diarrhea, and hematochezia. A previously validated (32) clinical disease activity index (DAI) that ranged from 0 to 4 was calculated based on the following parameters: stool consistency (0, normal; 2, loose; 4, diarrhea), gross bleeding (0, absence; 2, blood tinged; 4, presence), and weight loss (0, none; 1, 1%–5%; 2, 5%–10%; 3, 10%–20%; 4, >20%). The calculated DAI = (weight loss + stool consistency + gross bleeding)/3.

Microscopic Assessment and Histopathologic Analysis. The colon samples were fixed in 10% buffered formalin, were embedded in paraffin, and then were assessed in a blinded fashion with a scoring system described previously (33). Briefly, three parameters were measured: severity of inflammation (0, none; 1, slight; 2, moderate; 3, severe), extent of injury (0, none; 1, mucosal; 2, mucosal and submucosal; 3, transmural), and crypt damage (0, none; 1, basal one-third damaged; 2, basal two-thirds damaged; 3, only surface epithelium intact; 4, entire crypt and epithelium lost). The score of each parameter was multiplied by a factor that reflected the percentage of tissue involvement ($\times 1$, 0%–25%; $\times 2$, 26%–50%; $\times 3$, 51%–75%; $\times 4$, 76%–100%), and all numbers were summed. The combined histopathologic score ranged from 0 to 40. For the precise evaluation of colitis-associated cancer in colon tissue, the presence and tumor load was determined in the whole colon by a blinded pathologist.

Immunohistochemical Analysis for Ki-67, COX-2, and phospho-IKK α . To reveal the antigen, slides were immersed in a Tris/EDTA buffer (pH 9.0), were heated in a decloacking chamber at 125°C for 3 mins, and were cooled at room temperature for 20 mins. After adding 3% hydrogen peroxide, sections were incubated for 10 mins.

After washing the sections with Tris-buffered saline (TBS) Tween-20 (pH 7.6), they were stained with rabbit polyclonal antibody to Ki-67 (NB500–170, NOVUS Biologicals, Littleton, CO), to COX-2 (sc1745, Santa Cruz, CA), and to phospho-IKK α (Cell Signaling, Beverly, MA) in an autoimmunostainer (Autostainer 2D, Lab Vision Co., Fremont, CA) in accordance with the manufacturer's recommendations. Briefly, each tissue section was incubated with primary antibody to Ki-67 (1:50), COX-2 (1:25), and phospho-IKK α (1:50) for 1 hr at room temperature. The

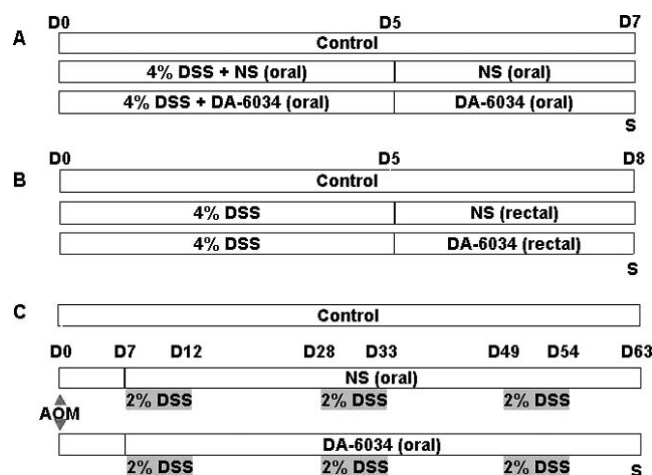


Figure 1. Experimental protocol. (A) Acute preventive model ($n = 5$ for each group). Mice were given 4% DSS in drinking water for 5 days before switching to regular drinking water. DA-6034 or 0.9% normal saline was administered twice daily by oral gavage until Day 7. (B) Acute therapeutic model ($n = 9$ for each group). Mice were fed 4% DSS in their drinking water for 5 days. Then, DA-6034 or 0.9% normal saline was administered once daily by rectal enema or by oral gavage. Normal control mice received autoclaved water alone. (C) Colitis-associated cancer model ($n = 5$ for each group). Experimental mice were given a single intraperitoneal injection of AOM on Day 0. DA-6034 or 0.9% normal saline was administered twice daily by oral gavage from Day 8 to the end of this study. Experimental mice were given three cycles of 2% DSS for 5 days followed by 2 weeks + 2 days of consumption of free water. Mice were sacrificed 10 days after the final 2% DSS administration.

slides were washed three times with TBS Tween-20 and were incubated with secondary antibody for 30 mins. After the slides were reacted with streptavidin for 20 mins, the reaction was visualized by 3,3'-diaminobenzidine tetrahydrochloride (DAB) for 5 mins, and the slides were counterstained with Mayer's hematoxylin.

The intensity and localization of immunoreactivities against all the primary antibodies used were examined on all sections with a microscope (Olympus BX41; Olympus Optical Co., Ltd., Tokyo, Japan). The number of Ki-67-positive cells was counted under a microscope at $\times 400$ magnification. The Ki-67 labeling index was defined as the percentage of Ki-67-positive cells among the counted epithelial cells. Each slide for COX-2 and phospho-IKK α immunohistochemistry was evaluated for intensity of immunoreactivity on a 0 to 4+ scale. The overall intensity of the staining reaction was scored as follows: 0 indicated no immunoreactivity and no positive cells; 1+ indicated weak immunoreactivity and <10% positive cells; 2+ indicated mild immunoreactivity and 10%–30% positive cells; 3+ indicated moderate immunoreactivity and 31%–60% positive cells; and 4+ indicated strong immunoreactivity and 61%–100% positive cells.

In situ TUNEL Assay. Apoptosis was determined by *in situ* TUNEL assay in mouse colon samples. Apoptotic cells in the colonic epithelial layer were detected with the TUNEL assay (ApopTaq Peroxidase *In situ* Apoptosis Detection Kit; Chemicon, Temecula, CA) by following the

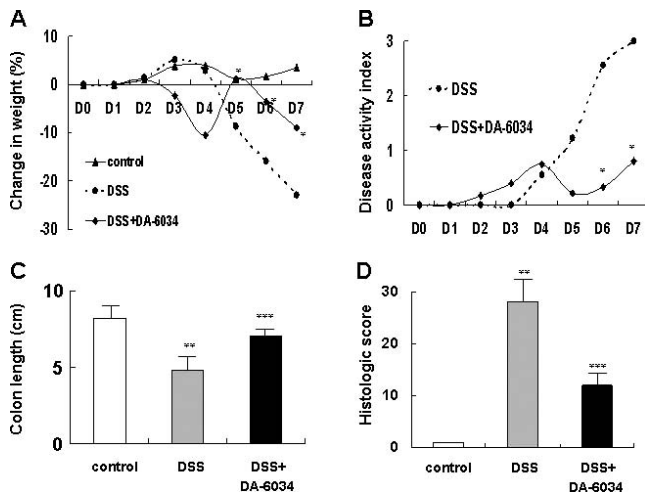


Figure 2. Preventive effects of DA-6034 in severe DSS-induced colitis. Treatment-group mice were administered 4% DSS dissolved in water for 5 days and normal saline or DA-6034 from Day 0 to Day 7. Control-group mice received water alone. (A) Changes in percentage of body weight. (B) Changes in the DAI. (C) Colon length. (D) Pathologic scores. Data are expressed as mean $\pm$ SD. * $P < 0.05$ compared with DSS; ** $P < 0.001$ compared with control; *** $P < 0.01$ compared with DSS.

manufacturer's instructions. Briefly, after equilibration with the terminal deoxynucleotidyl transferase buffer for 10 mins, sections were reacted with terminal deoxynucleotidyl transferase enzyme for 1 hr at 37°C. Antidigoxigenin peroxidase conjugates were added and incubated at room temperature for 30 mins. After adding DAB, a brown color change was noted. Sections were counterstained with methyl green and were mounted with histomount. The apoptotic cells were counted as follows: 300 epithelial cells were counted per high-power field and were scored for apoptosis; a total of three fields were counted per section of mouse colon (i.e., proximal, middle, and distal colon). The apoptotic index was determined by the ratio of TUNEL-positive nuclei to 100 total nuclei in the epithelial cells counted. The areas of necrosis (e.g., in an ulcer bed) were identified by examining the corresponding hematoxylin-eosin-stained slides and were excluded from the count for apoptotic cells, as previously described (34).

Statistical Analysis. Continuous data were expressed as the mean $\pm$ standard deviation and were compared by using the Mann-Whitney U test, the Wilcoxon rank sum test, or analysis of variance (ANOVA). All P values were two-tailed, and the level of statistical significance was specified as <0.05 .

Results

Starting Body Weight and DSS Load. There were no significant differences in the starting body weight between control and experimental groups in the acute preventive model (19.1 ± 0.8 g, 19.1 ± 0.3 g, and 19.4 ± 0.7 g in the control, DSS, and DSS + DA-6034 groups, respectively; $n = 5$ for each group) or in the acute therapeutic model (22.2 ± 1.6 g, 21.8 ± 1.2 g, and 22.6

± 1.1 g in control, DSS, and DSS + DA-6034 groups, respectively; $n = 9$ for each group). There were no significant differences in individual DSS loading in the acute preventive model (40.3 g and 42.5 g in the DSS and DSS + DA-6034 groups, respectively) or in the acute therapeutic model (47.8 g and 51.3 g in the DSS and DSS + DA-6034 groups, respectively).

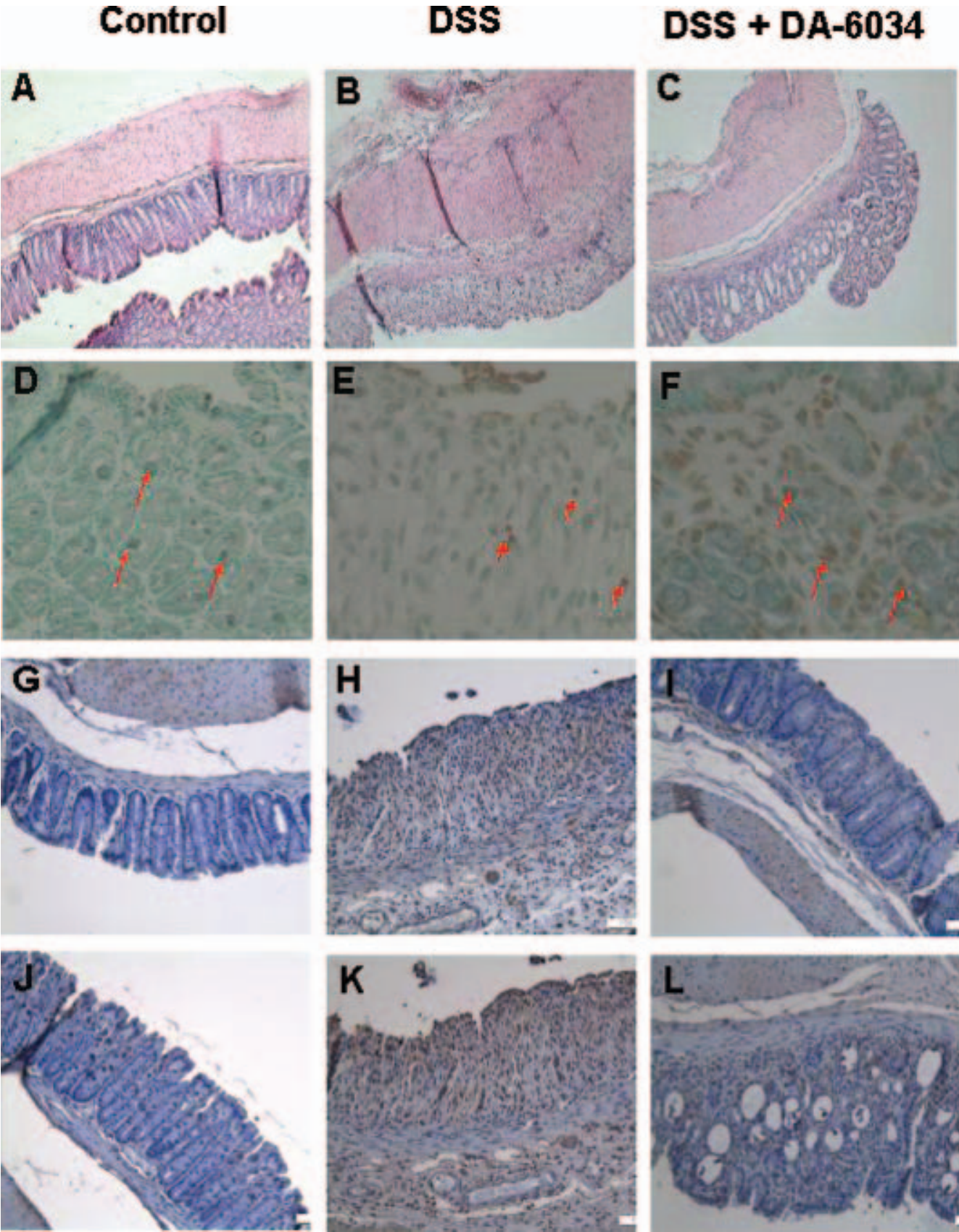
There were no significant differences in the starting body weight between control and experimental groups in the colitis-associated cancer model (19.3 ± 0.5 g, 19.1 ± 0.4 g, and 19.4 ± 0.7 g in the control, AOM + DSS, and AOM + DSS + DA-6034 groups, respectively; $n = 5$ for each group). There were no significant differences in individual DSS loading in the colitis-associated cancer model (first cycle, 25.1 g and 29.9 g; second cycle, 27.3 g and 25.2 g; third cycle, 20.9 g and 21.9 g; each in the AOM + DSS and AOM + DSS + DA-6034 groups, respectively).

DA-6034 Prevents DSS-Induced Acute Colitis in Mice. Orally administered DA-6034 (30 mg/kg bid) significantly attenuated DSS-induced weight loss, clinical DAI, and shortening of colon length (Fig. 2A, 2B, and 2C, respectively). Although DSS induced complete destruction of epithelial architecture with loss of crypts and epithelial integrity, submucosal edema, and intense infiltration of inflammatory cells including neutrophils and lymphocytes in all layers (Fig. 3B), DA-6034-treated mice maintained crypt architecture and showed scanty infiltration of inflammatory cells (Fig. 3C). Semiquantitative analysis of these changes showed that, in DA-6034-treated mice, the severity of colitis (as assessed by the total score of inflammation, extent, and crypt damage), was significantly lower than in mice with DSS-induced colitis (Fig. 2D).

DA-6034 Induces Apoptosis and Inhibits the Expression of COX-2 and Phospho-IKK α . In control mice, apoptotic cells were rare and were limited to the surface epithelium (Fig. 3D). In the mice with DSS-induced colitis (Fig. 3E), apoptotic cells were identified in the surface epithelium and crypt. There were numerous apoptotic cells in DA-6034-treated mice with DSS-induced colitis (Fig. 3F). There were significant increases of the apoptotic index in DA-6034-treated mice with DSS-induced colitis compared with the untreated mice with DSS-induced colitis ($P < 0.001$; Fig. 4A).

In mice with DSS-induced colitis, phospho-IKK α was strongly positive in both epithelial cells and submucosal inflammatory cells (Fig. 3H). Treatment with DA-6034 30 mg/kg twice a day (Fig. 3I) markedly reduced the degree of phospho-IKK α staining in the colon tissue. The scores for phospho-IKK α markedly decreased in DA-6034-treated mice with DSS-induced colitis compared with untreated mice with DSS-induced colitis ($P < 0.01$; Fig. 4B).

Although strong COX-2 expression was seen in both epithelial cells and submucosal inflammatory cells in untreated mice with DSS-induced colitis (Fig. 3K), weak immunoreactivity for COX-2 was observed in epithelial cells and in the basal layer of DA-6034-treated mice with



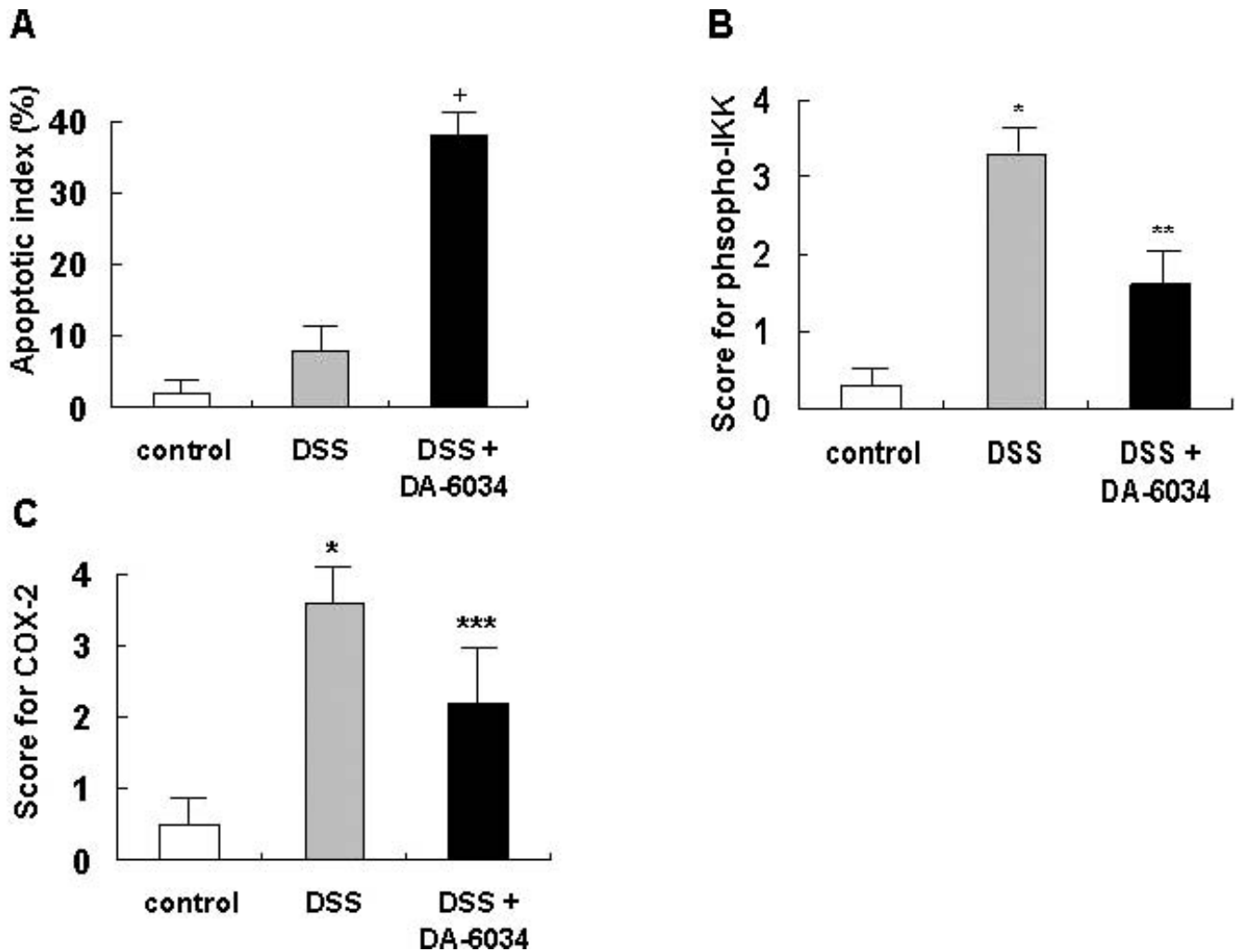


Figure 4. Immunoreactivity index in an acute preventive model. (A) Bars show the mean $\pm$ SD number of apoptotic cells per 100 total nuclei of the epithelial cells. ⁺ $P < 0.001$ compared with the DSS group. Bars show the mean $\pm$ SD of overall scores for phospho-IKK α (B) and for COX-2 (C). ^{*} $P < 0.001$ compared with the control-group mice; ^{**} $P < 0.01$ compared with the DSS group; ^{***} $P < 0.05$ compared with the DSS group.

DSS-induced colitis (Fig. 3L). The scores for COX-2 markedly decreased in DA-6034-treated mice with DSS-induced colitis compared with untreated mice with DSS-induced colitis ($P < 0.05$; Fig. 4C).

DA-6034 Ameliorates DSS-Induced Acute Colitis in Mice. Oral administration of DA-6034 (10 mg/kg qd) slightly reduced the weight loss (Fig. 5A) and improved the survival rate (Fig. 5B) of the mice, but it did not improve

the disease activity (data not shown). Weight loss continued to progress until all the mice were dead on Day 13. Compared with the DSS group, rectal administration of DA-6034 (10 mg/kg qd) increased the survival (Fig. 5D) and reduced the severity of DSS-induced colitis as assessed by body weight, DAI, and colon length (Fig. 5C, 5E, and 5F, respectively). After removal of 4% DSS on Day 5, colitis progressed, and most mice died before Day 9 in the DSS

Figure 3. Histopathology. (A–C) Hematoxylin-eosin stain (Magnification: $\times 100$). (D–F) TUNEL stain (Magnification: $\times 400$). (G–I) Immunohistochemistry for phospho-IKK α (Magnification: $\times 200$) and (J–L) for COX-2 (Magnification: $\times 200$) of colonic samples taken from mice that received water (A, D, G, and J), 4% DSS (B, E, H, and K), or 4% DSS + DA-6034 (C, F, I, and L) in the acute preventive model. The DSS group showed complete destruction of epithelial architecture with loss of crypts and epithelial integrity, submucosal edema, and intense inflammatory cellular infiltration in all layers (B). The DSS + DA-6034 group markedly reduced crypt and epithelial damage and showed mild cellular infiltration (C). Brown staining of nuclei indicates apoptotic cells (D–F); there are numerous apoptotic cells in the DSS + DA-6034 group (F). In the DSS group, phospho-IKK α is strongly positive in both epithelial cells and submucosal inflammatory cells (H), whereas phospho-IKK α is markedly attenuated in the DSS + DA-6034 group (I). Although strong COX-2 expression was seen both in epithelial cells and in submucosal inflammatory cells in the DSS group (K), weak immunoreactivity for COX-2 was observed in epithelial cells and basal layer in the DSS + DA-6034 group (L). Results are representatives of at least three separate experiments. A color version of the figure is available in the online version.

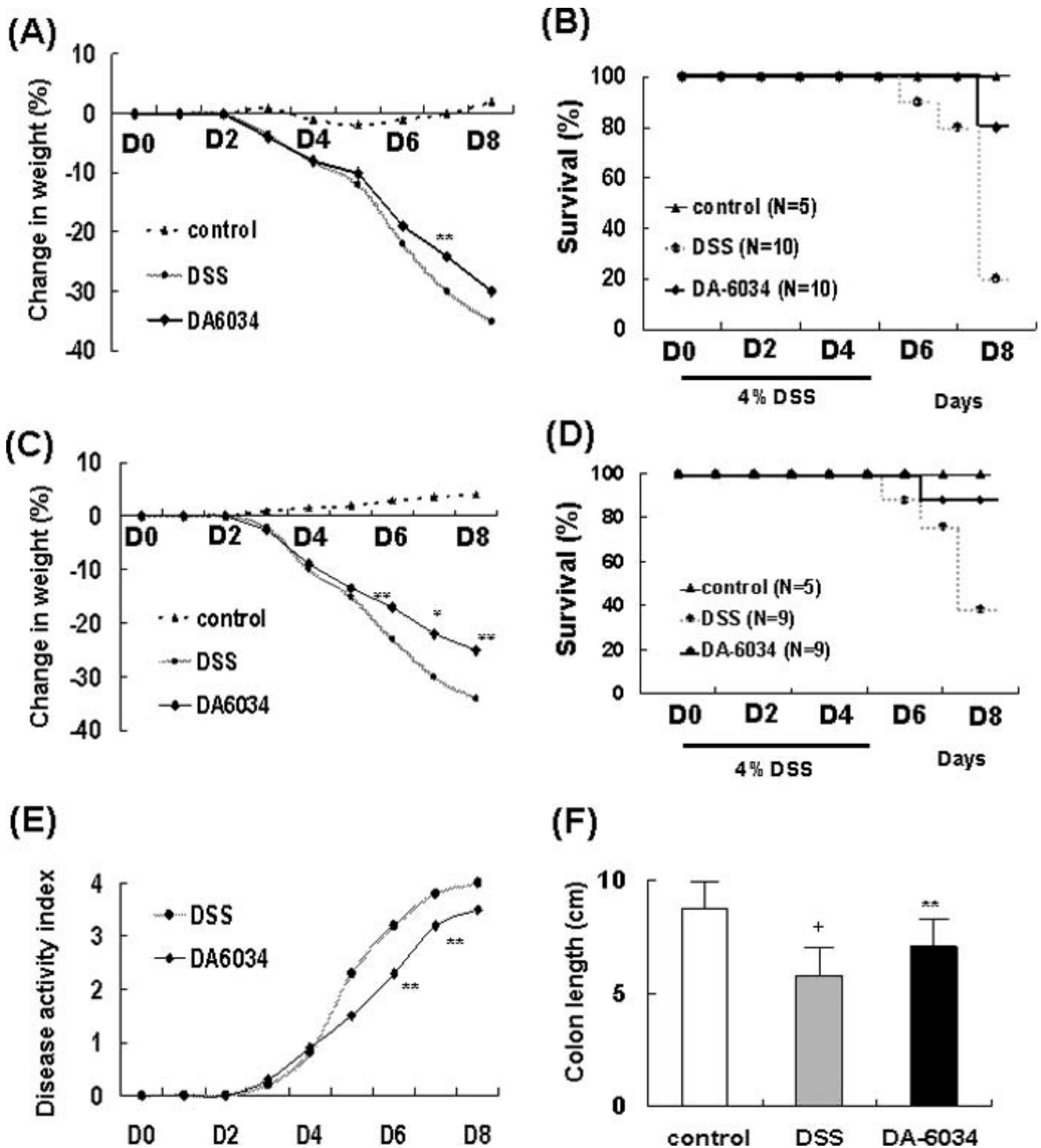


Figure 5. Therapeutic effects of DA-6034 in severe DSS-induced colitis. Mice were administered 4% DSS dissolved in water for 5 days and normal saline or DA-6034 orally (A, B) or rectally (C, D, E, F) on Day 5 to Day 8. Control-group mice received water alone. All mice were killed on Day 8, and clinical evaluation and severity were monitored by changes in percentage body weight (A, C), survival rate (B, D), DAI (E), and colon length (F). The weight changes (A, C) were expressed as a percentage of body weight on Day 0, and data represent the mean value. Colon lengths (F) are expressed as mean $\pm$ SD. * $P < 0.01$ compared with DSS; ** $P < 0.05$ compared with DSS; + $P < 0.001$ compared with control.

group; however, the DSS + DA-6034 group showed prolonged survival ($P < 0.05$; Fig. 5D).

DA-6034 Inhibits Development of Colitis-Associated Cancer. Weight change was examined daily

during the experimental period. Weight gain in DSS-treated mice was much lower than in control mice. Rapid weight reduction started 5 days after the second cycle of 2% DSS, and this reduction was sustained until 6 days after 2% DSS

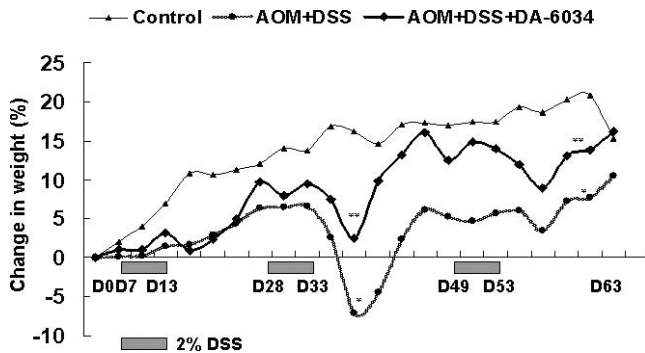


Figure 6. Change in body weight in a colitis-associated cancer model. Weight change was examined daily during the experimental period. Weight gain in the DSS group was much lower compared with the control-group mice. Weight reduction was noted 5 or 6 days after 2% DSS was started in both the AOM + DSS and the AOM + DSS + DA-6034 groups. DA-6034 markedly attenuated the DSS-induced weight reduction. * $P < 0.005$, the AOM + DSS group compared with the control mice; ** $P < 0.01$, the AOM + DSS + DA-6034 group compared with the AOM + DSS group.

cessation in both the AOM + DSS and the AOM + DSS + DA-6034 groups (Fig. 6). Rapid weight gain was noted afterward for 7 days in both experimental groups before the weights reached a plateau. Minimal weight reduction and later weight gain were observed after the third cycle of 2% DSS. DA-6034 markedly attenuated the DSS-induced weight reduction after the second cycle of 2% DSS ($P < 0.01$; Fig. 6). DA-6034 significantly reduced the number of colon tumors (Fig. 7A, B). Although the mice that received AOM + DSS showed a markedly short colon length (6.58 ± 0.52 cm, $P < 0.001$) compared with the control mice (9.1 ± 0.87 cm), the colon length of the mice that received AOM + DSS + DA-6034 (8.14 ± 0.19 cm) was comparable with that of the control mice and was markedly longer than the colon length in the AOM + DSS group ($P < 0.001$; Fig. 7D). Although partial destruction of epithelial architecture, submucosal edema, predominant lymphocytic infiltration, and several lymphoid follicles were observed in the 2% DSS + AOM group (Fig. 8A), the maintenance of crypt architecture, moderate infiltration of inflammatory cells, and several lymphoid follicles were observed in the 2% DSS + AOM + DA-6034 group (Fig. 8C).

DA-6034 Induces Apoptosis and Decreases Expression of Ki-67, phospho-IKK α and COX-2 in Colitis-Associated Cancer Model. DA-6034 strongly enhanced apoptosis in lamina propria mononuclear cells (Fig. 8G) compared with the AOM + DSS group (Fig. 8E). Although Ki-67 is expressed in the epithelial cells in mice with DSS-induced colitis (Fig. 8I), it is rarely expressed in the mice treated with DA-6034 30 mg/kg twice a day (Fig. 8K). In the AOM + DSS + DA-6034 group, the apoptotic index markedly increased (Fig. 9A), but the Ki-67 index significantly decreased (Fig. 9B). In untreated mice with DSS-induced colitis, phospho-IKK α is strongly positive in both destroyed epithelial cells and submucosal inflammatory cells (Fig. 8M). Treatment with DA-6034 30 mg/kg twice a

day markedly attenuates the degree of phospho-IKK α staining in the colon tissue (Fig. 8O). There was a significant decrease in scores for phospho-IKK α in the AOM + DSS + DA-6034 group compared with the AOM + DSS group ($P < 0.01$; Fig. 9C). Although strong COX-2 expression was seen in both epithelial cells and submucosal inflammatory cells in mice with DSS-induced colitis (Fig. 8Q), reduced immunoreactivity for COX-2 was observed in epithelial cells and in the basal layer in DA-6034-treated mice (Fig. 8S). The expression of COX-2 is increased in the cancer of DA-6034-treated mice (Fig. 8T). The scores for COX-2 markedly decreased in the AOM + DSS + DA-6034 group compared with the AOM + DSS group ($P < 0.01$; Fig. 9D).

Discussion

In the present study, we showed that orally administered DA-6034 prevented and improved DSS-induced murine colitis and inhibited inflammation-related colon carcinogenesis. Furthermore, it was clearly demonstrated by immunohistochemistry that the anti-inflammatory and anti-tumorigenic effects of DA-6034 were associated with the inhibition of IKK α activity and of COX-2 as well as with the induction of apoptosis. For the first time, these findings indicate the novel protective effect of DA-6034 against colitis and inflammation-related colon carcinogenesis and suggest that DA-6034 may be a potential therapeutic agent for IBD.

Recent studies for pharmacokinetics have shown low bioavailability of DA-6034 because of the intestinal first-pass effect (30). Thus, the therapeutic effect of oral DA-6034 may be caused by the local effect on the intestinal mucosa. Moreover, the disposition of DA-6034 was not affected considerably by DSS treatment in mice (35). According to previous studies, we used the appropriate dose to evaluate the preventive effect of DA-6034 (30 mg/kg bid; Refs. 30, 35). Orally administered DA-6034 significantly ameliorated DSS-induced weight loss, the increase in the clinical DAI, and the shortening of colon length. In the next step, we evaluated the efficacy of DA-6034 in the aspect of treatment for DSS-induced colitis after inflammation had been induced. We chose a different administrative route in the acute therapeutic model—a direct rectal administration of the agent. A series of our studies have shown that the effect and pharmacokinetics of DA-6034 are very similar to those of 5-ASA (28–30, 35).

Oral 5-ASA is effective for the treatment of mild-to-moderate active UC. In addition, rectal topical therapy with 5-ASA has been the treatment of choice in active distal UC (36). Combined oral and rectal treatment with 5-ASA is superior to oral therapy alone in patients with extensive, mild-to-moderate, active UC (37). Therefore, a therapeutic agent for UC should be developed as oral and topical forms. Our results suggest that both of oral and rectal administration of DA-6034 may be applied for the therapy of active distal and extensive UC. Despite the low dosage (10 mg/kg qd), rectal administration of DA-6034 increased the survival rate and reduced the severity of DSS-induced colitis

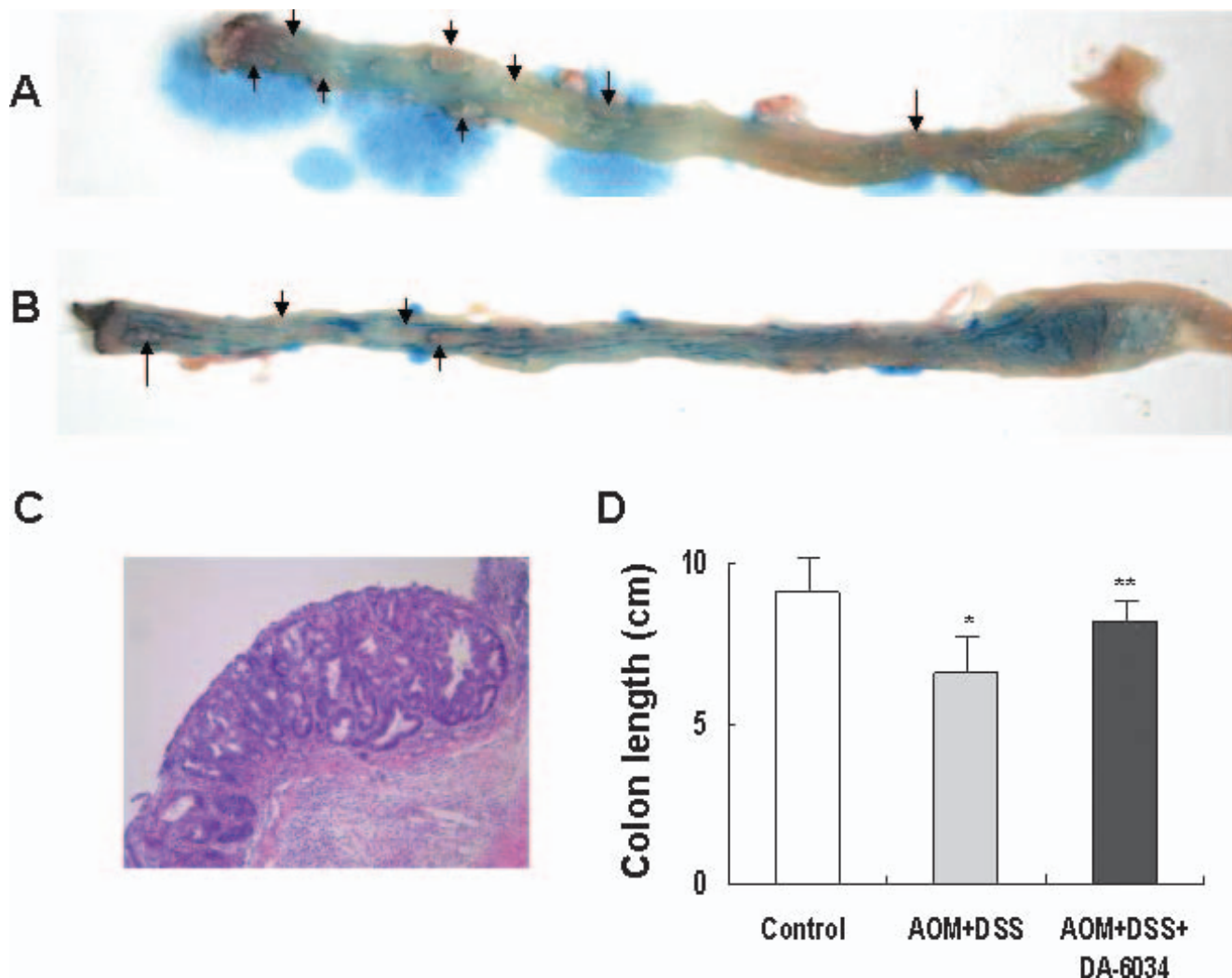


Figure 7. Colon cancer and dysplasia in a colitis-associated cancer model. Although numerous colon tumors developed and an edematous, shortened colon was observed in the mice treated with AOM + 2% DSS (A), several colon tumors and dysplastic lesions developed and a slightly edematous colon was noted in the mice treated with AOM + 2% DSS + DA-6034 (B). The histopathologic view of colon tumors developed in the mice treated with AOM + 2% DSS (C) shows the adenocarcinoma (hematoxylin-eosin stain; Magnification, originally: $\times 100$). Changes in colon length (D) are expressed as mean $\pm$ SD. * $P < 0.001$ AOM + DSS compared with control; ** $P < 0.001$ AOM + DSS + DA-6034 compared with AOM + DSS. A color version of the figure is available in the online version.

as assessed by body weight, DAI, and colon length compared with the DSS group, which suggests a strong direct effect of this agent in the local microenvironment. We are planning further studies of the therapeutic effect by oral administration of DA-6034 in DSS-induced colitis, TNBS-mediated colitis, and IL-10 gene-deficient mice.

DA-6034 reduced the number of colon tumors in an inflammation-related colon cancer model. IBD-associated colorectal carcinogenesis is probably promoted by chronic inflammation, but the mechanism is still unclear. However, mucosal inflammation may result in colonic carcinogenesis through several proposed mechanisms, such as induction of genetic mutations, increased-cryptal cell proliferation, changes in crypt cell metabolism, changes in bile acid enterohepatic circulation, and alterations in bacteria flora (38). Cell proliferation plays an important role in multistep

carcinogenesis (13). Suppressing apoptosis is one of the hallmarks of cancer, and the induction of apoptosis during tumor promotion represents a critical step for chemoprevention (14). Our findings that DA-6034 inhibits cell proliferation activity and induces apoptosis in colorectal mucosa are in accordance with these results. Thus, cellular responses, such as cell growth and/or apoptosis, to DA-6034 may contribute to chemopreventive effects against colorectal carcinogenesis.

NF- κ B, a key transcription factor for inflammatory responses, upregulates the expression of numerous proinflammatory genes involved in intestinal inflammation. The evidence for the association of NF- κ B activation and IBD suggests that the modulation of a NF- κ B signaling pathway could be the main target for the treatment of IBD (15, 16). Moreover, NF- κ B activates the transcription of numerous genes capable of suppressing apoptosis, suggesting a pivotal

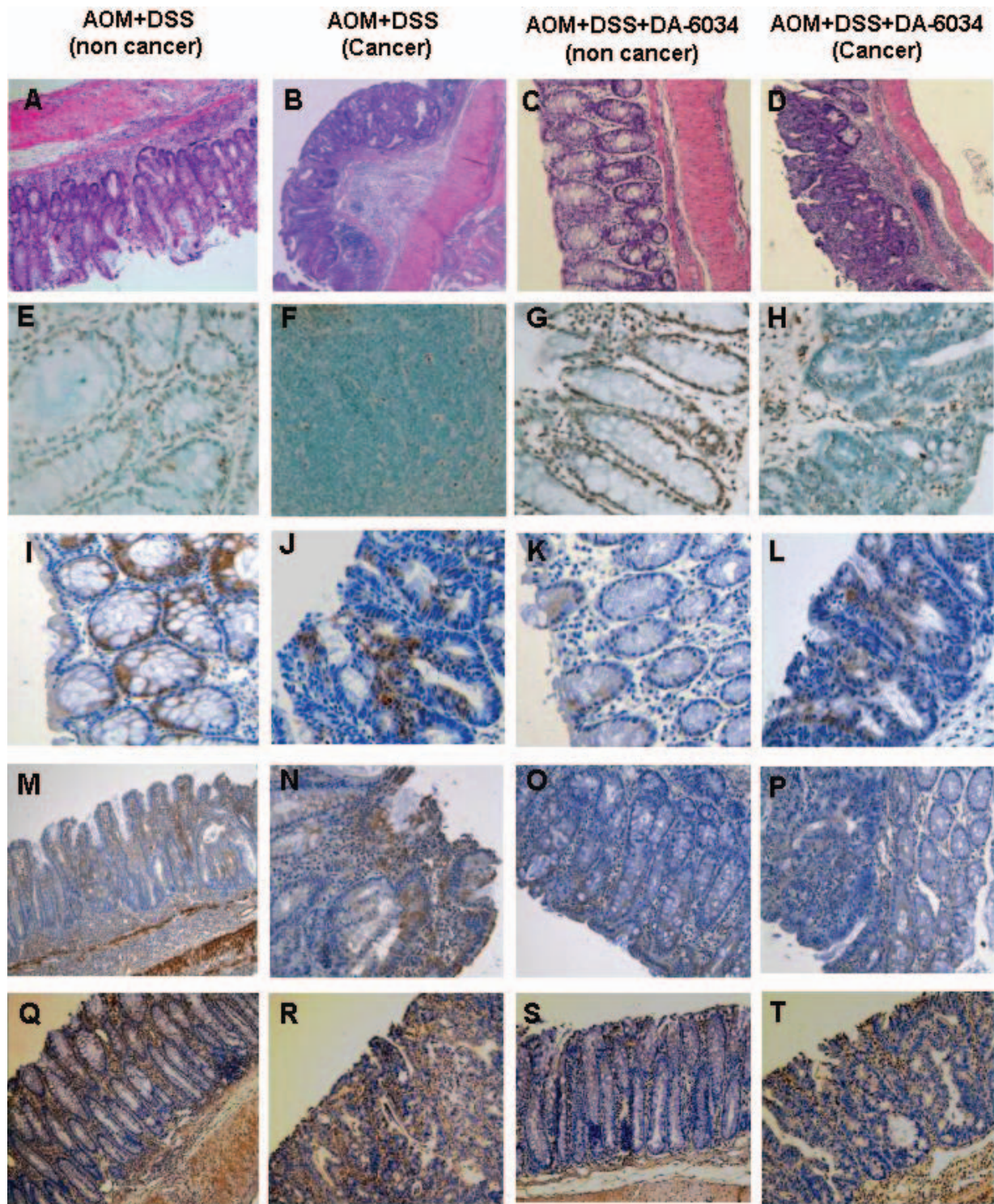


Figure 8. Histopathology. Hematoxylin-eosin stain (Magnification: $\times 100$; A–D). TUNEL staining (Magnification: $\times 400$; E–H). Immunohistochemical staining for Ki67 (Magnification: $\times 400$; I–L); for phospho-IKK α (Magnification: $\times 200$; M–P); and for COX-2 (Magnification: $\times 200$; Q–T) of inflamed colon tissue or cancer lesions taken from the AOM + DSS and the AOM + DSS + DA-6034 groups in a colitis-associated cancer model. Although partial destruction of epithelial architecture, submucosal edema, and predominant lymphocytic infiltration were observed in the AOM + DSS group (A), maintenance of crypt architecture and moderate infiltration of inflammatory cells were observed in the AOM + DSS + DA-6034 group (C). DA-6034 strongly enhanced apoptosis in lamina propria mononuclear cells (G) compared with the AOM + DSS group (E). Although Ki-67 is expressed in epithelial cells in the AOM + DSS group (I), it is rarely expressed in the AOM + DSS + DA-6034 group (K). In the AOM + DSS group, phospho-IKK α is strongly positive in both epithelial cells and submucosal inflammatory cells (M), whereas phospho-IKK α is markedly attenuated in the AOM + DSS + DA-6034 group (O). Although strong COX-2 expression was seen in both epithelial cells and submucosal inflammatory cells (Q) in the AOM + DSS group, reduced immunoreactivity for COX-2 was observed in epithelial cells and in the basal layer in the AOM + DSS + DA-6034 group (S). Results are representatives of at least three separate experiments.

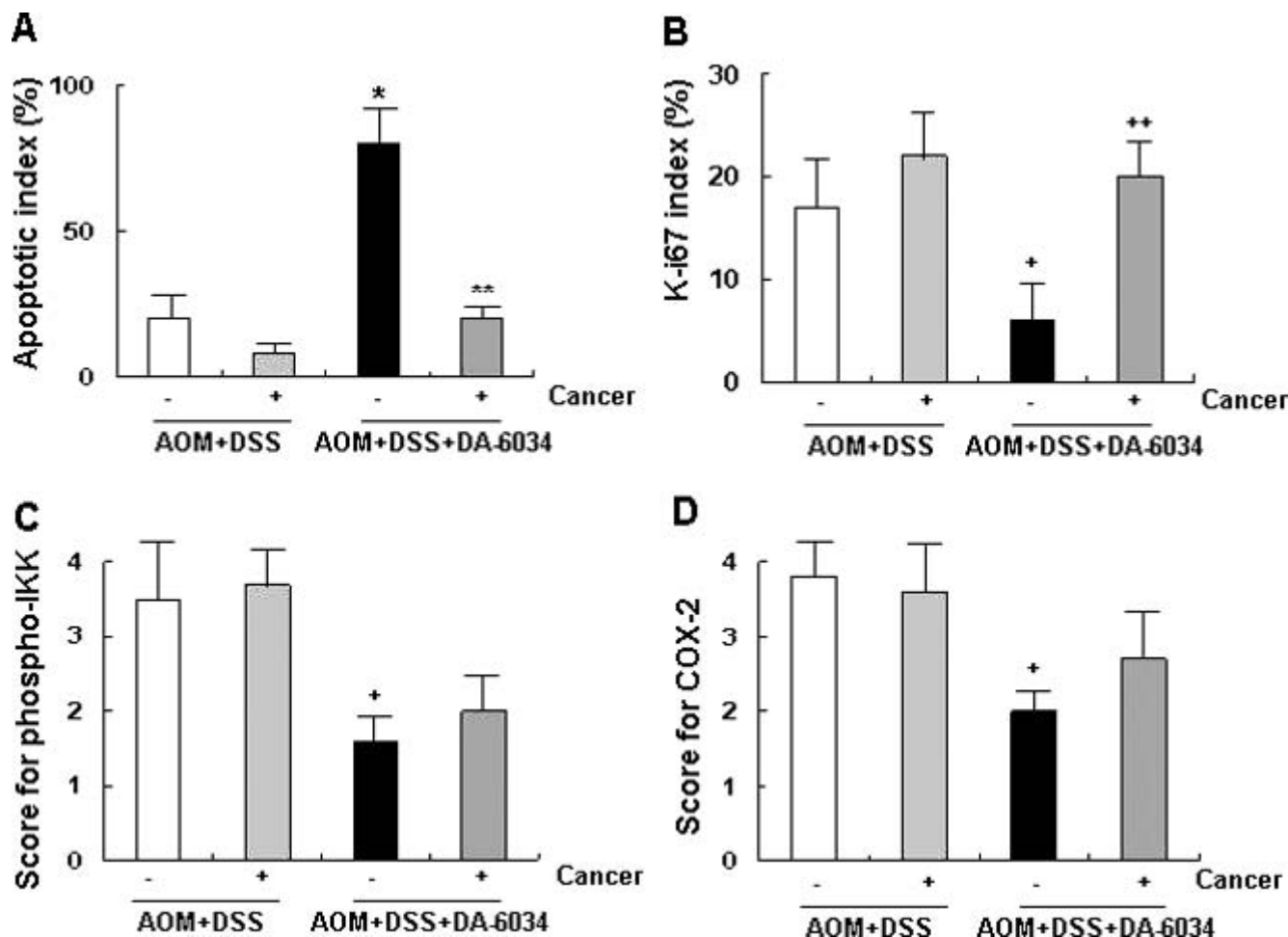


Figure 9. Immunoreactivity index in a colitis-associated cancer model. There were significant increases of apoptotic index (A) in the AOM + DSS + DA-6034 group compared with the AOM + DSS group (* $P < 0.001$). The index of Ki-67 (B) is decreased in the AOM + DSS + DA-6034 group compared with the AOM + DSS group (+ $P < 0.01$). The scores for phospho-IKK α (C) were markedly attenuated in AOM + DSS + DA-6034 group compared with the AOM + DSS group (+ $P < 0.01$). The scores for COX-2 (D) were markedly decreased in the AOM + DSS + DA-6034 group compared with the AOM + DSS group (+ $P < 0.01$). ** $P < 0.01$ compared with the noncancer lesion; ++ $P < 0.05$ compared with the noncancer lesion.

role in the inflammation-related carcinogenesis (17). In particular, a recent investigation demonstrated that IKK β is related to inflammation and tumorigenesis in murine models of colitis-associated cancer (18). Therefore, selective IKK-dependent NF- κ B activation may play a major role in oncogenesis. The anti-NF- κ B action of sulfasalazine, which is known to prevent colon cancer in patients with IBD, is mediated by the direct inhibition of IKK α and IKK β (39). In this study, DA-6034 inhibited the expression of COX-2, a NF- κ B-dependent mediator, in both of acute murine colitis and colitis-related cancer. We assessed the IKK α activation in a colon section by immunohistochemistry that used an anti-phospho-IKK α antibody to elucidate the target of action of DA-6034 more specifically *in vivo*. Our previous study showed that IKK α activity *in vitro* was well correlated with phospho-IKK α immunoreactivity in colon tissue *in vivo* (31). DA-6034 suppressed phospho-IKK α immunoreactivity in both acute colitis and colitic cancer, which suggests a comparable mechanistic effect to sulfasalazine.

In conclusion, DA-6034 prevents and improves acute

murine colitis and inhibits inflammation-related colon carcinogenesis by the inhibition of COX-2 and phospho-IKK α and by the induction of apoptosis. DA-6034 could be a potential therapeutic agent for IBD.

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