

3,3'-Diindolylmethane Attenuates Colonic Inflammation and Tumorigenesis in Mice

Yoon Hee Kim, MS,* Hyuck-Se Kwon, MS,* Dae Hwan Kim, MS,* Eun Kyung Shin, MS,*
Young-Hee Kang, PhD,[†] Jung Han Yoon Park, PhD,[†] Hyun-Kyung Shin, PhD,*[†] and Jin-Kyung Kim, PhD*

Background: 3,3-Diindolylmethane (DIM) is a major *in vivo* product of acid-catalyzed oligomerization of indole-3-carbinol (I3C) derived from *Brassica* food plants. Although DIM is known as a chemopreventive and chemotherapeutic phytochemical, the effects of DIM on inflammation *in vivo* are still unknown. In the present study we investigated the antiinflammatory effects of DIM on experimental colitis and colitis-associated colorectal carcinogenesis.

Methods: To determine if DIM has an antiinflammatory effect *in vivo*, we examined the therapeutic effects of DIM in dextran sodium sulfate (DSS)-induced experimental colitis and colitis-associated colon carcinogenesis induced by azoxymethane (AOM)/DSS in BALB/c mice.

Results: Treatment with DIM significantly attenuated loss of body weight, shortening of the colon, and severe clinical signs in a colitis model. This was associated with a remarkable amelioration of the disruption of the colonic architecture and a significant reduction in colonic myeloperoxidase activity and production of prostaglandin E₂, nitric oxide, and proinflammatory cytokines. Further, DIM administration dramatically decreased the number of colon tumors in AOM/DSS mice.

Conclusions: These results suggest that DIM-mediated antiinflammatory action at colorectal sites may be therapeutic in the setting of inflammatory bowel disease and colitis-associated colon cancer.

(*Inflamm Bowel Dis* 2009;15:1164–1173)

Key Words: inflammatory bowel diseases, colon cancer, 3,3'-diindolylmethane

Received for publication January 28, 2009; Accepted February 7, 2009.

From the *Center for Efficacy Assessment and Development of Functional Foods and Drugs, Hallym University, Chuncheon, South Korea, [†]Department of Food Science and Nutrition, Hallym University, Chuncheon, South Korea.

Supported by Regional Innovation Center (RIC) program of the Ministry of Commerce, Industry, and Energy, Republic of Korea and Korea Science and Engineering Foundation (KOSEF) grant funded by the Korea government (MOST)(R01-2007-000-20164-0).

Reprints: Jin-Kyung Kim, Center for Efficacy Assessment and Development of Functional Foods and Drugs, Hallym University, Chuncheon, 200-702 South Korea (e-mail: kimjin@hallym.ac.kr).

Copyright © 2009 Crohn's & Colitis Foundation of America, Inc.

DOI 10.1002/ibd.20917

Published online 30 March 2009 in Wiley InterScience (www.interscience.wiley.com).

The 2 major forms of inflammatory bowel disease (IBD)—Crohn's disease (CD) and ulcerative colitis (UC)—represent prototypical conditions whose most salient features are the presence of chronic inflammation involving various parts of the intestinal tract and an increased risk of cancer, which is a complication directly related to the duration and activity of gut inflammation.^{1–4} IBD is associated with intestinal and extraintestinal clinical manifestations, including weight loss, diarrhea accompanied by blood and/or mucus, fever, gastric dysmotility, and shortening of the colon.^{2,3} UC is a condition that primarily affects the superficial layer of the colon mucosa, and histological analyses have shown ulceration of the mucosa, blunting and loss of crypts, and inflammatory infiltrates.² The immune pathogenesis of IBD is associated with increased inflammatory mediators, including reactive oxygen species (ROS) such as nitric oxide (NO),^{5,6} prostaglandins (PG),^{7,8} and inflammatory cytokines such as tumor necrosis alpha (TNF)- α , interleukin (IL)-6, and IL-23.^{9–11}

IBD therapy has improved and expanded as understanding of the disease mechanisms has evolved. Pharmacologic agents such as aminosalicylates, azathioprine/6-mercaptopurine, or steroids are the mainstays of therapy.^{12–15} Newer agents, including monoclonal antibodies targeted to specific proinflammatory cytokines such as TNF- α , have emerged and provided great clinical benefit. However, unknown long-term toxicity and immunogenicity may limit their use.¹⁶ Although many types of IBD treatments have been proposed and clinically implemented, additional therapeutic approaches are needed, as many patients either fail to respond to the currently available options or show significant side effects. Alternative medicine is becoming an increasingly attractive approach for the treatment of various inflammatory disorders among patients who are unresponsive to, or unwilling to take, standard medications. Among these alternative approaches is the use of food derivatives, which have the advantage of being relatively nontoxic. However, limited scientific evidence regarding the effectiveness of these natural derivatives, in conjunction with a lack of understanding of their action mechanisms, has prevented their incorporation into mainstream medical care.

3,3-Diindolylmethane (DIM) is a major dimer formed in acidic conditions from indole-3-carbinol (I3C), an autolysis product of the abundant glucobrassicin in cruciferous

vegetables.¹⁷ DIM has antiproliferative effects against various cancers, including prostate,¹⁸ pancreatic,¹⁹ and breast cancer.²⁰ Moreover, a previous study showed that DIM inhibits lipopolysaccharide (LPS)-induced production of inflammatory mediators in murine macrophages via downregulation of nuclear factor-kappaB (NF- κ B) signaling.²¹ These previous observations led us to examine the therapeutic effects of DIM on intestinal inflammation and inflammation-related colon cancer. In order to investigate the effects of DIM, a dextran sodium sulfate (DSS)-induced mouse colitis model and an azoxymethane (AOM)/DSS-induced colon cancer model were selected as the subjects for this study. We showed that DIM administration dramatically attenuated weight loss, colon shortening, and severe clinical signs in a colitis model. Further, DIM dramatically suppressed tumor formation in AOM/DSS BALB/c mice.

MATERIALS AND METHODS

Reagents and Cell Line

All reagents were obtained from Sigma-Aldrich (St. Louis, MO) unless otherwise indicated. DSS (MW 40,000–50,000) and DIM were purchased from ICN Biochemicals (Aurora, OH) and LKT Laboratories (St. Paul, MN), respectively. RPMI 1640 medium, fetal bovine serum (FBS), penicillin, and streptomycin were obtained from Hyclone (Logan, UT). Griess reagent was obtained from Promega (Madison, WI). PGE₂ enzyme-linked immunosorbent assay (ELISA) kits were manufactured by R&D systems (Minneapolis, MN). Multiplex bead array instruments and cytokine kits were purchased from Bio-Rad (Hercules, CA). The RNAeasy mini-kit and SYBR-Green II master mix were obtained from Qiagen (Hilden, Germany). Superscript reagents were purchased from Invitrogen (Carlsbad, CA). The antibodies (Abs) used in this study were: anti-inhibitor of NF- κ B (I κ -B) α rabbit polyclonal, anti-NF- κ B p65 rabbit polyclonal, anti-laminB rabbit polyclonal (Santa Cruz Biotechnology, Santa Cruz, CA), anti- β -actin rabbit polyclonal (Cell Signaling Technology, Danvers, MA), anti-inducible NO synthase (iNOS) rabbit polyclonal (BD Biosciences Pharmingen, San Diego, CA), and cyclooxygenase (COX)-2 goat polyclonal (Santa Cruz Biotechnology). IEC-6 cells were acquired from the Korea Cell Bank (Seoul, Korea).

Induction of Experimental Colitis

Six-week-old BALB/c mice were purchased from SLC (Hamamatsu, Japan) and maintained under specific pathogen-free conditions with free access to food and water during the course of the experiments at the animal facility of Hallym University (Chuncheon, Korea).

In order to induce experimental colitis, we gave the mice 5% DSS dissolved in water that was filter-purified (Millipore, Bedford, MA), for 7 days. DIM was dissolved in

corn oil. DIM (1, 5, 10, and 20 mg/kg) or the vehicle (corn oil) was administered orally for 7 days, commencing at the same time DSS exposure began. The mice were checked daily for colitis through monitoring of body weight, gross rectal bleeding, stool consistency, and survival. The overall disease severity was assessed by a clinical scoring system with a scale of 0–4.²² After 10 days of DSS treatment, the mice were killed by cervical dislocation and their colons were removed. All animal experiments were approved by the Committee on Animal Experimentation of Hallym University and performed in compliance with the university's Guidelines for the Care and Use of Laboratory Animals.

Measurement of Cytokines, NO, and PGE₂

Colon tissues corresponding to the mid-colon were washed with RPMI 1640 medium containing 2% FBS and penicillin and streptomycin before being cut into smaller pieces. Then \approx 0.5 cm of tissue was placed in 0.5 mL of 0.1% FBS containing RPMI 1640 medium, distributed into 48-well tissue culture plates, and incubated for 24 hours at 37°C in 5% CO₂. The IL-1 β , TNF- α , IL-6, and interferon gamma (IFN)- γ concentrations in the cell-free culture supernatants of the colon tissues were measured in triplicate using a Bio-Rad Multiplex bead array instrument and cytokine kits according to the manufacturer's protocol. The cytokine concentration was adapted to the protein content of the colon tissue used in the culture.

The amount of nitrite and PGE₂ was measured using the Griess reagent system (Promega) and an ELISA kit (R&D systems) according to the manufacturer's instructions, respectively.

Determination of Myeloperoxidase (MPO) Activity in the Colon

The mouse colons were rinsed with cold phosphate-buffered saline (PBS), blotted dry, and immediately frozen in liquid nitrogen. They were then stored at –80°C until being assayed for MPO activity using the *o*-dianisidine method.²³ To perform the assay, the tissue samples were thawed and weighed, then suspended (10% wt/vol) in 50 mM potassium phosphate buffer (pH 6.0) containing 0.5% hexadecyltrimethylammonium bromide, and homogenized. A sample of the homogenate (1 mL) was sonicated for 30 seconds and centrifuged at 200g for 10 minutes at 4°C. The reaction was started by mixing and incubating the supernatant (100 μ L) at 20°C for 10 minutes with a solution composed of 2,810 μ L of 50 mM potassium phosphate, 30 μ L of 20 mg/mL *o*-dianisidine dihydrochloride, and 30 μ L of 20 mM hydrogen peroxide. After 10 minutes the reaction was terminated by the addition of 30 μ L of 2% sodium azide. The change in absorbance was read at 460 nm using a SpectraMax M2 Microplate Reader (Molecular Devices, Sunnyvale, CA). MPO activity was expressed as the amount of enzyme nec-

essary to produce a change in absorbance of 1.0 unit per minute per g of tissue (wet weight).

Quantitative Real-time Reverse-transcriptase Polymerase Chain Reaction (RT-PCR)

The total RNA from the colon homogenates was isolated with an RNaseasy mini kit. After RNA preparation, the cDNA was transcribed using a single reverse transcriptase synthesis step with Superscript reagents. Quantitative PCR was performed with the incorporation of SYBR green into the double-stranded PCR products. The primers used in quantitative PCR were as follows: iNOS, forward 5'-CAG CTG GGC TGT ACA AAC CTT-3' and reverse 5'-CAT TGG AAG TGA AGC GTT TCG-3'; COX-2, forward 5'-CAT CCC AGG CCG ACT AAA TG-3' and reverse 5'-TTT CAG AGC ATT GGC CAT AGA A-3'; TNF- α , forward 5'-TAC CTT GTC TAC TCC CAG GTT CTC T-3' and reverse 5'-GTG TGG GTG AGG AGC ACG TA-3'; IL-6, forward 5'-CTT CCT ACC CCA ATT TCC AAT G-3' and reverse 5'-ATT GGA TGG TCT TGG TCC TTA GC-3'; IFN- γ , forward 5'-ATC TGG AGG AAC TGG CAA AA and reverse 5'-TGA GCT CAT TGA ATG CTT GG-3'; IL-10, forward 5'-GGT TGC CAA GCC TTA TCG GA-3' and reverse 5'-ACC TGC TCC ACT GCC TTG CT-3'; β -actin, forward 5'-AGA GGG AAA TCG TGC GTG AC-3' and reverse 5'-CAA TAG TGA TGA CCT GGC CGT-3'. The specificity of the reaction was tested via product separation by gel electrophoresis, or by melting curve analysis when SYBR green was incorporated. The mean relative expressions of the cytokine genes were calculated, and differences were determined using the $2^{-\Delta C(t)}$ method.²³

Immunohistochemical Study

The colonic tissues were fixed in 4% buffered paraformaldehyde, dehydrated through graded concentrations of ethanol, embedded in paraffin, and sectioned. The 5 μ m-thick sections were mounted on slides, cleared, and hydrated; all were treated with a buffered blocking solution (3% BSA) for 15 minutes. The sections were then coincubated with primary antibodies to NF- κ B-p65 at a dilution of 1:25 at room temperature for 1 hour. The sections were washed with PBS and then coincubated with a secondary antibody of peroxidase-conjugated antirabbit IgG (1:500 in PBS, v/v) at room temperature for 1 hour. Thereafter, the sections were washed as before with 0.05 M Tris-HCl, pH 7.66, then coincubated with 3,3-diaminobenzidine solution in the dark at room temperature for 10 minutes. Finally, the sections were washed with Tris-HCl and stained with hematoxylin according to standard protocols.

NF- κ B Activity

IEC-6 cells were placed in a 24-well cell culture plate at a density of 1×10^5 cells in 0.5 mL of culture medium and

incubated for 12 hours. The cells were then exposed to various concentrations of DIM and 10 ng/mL of recombinant rat TNF- α (R&D systems) for 6 hours. In addition, colonic tissues were obtained 10 days after DSS treatment, and nuclear protein was isolated according to the manufacturer's protocol (TransAM Nuclear extraction kit, Active Motif, Carlsbad, CA). The transcription NF- κ B assay was then carried out using TransAM (Active Motif) according to the manufacturer's instructions. In order to observe I κ -B α degradation and nuclear translocation of NF- κ B-p65, IEC-6 cells were incubated with various concentrations of DIM for 1 hour followed by adding 10 ng/mL of recombinant rat TNF- α . After 30 minutes, whole cell, cytoplasmic, and nuclear lysates were obtained and electrophoresed on 10% sodium dodecyl sulfate (SDS) polyacrylamide gels for the detection of I κ -B α and NF- κ B-p65, as described previously.²⁴

Induction of Colon Cancer

To induce colon cancer, mice were injected intraperitoneally with 12.5 mg/kg body weight of AOM dissolved in physiological saline. Five days later, 2% DSS was given in the drinking water over 5 days, followed by 16 days of regular water. This cycle was repeated twice, for a total of 3 cycles. Mice were given DIM 0, 1, 5, 10, and 20 mg/kg of body weight 3 times a week via gastric intubation starting 1 week after the cessation of DSS administration. Body weight was measured every week, and animals were sacrificed at 56 days. The colons were removed, rinsed in ice-cold PBS, slit open longitudinally, and fixed flat between wet filter papers for 48 hours in 10% neutral buffered formalin prior to 30-second staining with 0.2% methylene blue dissolved in the same formalin solution.

Statistical Analysis

The data are depicted as means \pm SEM from at least 3 independent experiments. The values were evaluated by 1-way analysis of variance (ANOVA) with Bonferroni multiple comparison post tests using GraphPad Prism 4.0 software (GraphPad Software, San Diego, CA). Differences with P -values < 0.05 were considered statistically significant.

RESULTS

DIM Administration Caused a Reduction in Weight Loss, Clinical Signs, and Shortening of Colon Length in a DSS-induced Colitis Model

We first noted symptomatic parameters such as body weight loss, clinical scoring, and shortening of colon length caused by colitis 10 days after the start of the 5% DSS oral challenge. As shown in Figure 1A, the body weights of DSS-exposed mice were drastically decreased at day 10. However, treatment with 10 or 20 mg/kg DIM reduced

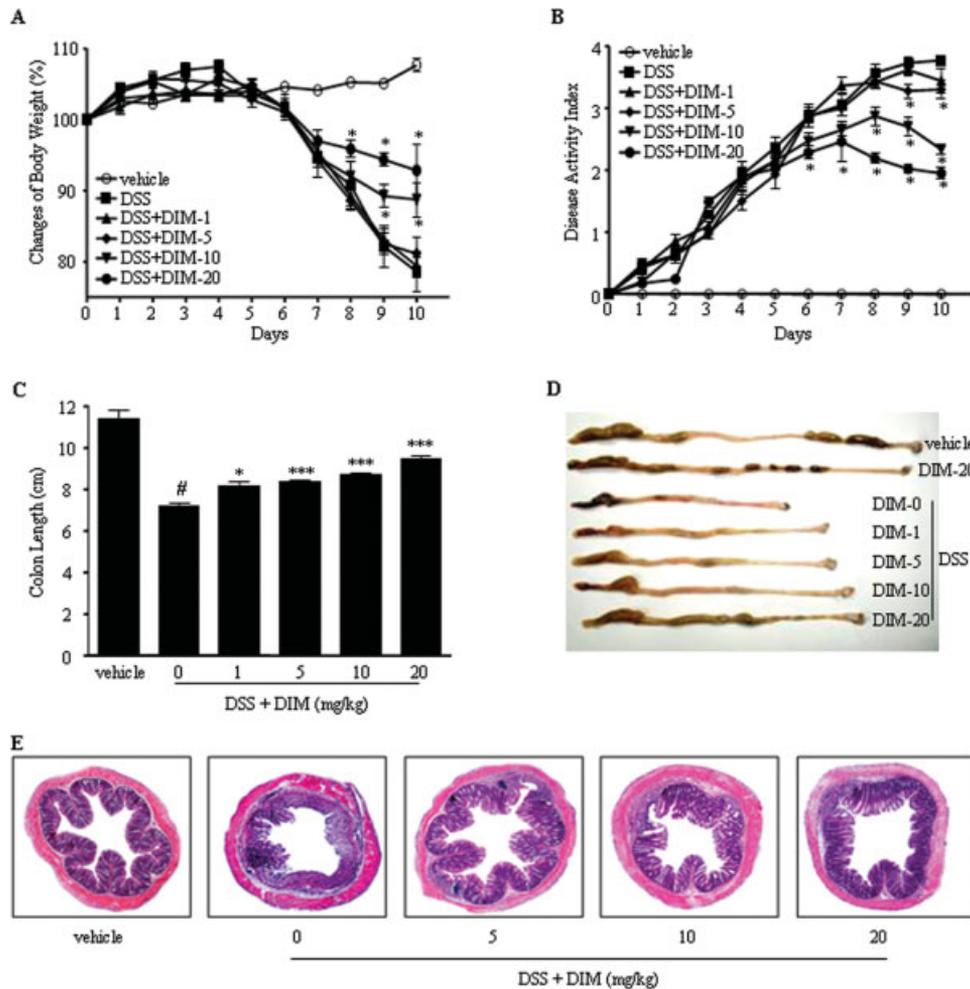


FIGURE 1. DIM attenuates clinical signs in DSS-induced colitis mice. (A) Changes in the body weights and (B) clinical scores of DIM-treated mice and control mice administered 5% DSS were monitored every day. Body weight values are expressed as a percentage of body weight on day 0. (C) Colons were obtained 10 days after DSS administration and their lengths were measured. (D) Macroscopic features of colon. Data are the sum of the results of 3 independent experiments and are expressed as means \pm SEM ($n = 10$ per group). Significant differences in the DSS group, * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$. The number sign (#) represents a significant difference between the vehicle and DSS treatment groups, $P < 0.001$. (E) Colons were obtained 10 days after DSS administration, then sectioned, stained with hematoxylin and eosin, and analyzed histopathologically (original magnification $\times 40$). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

weight loss in mice with colitis. The clinical score, another common feature of the DSS-induced model of colitis, also decreased significantly following DIM administration (5, 10, and 20 mg/kg) compared to the DSS-exposed mice (Fig. 1B). In addition, we found that colon length in the DSS-administered mice was significantly shorter than that in DIM-treated mice (Fig. 1C,D) at day 10 following DSS administration.

Histological examination of the colonic sections was used to assess intestinal inflammatory status. Microscopically, the samples from DSS-induced colitis mice showed typical inflammatory changes in colonic architecture, such as ulceration, crypt dilation, and goblet cell depletion, as well as mixed cell infiltration composed mainly of macrophages, lymphocytes, plasma cells, and granulocytes (Fig. 1E). Con-

versely, histological analysis of the colons from DIM-treated mice showed greatly reduced numbers of infiltrating cells and a lesser degree of mucosal injury and edema (Fig. 1E). In order to determine the side effects of DIM in the colon, we administered 20 mg/kg of DIM for 7 days without DSS exposure. Macroscopic and microscopic features did not change with DIM treatment (Fig. 1D and data not shown).

DIM Administration Reduced MPO Activity and Production of Proinflammatory Mediators in Colon Tissues

Since DIM administration reversed the severe clinical signs caused by DSS exposure, we next measured the effects of DIM on the production of inflammatory mediators. MPO is

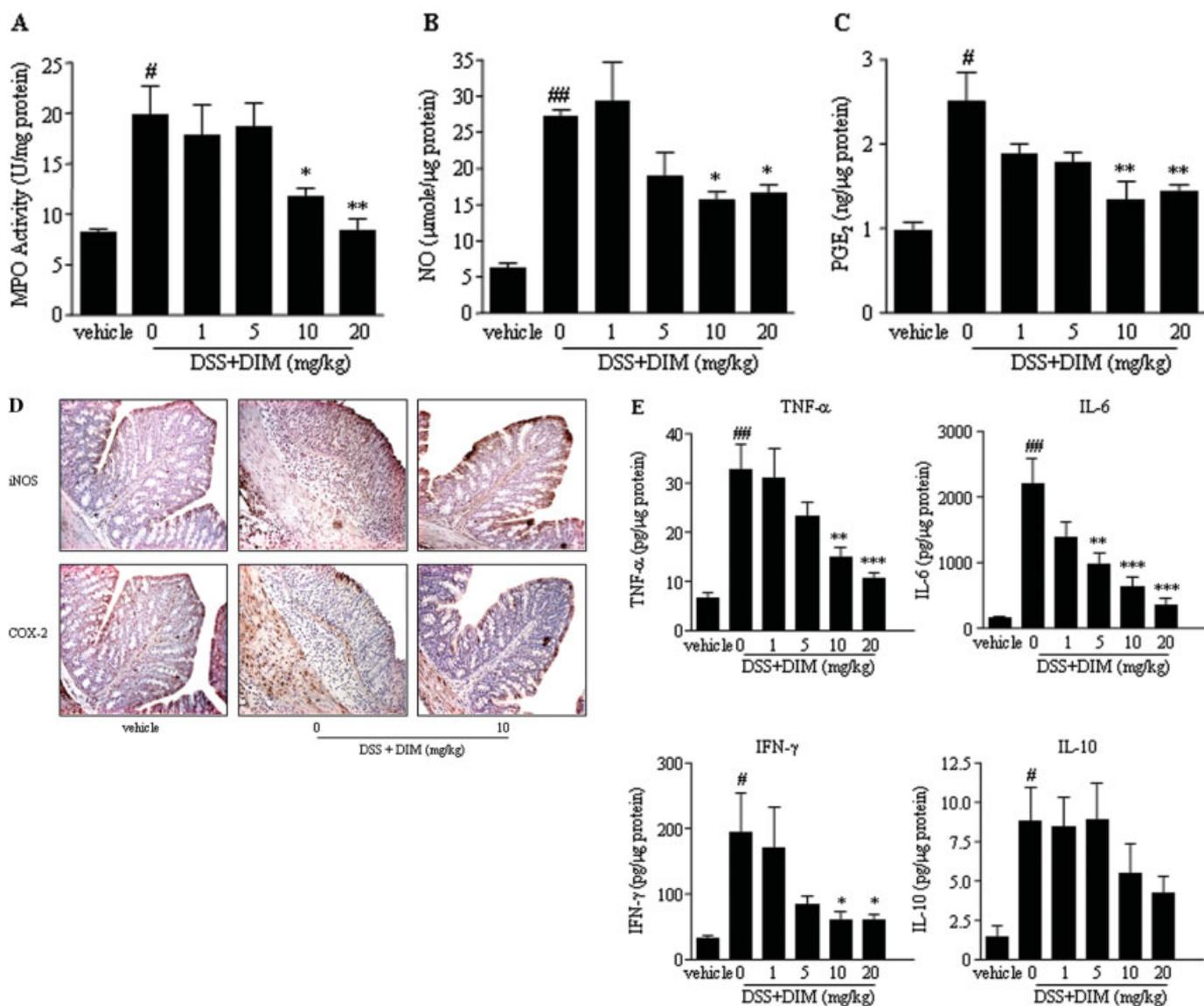


FIGURE 2. DIM suppresses the production of inflammatory mediators in colon. (A) MPO activity, (B) NO production, (C) PGE₂ production, and (E) inflammatory cytokine production in colonic tissue from dextran sodium sulfate (DSS)-exposed, DIM-treated mice. Segments of colon from DSS-exposed, DIM-treated mice and from vehicle-treated mice were prepared on day 10 after DSS exposure and cultured without any stimulation for 24 hours at 37 °C. Values are means \pm SEM ($n = 5$ per group). Significant differences in the DSS group, $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$). Significant difference between vehicle and DSS treatment alone are indicated, $#P < 0.01$ and $##P < 0.001$. (D) Colons in DSS-administered DIM-treated mice (10 mg/kg) and vehicle-treated control mice were prepared on day 10 after DSS exposure, and immunostaining was performed as described in Materials and Methods (original magnification $\times 100$). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

an enzyme produced mainly by polymorphonuclear leukocytes. It is correlated with the degree of neutrophil infiltration in tissues. Following 7 days of DSS treatment via drinking water, MPO activity was markedly increased, to a level ≈ 2.5 times higher than that in the control group (Fig. 2A). This increase in MPO activity was significantly reduced by DIM administration (10 and 20 mg/kg) at day 10, although the

inhibitory effect was absent at low doses of DIM treatment (1 and 5 mg/kg).

In recent years, NO has been considered an important inflammatory mediator playing a key role in the pathogenesis of IBD.^{5,6} In addition, the dramatic increase in mucosal PG synthesis during inflammation has been shown to correlate with human IBD activity and experimental coli-

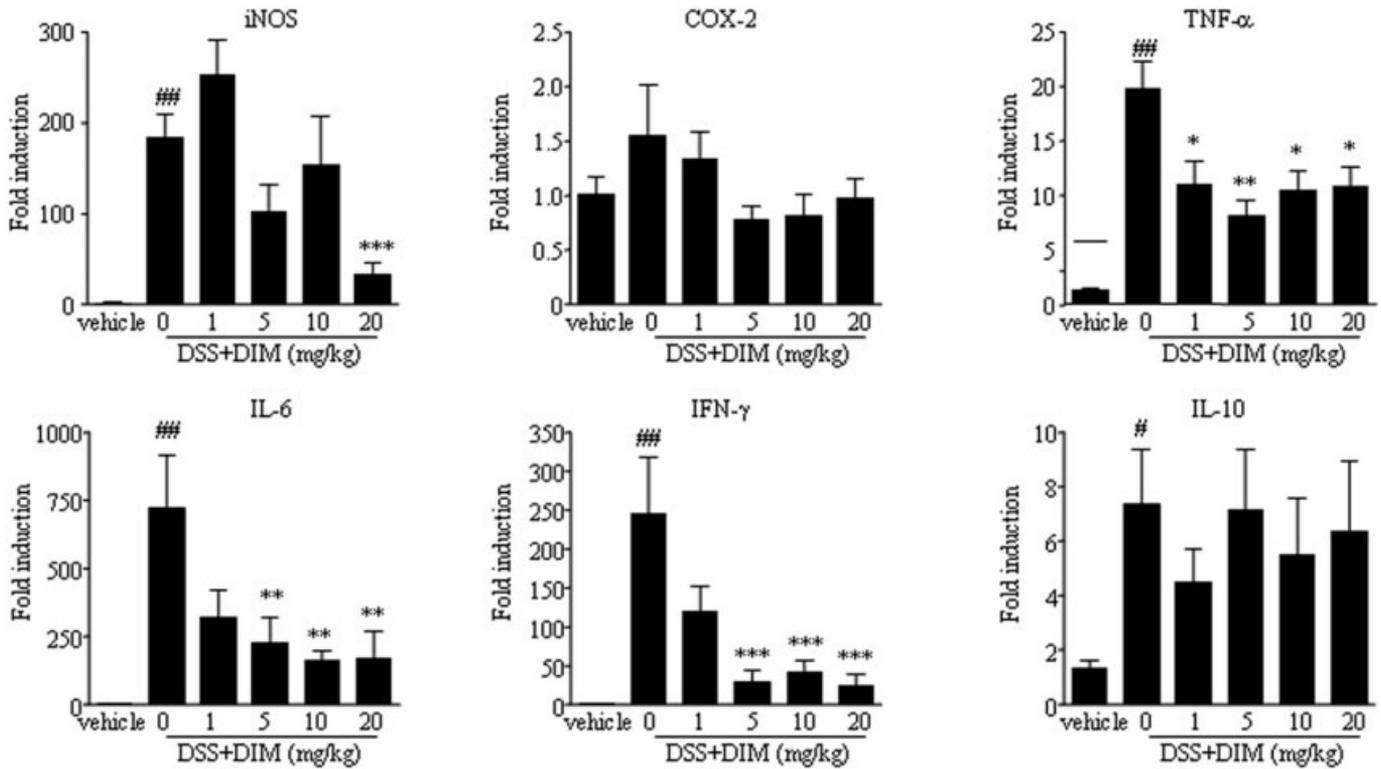


FIGURE 3. Colonic mRNA expression of inflammatory mediators in colons of DSS-induced colitis in DIM-treated mice. Colons in DSS-administered DIM-treated mice and DSS-treated control mice were prepared on day 10 after DSS exposure, and real-time RT-PCR was performed as described in Materials and Methods. Values are means \pm SEM ($n = 5$ per group). Significantly different DSS group, * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$. Significant differences between the vehicle and DSS treatment groups are indicated, # $P < 0.01$ and ## $P < 0.001$.

tis.^{7,8} For this reason, we evaluated the effect of DIM on colonic NO and PGE₂ production in DSS-induced experimental colitis. As shown in Figure 2B,C, oral administration of DIM reversed the increase in NO and PGE₂ production in a concentration-dependent manner at day 10 after DSS exposure.

Next we examined the effects of DIM on the expression of iNOS and COX-2 in the colon because iNOS and COX-2 are responsible for inducing NO and PGE₂, respectively. In the vehicle-treated noncolitic animals, iNOS and COX-2 immunoreactivities were detectable only in the epithelial cells located in the apical side of the villi, as shown in Figure 2D. In animals sampled 10 days after DSS administration, marked iNOS and COX-2 immunoreactivities were evident throughout the epithelial cells, as well as in the muscularis mucosa and submucosa. However, immunoreactivity was particularly noticeable in the infiltrating inflammatory cells. Administration of DIM significantly decreased the number of iNOS- and COX-2-producing cells, when compared with the DSS-treated group. These results suggest that infiltrating inflammatory cells may play a major role in the production of excess inflammatory mediators such as NO and PGE₂ in DSS-exposed colon.

To determine the effect of DIM on major inflammatory cytokine production in the colon, we measured the TNF- α , IL-6, IFN- γ , and IL-10 levels (Fig. 2E). After 10 days of DSS administration, TNF- α , IL-6, and IFN- γ levels had increased significantly to 32.6 ± 5.3 , 2196.0 ± 395.1 , and 193.4 ± 60.1 pg/mg of protein, respectively. This contrasted with the control group TNF- α , IL-6, and IFN- γ levels of 6.5 ± 1.1 , 144.3 ± 37.3 , and 31.6 ± 4.1 pg/mg of protein, respectively. DIM administration (20 mg/kg) prevented significant increases in TNF- α , IL-6, and IFN- γ levels (10.6 ± 1.1 , 347.4 ± 97.6 , and 60.2 ± 8.7 pg/mg of protein, respectively, at day 10). However, the IL-10 level was not significantly altered by DIM treatment.

To gain further insight into the molecular mechanisms underlying the suppression of colitis by DIM, we measured the mRNA expression levels of iNOS, COX-2, and inflammatory cytokines in the colon using real-time RT-PCR. As shown in Figure 3, the mRNA level for iNOS was significantly increased 10 days after the start of DSS exposure. The increases in iNOS were attenuated by 65.3% after DIM administration (20 mg/kg). In addition, the DIM treatment-induced reduction in COX-2 mRNA expression was shown to be statistically insignificant. DSS-induced

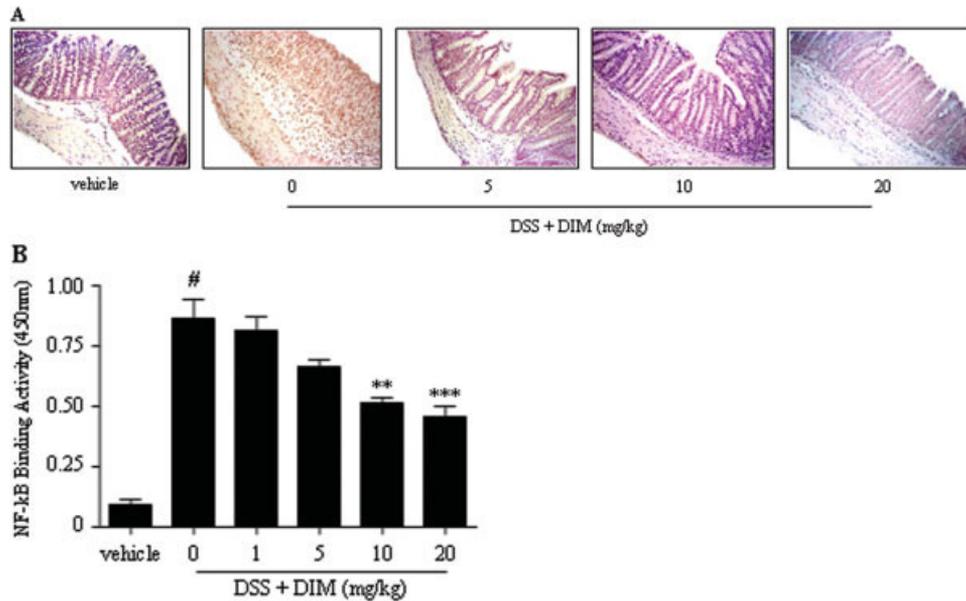


FIGURE 4. DIM attenuates NF- κ B activation in DSS-induced colitis mice. (A) Colons were obtained 10 days after DSS administration and NF- κ B-p65 staining was performed as described in Materials and Methods. (B) Nuclear cell extracts were obtained from colonic tissues of mice, and activated NF- κ B was measured using an oligonucleotide-based specific ELISA as described in Materials and Methods. Values are means \pm SEM ($n = 5$ per group). Significant differences in the DSS group are indicated, ** $P < 0.01$ and *** $P < 0.001$. The number sign (#) represents a significant difference between the vehicle and DSS treatment groups, $P < 0.001$. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

mRNA expression of TNF- α , IL-6, and IFN- γ also decreased in DIM-treated colons. Consistent with the production of IL-10, colonic IL-10 mRNA expression was not changed by DIM treatment.

DIM Inhibited NF- κ B Activation

To investigate the cellular mechanisms by which treatment with DIM attenuated DSS-induced intestinal inflammation, we evaluated the influence of DIM on the activation of NF- κ B in colonic tissues and intestinal epithelial cells, IEC-6. NF- κ B expression (p65) was detected in the nuclei of vascular endothelial and inflammatory cells, especially in the mucosa and submucosa, but such positive cells were seldom observed in the DIM-treated group (Fig. 4A). Similarly, in colonic tissues, exposure to DSS for 7 days stimulated the DNA-binding activity of NF- κ B compared with vehicle-treated animals, whereas treatment with DIM significantly decreased NF- κ B DNA-binding activity at day 10 (Fig. 4B).

In the healthy colon, intestinal epithelial cells form a physical barrier separating the myriad of gut antigens from the cells of the immune system. Moreover, intestinal epithelial cells play a secondary role in maintaining immune tolerance, while simultaneously initiating immune responses when potential pathogens are detected. In IBD, the line of defense provided by the intestinal epithelial cell is breached, resulting in an uncontrolled immune response.²⁵⁻²⁷ As intestinal epithelial cells are a principal mediator of immune

responses in the gut, we sought to determine if DIM affects NF- κ B activation in intestinal epithelial cells using IEC-6 cells. Consistent with NF- κ B expression and DNA binding activity in the colon, DIM treatment blocked I κ -B α degradation and NF- κ B nuclear translocation (Fig. 5A) in IEC-6 cells. Moreover, reduced NF- κ B DNA binding was observed in DIM-treated IEC-6 cells (Fig. 5B).

DIM Administration Suppressed Colitis-associated Colon Carcinogenesis

Finally, we examined the impact of DIM on colitis-associated cancer. The link between chronic inflammation and tumorigenesis has been well established for colorectal cancer.²⁸ Because DIM plays an important role in reducing intestinal inflammation, we believe DIM should impact inflammation-induced cancer in colon tissues. To test this hypothesis, we employed a mouse model of colitis-associated cancer, injecting mice with procarcinogen AOM followed by 3 cycles of oral DSS administration.^{29,30} The average tumor number per vehicle-treated mouse (32.4 ± 2.3) was 3 times greater than that in 10 and 20 mg/kg DIM-treated mice (10.3 ± 1.6 and 11.4 ± 1.5 , respectively; Fig. 6B). These results showed that tumor incidence was suppressed in DIM-treated mice, indicating that DIM administration reduced colitis-associated tumor induction.

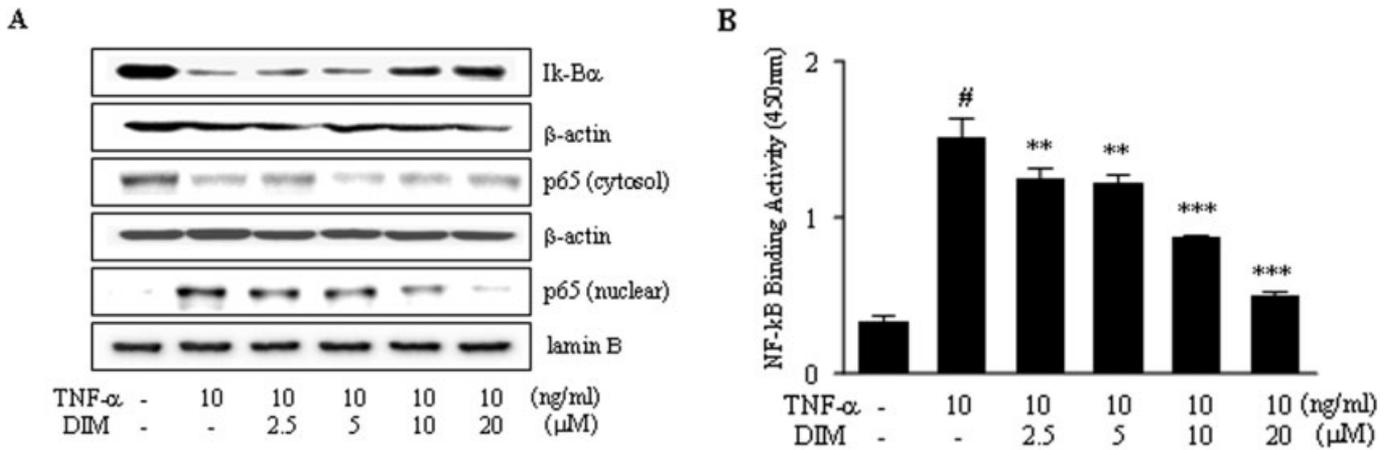


FIGURE 5. DIM attenuates NF-κB activation in intestinal epithelial cells. (A) IEC-6 cells, rat intestinal epithelial cell line, were preincubated for 1 hour with increasing DIM concentrations (0, 2.5, 5, 10, or 20 μM) and stimulated with 10 ng/mL TNF-α for 30 minutes. Whole cell extracts were isolated and the degradation of inhibitor of κB was determined by immunoblot analysis. (B) Nuclear cell extracts were obtained and activated NF-κB was measured using an oligonucleotide-based specific ELISA as described in Materials and Methods. Experiments were performed in quadruplicate, with results expressed as means ± SEM. Significant differences in the DSS group are indicated, **P* < 0.05, ***P* < 0.01, and ****P* < 0.001. The number sign (#) represents a significant difference between the vehicle and DSS treatment groups, *P* < 0.001.

DISCUSSION

Many previous investigations have shown that DIM, a major in vivo acid-catalyzed condensation product of indole-

3-carbinol, effectively induces apoptosis and inhibits tumor growth in several tumor cell lines and a variety of animal models.^{18–20,31–33} However, its effects on immune function,

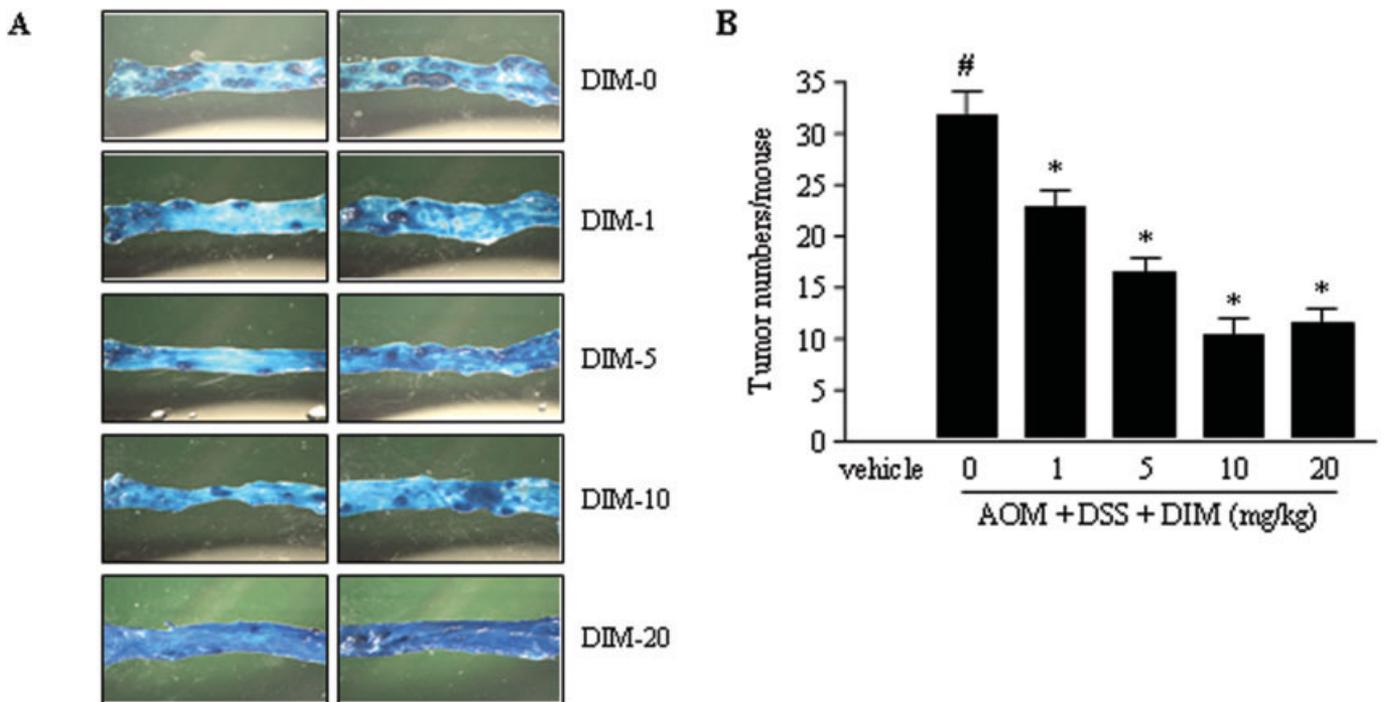


FIGURE 6. Reduced tumor formation after DIM treatment. (A) Macroscopic features in the colon. Colons were removed at day 56 and stained with 0.2% methylene. Representative results from 10 independent animals are shown. (B) Number of tumors. Colons were removed at day 56 to determine the numbers of macroscopic tumors. Results are expressed as means ± SEM (*n* = 10 per group). Significant differences in the AOM/DSS group are indicated, **P* < 0.001. The number sign (#) represents a significant difference between the vehicle and DSS treatment groups, *P* < 0.001. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

especially inflammation, remain largely unknown. Only 1 previous study showed that DIM exerts antiinflammatory effects in the LPS-stimulated murine macrophage cell line, RAW264.7.²¹ In the present study, we elucidated for the first time that DIM has therapeutic effects in the treatment of IBD, an inflammatory disease, through the reduction of inflammatory mediators via blockage of NF- κ B activation. Furthermore, DIM treatment also inhibited tumor formation in an AOM/DSS-induced colon cancer model.

In the healthy gut, the mucosal immune system ensures a balance between pro- and antiinflammatory mediators and thereby allows for effective defense against luminal pathogens. However, at the same time, the mucosal immune system prevents a devastating immune reaction directed against the huge amount of harmless luminal antigens, such as food components and nonpathological bacteria.¹ In IBD, this immunological balance is severely impaired and shifted toward the proinflammatory side.^{2,3} The chronic mucosal inflammation in IBD is caused by hyperactivation of immune cells, which produce high levels of proinflammatory cytokines like TNF- α , IL-6, and IFN- γ , resulting in colonic tissue damage.^{2,3,9–11} Although the pathogenesis of DSS-induced colitis appears to be T-cell-independent,³⁴ cytokine production plays a major role in the development of colitis in this model.^{35,36} In fact, it has been shown that cytokines are important mediators of inflammation.³⁷ Elevated production of proinflammatory cytokines is found in the inflamed colons of patients with IBD, as well as in animals with DSS colitis.^{35,36,38} Further evidence for the involvement of these cytokines came from the observation that antibodies against TNF- α and IL-12 reduced the severity of the disease in animals with DSS-induced colitis, as well as in patients with CD.^{39,40} Thus, reduction of proinflammatory cytokine production appears to be an effective approach to the prevention and treatment of IBD. In the present study the colons of DIM-administered mice exhibited significantly lower amounts of proinflammatory cytokines such as TNF- α , IL-6, and IFN- γ , compared with those from vehicle-administered animals, and were protected from DSS-induced colitis. This finding suggests that the inflammation protection observed in DIM-treated mice may, in part, be the result of reduced cytokine production.

NF- κ B was identified as 1 of the key regulators in inflammation. Its activation is markedly induced in IBD patients. Through its ability to promote the expression of various proinflammatory genes, NF- κ B strongly influences the course of mucosal inflammation.^{41,42} These previous observations suggest that DIM may exert an antiinflammatory effect by blocking the NF- κ B signaling pathway, and this has been the mechanism most commonly proposed in the past to explain the biological effectiveness of DIM.^{21,32,43–45} In this study, we noted a remarkable suppression of NF- κ B DNA binding activity in DIM-treated IEC-6 cells stimulated by TNF- α , as well as in colonic tissues challenged by DSS.

These results strongly suggest that DIM suppressed inflammatory reactions in DSS-induced colonic inflammation by inhibiting NF- κ B activation and indicate that suppressed production of NO, PGE₂, and proinflammatory cytokines such as IL-6, TNF- α , and IFN- γ in DIM-treated colons might be the result of inhibited NF- κ B activation. In addition, the role of NF- κ B activation in tumor development has been demonstrated using various animal models, including a mouse model of colitis-associated cancer.⁴⁶ Deletion of I κ B kinase (IKK β) in intestinal epithelial cells leads to a dramatic decrease in tumor incidence in such models, whereas deletion of IKK β in myeloid cells in these mice results in decreased tumor size through diminished expression of proinflammatory cytokines that act as tumor growth factors.⁴⁶ These results highlight the ability of NF- κ B to link inflammation and cancer and provide additional evidence for the specific inactivation of NF- κ B as a promising means by which to attenuate the formation of inflammation-associated tumors.

How DIM suppresses the DNA binding activity of NF- κ B is unclear. Staub et al.⁴⁷ demonstrated that DIM was rapidly transferred to the nuclear and membrane fractions of MCF-7 breast cancer cells and then was slowly metabolized by P450 oxidation to hydroxylated products that are conjugated with sulfate. They also observed DIM partitioned directly into isolated nuclei. Within 30 minutes of the treatment of MCF-7 cells with ³H-DIM, up to 40% of the dose had accumulated in the nuclei. DIM seems to be directly associated with the bilipid membranes since DIM is recovered in the membrane pellet following centrifugation (100,000g) after homogenization and disruptive sonication of the isolated nuclei. This finding indicates that the partitioning of DIM into membranes may contribute to its biological activities. Importantly, a massive increase in the localized concentration of DIM in nuclei may be in part responsible for its biological activity.

In conclusion, our findings suggest that DIM has potential applications in the treatment of IBD and inflammation-associated tumors.

REFERENCES

1. Strober W, Fuss IJ, Blumberg RS. The immunology of mucosal models of inflammation. *Annu Rev Immunol.* 2002;20:495–549.
2. Fiocchi C. Inflammatory bowel disease: etiology and pathogenesis. *Gastroenterology.* 1998;115:182–205.
3. Hendrickson BA, Gokhale R, Cho JH. Clinical aspects and pathophysiology of inflammatory bowel disease. *Clin Microbiol Rev.* 2002;15:79–94.
4. Herszenyi L, Miheller P, Tulassay Z. Carcinogenesis in inflammatory bowel disease. *Dig Dis.* 2007;25:267–269.
5. Araki Y, Sugihara H, Hattori T. The free radical scavengers edaravone and tempol suppress experimental dextran sulfate sodium-induced colitis in mice. *Int J Mol Med.* 2006;17:331–334.
6. Kolios G, Valatas V, Ward SG. Nitric oxide in inflammatory bowel disease: a universal messenger in an unsolved puzzle. *Immunology.* 2004;113:427–437.
7. Martín AR, Villegas I, Alarcón de la Lastra C. The COX-2 inhibitor,

- rofecoxib, ameliorates dextran sulphate sodium induced colitis in mice. *Inflamm Res*. 2005;54:145–151.
8. Halter F, Tarnawski AS, Schmassmann A, et al. Cyclooxygenase 2-implications on maintenance of gastric mucosal integrity and ulcer healing: controversial issues and perspectives. *Gut*. 2001;49:443–453.
 9. Rojas-Cartagena C, Flores I, Appleyard CB. Role of tumor necrosis factor receptors in an animal model of acute colitis. *Cytokine*. 2005;32:85–93.
 10. Ito R, Shin-Ya M, Kishida T, et al. IL-6 signaling in inflammatory bowel disease: pathophysiological role and clinical relevance. *Inflamm Bowel Dis*. 2007;13:1016–1023.
 11. Yen D, Cheung J, Scheerens H, et al. IL-23 is essential for T cell-mediated colitis and promotes inflammation via IL-17 and IL-6. *J Clin Invest*. 2006;116:1310–1316.
 12. Gisbert JP, González-Lama Y, Maté J. 5-Aminosalicylates and renal function in inflammatory bowel disease: a systematic review. *Inflamm Bowel Dis*. 2007;13:629–638.
 13. Ananthkrishnan AN, Attila T, Otterson MF, et al. Severe pulmonary toxicity after azathioprine/6-mercaptopurine initiation for the treatment of inflammatory bowel disease. *J Clin Gastroenterol*. 2007;41:682–688.
 14. Giampaolo B, Giuseppe P, Michele B, et al. Treatment of active steroid-refractory inflammatory bowel diseases with granulocytapheresis: our experience with a prospective study. *World J Gastroenterol*. 2006;12:2201–2204.
 15. Brown SJ, Mayer L. The immune response in inflammatory bowel disease. *Am J Gastroenterol*. 2007;102:2058–2069.
 16. Stokkers PC, Hommes DW. New cytokine therapeutics for inflammatory bowel disease. *Cytokine*. 2004;28:167–173.
 17. De Kruif CA, Marsman JW, Venekamp JC, et al. Structure elucidation of acid reaction products of indole-3-carbinol: detection in vivo and enzyme induction in vitro. *Chem Biol Interact*. 2001;80:303–315.
 18. Nachshon-Kedmi M, Yannai S, Haj A, et al. Indole-3-carbinol and 3,3'-diindolylmethane induce apoptosis in human prostate cancer cells. *Food Chem Toxicol*. 2003;41:745–752.
 19. Ali S, Banerjee S, Ahmad A, et al. Apoptosis-inducing effect of erlotinib is potentiated by 3,3'-diindolylmethane in vitro and in vivo using an orthotopic model of pancreatic cancer. *Mol Cancer Ther*. 2008;7:1708–1719.
 20. Hong C, Kim HA, Firestone GL, et al. 3,3'-Diindolylmethane (DIM) induces a G(1) cell cycle arrest in human breast cancer cells that is accompanied by Sp1-mediated activation of p21(WAF1/CIP1) expression. *Carcinogenesis*. 2002;23:1297–1305.
 21. Cho HJ, Seon MR, Lee YM, et al. 3,3'-Diindolylmethane suppresses the inflammatory response to lipopolysaccharide in murine macrophages. *J Nutr*. 2008;138:17–23.
 22. Copper HS, Murthy SNS, Shah RS, et al. Clinicopathologic study of dextran sulfate sodium experimental murine colitis. *Lab Invest*. 1993;66:635–639.
 23. Kwon HS, Oh SM, Kim JK. Glabridin, a functional compound of licorice, attenuates colonic inflammation in mice with dextran sulfate sodium-induced colitis. *Clin Exp Immunol*. 2008;151:165–173.
 24. Kim JK, Oh SM, Kwon HS, et al. Anti-inflammatory effect of roasted licorice extracts on lipopolysaccharide-induced inflammatory responses in murine macrophages. *Biochem Biophys Res Commun*. 2006;345:1215–1223.
 25. Lichtenstein GR. Chemokines and cytokines in inflammatory bowel disease and their application to disease treatment. *Curr Opin Gastroenterol*. 2000;16:83–88.
 26. Danese S, Gasbarrini A. Chemokines in inflammatory bowel disease. *J Clin Pathol* 2005;58:1025–1027.
 27. Keates S, Keates AC, Mizoguchi E, et al. Enterocytes are the primary source of the chemokine ENA-78 in normal colon and ulcerative colitis. *Am J Physiol*. 1997;273:G75–G82.
 28. Itzkowitz SH, Yio X. Inflammation and cancer. IV. Colorectal cancer in inflammatory bowel disease: the role of inflammation. *Am J Physiol Gastrointest Liver Physiol*. 2004;287:G7–17.
 29. Popivanova BK, Kitamura K, Wu Y, et al. Blocking TNF-alpha in mice reduces colorectal carcinogenesis associated with chronic colitis. *J Clin Invest*. 2008;118:560–570.
 30. Suzuki R, Kohno H, Sugie S, et al. Sequential observations on the occurrence of preneoplastic and neoplastic lesions in mouse colon treated with azoxymethane and dextran sodium sulfate. *Cancer Sci*. 2004;95:721–727.
 31. Sarkar FH, Rahman KM, Li Y. Bax translocation to mitochondria is an important event in inducing apoptotic cell death by indole-3-carbinol (I3C) treatment of breast cancer cells. *J Nutr*. 2003;133:2434S–2439S.
 32. Aggarwal BB, Ichikawa H. Molecular targets and anticancer potential of indole-3-carbinol and its derivatives. *Cell Cycle*. 2005;4:1201–1215.
 33. Nachshon-Kedmi M, Fares FA, Yannai S. Therapeutic activity of 3,3'-diindolylmethane on prostate cancer in an in vivo model. *Prostate*. 2004;61:153–160.
 34. Dieleman LA, Ridwan BU, Tennyson GS, et al. Dextran sulfate sodium-induced colitis occurs in severe combined immunodeficient mice. *Gastroenterology*. 1994;107:1643–1652.
 35. Siegmund B, Fantuzzi G, Rieder F, et al. Neutralization of interleukin-18 reduces severity in murine colitis and intestinal IFN-gamma and TNF-alpha production. *Am J Physiol* 2001;281:R1264–R1273.
 36. Dieleman LA, Palmen MJ, Akol H, et al. Chronic experimental colitis induced by dextran sulphate sodium (DSS) is characterized by Th1 and Th2 cytokines. *Clin Exp Immunol*. 1998;114:385–391.
 37. Pullman WE, Elsbury S, Kobayashi M, et al. Enhanced mucosal cytokine production in inflammatory bowel disease. *Gastroenterology*. 1992;102:529–537.
 38. Murthy S, Cooper HS, Yoshitake H, et al. Combination therapy of pentoxifylline and TNFalpha monoclonal antibody in dextran sulphate-induced mouse colitis. *Aliment Pharmacol Ther*. 1999;13:251–260.
 39. Neurath MF, Fuss I, Kelsall BL, et al. Antibodies to interleukin 12 abrogate established experimental colitis in mice. *J Exp Med*. 1995;182:1281–1290.
 40. Sandborn WJ, Hanauer SB. Antitumor necrosis factor therapy for inflammatory bowel disease: a review of agents, pharmacology, clinical results, and safety. *Inflamm Bowel Dis*. 1999;5:119–133.
 41. Atreya I, Atreya R, Neurath MF. NF-kappaB in inflammatory bowel disease. *J Intern Med*. 2008;263:591–596.
 42. Schottelius AJ, Dinter H. Cytokines, NF-kappaB, microenvironment, intestinal inflammation and cancer. *Cancer Treat Res*. 2006;130:67–87.
 43. Rahman KM, Ali S, Aboukameel A, et al. Inactivation of NF-kappaB by 3,3'-diindolylmethane contributes to increased apoptosis induced by chemotherapeutic agent in breast cancer cells. *Mol Cancer Ther*. 2007;6:2757–2765.
 44. Rahman KW, Sarkar FH. Inhibition of nuclear translocation of nuclear factor-kappaB contributes to 3,3'-diindolylmethane-induced apoptosis in breast cancer cells. *Cancer Res*. 2005;65:364–371.
 45. Kong D, Li Y, Wang Z, et al. Inhibition of angiogenesis and invasion by 3,3'-diindolylmethane is mediated by the nuclear factor-kappaB downstream target genes MMP-9 and uPA that regulated bioavailability of vascular endothelial growth factor in prostate cancer. *Cancer Res*. 2007;67:3310–3319.
 46. Greten FR, Eckmann L, Greten TF, et al. IKKbeta links inflammation and tumorigenesis in a mouse model of colitis-associated cancer. *Cell*. 2004;118:285–296.
 47. Staub RE, Onisko B, Bjeldanes LF. Fate of 3,3'-diindolylmethane in cultured MCF-7 human breast cancer cells. *Chem Res Toxicol*. 2006;19:436–442.