Pharmacological Reports
2011, 63, 1210–1221
ISSN 1734-1140

Chemopreventive effects of NSAIDs on cytokines and transcription factors during the early stages of colorectal cancer

Vivek Vaish, Sankar N. Sanyal

Department of Biophysics, Panjab University, Chandigarh 160014, India

Correspondence: Sankar N. Sanyal, e-mail: sanyalpu@gmail.com

Abstract:
The earliest stages of colorectal cancer have been linked to inflammatory responses caused by carcinogens, but the molecular signaling of various pro- and anti-inflammatory cytokines and transcription factors in colorectal cancer remains unknown. The higher expression and secretion of various pro-inflammatory cytokines and their autocrine and paracrine functions play an important role in the activation of transcription factors, which in turn promote tumorigenesis. NF-κB serves as a vital biomolecule that transcribes a number of pro-inflammatory cytokines and anti-apoptotic proteins. Pro-inflammatory cytokines can also activate Jak3/Stat3 signaling pathways, thereby increasing inflammation and cell survival. In the present study, the expression of IL-1β, IL-2, IL-4, IFNγ, TNF-α, iNOS, COX-2, Jak3, Stat3 and NF-κB were increased in the early stages of experimental colorectal cancer. The increased expression of these inflammatory molecules was reversed by the simultaneous administration of non-steroidal anti-inflammatory drugs (NSAIDs; sulindac and celecoxib). The anti-inflammatory activity of the NSAIDs was found to be mediated by the inhibition of NF-κB (p65) DNA-binding activity. The anti-neoplastic end effect of the NSAIDs in the isolated colonocytes was demonstrated by an increased level of apoptosis. This study suggests that NSAIDs inhibit NF-κB and Jak3/Stat3 signaling and down-regulate pro-inflammatory cytokines to a level that inhibits inflammation and carcinogenesis.

Key words:
carcinogenesis, cytokines, inflammation, NSAIDs, transcription factors

Introduction

Colorectal cancer has become a leading cause of cancer mortality worldwide. The progression of adenomatous polyps to late stage cancer is a relatively slow process and suggests an opportunity for chemoprevention at an early stage [37]. The levels of prostaglandins (PGs), which are synthesized by the enzyme cyclooxygenase (COX), have been shown to be higher in human colorectal tumors [6, 26]. Among the two isoforms of COX, (COX-1 and COX-2), evidence suggests that COX-2 is primarily involved in the propagation of malignancies [16].

The interactions between tumor and host cells are mediated by growth factors and cytokines, which function as paracrine factors for endothelial and stromal cells [8]. Interleukin-1 (IL-1), which is a pro-inflammatory cytokine, increases the expression of COX-2 and the production of PGE_2 at the site of inflammation. The inhibition of COX-2 can also suppress the inflammatory effects of IL-1, suggesting that the COX-2/PGE_2 pathway is involved in the pro-inflammatory signaling of cytokines [28]. IL-1β in

particular has been documented to induce the expression of COX-2 in colorectal cancer [13, 14].

The higher expression of COX-2 in malignant tissues is also related to nuclear factor-κB (NF-κB), which positively regulates the *cox-2* gene. Under physiological conditions, NF-κB usually remains inactive in the cytoplasm because it is bound to the inhibitory protein IκB. NF-κB is released due to the phosphorylation of IκB by IκB kinase (IKK) during an inflammatory response. The major downstream signaling effect of NF-κB activation is the binding of tumor necrosis factor-α (TNF-α) and IL-1 to their receptors [22, 38]. The activation of NF-κB during an inflammatory response leads to the expression of various other pro-inflammatory cytokines, including interleukin-2 (IL-2), which may cause activation-induced cell death (AICD) in antigen-generated cytotoxic lymphocytes after recognizing the tumor as self [39]. However, the actual role of IL-2 in colon cancer progression or regression is still unknown.

IL-2 can activate Janus kinase 3 (Jak3) by autotransphosphorylation [4]. Jak3 functions as an upstream kinase that activates a signal transducer and activator of transcription 3 (Stat3) [2]. The activation of Stat is also mediated by interferons [17], although the role of interferon-γ (IFNγ) in Stat3 activation in colon cancer progression is unknown. Overexpression and aberrant expression of Jak3/Stat3 have been observed in human colon cancer *in vivo* and *in vitro* and have been shown to prevent apoptosis in the tumor, leading to poor prognosis [12, 33].

Non-steroidal anti-inflammatory drugs (NSAIDs) are a class of promising antineoplastic agents for the suppression of tumor progression induced by the inflammatory response [1]. They may exert their effects by down-regulating pro-inflammatory cytokines and/or growth factors and transcription factors, the mechanism of which is not well understood. In an earlier study by our laboratory, we linked the inhibitory effects of NSAIDs to NF-κB and COX-2 expression in the early neoplasm of colon [35].

The present study investigated the chemopreventive effects of two NSAIDs (sulindac and celecoxib) on the expression of pro- and anti-inflammatory cytokines, transcription factors and COX-2. The expression of iNOS protein was measured as a molecular marker for inflammation in colorectal cancer. For this study, an *in vivo* model of inflammatory colorectal cancer was generated in Sprague-Dawley rats after the administration of 1,2-dimethylhydrazine dihydrochloride (DMH), which is a colon-specific carcinogen.

Materials and Methods

Chemicals

DMH, sulindac and the Bradford reagent were purchased from Sigma-Aldrich (St. Louis, MO, USA). Celecoxib was a generous gift from Ranbaxy Pharmaceuticals (Gurgaon, India). Primary antibodies against COX-2, IL-1β, IL-2, IL-4, TNF-α, IFNγ, iNOS, Jak3, β-actin and Stat3 were purchased from Santa Cruz Biotechnology (CA, USA) and BD Biosciences (CA, USA). Alkaline phosphatase-conjugated secondary antibodies, fluorescein isothiocyanate (FITC)-conjugated secondary antibodies and 5-bromo-4-chloro-3-indolyl phosphate – nitroblue tetrazolium chloride (BCIP-NBT) were purchased from Genei (Bangalore, India). All of the other chemicals and reagents used in the present study were of analytical grade and purchased from reputed Indian manufacturers.

Animal procurement

Male Sprague-Dawley rats with a body weight between 150 and 200 g were obtained from the inbred population of the Central Animal House, Panjab University, Chandigarh. They were acclimatized to the control diet (rodent chow) and water *ad libitum* for at least 1 week. They were maintained according to the principles and guidelines of the Ethics Committee of Animal Care of Panjab University in accordance with the Indian National Law on animal care and use. They were housed three per cage in polypropylene cages with a wire mesh top and a hygienic bed of husk (regularly changed) in a well-ventilated animal room until the end of the experimental period. The animals were also maintained at ambient temperature and humidity and under a 12 h light/dark period.

Treatment schedule

The animals were assorted into the following groups:

Control group, vehicle-treated

The animals were administered the vehicle (1 mM EDTA-saline subcutaneously (*sc*) *via* weekly injection and received 0.5% carboxymethylcellulose sodium salt (CMC) daily per oral (*po*).

1,2-Dimethylhydrazine dihydrochloride (DMH) group

The animals were administered DMH weekly at a dose of 30 mg/kg body weight (*sc*). This dose of DMH has been previously established by our laboratory [10, 34]. DMH was freshly prepared in 1 mM EDTA-saline, and the pH was adjusted to 7.0 using a dilute NaOH solution.

DMH + sulindac group

Sulindac was given daily *po* within its therapeutic anti-inflammatory dose (ED_{50} for rats, 20 mg/kg b.w.) to the animals along with weekly DMH administration of 30 mg/kg b.w.

DMH + celecoxib group

Celecoxib was administered daily *po* (ED_{50} for rats, 6 mg/kg b.w.) to the animals along with weekly DMH administration of 30 mg/kg b.w.

After six weeks, the animals fasted overnight with drinking water available *ad libitum* and were sacrificed the next day by an overdose of ether anesthesia. The body weights of the animals in all of the groups were recorded every week until the termination of the experiment.

Gross morphological observations

The colons were removed and flushed clear with ice-cold physiological saline (NaCl solution, 9 g/l). The colons were opened longitudinally along the median and laid flat to examine the incidence of macroscopic neoplastic lesions/plaques, known as multiple plaque lesions (MPLs). The colons were divided into proximal, medial and distal segments for the examination.

Aberrant crypt foci (ACF)

The colons were removed, flushed clean, cut open and divided into different parts as described above. After a minimum of 24 h fixation in 10% buffered formalin, the colons were stained with 0.2% methylene blue in Krebs-Ringer solution for 5–10 min [23]. The mucosal surface of the colon was evaluated for the number of ACF in the stained colon under 40× magnification using a light microscope. Enlarged and slightly elevated lesions with increased staining were readily identifiable in comparison to the normal adjacent mucosa. These lesions were classified as single enlarged crypts or foci containing two or more abnormal crypts.

Isolation of colonic epithelial cells

Colonic epithelial cells (colonocytes) were obtained from freshly isolated colons by the method of Mouille et al. [18], as originally described by Roediger and Truelove [25]. The colonic segments starting from the cecum to the rectal ampulla were rapidly removed and flushed clear with chilled physiological saline (NaCl solution, 9 g/l) followed by calcium- and magnesium-free Krebs-Hanseleit (K-H) bicarbonate buffer (pH 7.4) containing 10 mM HEPES, 5 mM dithiothreitol (DTT) and 2.5 g/l bovine serum albumin (BSA). The K-H buffer was equilibrated with a mixture of O_2 and CO_2 (19:1, v/v). Each colon was everted, distally ligated and distended as much as possible using a syringe containing calcium-free K-H saline with 0.25% w/v BSA. These sacs were then placed in a plastic flask containing 100 ml calcium-free K-H buffer, 5 mM EDTA and 0.25% BSA. The flask was gassed with O_2 and CO_2 (19:1 v/v) and incubated at 37°C in a shaking water bath at 60–70 oscillations per minute for 30 min. Thereafter, the colons were rinsed in fresh calcium-free saline to remove the excess EDTA and placed in a plastic beaker with 50 ml K-H buffer containing 0.25% w/v BSA. Manual stirring with a plastic stirrer for 2 min readily disaggregated the colonocytes, which were then separated by centrifugation at 500 × g for 60 s. The cells were washed two times in oxygenated K-H buffer containing normal amounts of calcium (2.5 mM $CaCl_2$), 5 mM DL-DTT and 2.5% w/v BSA. The cells were washed and resuspended in PBS for further studies.

Morphological analysis of apoptosis (fluorescence microscopy)

Acridine orange/ethidium bromide co-staining

Fluorescence microscopy using the DNA-binding fluorescent dyes acridine orange and ethidium bromide was employed to study the morphology of the isolated colonocytes undergoing apoptosis [5]. Briefly, 10 µl of the cell suspension (10^6 cells/ml) was mixed in PBS (pH 7.4) containing acridine orange (1 µg/ml) and ethidium bromide (1 µg/ml) [27]. Ten microliters of this mixture was placed on a clean glass

slide and covered with a coverslip. A total of 300 cells from four different slides were observed for each animal group at 400× magnification using a fluorescence microscope (Axioscope A1, Carl Zeiss, Germany). The index of apoptosis was calculated as the percentage of the total number of cells with apoptotic nuclei to the total number of cells counted.

NF-κB assay

An NF-κB (p65) transcription factor assay kit (Catalogue No. 10007889) was purchased from the Cayman Chemical Company (MI, USA). 1×10^7 cells were used for the extraction of approximately 50 μg of nuclear protein and the detection of NF-κB, according to the manufacturer's protocol. A graph was plotted for the NF-κB activity [absorbance at 450 nm/cell extract (50 μg/well)] against all of the groups.

Western blot analysis

Protein samples and nuclear extracts (50 μg) from each group were separated on a 10% gel by SDS-PAGE. The separated proteins were electrophoretically transferred to a nitrocellulose membrane (Millipore, Bangalore, India), and the transfer was checked by staining with Ponceau S. Immunoblotting was conducted using primary antibodies (COX-2: 1:1,000, IL-1β: 1:1,000, IL-2: 1:1,000, IL-4: 1:1,000, IFNγ: 1:1,000, TNF-α: 1:500, iNOS: 1:500, Jak3: 1:1,000, Stat3: 2 μg/ml and β-actin: 1:10,000) and alkaline phosphatase-conjugated IgG secondary antibodies. The BCIP-NBT detection system was used to develop the blot. The bands were analyzed using ImageJ software (NIH, Bethesda, MD, USA), and the density was expressed in gray values.

For the preparation of cell lysates, the colons were removed and rinsed from the different groups after completion of 6 weeks of treatment. Total lysates were prepared in fresh ice-cold lysis buffer (10 mM Tris, 100 mM NaCl, 5 mM EDTA, 1% Triton-X100, 1 mM PMSF and 2 mM DTT (pH 8)). The extracts were cleared by centrifugation at 10,000 × g for 10 min at 4°C, and the supernatants were collected.

For the nuclear extracts, the nuclei from the colonic tissues were suspended at 0–4°C in 50 mM NaCl, 10 mM HEPES, pH 7.6, 0.1 mM EDTA, 25% glycerol and 0.5 mM PMSF and pelleted by centrifugation in an Eppendorf centrifuge at 5,000 × g for 15 min at 4°C. The resulting nuclear debris was incubated in the same buffer for 30 min on ice and centrifuged at 10,000 × g at 4°C for 10 min. The resultant supernatant was used as the nuclear extract in the present study. The protein concentration was determined by the Bradford method [3].

Immunofluorescence analysis

Five-micrometer thick paraffin sections were deparaffinized in mixed xylenes (CAS No. 1330-20-7) for

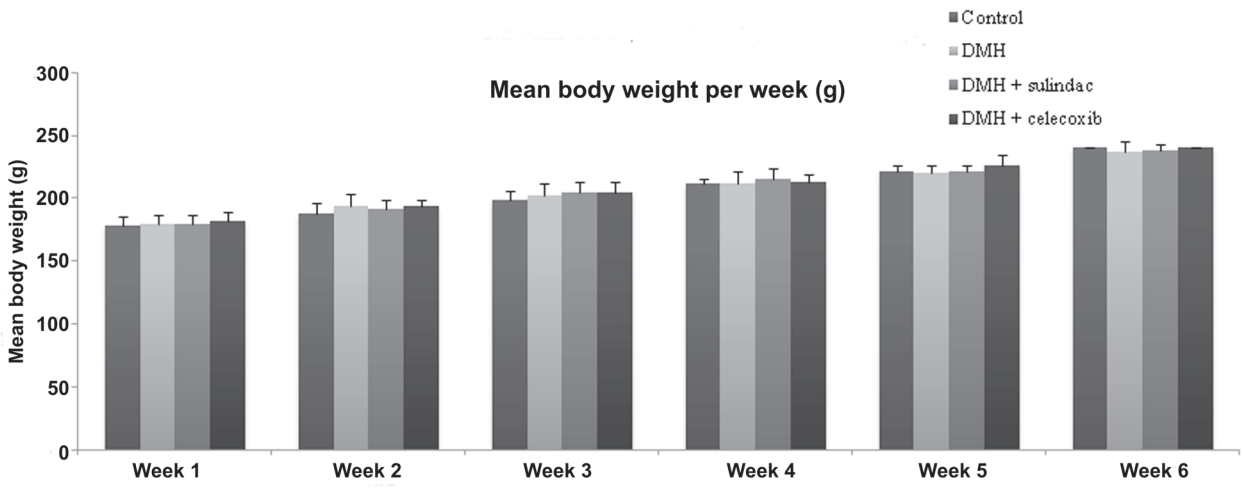


Fig. 1. This figure shows the mean body weight (g) of the animals segregated into 4 different groups for each week of the 6-week treatment schedule

Tab. 1. The occurrence of multiple plaque lesions (MPLs) after 6 weeks of treatment in the different treatment groups

Animal groups	No. of MPLs in different regions of the colon			Total no. of MPLs	No. of rats with MPL/total no. of rats	MPL incidence (%)	MPL burden	MPL multiplicity
	Proximal	Middle	Distal					
Control	0	0	0	0	No MPL	0	0	0
DMH	7 ± 0.87	10 ± 1.25	6 ± 0.75	23 ± 1.70	8/8	100	2.87	2.87
DMH + sulindac	2 ± 0.25	4 ± 0.5	3 ± 0.37	9 ± 1.25	5/8	62.5	1.125	1.8
DMH + celecoxib	3 ± 0.37	4 ± 0.5	3 ± 0.37	10 ± 1.08	5/8	62.5	1.25	2.00

MPL incidence = the percentage of animals with MPLs. MPL burden = the total number of MPLs counted/total number of rats. MPL multiplicity = the total number of MPLs counted/number of MPL-bearing rats. The values represent the mean ± SD of 8 animals from each group

30 min, gradually hydrated in a descending series of alcohol (100%, 90%, 70%, 50%, and 30%) and subsequently hydrated in water. The sections were incubated at 95°C for 5 min in 50 mM glycine-HCl buffer (pH 3.5) with 0.01% (w/v) EDTA for antigen retrieval. Fresh buffer (10 ml) was added, and the sections were incubated in this solution at 95°C for 5 min. The slides were allowed to cool for approximately 20 min and washed in three changes of distilled water. Non-specific binding was blocked by incubating the sections in 2% bovine serum albumin (BSA) in phosphate buffered saline (PBS; 10 mM, pH 7.2) for 30 min at 37°C. The sections were then incubated in 1.5% BSA with primary antibodies against IL-1β (1:1,000), IL-2 (1:1,000), IL-4 (1:1,000), IFNγ (1:1,000), TNF-α (1:500) and iNOS (1:500) in a moist chamber for 2 h at 37°C. For the negative control, 1.5% BSA was added without primary antibody. After the incubation, the sections were washed three times with PBS, PBS Tween (PBS with 0.05% Tween 20) and PBS, successively, for 5 min each. The sections were then incubated with fluorescein isothiocyanate (FITC)-conjugated secondary antibodies at a dilution of 1:10,000 for 2 h at 37°C in the dark. The sections were washed again in the same manner described above, counterstained with propidium iodide (PI) for 20 min at 37°C in the dark and washed as described previously. The sections were mounted in glycerol (1:10 in PBS), sealed with nail polish and observed under a fluorescence microscope at 400× magnification.

Statistical analysis

The data were expressed as the mean ± SD of four independent observations for each group. A one-way analysis of variance (ANOVA) was used to compare the means between the different treatments with the least significant difference (LSD) *post-hoc* comparison. The statistical software package SPSS v14 for Windows was used for this purpose. A value of $p < 0.05$ was considered significant in the present study.

Results

There were no significant differences in the body weights of the animals among all 4 of the groups during the 6-week treatment schedule (Fig. 1).

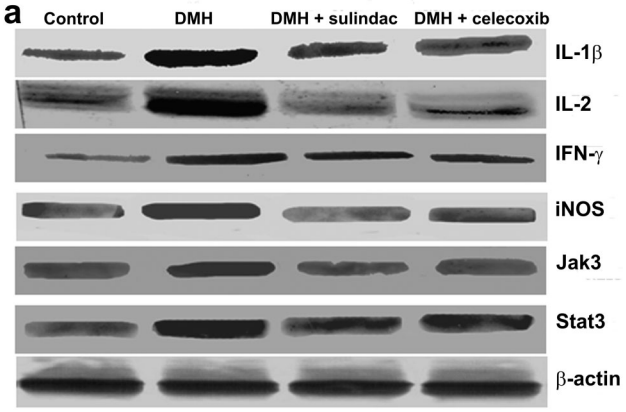
The colons were examined by a handheld lens to determine the incidence of MPLs. The MPL incidence was 100% in the DMH-treated group, while an incidence of 62.5% was observed in the groups co-administered NSAIDs (Tab. 1). The MPLs in rats largely resemble polyps in humans, which occur as either large, single outgrowths or small, grouped outgrowths of tissue that may or may not be raised. No MPLs were observed in the control group.

Aberrant crypts (ACs) and ACFs were counted after methylene blue staining under a light microscope at 40× magnification. These ACs and ACFs are thought to be pre-neoplastic lesions that respond to carcinogen treatment and stain more darkly than normal crypts. The microscopic plane of the ACs and the ACFs was higher than the normal crypts because of the thickening of the epithelial layer. ACs are generally larger than ACFs and are present as single lesions. In the DMH-treated group, the number of ACs and ACFs was higher than in the other groups; no ACs or ACFs were present in the control group (Tab. 2).

Tab. 2. The occurrence of aberrant crypt foci (ACFs) after 6 weeks of treatment in the different treatment groups

Groups	ACF/colon			
	Small (1–3 crypts)	Medium (4–6 crypts)	Large (> 6 crypts)	Total
Control	0	0	0	0
DMH	8 ± 0.8	5 ± 0.5	6 ± 0.6	19 ± 1.70
DMH + sulindac	5 ± 0.5	3 ± 0.3	2 ± 0.2	10 ± 1.29
DMH + celecoxib	7 ± 0.7	4 ± 0.4	3 ± 0.3	14 ± 1.29

The values represent the mean ± SD of 10 animals from each group



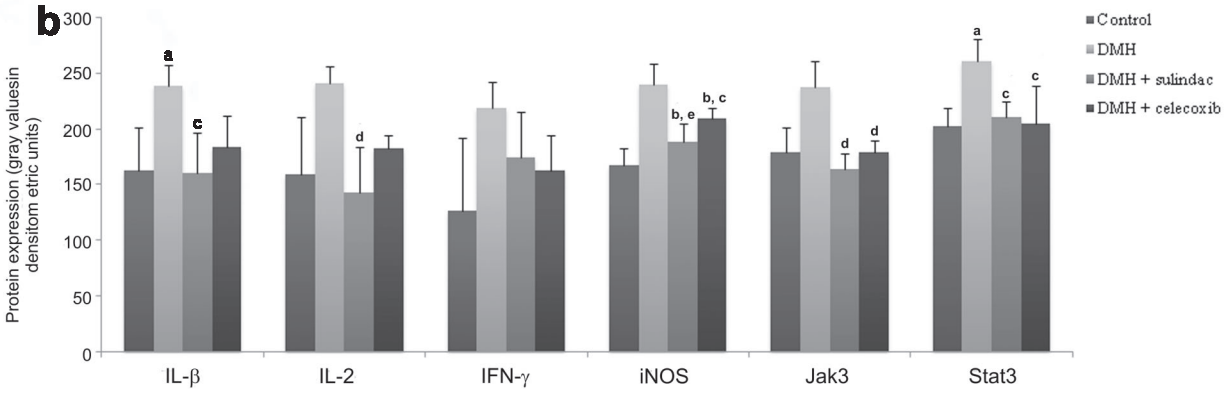


Fig. 2. (**a**) Western blot analysis showing the effects of DMH and DMH co-administered with either sulindac and celecoxib on the protein expression of IL-1β, IL-2, IFNγ, iNOS, Jak3 and Stat3. (**b**) The densitometric analysis of the blots. The values represent the mean ± SD of 4 animals from each group. [a] $p < 0.05$ and [b] $p < 0.01$ in comparison to the control and [c] $p < 0.05$, [d] $p < 0.01$ and [e] $p < 0.001$ in comparison to the DMH alone group by a one-way ANOVA

The levels of the pro-inflammatory cytokines IL-1β, IL-2 and IFNγ were higher in the DMH-treated group as compared with the control group. The DMH + sulindac and DMH + celecoxib groups showed lower expression of IL-1β, IL-2 and IFNγ as compared to the DMH alone group (Fig. 2a). Higher expression and localization of IL-1β, IL-2 and IFNγ in the DMH alone group were also observed by immunofluorescence in paraffin sections (Figs. 3a, 3b and 4a).

The higher expression of iNOS in the DMH-treated group confirms that DMH, which is a carcinogen in the colon, initiates inflammatory pathways that result in the origin of neoplasms because higher levels of iNOS are known to promote carcinogenesis. Sulindac and celecoxib reduced the expression of iNOS (Fig. 2a). The immunofluorescence study also confirmed the higher expression and localization of iNOS in the stromal and epithelial cells of the colon in the DMH-treated group as compared to the control group, while a lower expression of iNOS was observed in the groups co-administered NSAIDs as compared to the DMH alone group (Fig. 4b).

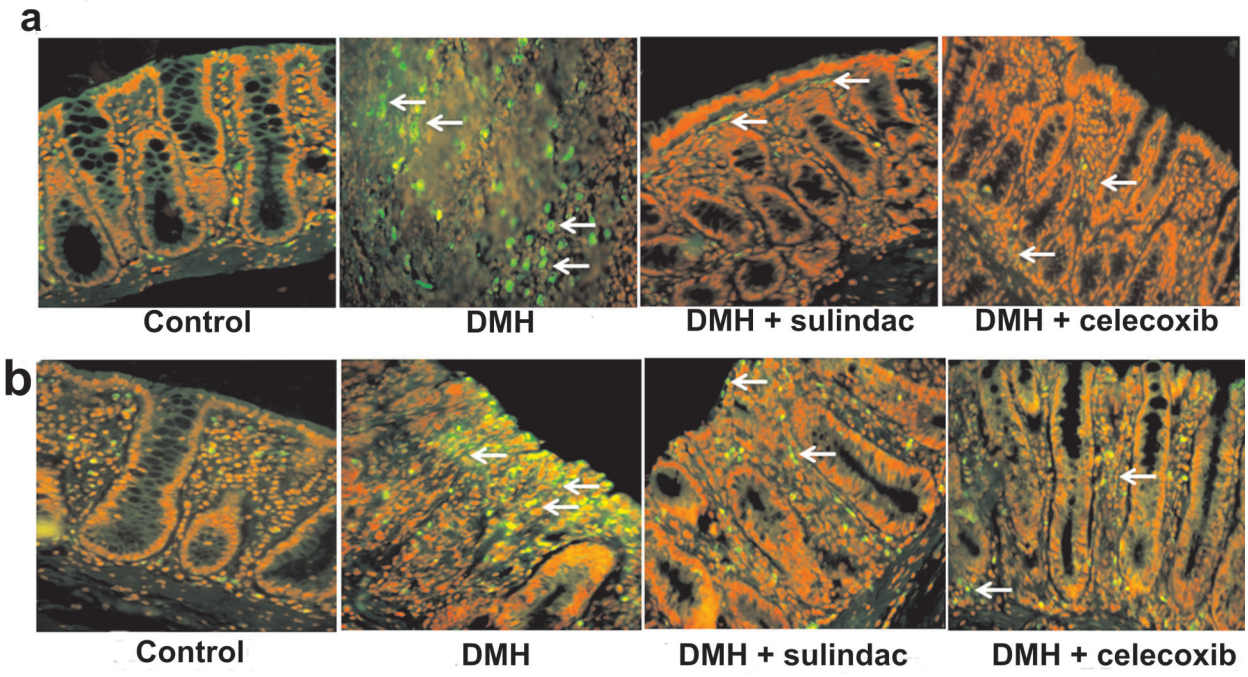


Fig. 3. (**a**) Photomicrographs showing the expression and localization of IL-1β in paraffin sections from different treatment groups using immunofluorescence at 400× magnification. Colonocytes expressing IL-1β (arrows) can be observed with bright fluorescence due to the FITC-conjugated secondary antibody. (**b**) The expression and localization of IL-2 in the colonocytes (arrows) of different treatment groups were detected *via* immunofluorescence in paraffin sections at 400× magnification

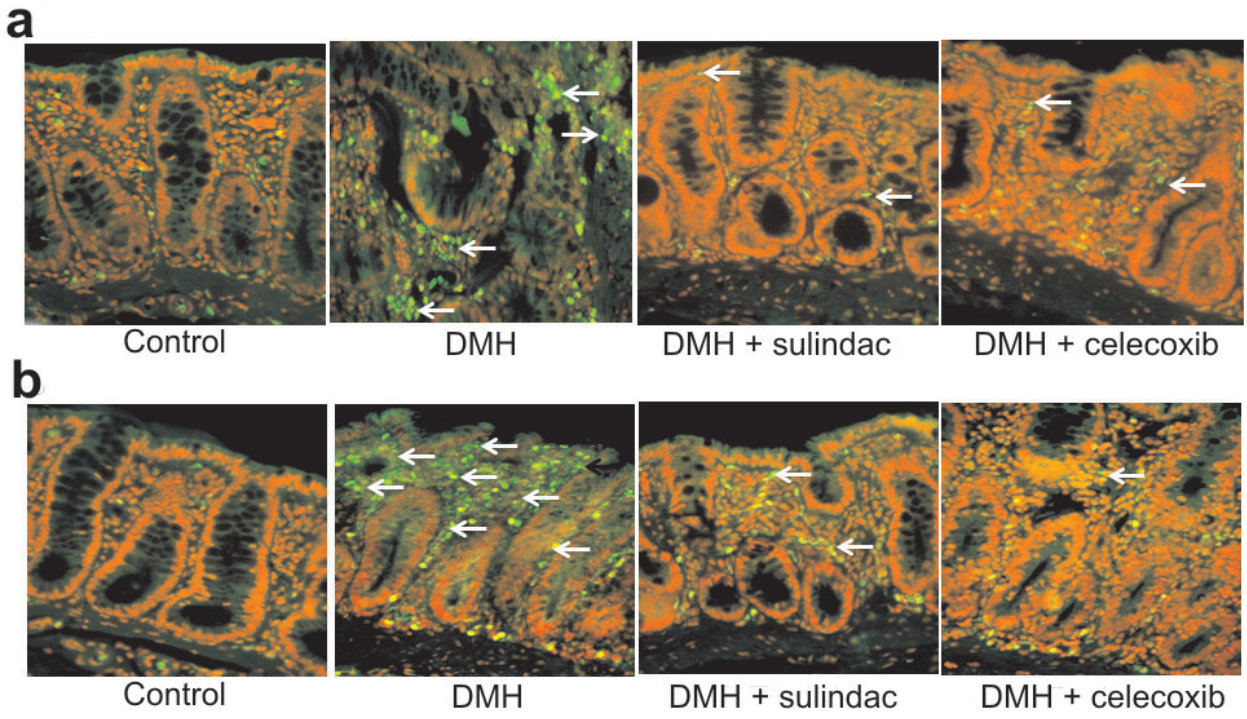


Fig. 4. (**a**) Photomicrographs showing the expression and localization of INF-γ (arrows) in different treatment groups *via* immunofluorescence in paraffin sections at 400× magnification. (**b**) The expression and localization of iNOS (arrows) in paraffin sections from all of the treatment groups observed by immunofluorescence at 400× magnification

The expression of Jak3 and Stat3, which are anti-apoptotic transcription factors induced by pro-inflammatory cytokines, were higher in the DMH alone group as compared to the control group. Jak3 and Stat3 further enhance the inflammatory pathways activated by DMH. The groups co-administered NSAIDs showed lower expression of Jak3 and Stat3 with respect to the DMH alone group, which supports the apoptotic potential of sulindac and celecoxib (Fig. 2a).

The differential DNA-binding activities of acridine orange and ethidium bromide showed that the NSAID co-administered groups had more apoptotic cells with respect to the DMH alone group (Fig. 5a).

NF-κB, which is a transcription factor that promotes the expression of both pro-inflammatory and anti-apoptotic proteins, plays a role in tumor progression. The DMH alone group showed higher NF-κB (p65) DNA-binding activity compared with the control group, whereas the DNA-binding activity of NF-κB (p65) was suppressed by sulindac and celecoxib when co-administered with DMH (Fig. 5c). This shows that the anti-inflammatory and anti-

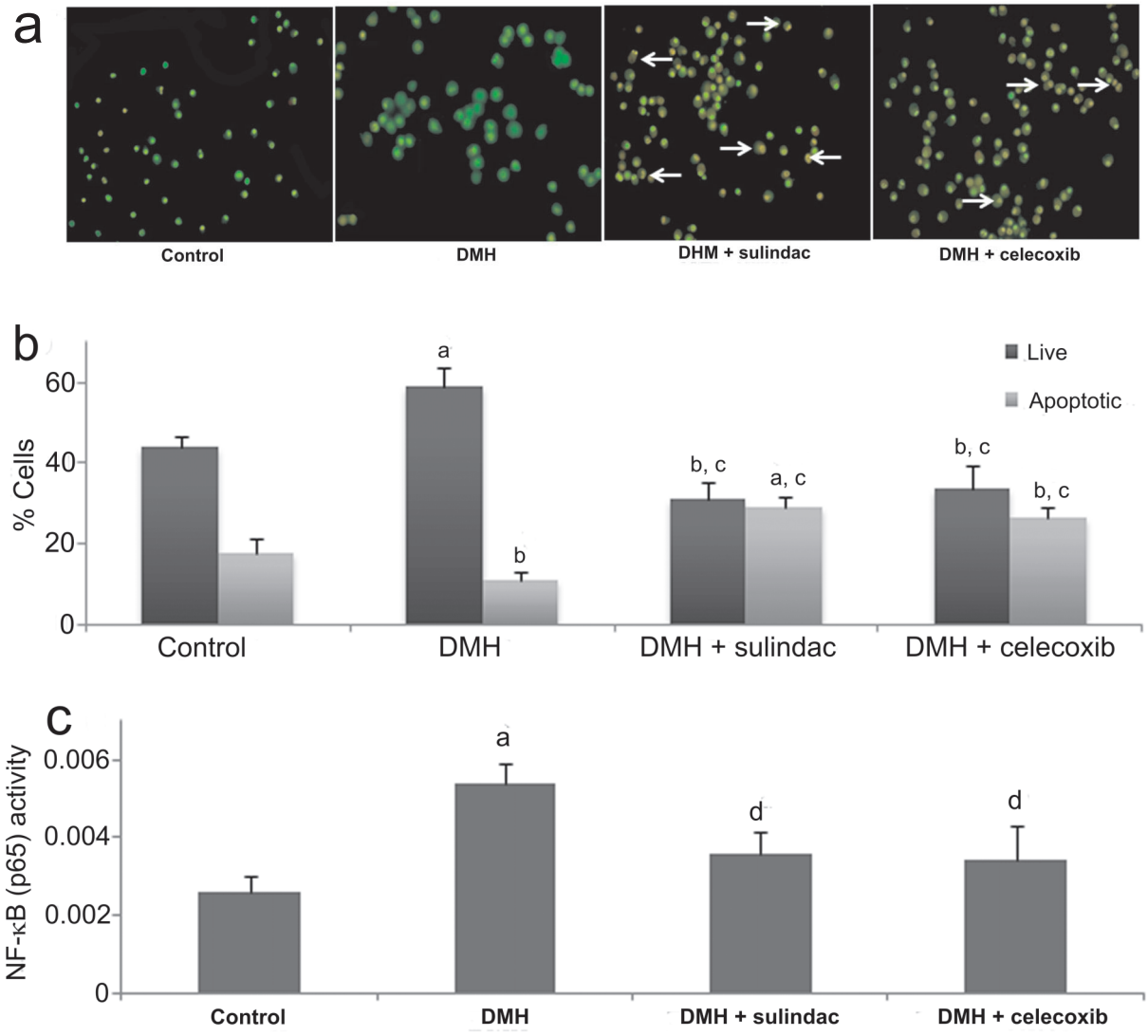


Fig. 5. (**a**) Apoptotic potential analysis using the DNA-binding dyes acridine orange and ethidium bromide, which were observed under a fluorescent microscope at 400× magnification. The apoptotic cells have nuclei that are brightly stained with ethidium bromide (arrows). (**b**) A graph representing the percent apoptotic cell counts in different treatment groups. (**c**) The quantitation of NF-κB (p65) DNA-binding activity was calculated by the absorbance at 450 nm/cell extract (50 μg/well). The graph represents the relative transcriptional activity of NF-κB in all of the treatment groups. The values represent the mean ± SD of 4 animals from each group. [a] $p < 0.001$ and [b] $p < 0.01$ in comparison to the control group and [c] $p < 0.001$ and [d] $p < 0.01$ in comparison to the DMH alone group by a one-way ANOVA

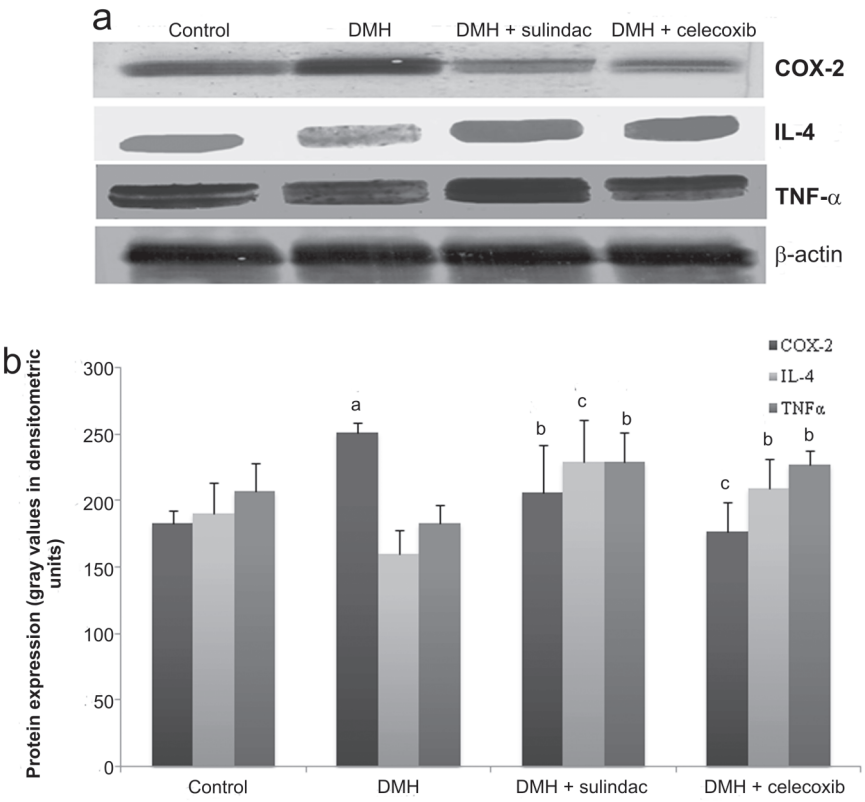


Fig. 6. (**a**) Western blot analysis showing the effect of DMH and DMH co-administered with either sulindac and celecoxib on the protein expression of COX-2, IL-4 and TNF-α. (**b**) The densitometric analysis of the blots. The values represent the mean ± SD of 4 animals from each group. [a] $p < 0.01$ in comparison to the control group and [b] $p < 0.05$ and [c] $p < 0.01$ in comparison to the DMH alone group by a one-way ANOVA

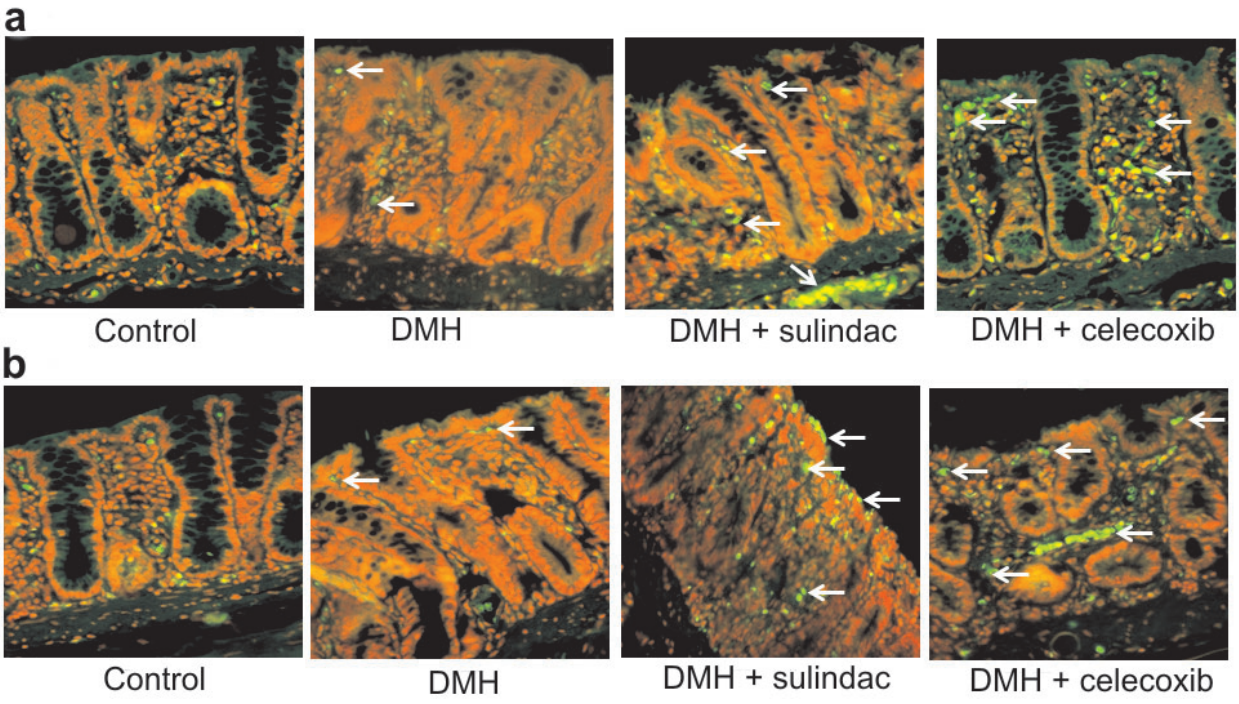


Fig. 7. (**a**) Immunofluorescence analysis of paraffin sections at 400× magnification showing the expression and localization of IL-4-expressing colonocytes (arrows) in the different groups. (**b**) TNF-α expression and localization in colonocytes (arrows) in paraffin sections of the different groups observed by immunofluorescence at 400× magnification

carcinogenic properties of sulindac and celecoxib are mediated *via* suppression of the transcriptional activity of NF-κB.

The expression of COX-2 was also higher in the DMH alone group with respect to the control group, suggesting that COX-2 is a cause of inflammation in colonic tissue. However, both of the NSAIDs suppressed the inflammatory action of COX-2 by lowering its expression (Fig. 6a).

The anti-inflammatory and apoptotic actions of the NSAIDs were demonstrated by the higher expression of the cytokines IL-4 and TNF-α following co-administration with DMH as compared to the DMH alone group (Fig. 6a). The expression and localization of IL-4 and TNF-α were also shown by immunofluorescence. Figure 7a shows a blood vessel localized in the sub-mucosal layer (arrow) containing IL-4-secreting leukocytes in the DMH + sulindac group, and Figure 7b shows a blood vessel generated due to angiogenesis in the stromal layer (arrow), which also has leukocytes that are known to secrete pro-inflammatory cytokines, such as IL-4 and TNF-α. These results suggest an anti-carcinogenic effect of NSAIDs when co-administered with DMH by negatively regulating the pro-inflammatory cytokines.

Discussion

The present *in vivo* experimental colon cancer model demonstrates the role of inflammatory pathways in rats in the initiation of colon tumorigenesis when treated with DMH and the chemopreventive role of NSAIDs (sulindac and celecoxib). NSAIDs have long been used to cure inflammatory diseases; however, they have recently gained interest in the chemoprevention of various cancers, especially in the chemoprevention of colorectal cancer [24].

At an early stage of neoplasia, the presence of MPLs suggests macroscopic sites of tumor growth [30]. In our study, MPLs appeared as bulges of the epidermis in the DMH-treated group. When DMH was co-administered with an NSAID, the MPLs were small and few in number, suggesting that NSAIDs may inhibit tumor growth in early stages of neoplasia. Similarly, ACFs are characterized as areas of thick-walled cells that are composed of one or multiple enlarged crypts elevated above the surrounding mucosa and are considered to be putative pre-neoplastic lesions of the colon during inflammation. In these lesions, multiple molecular changes are likely to drive the cell proliferation toward committed malignancy [7, 20].

The higher expression of the pro-inflammatory cytokines IL-1β, IL-2 and IFNγ suggest that the colon is under inflammatory stress. IL-1β has been demonstrated to increase in colonic cells and in the surrounding immune cells found either in blood vessels or in lymphoid tissue [36]. This secreted IL-1β works as a paracrine factor by binding to its receptors and initiating inflammatory responses that induce COX-2 expression; the induction of COX-2 is likely caused by activation of the NF-κB pathway [15]. Co-administration of sulindac and celecoxib with DMH reduced the DMH-elevated levels of IL-1β and thereby prevented the inflammatory response.

IL-1β may not be the only molecule responsible for the inflammation observed in this study because there are many pro-inflammatory cytokines that may also play a role in the inflammatory response *via* other signaling pathways. A higher expression of IL-2 was observed in the DMH-treated group, which may contribute toward the non-recognition of tumor cells by cytotoxic lymphocytes. Elevated IL-2 also causes AICD of the cytotoxic immune cells that are generated during the inflammatory response to fight the pathogen and kill malignant cells with undefined cell surface markers [4]. IL-2 also functions in a paracrine manner and acts as an activator of Jak3 by auto-phosphorylation in cancer cells [11]. In turn, the activation of Jak3 leads to the activation of Stat3, which is a transcription factor that positively regulates various pro-inflammatory cytokines, growth factors and anti-apoptotic agents [17, 33]. The expression of both Jak3 and Stat3 was higher in the DMH-treated group. With the co-administration of NSAIDs, the expression of IL-2, Jak3 and Stat3 was reduced with respect to the DMH alone group. Similarly, down-regulation of IFNγ expression in the DMH + sulindac and DMH + celecoxib groups with respect to the DMH alone group may contribute toward the anti-inflammatory actions of these NSAIDs in the colon.

The higher expression of iNOS is related to the inflammatory response in the colon because its expression increases following the activation of Stat3, NF-κB and COX-2 [29, 32, 41]. However, sulindac and celecoxib efficiently suppressed the activation and the transcriptional activity of NF-κB; therefore, the levels of COX-2 and iNOS were lower in the groups co-administered NSAIDs.

The activation of NF-κB is a multi-signaling pathway, which can also be activated by IL-1β and TNF-α. The anti-apoptotic properties of TNF-α are known to be mediated *via* NF-κB activation [31, 40], but if NF-κB is suppressed by other means, the elevated levels of TNF-α may also induce apoptosis [21]. In the present study, the transcriptional activity of NF-κB was suppressed by sulindac and celecoxib; thus, the higher levels of TNF-α may support the anti-neoplastic activity of these agents.

IL-4 functions as a growth and differentiation factor for B- and T-lymphocytes; however, it also has an inhibitory effect on human cancers [19]. Higher expression of IL-4 was observed in the groups that were co-administered NSAIDs as compared with the DMH alone group, which suggests that the anti-neoplastic activity of the NSAIDs may be mediated through the inhibitory effects of IL-4 on cell-cell adhesion to prevent tumor invasion and metastasis [9].

The higher expression of pro-inflammatory cytokines and transcription factors observed in the DMH-treated group gives rise to an imbalance in cell proliferation and apoptosis that may lead to cancer. However, NSAIDs may induce apoptosis by suppressing anti-apoptotic gene transcription while increasing the expression of anti-inflammatory cytokines to counter the neoplastic commitment of the host cells.

In conclusion, the present study demonstrated the molecular effects of pro-inflammatory cytokines and transcription factors in the initiation of DMH-induced colorectal cancer. Further, sulindac and celecoxib decreased the levels of pro-inflammatory cytokines while elevating the expression of anti-inflammatory cytokines in DMH induced colorectal cancer, suggesting an anti-inflammatory role for NSAIDs in colorectal cancer. Thus, NSAIDs represent promising agents for the chemoprevention of colorectal cancer in early neoplasm stages.

Acknowledgment:
Financial assistance from the Department of Science and Technology, Government of India (SR/SO/BB-05/2008) is gratefully acknowledged.

References:

1. Baek SJ, Wilson LC, Lee CH, Eling TE: Dual function of nonsteroidal anti-inflammatory drugs (NSAIDs): inhibition of cyclooxygenase and induction of NSAID-activated gene. J Pharmaco Exp Ther, 2002, 301, 1126–1131.
2. Bill MA, Fuchs JR, Li C, Yui J, Bakan C, Benson DM Jr, Schwartz EB et al.: The small molecule curcumin analog FLLL32 induces apoptosis in melanoma cells via STAT3 inhibition and retains the cellular response to cytokines with anti-tumor activity. Mol Cancer, 2010, 9, 165d.
3. Bradford MM: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem, 1976, 72, 248–254.
4. Cornejo MG, Boggon TJ, Mercher T: JAK3: A two-faced player in hematological disorders. Int J Biochem Cell Biol, 2009, 41, 2376–2379.
5. Duke RC, Cohen JJ: Morphological and biochemical assays of apoptosis. Curr Prot Immunol, 1992, 13, 1713–1716.
6. Ghosh N, Chaki R, Mandal V, Mandal SC: COX-2 as a target for cancer chemotherapy. Pharmacol Rep, 2010, 62, 233–244.
7. Hirose Y, Kuno T, Yamada Y, Sakata K, Katayama M, Yoshida K, Qiao Z et al.: Azoxymethane-induced beta-catenin-accumulated crypts in colonic mucosa of rodents as an intermediate biomarker for colon carcinogenesis. Carcinogenesis, 2003, 24, 107–111.
8. Kai H, Kitadai Y, Kodama M, Cho S, Kuroda T, Ito M, Tanaka S et al.: Involvement of proinflammatory cytokines IL-1 and IL-6 in progression of human gastric carcinoma. Anticancer Res, 2005, 25, 709–714.
9. Kanai T, Watanabe M, Hayashi A, Yajima T, Okazawa A, Yamazaki M, Ishii H, Hibi T: Regulatory effect of interleukin-4 and interleukin-13 on colon cancer cell adhesion. Br J Cancer, 2000, 82, 1717–1723.
10. Kanwar SS, Vaipei K, Nehru B, Sanyal SN: Antioxidative effects of non-steroidal anti-inflammatory drugs during the initiation stages of experimental colon carcinogenesis in rat. J Environ Pathol Toxicol Oncol, 2008, 27, 89–100.
11. Kirken RA, Rui H, Malabarba MG, Howard OM, Kawamura M, O'Shea JJ, Farrar WL: Activation of JAK3, but not JAK1, is critical for IL-2-induced proliferation and STAT5 recruitment by a COOH-terminal region of the IL-2 receptor β-chain. Cytokine, 1995, 7, 689–700.
12. Lin Q, Lai R, Chirieac LR, Li C, Thomazy VA, Grammatikakis I, Rassidakis GZ et al.: Constitutive activation of JAK3/STAT3 in colon carcinoma tumors and cell lines. Am J Pathol, 2005, 167, 969–980.
13. Liu W, Reinmuth N, Stoeltzing O, Parikh AA, Tellez C, Williams S, Jung YD et al.: Cyclooxygenase-2 is up-regulated by interleukin-1β in human colorectal cancer cells via multiple signaling pathways. Cancer Res, 2003, 63, 3632–3636.
14. Maihöfner C, Charalambous MP, Bhambra U, Lightfoot T, Geisslinger G, Gooderham NJ and The Colorectal Cancer Group: Expression of cyclooxygenase-2 parallels expression of Interleukin-1beta, Interleukin-6 and NF-kappaB in human colorectal cancer. Carcinogenesis, 2003, 24, 665–671.
15. McConnell BB, Yang VW: The role of inflammation in the pathogenesis of colorectal cancer. Curr Colorectal Cancer Rep, 2009, 5, 69–74.
16. Mitchell JA, Warner TD: Cyclo-oxygenase-2: pharmacology, physiology, biochemistry and relevance to NSAID therapy. Br J Pharmacol, 1999, 128, 1121–1132.

17. Mora LB, Buettner R, Seigne J, Diaz J, Ahmad N, Garcia R, Bowman T et al.: Constitutive activation of Stat3 in human prostate tumors and cell lines: direct inhibition of Stat3 signaling induces apoptosis of prostate cancer cells. Cancer Res, 2002, 62, 6659–6666.
18. Mouillé B, Robert V, Blachier F: Adaptive increase of ornithine production and decrease of ammonia metabolism in rat colonocytes and hyperproteic diet ingestion. Am J Physiol Gastrointest Liver Physiol, 2004, 287, G344–G351.
19. Murata T, Noguchi PD, Puri RK: Receptors for interleukin (IL)-4 do not associate with the common γ chain, and IL-4 induces the phosphorylation of Jak2 tyrosine kinase in human colon carcinoma cells. J Biol Chem, 1996, 270, 30829–30836.
20. Ochiai M, Ushigome M, Fujiwara K, Ubagai T, Kawamori T, Sugimura T, Nagao M, Nakagama H: Characterization of dysplastic aberrant crypt foci in the rat colon induced by 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine. Am J Pathol, 2003, 163, 1607–1614.
21. Onizawa M, Nagaishi T, Kanai T, Nagano K, Oshima S, Nemoto Y, Yoshioka A et al.: Signaling pathway via TNF-α/NF-κB in intestinal epithelial cells may be directly involved in colitis-associated carcinogenesis. Am J Physiol Gastrointest Liver Physiol, 2009, 296, G850–G859.
22. Poligone B, Baldwin AS: Positive and negative regulation of NF-κB by COX-2. J Biol Chem, 2001, 276, 38658–38664.
23. Rao CV, Indranie C, Simi B, Manning PT, Connor JR, Reddy BS: Chemopreventive properties of a selective inducible nitric oxide synthase inhibitor in colon carcinogenesis, administered alone or in combination with celecoxib, a selective cyclooxygenase-2 inhibitor. Cancer Res, 2002, 62, 165–170.
24. Rayburn ER, Ezell SJ, Zhang R: Anti-inflammatory agents for cancer therapy. Mol Cell Pharmacol, 2009, 1, 29–43.
25. Roediger WE, Truelove SC: Method of preparing isolated colonic epithelial cells (colonocytes) for metabolic studies. Gut, 1979, 20, 484–488.
26. Sano H, Kawahito Y, Wilder RL, Hashiramoto A, Mukai S, Asai K, Kimura S et al.: Expression of cyclooxygenase-1 and -2 in human colorectal cancer. Cancer Res, 1995, 55, 3785–3789.
27. Schwartz LM, Obsorne BA: Cell death. Method Cell Biol, 1995, 46, 15–18.
28. Shao J, Sheng H: Prostaglandin E_2 induces the expression of IL-1α in colon cancer cells. J Immunol, 2007, 178, 4097–4103.
29. Song ZJ, Gong P, Wu YE: Relationship between the expression of iNOS, VEGF, tumor angiogenesis and gastric cancer. World J Gastroenterol, 2002, 8, 591–595.
30. Takeda H, Sonoshita M, Oshima H, Sugihara K, Chulada PC, Langenbach R, Oshima M, Taketo MM: Cooperation of cyclooxygenase 1 and cyclooxygenase 2 in intestinal polyposis. Cancer Res, 2003, 63, 4872–4877.
31. Takenouchi-Ohkubo N, Moro I, Mukae S, Kaneko Y, Komiyama K: Tumour necrosis factor-α-mediated human polymeric immunoglobulin receptor expression is regulated by both mitogen-activated protein kinase and phosphatidylinositol-3-kinase in HT-29 cell line. Immunology, 2008, 123, 500–507.
32. Timoshenko AV, Lala PK, Chakraborty C: PGE_2-mediated upregulation of iNOS in murine breast cancer cells through the activation of EP4 receptors. Int J Cancer, 2004, 108, 384–389.
33. Tsareva SA, Morigglz R, Corvinus FM, Wiederanders B, Schütz A, Kovacic B, Friedrich K: Signal transducer and activator of transcription 3 activation promotes invasive growth of colon carcinomas through matrix metalloproteinase induction. Neoplasia, 2007, 9, 279–291.
34. Vaish V, Tanwar L, Kaur J, Sanyal SN: Chemopreventive effects of non steroidal anti-inflammatory drugs in early neoplasm of experimental colorectal cancer: An apoptosome study. J Gastrointest Canc, 2011, 42, 195–203.
35. Vaish V, Tanwar L, Sanyal SN: The role of NF-κB and PPARγ in experimentally induced colorectal cancer and chemoprevention by cyclooxygenase-2 inhibitors. Tumour Biol, 2010, 31, 427–436.
36. Voronov E, Shouval DS, Krelin Y, Cagnano E, Benharroch D, Iwakura Y, Dinarello CA, Apte RN: IL-1 is required for tumor invasiveness and angiogenesis. Proc Natl Acad Sci USA, 2003, 100, 2645–2650.
37. Xiao H, Zhang Q, Lin Y, Reddy BS, Yang CS: Combination of atorvastatin and celecoxib synergistically induces cell cycle arrest and apoptosis in colon cancer cells. Int J Cancer, 2008, 122, 2115–2124.
38. Yasui H, Adachi M, Imai K: Combination of tumor necrosis factor-α with sulindac augments its apoptotic potential and suppresses tumor growth of human carcinoma cells in nude mice. Cancer, 2003, 97, 1412–1420.
39. Zhang M, Yao Z, Dubois S, Ju W, Müller JR, Waldmann TA: Interleukin-15 combined with an anti-CD40 antibody provides enhanced therapeutic efficacy for murine models of colon cancer. Proc Natl Acad Sci USA, 2009, 106, 7513–7518.
40. Zhou A, Scoggin S, Gaynor RB, Williams NS: Identification of NF-κB-regulated genes induced by TNFα utilizing expression profiling and RNA interference. Oncogene, 2003, 22, 2054–2064.
41. Ziesché E, Bachmann M, Kleinert H, Pfeilschifter J, Mühl H: The IL-22/STAT3 pathway potentiates expression of iNOS in human colon carcinoma cells. J Biol Chem, 2007, 282, 16006–16015.

Received: February 16, 2011; **in the revised form:** May 2, 2011; **accepted:** May 16, 2011.