RESEARCH COMMUNICATION

Chemoprevention of 1,2-Dimethylhydrazine-Induced Colon Carcinogenesis by a Non-Steroidal Anti-Inflammatory Drug, Etoricoxib, in Rats: Inhibition of Nuclear Factor κB

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Abstract

Etoricoxib, a highly selective cyclooxygenase- 2 (COX-2) inhibitor (a non steroidal anti-inflammatory drug) used for the treatment of rheumatoid arthritis and osteoarthritis, has been newly marketed and studied for the chemopreventive response in the 1,2-dimethylhydrazine dihydrochloride (DMH) induced rat colon cancer model. Male Sprague-Dawley rats were divided into four groups. Group I served as the Control and received the vehicle treatment, while Groups 2 and 3 were administered freshly prepared DMH (30 mg/kg body weight, subcutaneously) in 1mM EDTA-saline (pH 7.0). Groups 3 and 4 received Etoricoxib (0.64 mg/kg body weight, orally) daily prepared in 0.5% carboxymethyl cellulose. After a 6 week treatment period, animals were sacrificed and the colons were subjected to macroscopic and histopathological studies. Well characterized pre-neoplastic features such as multiple plaque lesions (MPLs), aberrant crypts (ACs) and aberrant crypt foci (ACF) were found in the DMH group. The number was reduced in DMH + Etoricoxib group, while very few MPLs and ACFs were recorded in the Etoricoxib only group. Also, histologically well characterized dysplasia and hyperplasia were observed in DMH treated group. The simultaneous administration of DMH and Etoricoxib reduced these features. To study apoptosis, colonocytes were isolated by metal chelation from colonic sacs and studied by fluorescent staining. The DMH treated animals produced much less apoptotic nuclei as compared to the Control. The number of apoptotic nuclei was also found higher in the DMH + Etoricoxib group as well as in Etoricoxib only group. Studies of a nuclear transcription factor (NF-κB) and COX-2 by Western blot analysis and immunohistochemistry demonstrated expression of both to be elevated in the DMH treated group but reduced in the DMH + Etoricoxib group. Expression was also low in the Etoricoxib only group. It may be concluded that the drug, Etoricoxib, has the potential to reduce DMH induced colon cancer development.

Key Words: Etoricoxib - NSAID - colon cancer - ACF - COX-2 expression - NFκB - apoptosis

Introduction

Non steroidal anti-inflammatory drugs (NSAIDs), which block both COX-1 and COX-2 isoenzymes of cycloxygenase, had also show surprisingly effective chemopreventive activity against colon and other cancers (Bodelon et al., 2009; Khan and Lee, 2009). Numerous studies have shown that NSAIDs, including aspirin, sulindac, piroxicam, ibuprofen, celecoxib and indomethacin, are suitable chemopreventive agents against carcinogen-induced and genetically manipulated animal models of colon carcinogenesis (Moorghen et al., 1988; Craven and DeRobertis, 1993; Reddy et al., 1993). NSAIDs are also the best found chemopreventive agents against colorectal cancer in epidemiological studies (Marnett, 1992; Smalley and DuBois, 1997). However, long-term use of these agents is limited by gastric and renal toxicity caused by inhibition of the constitutive COX-1 enzyme. COX-2 inhibitors on the other hand retain full anti-inflammatory activity, while exerting no ulcerogenic effect in the stomach (Wong et al., 1992; Donnelly and Hawkey, 1997). Therefore, selective inhibition of COX-2 is a promising approach in the development of cancer chemoprevention, as specific inhibition of COX-2 may permit lower doses and a greater margin of safety than possible with the current non-specific COX inhibitors.

The nuclear transcription factor κB (NF-κB) is a well established regulator of genes encoding cytokines, cytokine receptors and cell adhesion molecules that drive immune and inflammatory responses (Bonizzi and Karin, 2004). Recently, NF-κB activation has also been connected with multiple aspects of oncogenesis, including the control of apoptosis, cell cycle, differentiation and cell migration (Baldwin, 2001). Expression of several genes, such as COX-2, matrix metalloproteinase (MMP-9), inducible nitric oxide synthase, tumor necrosis factor (TNFα), interleukin (IL-8), cell surface adhesion

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molecules and anti-apoptotic proteins which are involved in tumor initiation, tumor promotion and metastasis are regulated by NF-κB (Pahl, 1999). This ubiquitous nuclear transcription factor resides in the inactive state in cytoplasm as a heterotrimer consisting of p50, p65 and IκBα subunits. As a transcription factor, it assumes a dimeric form composed of different members of the Rel/NF κB family of polypeptides (Ghosh et al., 1998). The p50-p65 heterodimer is retained in the cytoplasm by the inhibitory subunit IκBα. Few studies have shown that NF-κB pathway is activated and COX-2 expression is upregulated in stromal myofibroblasts surrounding the colon adenocarcinomas (Vandoros et al., 2006). Sawhney et al, (2007) also co-relates between the over expression of NF-κB and COX-2 in early precancerous stages of development of oral cancer and sustained elevation down the tumorigenic pathway.

The primary aim of the present work was to investigate chemopreventive response of Etoricoxib (a non steroidal anti-inflammatory drug), a selective COX-2 inhibitor in the 1,2-dimethyl hydrazine dihydrochloride (DMH) induced rat colon cancer and to study the role of COX-2 and NF-κB in colon carcinogenesis.

Materials and Methods

Chemicals

1,2-dimethylhydrazine dihydrochloride (DMH), Hoechst 33342, Propidium iodide and Bradford reagent were purchased from Sigma-Aldrich (St. Louis, MO, USA). Primary antibody against COX-2, NF-κB (p65) and anti-mouse β-actin were purchased from Santa Cruz Biotechnology Inc., CA (USA). Alkaline phosphatase – conjugated secondary antibodies and BCIP-NBT were purchased from Genei, Bangalore (India). Etoricoxib was obtained from Ranbaxy Research Lab (Gurgaon, India). All other chemicals and reagents used in the present study were of the analytical grade and procured from the reputed Indian manufacturers.

Animal Procurement

Male adult Sprague-Dawley rats of body weight between 200-300g were obtained from the inbred population of Central Animal House, Panjab University, Chandigarh. These were acclimatized to the control diet (rodent chow) and water *ad libitum* for at least 1 week. Animals were maintained as per the principles and guidelines of the Ethics Committee of the Animal Care of Panjab University in accordance with the Indian national law on animal care and use. The animals were housed 4 per cage in polypropylene cages with a wire mesh top and a hygienic bed of husk (regularly changed) in a well ventilated animal room till the end of the experimental period. The animals were also maintained under a 12hr photoperiod of light and darkness, respectively.

Treatment Schedule

Animals were assorted into the following groups with four to six animals in each group: Control Group: Animals were administrated the vehicle (1mM EDTA-saline subcutaneously s.c.) in weekly injection and 0.5%

Carboxymethyl cellulose (CMC) per oral (p.o.) daily. DMH Group: Animals were administrated with DMH weekly at a dose of 30 mg/kg body weight (s.c.), as had been established in our laboratory earlier (Kanwar et al., 2008). DMH was freshly prepared in 1mM EDTA-saline, pH adjusted to 7.0 using dilute NaOH solution. DMH + Etoricoxib Group: Etoricoxib was given daily per oral (p.o.) at its therapeutic anti-inflammatory dose (ED50 for rats, 0.64 mg/kg body weight) to the animals along with the weekly administration of DMH (Riendeau et al., 2001). Etoricoxib Group: Etoricoxib alone was administered p.o daily (0.64 mg/kg body weight). The anti-inflammatory dose of Etoricoxib had been established earlier in a model of carragenan induced oedema in rat hind paw (Sharma et al., 2010).

After six weeks, animals were kept on overnight fasting with drinking water *ad libitum* and sacrificed the next day. The animal body weights in all the groups were recorded once in a week till the termination of the experiment.

Tissue Pathology

The colons were removed and flushed clear with ice-cold physiological saline (0.9% NaCl solution). These were opened longitudinally along the median and laid flat to examine the incidence of macroscopic lesions/plaques called the multiple plaque lesions (MPLs). The colons were divided into proximal, medial and distal segments for the examination.

Aberrant Crypt Foci (ACF) Analysis

The colons were removed, flushed clean, cut open and divided into different parts as above. After a minimum of 24 hrs fixation in 10% buffered formalin, the colons were stained with 0.2% methylene blue in Krebs Ringer solution for 5-10 min (Park et al., 1997). The mucosal surface of the colon was evaluated for the number of aberrant crypt foci (ACF) in the stained colon under 40X magnification using a light microscope. Enlarged and slightly elevated lesions with increased staining were readily identifiable in comparison to normal adjacent mucosa. These lesions were classified as single enlarged crypts or foci containing two or more abnormal crypts.

Histopathological analysis

Colonic pieces were cut from the sacrificed rats and immediately fixed in 10% buffered formalin for 24 hrs. The tissues were dehydrated in ascending series of alcohol and kept in 1:1 mixture of absolute alcohol and benzene for 1 hr. For embedding the tissues in wax, they were kept in benzene for 40-45 min and transferred sequentially to 1:1 benzene and wax mixture at 60°C for 1hr and then pure wax for 6 hr at 60°C with two changes. The tissues were embedded in wax and five micron sections were cut using a hand driven microtome and transferred to the egg albumin coated slides. Sections were then dewaxed in xylene, stained in haematoxylin and eosin, mounted in DPX viewed under a light microscope and photographed.

Western Blot Analysis

Protein samples/nuclear extracts (100 μg) from each

treatment group were separated on 10% SDS-PAGE. The separated proteins were electrophoretically transferred to nitrocellulose membrane (Genei, Bangalore, India). Immunoblot was prepared using primary antibodies (COX-2-1:1000, NF-κB (p65)-1:1000) from Santa Cruz Biotechnology Inc., CA (USA) and alkaline phosphatase-conjugated secondary antibody at a dilution of 1:10,000 (Genei, Bangalore, India). BCIP-NBT detection system was used to develop the blots. Bands obtained were densitometrically analysed using Image J software and the density expressed as gray values in the densitrometric units.

For preparation of protein extracts, colons were removed and rinsed from the different treatment groups after completion of 6 weeks. Total lysates were prepared in fresh ice-cold protein lysis buffer (10mM Tris NaCl, 5mM EDTA, 1% Triton X-100, 1mM PMSF and 2mM DTT (pH 8.0)). The extracts were cleared by centrifugation at 10,000 rpm for 10 min at 4°C. The supernatants were collected as the total lysate. Protein concentrations were determined by the method of Bradford (1976).

Immunohistochemical Localization Studies

5µm thick paraffin sections of rat colon were deparaffinised in two changes of xylene for 10 min each. The sections were then gradually hydrated to water. The non-specific staining was blocked by incubating the sections with 2% BSA in phosphate buffered saline (PBS 10mM, pH 7.2). The sections were then incubated with polyclonal antibodies against COX-2 -1:1000 and NF-κB (p65) - 1:1000 in a moist chamber for 2hr at 37°C. After incubation, the sections were then incubated with alkaline phosphatase-labelled secondary antibody for 2hr. Sections were washed again in the same manner as described above and the reaction product developed using BCIP-NBT. Reaction was terminated by washing with distilled water after which sections were counterstained with methyl green and mounted in DPX.

Isolation of Colonocytes

Colonic epithelial cells (colonocytes) were obtained from the freshly isolated colons by the method of Mouille et al (2004), as originally described by Roediger and Truelove (1979). The colonic segments were removed and flushed with chilled physiological saline (NaCl solution, 9g/l) and then with a Ca^{++}- and Mg^{++}- free Krebs-Hanseleit (K-H) bicarbonate buffer (pH 7.4). The K-H buffer was equilibrated against a mixture of O_2 and CO_2 (19:1, vol/vol). Then, each colon was everted, distally ligated, and distended as much as possible by means of a syringe

containing calcium free K-H saline with 0.25% w/v BSA. The proximal end of the colon was now ligated and placed in a plastic flask containing 100ml calcium-free K-H buffer, 5mMole/l EDTA, and 0.25% BSA. The flask was gassed with O_2 - CO_2 (19:1 v/v) and incubated at 37°C in a shaking water bath at 60-70 oscillations per min 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 50ml K-H buffer with 0.25% w/v BSA. Manual stirring with a plastic stirrer for two min readily disaggregated the colonocytes, which were then separated by centrifugation at 500g for 60 sec. The pellet was resuspended in 2 ml of the phosphate buffered saline by being taken up several times into a 1 ml polypropylene pipette.

Apoptotic Studies by Fluorescence Microscope

Hoechst 33342 dye is used to stain the DNA where the staining procedure was performed by the method of Yuan et al. (2002). Briefly, to 10 µl of the harvested cells, 10µl of 1mg/ml propidium iodide (PI) and Hoescht 33342 mixture (Sigma, St. Louis, USA) was added. Suspension was placed on a clean glass slide and examined under a fluorescence microscope. The percentage of apoptotic cells was calculated by counting 100 cells on separate slides as above for individual animals.

Microscopy and Data Analysis

Sections were viewed and photographed at 200X and 100X with a Leica Optiphot microscope to which was attached a Leica Digital Camera. The data expressed as means ± SD were analysed by one way analysis of variance (ANOVA) and post hoc comparison between the means were made by the method of least significant difference (LSD) using the statistical software package SPSS 12.0 for Windows. The values were considered significant if the p value was found to be 0.05 or less.

Results

Morphological and Histopathological Study

All the groups showed normal body growth in this time and gained almost equal weight when compared to their initial body weights.

Multiple plaque lesions (MPLs) were recognized as either raised or non-raised lesions with identifiable tissue growth in carcinogen treated animals, often appearing singly or in multiple forms throughout the length of the colon (see Table 1). A few MPLs were also observed in Control and Etoricoxib animals as well. Maximum numbers of MPLs however, were observed in DMH

Table 1. Mean scores for Multiple Plaque Lesions (MPLs)

Animal groups	No. of MPLs Proximal	Middle	Distal	Total No. of MPLs	No. of rats with MPL /total no. of rats	MPL Incidence (%)	MPL burden	MPL multiplicity
Controls	2	1	1	4	2/5	40	0.8	2.0
DMH	6	8	9	23	5/5	100	4.6	4.6
DMH + Etoricoxib	2	5	3	10	3/5	60	2.0	3.3
Etoricoxib	1	2	1	4	2/5	40	0.8	2.0

MPL incidence= the percentage of animals having MPLs;MPL burden= total number of MPLs counted/ total number of rats; MPL multiplicity= total number of MPLs counted / number of MPLs bearing rats
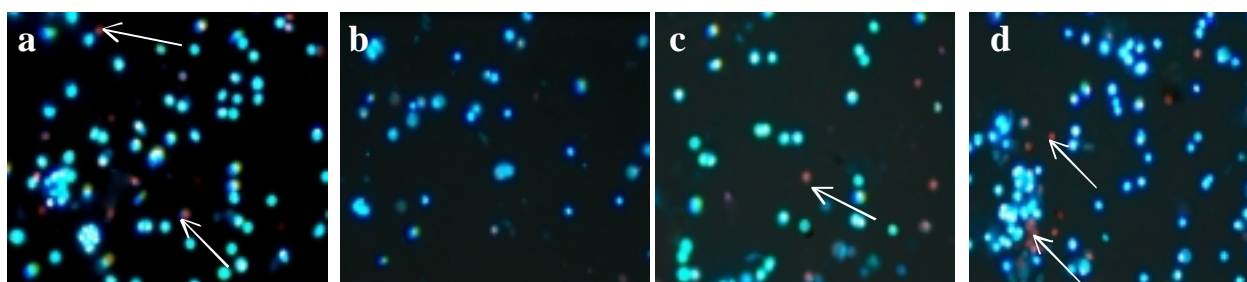
Figure 1. Apoptotic (shown by arrows) and Live Cells Visualized with Propidium Iodide / Hoechst 33342 Co-Staining under a Fluorescence Microscope. a) Control; b) DMH; c) DMH + Etoricoxib; d) Etoricoxib only (x400)
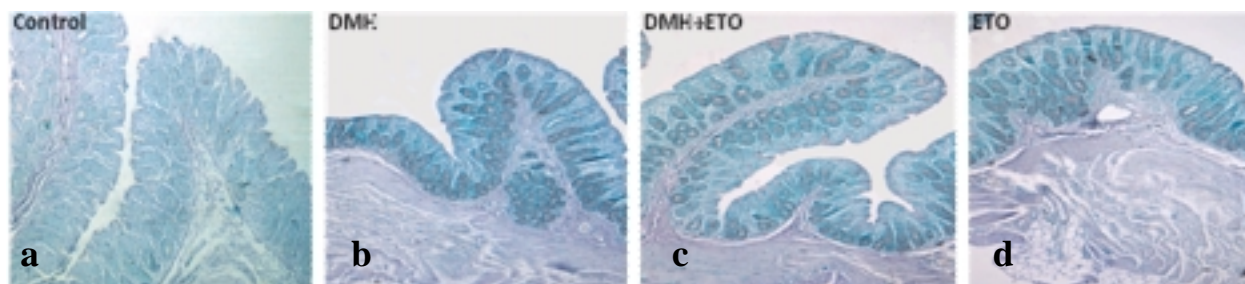

Figure 2. NF-κB Immunohistochemical Staining. a) Control; b) DMH; c) DMH+Etoricoxib; d) Etoricoxib only (x100)

treated animals but the number was found much less in DMH + Etoricoxib treated animals (Table 1).

Aberrant crypts (ACs) were identified as the crypts that were 2 to 3 times larger than the surrounding normal crypts. ACFs are early appearing lesions recognized on the colonic lumen surface in the treated rats. These ACFs are characterized by crypts with altered luminal epithelia and being larger than the adjacent normal crypts. ACF frequencies were significantly higher in the DMH treated rats. Control and Etoricoxib treated rats were free from the aberrant crypts. ACFs were also reduced with the simultaneous treatment of Etoricoxib and DMH (Table 2).

Histopathological Changes

DMH treated animals showed severe dysplasia and hyperplasia of crypt cells. The nuclei were also deeply stained in DMH treated animals, whereas in control

animals no such features were observed. The simultaneous administration of DMH and Etoricoxib reduced these features of dysplasia and hyperplasia. No such features were observed in the Etoricoxib only group.

Apoptotic Study

To study apoptosis, DNA binding dyes Hoechst 33342-Propidium iodide were used. Figure 1 shows photomicrographs of live (blue) and apoptotic (pink) cells in all the treatment groups. The number of apoptotic cells was observed under fluorescence microscope at 400X. The control group showed maximum number of apoptotic cells whereas the number of such cells was highly reduced in DMH treated animals (see Table 3). Increase in count of the apoptotic cells was observed in DMH + Etoricoxib group. Etoricoxib only group showed a few apoptotic cells which in general comparable to the Control group.

Western Blot Analysis and Immunohistochemistry

The expression of NF-κB and COX-2 was studied by Western blot analysis and immunohistochemistry. The expression of both the proteins was found to be higher in the paraffin tissue sections in DMH treated group as compared to the Control (see Figure 2). The expression was found to be low in Etoricoxib only group. In DMH + Etoricoxib group also, the expression was found to be high as compared to control, whereas it was much less than the DMH group . When analysed by Western blot, NF-κB and COX-2 expression was high in DMH treated group as compared to the control and the expression of these two proteins was found to be low in DMH + Etoricoxib treated group. The expression was also found to be low in Etoricoxib only group.

Discussion

The present study was undertaken to evaluate the chemopreventive response of Etoricoxib, a highly selective COX-2 inhibitor in the initiation stage (6 week

Table 2. Numbers of Aberrant Crypt Foci (ACF) of Each Size in the Different Groups

Group	Small	Medium	Large	Total
Control	0	0	0	0
DMH	12	13	7	32
DMH+ Etoricoxib	4	3	3	10
Etoricoxib	5	2	1	8

ACF were classified as small (1-3), medium (4-6), or large (>6) by the number of crypts

Table 3. Percentages of Apoptotic Nuclei Studied by Propidium Iodide / Hoeschst 33342 Staining

Group	Apoptotic nuclei/100 nuclei
Control	19 ± 2
DMH	4 ± 1^a
DMH + ETO	17 ± 3^b
ETO	20 ± 4^b

Data expressed as Mean ±S.D (n=4-6). Statistical significance was analysed by one way ANOVA; $^a p<0.001$ when compared to Group Control; $^b p<0.001$ respectively when compared to Group DMH

treatment) of colon carcinogenesis induced by DMH and the correlation of COX-2 with the NF-κB.

The initiation of colon cancer is thought to begin with a single mutational event within an isolated aberrant colon crypt. Colon carcinogenesis is a long process, taking months to develop in rodents. Preneoplastic lesions such as ACF, which occurs in 30-45 days after carcinogen administration or even earlier, have been extensively, used as endpoint in short term carcinogenesis and chemopreventive studies (Pereira et al., 1994; Corpet and Tache, 2002). In fact, ACFs are considered the 'gold standard' of colon carcinogenesis biomarkers (Pretlow et al., 1992; Takayama et al., 1998). In the present study, DMH treatment led to a marked increase in the grossly visible multiple plaque lesions (MPLs), aberrant crypts (ACs) and aberrant crypt foci (ACF) after a period of six weeks. Our study demonstrates that Etoricoxib treated animals were showing a decreased number of ACF.

Histopathologically also, there was seen a marked dysplasia as well as hyperplasia. Oral administration of Etoricoxib was able to weaken these features prominently indicating its efficiency as a chemopreventive agent at the present dose. The result of the present study thus clearly demonstrates that regular administration of a specific COX-2 inhibitor at its anti-inflammatory dose brings lesser MPLs, ACFs and dysplastic changes in the colon at the initial stages of carcinogenesis. It may be through the blockage of COX enzyme, which in turn suppresses the eicosanoid production of prostaglandins that affect the cell proliferation, tumor growth and immune responsiveness (Sheng et al., 1998). Free arachidonic acids are known to promote apoptosis in cancer cells (Higuchi et al., 2007) and apoptosis plays an important role in the regulation of normal and cancer cells (Wyllie et al., 1972). The characteristic features of apoptosis which distinguish it from necrosis are cell shrinkage, cytoplasmic blebbing, loss of membrane architecture, chromatin condensation, fragmentation of DNA into oligonucleosome-sized fragments, and formation of apoptotic bodies (Kerr et al., 1994).

NF-κB is an inducible and ubiquitously expressed transcription factor, which regulates the expression of numerous genes (including COX-2), associated with inflammation and cellular apoptosis, adhesion, differentiation and growth (Baeuerle and Baltimore, 1996). In view of the importance of NF-κB activated pathways in proliferative and anti-apoptotic responses, agents that suppress these pathways are anticipated to be useful in the prevention and treatment of cancer (Aggarwal, 2004). It is known that COX-2 gene has been shown to be regulated at the promoter level by NF-κB (Stark et al., 2007, St-Germain et al., 2004). The COX-2 promoter contains two potential NF-κB binding sites and NF-κB is a positive regulator of COX-2 expression in response to various cytokines and growth factors (Tanabe and Tohnai, 2002).

Immunohistochemical analysis of COX-2 protein in the colonic segments of different groups revealed the low expression of COX-2 protein in the control, while sections from DMH treated animals showed intense cytoplasmic expression of COX-2 in various locations of mucosal surface. Selective COX-2 inhibitor, Etoricoxib reduced the expression of COX-2 protein in the DMH treated animals. Western Blot analysis of COX-2 protein and NF-κB also revealed the same results as shown by immunohistochemistry. Our results suggest that DMH might have induced COX-2 expression and is mediated through the activation of the NF-κB associated pathways. These findings are consonant with the previous results reported by Shattuck-Brandt and colleagues (2000), who also detected COX-2 expression in stromal myofibroblasts in various colon tumor models in mice.

From the present study we conclude that, the anti-inflammatory efficacy of COX-2 selective NSAID Etoricoxib had the potential to reduce the number of MPLs and ACFs in colon and also reduce the dysplastic neoplasia of the crypt cells. This study also emphasizes the role of NF-κB and COX-2, which are important molecular targets of chemoprevention and are important players in the process of colon carcinogenesis.

Acknowledgement

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