Activation of Mitochondrial Apoptosis and Regulation of Ceramide Signalling by COX-2 Inhibitors in Colon Cancer

SN Sanyal*, Shelly Jain, Preety Ghanghas and Chandan Rana
Department of Biophysics, Panjab University, Chandigarh, India 160014

Abstract
Bcl-2 family of proteins is implicated in the malignant tumors including colorectal cancer. Activation of Bcl-2 inhibits the pro-apoptotic proteins (Bax and Bad) and regulates many biological processes such as apoptosis, cell proliferation and cell growth. As the mitochondrial enzymes are involved in sphingolipid metabolism, it can regulate ceramide formation and in turn mitochondria play a central role for the regulation of ceramide induced apoptosis. Bcl-2 Bcl-XL activates sphingosine kinases (SKs), resulting in the accumulation of S1P (sphingosine-1-phosphate), thereby reducing apoptosis. In the present study, the anti-neoplastic effects have been observed of Etoricoxib and Celecoxib, two COX-2 selective non-steroidal anti-inflammatory drugs (NSAIDs), and Diclofenac, a preferential COX-2 inhibitory NSAIDs, in the early stage of colon cancer in rats. These NSAIDs regress the expressions of Bcl-2 and SK-1 and promote apoptosis. Gross morphological analysis revealed the occurrence of raised mucosal lesions called MPL or multiple plaque lesions, which were maximum in the 1, 2-Dimethylhydrazine (DMH) treated group and their number regressed with the co-administration of the NSAIDs. An abnormal histo-architecture like hyperplasia and dysplasia were evident in the carcinogenic group, which were reduced with NSAIDs co-administration.

Keywords: Apoptosis, Bcl-2, Colorectal cancer; DMH; NSAIDs; SK-1

Introduction
Cells die in response to various stimuli and during apoptosis or programmed cell death this happens in a controlled way to maintain homeostasis in constantly replicating tissues such as the colon [1]. The pro-apoptotic Bax and Bad protein form important components of Bcl-2 family which is one of the dominant member in the mitochondrial form of apoptosis [2, 3]. It also involves down-modulation of Bax-antagonists such as Bcl-2 [4]. Translocation of pro-apoptotic Bax and Bak from the cytoplasm to the mitochondria is a crucial event in apoptosis cascades and therefore Bax has been recognized as an important mediator of anticancer drug-induced cell death [5]. Cytochrome c interacts with Atp-1 and triggers the formation of apoptosome protein complex in the cytosol and in the presence of ATP and Apaf-1 then becomes an allosteric activator of the caspase cascade and the proteolytic demolition of the cell [6, 7]. Caspases are the family of endoproteases, it provides critical links in cellular regulatory networks to control inflammation and cell death. Activation of apoptotic caspases results in either the inactivation or activation of the substrates, and the production of a cascade of signalling events permitting to the controlled demolition of cellular components [8]. Stimulation of inflammatory caspases promotes the production of active pro-inflammatory cytokines and promotes apoptosis. Caspases are critical links as their dysregulation underlies human diseases which includes inflammatory disorders and cancer [9].

Cell signalling pathway mediated by ceramide has been shown to contribute to terminal cell differentiation, cell cycle arrest and apoptosis [10] as well as to cell proliferation [11]. Ceramide may modify the relationship between pro-apoptotic (i.e., Bax and Bad) and anti-apoptotic (i.e., Bcl-2 and Bcl-XL) members of the Bcl-2 family of proteins. Key components of the sphingolipid metabolic pathway, are the ceramide and its metabolites, which includes sphingosine and sphingosine-1-phosphate (S1P). S1P has been exhibited to stimulate cell growth and prevent apoptosis [12]. Sphingosine kinase (SK) catalyse the phosphorylation of sphingosine that results in the synthesis of S1P. Sphingosine kinase (SK1) can be stimulated by a variety of growth factors, cytokines and mitogens [13].

Presently, in an experimental colorectal cancer (CRC) in rodents, apoptotic proteins and sphingosine kinase have been targeted in the COX-2 inhibitors mediated cancer cell killings. Three COX-2 inhibitors used on comparable basis are 1) Celecoxib, a methylphenyl trifluoromethyl pyrazolyl benzene sulfonamide, 2) Etoricoxib, a chloromethyl methysulfonyl phenyl bipyriridine and 3) Diclofenac, a dichloro anilinophenyl acetic acid.

Materials and Methods

Chemicals

1, 2-Dimethylhydrazine (DMH) and Bradford reagent have been purchased from Sigma Aldrich (St. Louis, MO, USA). Celecoxib and Diclofenac were a generous gift from Ranbaxy Pharmaceuticals (Gurgaon, India). The primary antibody against Bcl-2, Bax, Bad, Apaf-1, Cyt c, Caspase-9, Caspase-3, SK-1 and β-actin was purchased from Santa Cruz Biotechnology (USA). Anti-caspase-3 phosphatase conjugated secondary antibodies and BCIP-NBT were purchased from Genet, Bangalore, India. All other chemicals and reagents used in the present study were of analytical grade and purchased from the reputed Indian manufacturers.

Animal Procurement

Female Sprague-Dawley rats of body weight between 100-150g were obtained from the inbred population of the Central Animal House, Panjab University, Chandigarh. The animals were acclimatized for at least 1 week and given normal diet (rodent chow) and water ad libitum. They were maintained as per the principles and guidelines of the Ethics Committee of Animal Care of Panjab University and in general, followed the NIH guidelines (Rule No. 23-85, as revised in 1985). They
were housed three/four per cage in polypropylene cages with a wire mesh top and kept in a hygienic bed of husk (regularly changed) in a well-ventilated animal room. The animals were also maintained at the ambient temperature and humidity, and under a 12 hr photoperiod of light and darkness, respectively. They were provided with a room cooler and room heater in the summer and winter months, respectively.

**Treatment Schedule**

Animals were assorted into the following groups

Group 1 (control): Animals received food (rat chow) and water *ad libitum* daily.

Group 2 (Vehicle Treated): Animals were administered the vehicle (1 mM EDTA–saline) subcutaneously (s.c.) in weekly injection and 0.5% carboxymethyl cellulose (CMC) sodium salt per oral (p.o.) daily.

Group 3 (1, 2-Dimethylhydrazine dihydrochloride (DMH)): Animals were administered with DMH weekly at a dose of 30 mg/kg body weight (s.c.). The dose of DMH in early carcinogenesis has been established in our laboratory earlier [14]. DMH was freshly prepared in 1 mM EDTA–saline and pH adjusted to 7.0 using dilute NaOH solution.

Group 4 (DMH + Celecoxib): celecoxib was given daily (p.o.) within its therapeutic anti-inflammatory dose (ED₅₀ for rats, 6 mg/kg body weight) along with the weekly administration of 30 mg/kg body weight of DMH [15].

Group 5 (DMH + Etoricoxib): Etoricoxib was given daily (p.o.) within its therapeutic anti-inflammatory dose (ED₅₀ for rats, 0.6 mg/kg body weight) along with the weekly administration of 30 mg/kg body weight of DMH.

Group 6 (DMH + Diclofenac): Diclofenac was given daily (p.o.) within its therapeutic anti-inflammatory dose (ED₅₀ for rats, 8 mg/kg body weight) along with the weekly administration of 30 mg/kg body weight of DMH.

Group 7 (Celecoxib): Animals received only celecoxib (6 mg/kg) dissolved in 0.5% CMC per oral daily.

Group 8 (Etoricoxib): Animals received only etoricoxib (0.6 mg/kg) dissolved in 0.5% CMC per oral daily.

Group 9 (Diclofenac): Animals received only diclofenac (8 mg/kg) dissolved in 0.5% CMC per oral daily.

Four animals were taken in each treatment group. Body weight of the animals was recorded weekly till the termination of the treatment. After 6 week of treatment schedule, the animals were kept on overnight fasting with drinking water *ad libitum* and sacrificed the next day under an over anesthesia with ether.

**Gross Morphological Observation**

The colons were removed and flushed clean with ice-cold physiological saline (NaCl solution, 9 g/L). These were opened longitudinally along the median and laid flat to examine with a hand held lens for the incidence of macroscopic neoplastic lesions/plaques called the multiple plaque lesions (MPLs) [17].

**Histopathology**

The Colonic pieces were divided into proximal, middle and distal regions and immediately fixed in 10% buffered formalin for 24 hrs. The tissues were dehydrated in ascending series of alcohol (30-90%) 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 hour at 60°C with two changes. The tissues were embedded in wax and set in the wooden blocks, then five micron sections were cut using a hand driven microtome, stretched in warm water bath (58°C) and transferred to egg albumin coated slides. Slides were dewaxed in xylene, then hydrated in descending series of alcohol, stained with hematoxylin and dipped in amonia water till blue colour is developed. In case of being over stained the slides are dipped in acid water. The slides were dehydrated in ascending series of alcohol and brought to 70% alcohol and then stained with eosin and differentiated in 90% alcohol. Slides were dipped in absolute alcohol, cleared in xylene and finally mounted in DPX, and viewed under a light microscope and photographed at 200X with a Carl Zeiss, Axioscope AI microscope to which was attached a Digital Camera (Jenoptix) [18].

**Isolation of Colonocytes**

Colonic epithelial cells (colonocytes) were obtained from the freshly isolated colons by the method of Mouille et al [19] as described earlier [14,16]. Trypan blue dye exclusion was performed each time for every group of isolated colonocytes and the viability of the cells observed ~90%.

**Western Blot Analysis**

Cell lysate preparation and western blots were performed as described earlier [20]. Protein samples (50 µg) from each treatment group were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis. The separated proteins were electrophoretically transferred to nitrocellulose membrane. Immunoblot was prepared using primary antibodies (Bcl-2: 1:1,000, β-actin: 1:1,000) from Santa Cruz Biotechnology, Inc., (USA) and alkaline phosphatase conjugated respective IgG secondary antibody at a dilution of 1:10,000 (Genei, Bangalore, India). A BCIP-NBT detection system was used to develop the blots. Bands obtained were densimetrically analysed using Image J software (NIH, Bethesda, Maryland, USA), and the density was expressed as grey values in densimetric units. β-actin has been used in the blots as the protein loading control. For the preparation of protein extracts, colons were removed at the end of the 6th week treatment period and rinsed. Total lysates were prepared in fresh ice-cold protein lysis buffer. The extracts were cleared of nuclei and residual mucosa by centrifugation at 10,000g at 4°C. The supernatant was collected, and protein concentration determined by the method of Bradford [21].

**Immunofluorescence**

Immunofluorescence analysis was done as described earlier with primary antibodies (Bcl-2: 1:1,000, Bax: 1:1,000, Bad: 1:1,000, Apaf-1: 1:1,000, Cyt c: 1:1,000, Caspase-9: 1:1,000, Caspase-3: 1:1,000 and SK-1: 1:1,000), then incubated with respective FITC-conjugated secondary antibody and observed under a fluorescence microscope (AxioScope A1, Carl Zeiss, Germany).

**Immunofluorescence scores** were calculated as follows [22]

IF score = % of positive cells X Intensity of fluorescence

Where, <10% positive cells = 0, 11-50 % positive cells = 2, 51-100% positive cells = 4, No staining = 0, Light Staining = 1, Moderate Staining = 2 and Bright Staining = 4

**Apoptotic Studies**

**Acridine orange/ ethidium bromide co-staining**

Fluorescence microscopy using the DNA binding fluorescent dyes, Acridine orange and Ethidium bromide was employed to study the
morbidity of the isolated colonocytes undergoing apoptosis. 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) [23]. Of this mixture, 10 μl was placed on a clean glass slide, covered with coverslip, and a minimum of 300 cells were counted (X200), using a fluorescence microscope (Axioskope 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.

Terminal deoxynucleotidyl transferase (TdT) dUTP Nick End Labelling (TUNEL) Assay

Cells undergoing apoptosis were detected in paraffin embedded tissue sections (5 μm thick) by the TUNEL method, using TUNEL apoptosis detecting kit (GenScript, NJ, USA) according to the manufacturer’s protocol. A minimum of 200 cells per field were counted in four randomly selected fields and the percentage of apoptosis calculated.

Statistical Analysis

For analyzing the data, one way analysis of variance (ANOVA) was performed using statistical software package SPSS v 16 for Windows. The post-hoc comparisons of means from different groups were made by the Duncan’s test, where the preliminary analysis of variance indicated significant treatment effects. This is a multi-parametric test. Significant at p<0.01.

Results and Discussion

Morphological analysis of colons

Among all the nine groups, DMH alone showed higher number of MPLs, with a significant appearance of MPLs in all the three regions of colon; proximal, middle and distal, as shown in Figure1. MPLs were recognized as either raised or non-raised lesions with identifiable tissue growth in carcinogen treated animals. MPL often appearing singly, but sometimes in multiples throughout the length of the colon. Appearance of MPLs at an early stage of neoplasia suggests that MPLs are the macroscopic sites for tumor growth. The control and vehicle treated animals from 6 week study show no such carcinogenic changes. In the Celecoxib, Etoricoxib and Diclofenac sections showing normal histological morphology of colon sections comparable to the control sections. DMH sections showing severe hyperplasia, an early neoplastic alteration in the mucosa characterized by abnormal cell division and loss of mucin producing cells, nuclei enlarged and deeply stained. DMH + Celecoxib, DMH + Etoricoxib and DMH + Diclofenac as visible in the connective tissue layer and less number of mitotic cells. As shown in Figure 2, the sections from vehicle treated and NSAID alone groups were showing normal crypt structure similar to those of the control group. Results from the present study showed the occurrence of invasive carcinomas leading to the muscularis mucosa both in tumors and in flat, non-raised mucosa of the colon. Most of the features of DMH-induced adenocarcinomas of the rat colon in the present study are similar to those of human colon carcinoma [24]. Invading crypts associated with the lymph nodes as observed in the present study were also similar to those carcinogenic changes. However, mild grade hyperplasia can still be evident. There were less dysplastic changes in DMH + Celecoxib, DMH + Etoricoxib and DMH + Diclofenac as visible in the connective tissue layer and less number of mitotic cells. As shown in Figure 2, the sections from vehicle treated and NSAID alone groups were showing normal crypt structure similar to those of the control group. Results from the present study showed the occurrence of invasive carcinomas leading to the muscularis mucosa both in tumors and in flat, non-raised mucosa of the colon. Most of the features of DMH-induced adenocarcinomas of the rat colon in the present study are similar to those of human colon carcinoma [24]. Invading crypts associated with the lymph nodes as observed in the present study were also similar to those
Activation of Mitochondrial Apoptosis

Inhibition of mitochondrial apoptosis is common in cancers and have a role in various cellular functions including, cell growth, differentiation, development and apoptosis [25]. Initiation of mitochondrial-based apoptosis with the use of NSAIDs supports their chemoprevention and anti-cancerous potential [14,16]. The protein expression as observed by western immunoblot analysis shows that administration of DMH leads to an up-regulation of Bcl-2 (arrows), the anti-apoptotic mitochondrial membrane guard proteins, as compared to control while NSAIDs co-administration had significantly reduced it upto the basal level (Figures 3a and 3b). Immunofluorescence further confirms the higher amount of translated Bcl-2 in DMH group as compared to Control whereas DMH + Celecoxib, DMH + Etoricoxib and DMH + Diclofenac were having lesser Bcl-2 levels as compared to DMH group, as shown in (Figures 3c and 3d). Along with the upregulation of Bcl-2 levels, the expressions of Bad and Bax were downregulated in DMH group as compared to control (Figures 4a-c). Celecoxib, Etoricoxib and Diclofenac administrations were able to upregulate the Bad and Bax expression, and thus triggered the mitochondrial pathway of apoptosis in colon cancer. Vehicle treated or alone NSAID groups (Celecoxib, Etoricoxib and Diclofenac) also showed significant expression of Bax, Bad and a low expression of Bcl-2.

The immunofluorescence localization of Cyt c and Apaf-1 (arrows) in NSAIDs co-administration group is higher as compared to DMH alone group, as shown in Figure 5a-c. With the elevated cytoplasmic expression and localization of Cyt c and Apaf-1 in NSAIDs co-administration groups, it is clear that all the three NSAIDs are promoting mitochondrial mediated pathway of apoptosis. Apoptosis proceeds when activated caspase 9 cleaves pro-caspase3 to produce active caspase3 which is a known final executioner of apoptosis [26]. In the present study the expression levels of caspase9 and active caspase3 is higher in NSAIDs co-administration groups as compared to DMH alone group (Figures 6a-c), which suggests that NSAIDs may prevent carcinogenesis via activating the mitochondrial cascade of apoptosis. As caspase9 is involved in the activation of the caspase cascade promoting mitochondrial mediated pathway of apoptosis. Apoptosis proceeds when activated caspase9 cleaves pro-caspase3 to produce active caspase3 which is a known final executioner of apoptosis [26]. In the present study the expression levels of caspase9 and active caspase3 is higher in NSAIDs co-administration groups as compared to DMH alone group (Figures 6a-c), which suggests that NSAIDs may prevent carcinogenesis via activating the mitochondrial cascade of apoptosis.

Figure 3: Expression of Bcl-2 protein in various treatment groups.a. Western immunoblot analysis of Bcl-2 showed higher expression in DMH group as compared to Control, whereas DMH + Celecoxib, DMH + Etoricoxib and DMH + Diclofenac groups were having lesser Bcl-2 levels as compared to DMH group. β-actin is serving as loading control.b. Quantitative analysis of the blots in densitometric units normalized with β-actin. (a,b,c,d,e) represents in each segment not sharing a common superscript letter that differed significantly (Duncan’s test: p < 0.01).c. Immunofluorescence analysis of Bcl-2 for its expression and localization studies in colonic tissue sections of 6 weeks treatment schedule (X400). DMH + Celecoxib, DMH + Etoricoxib and DMH + Diclofenac groups showing increased localization of Bcl-2 (arrows) as compared to DMH alone group. b. Expression of Bcl-2 in the colonic tissue sections (arrows), analyzed by immunofluorescence in colonic tissue sections of 6 weeks treatment schedule (X400). DMH + Celecoxib, DMH + Etoricoxib and DMH + Diclofenac group showing increased localization of Bad (arrows), whereas DMH group is showing decreased expression of Bax. c. Shows the immunofluorescent staining score of Bad and Bax. (a,b,c,d,e) represents in each segment not sharing a common superscript letter that differed significantly (Duncan’s test: p < 0.01).

Figure 4: Immunofluorescence analysis Bax and Bad.a. Expression of Bax in the colonic tissue sections (arrows), analyzed by immunofluorescence in colonic tissue sections of 6 weeks treatment schedule (X400). DMH + Celecoxib, DMH + Etoricoxib and DMH + Diclofenac group showing increased localization of Bax (arrows) as compared to DMH alone group. b. Expression of Bax in the colonic tissue sections (arrows), analyzed by immunofluorescence in colonic tissue sections of 6 weeks treatment schedule (X400). DMH + Celecoxib, DMH + Etoricoxib and DMH + Diclofenac group showing increased localization of Bad (arrows), whereas DMH group is showing decreased expression of Bax. c. Shows the immunofluorescent staining score of Bad and Bax. (a,b,c,d,e) represents in each segment not sharing a common superscript letter that differed significantly (Duncan’s test: p < 0.01).

Figure 5: Expression of Apaf-1 and Cyt-c analysed by immunofluorescence. a. Expression of Apaf-1 in the colonic tissue sections (arrows), analyzed by immunofluorescence. DMH + Celecoxib, DMH + Etoricoxib and DMH + Diclofenac groups showing increased localization of Apaf-1(arrow), whereas expression is decreased in DMH alone group. b. Immunofluorescence analysis of Cyt-c for its expression and localization studies in colonic tissue sections of 6 weeks treatment schedule (X400). DMH + Celecoxib, DMH + Etoricoxib and DMH + Diclofenac group showing increased localization of Cyt-c (arrows) as compared to DMH alone group. c. Shows the immunofluorescent staining score of Apaf-1 and Cyt-c. (a,b,c,d,e) represents in each segment not sharing a common superscript letter that differed significantly (Duncan’s test: p < 0.01).
phosphorylates sphingosine to sphingosine-1-phosphate protects anti-
induced apoptosis [27]. However, over-expression of SK-1 which
regulates mitochondria plays a central role for the regulation of ceramide
induced apoptosis specifically when generated in mitochondria and in
sphingolipid metabolism, it can regulate ceramide formation which can
administered groups. As the mitochondrial enzymes are involved in
control, vehicle treated and NSAID alone groups (Figure 7a & b). The
expression of SK-1 is higher in DMH alone group as compared to
other groups. In the present study, sphingosine kinase (SK-1) protein was checked. In the present study,
accumulation of S1P had been seen in DMH group and corrected by
levels of apoptosis (Figures 9a and 9b). The number of TUNEL positive
cells undergoing apoptosis was significant and the intensity of staining
was high in these groups as compared to DMH. DMH treated group
showed a considerable decrease in TUNEL positive cells and an
increase in the number of apoptotic cells after Celecoxib, Etoricoxib
and Diclofenac co-administration.

Regulation of Apoptosis by NSAIDs

The acridine orange and ethidium bromide co-staining of isolated
colonocytes showed that the NSAIDs co-administered groups; DMH +
Celecoxib, DMH + Etoricoxib and DMH + Diclofenac had more
apoptotic cells with respect to the DMH alone group, as shown in Figure
8a-e. Viable cells glow green with acridine orange whereas the apoptotic
cells were characterized by yellow colour with ethidium bromide.
Apoptotic cell number in the control, vehicle treated and alone NSAID
groups i.e., Celecoxib, Etoricoxib and Diclofenac was significantly
higher in comparison to NSAIDs co-administration groups and DMH
alone (Figures 8a-e).

The results from the TUNEL assay showed that the colonic sections
of control, vehicle treated and alone NSAID groups detected significant
level of apoptosis (Figures 9a and 9b). The number of TUNEL positive
cells undergoing apoptosis was significant and the intensity of staining
was high in these groups as compared to DMH. DMH treated group
also showed a considerable decrease in TUNEL positive cells and an
increase in the number of apoptotic cells after Celecoxib, Etoricoxib
and Diclofenac co-administration.

Conclusion

Ceramide induces apoptosis, while sphingosine-1 phosphate (SIP)
functions as a survival factor in colon cancer. Mitochondria play a
central role in ceramide induced apoptosis and regulated by Bcl-2 family
of proteins. Presently, a high level of SK-1 which is responsible for the
accumulation of SIP had been seen in DMH group and corrected by
Ceramide/ S1P pathway

To further elucidate the role of Bcl-2 in promoting cell survival
and blocking apoptosis in colon carcinogenesis, the expression of
sphingosine kinase (SK-1) protein was checked. In the present study,
expression of SK-1s is higher in DMH alone group as compared to
control, vehicle treated and NSAID alone groups (Figure 7a & 8b). The
higher expression of SK-1 is significantly reduced in the NSAIDs co-
administration groups. As the mitochondrial enzymes are involved in
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NSAIDs. This may lead to a novel chemopreventive strategy targeting the Bcl-2 proteins, regulating the enzymes involved in the ceramide metabolism, and downregulating the pro-survival growth factors in colon carcinogenesis.

References