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Original article

Chemopreventive action of non-steroidal anti-inflammatory drugs on the inflammatory pathways in colon cancer

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ABSTRACT

Non-steroidal anti-inflammatory drugs (NSAIDs) are emerging as novel chemopreventive agents against a variety of cancers owing to their capability in blocking the tumor development by cellular proliferation and by promoting apoptosis. Inflammation is principal cause of colon carcinogenesis. A missing link between inflammation and cancer could be the activation of NF-κB, which is a hallmark of inflammatory response, and is commonly detected in malignant tumors. Therefore, targeting pro-inflammatory cyclooxygenase enzymes and transcription factors will be profitable as a mechanism to inhibit tumor growth. In the present study, we have studied the role of various pro-inflammatory enzymes and transcription factors in the development of the 1,2-dimethylhydrazine dihydrochloride (DMH)-induced colorectal cancer and also observed the role of three NSAIDs, viz., Celecoxib, Etoricoxib and Diclofenac. Carcinogenic changes were observed in morphological and histopathological studies, whereas protein regulations of various biomolecules were identified by immunofluorescence analysis. Apoptotic studies was done by TUNEL assay and Hoechst/PI co-staining of the isolated colonocytes. It was found that DMH-treated animals were having an over-expression of pro-inflammatory enzymes, aberrant nuclear localization of activated cell survival transcription factor, NF-κB and suppression of anti-inflammatory transcription factor PPAR-γ, thereby suggesting a marked role of inflammation in the tumor progression. However, co-administration of NSAIDs has significantly reduced the inflammatory potential of the growing neoplasm.

1. Introduction

Nonsteroidal anti-inflammatory drugs (NSAIDs) are a class of compounds that provide analgesic (pain-killing) and antipyretic (fever-reducing) effects, and in higher doses can show anti-inflammatory effects. The importance of NSAIDs in reducing cancer risk have emerged in recent times and been clearly shown by several preclinical and clinical studies [1]. These also control a number of signal transduction pathways that may affect cellular proliferation, immune response, differentiation, programmed cell death, angiogenesis, cellular adhesion and tumor invasion. In 1970, John Vane proposed that NSAIDs mediate their anti- inflammatory effects by restraining the enzyme activity of the cyclooxygenase (COX), when the association between NSAIDs and the COX pathway was made – a work for which he was later awarded the Nobel Prize for physiology and medicine. Subsequently, the landmark study by Kune et al., 1988 showed that a considerably lower incidence of colon cancer was observed in those subjects using NSAIDs for various clinical indications accompanied a new era in cancer prevention. NSAIDs are strong chemopreventive agents for colon cancer and inhibition of the COX enzyme, which catalyzes the synthesis of prostaglandins which is their best recognized pharmacological property [2].

The first clue to the role of inflammation in cancer is given by Virchow in 1863 who hypothesized that some classes of irritants at the site of tissue injury and inflammation may enhance cell proliferation [3]. Inflammation is a vital component of tumor progression supported by various studies which show the tumor initiation and progression from the site of chronic irritation, infection and inflammation. The role of inflammatory in maintenance of tissue homeostasis and repair is well known but how the disrupted homeostasis in the inflammatory microenvironment may ultimately lead to carcinogenesis is not yet clear. Inflammatory microenvironment obstructing a network of signaling molecules and various inflammatory cells are crucial for the neoplastic growth of transformed cells, which at the site of chronic inflammation, is accredited to the mutagenic tendency of persistent infection fighting agents [4]. DNA damage promoting agents along with the sustained cell proliferation is required for the

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neoplastic growth, a fact supported by various epidemiological evidences which point to a connection between inflammation and a predisposition for the progression of cancer, i.e., long-term inflammation leading to the development of dysplasia [5].

Nuclear factor kappa B (NF-κB) is a transcription factor that plays an important role at the sites of inflammation and cancer [6]. NF-κB was discovered in the B cells where it was found bound to the kappa immunoglobulin gene enhancer [7]. It exists as a trimer with RelA and its inhibitory subunit, IκBα in the cytoplasm in an inactive state. However, various signals activate IKK complex, a kinase which phosphorylates IKKα the inhibitory subunit of NF-κB leading to its degradation by proteasome complex, thus allowing the translocation of NF-kB to the nucleus and induces the transcription of various genes encoding cytokines, growth factors, cell adhesion molecules and prooncogene proteins [8,9]. Activation of NF-κB mediates cellular proliferation, induces cellular transformation and prevents the elimination of pre-neoplastic and malignant cells by upregulation of the anti-apoptosis proteins [10]. NF-κB contribute to the development of various types of cancers, including colon cancer by regulating the expression of genes that are involved in cell proliferation (Cyclin D1), angiogenesis (VEGF, IL-8), and metastasis (MMP9) [11,12]. As NF-κB is a positive regulator of COX-2 expression, Aspirin and COX-2-specific NSAIDs had been observed to diminish COX-2 expression through NF-κB inactivation pathway [13]. These findings implicate NF-κB inhibition as an important target in the chemoprevention of colon cancer.

Peroxisome proliferator-activated receptors (PPARs) are ligand-activated transcription factors that belong to the nuclear hormone receptor family. Three different types of PPARs have been identified which are alpha, delta/beta and gamma. They play significant role in glucose and lipid metabolism, and cell differentiation and proliferation [14,15]. PPAR-γ isoform behaves as a "molecular sensor", binding a broad range of molecules associated with metabolism, and has been studied comprehensively in diabetes and obesity due to its role in mediating glucose metabolism [16,17].

In the present study we studied the inflammatory role of Celecoxib, Etoricoxib and Diclofenac at the initial stage of experimental colon cancer which could be mediated via the regulation of the proteins of inflammatory pathway and apoptosis. Celecoxib, a methylphenyl trifluoromethyl benzenesulfonimide, is a COX-2 selective inhibitor and found to lessen the numbers of colon and rectal polyps in patients whereas Etoricoxib, a second generation selective cyclooxygenase-2 (COX-2) inhibitor having chloromethyl sulfonyl bipyridine group and found to be highly effective in reducing inflammation [18]. Diclofenac is a preferential COX-2 selective NSAID contains dichloro anilinophenyl acetic acid group and has anti-inflammatory, antipyretic and analgesic properties [19].

2. Materials and methods

2.1. Chemicals

1,2-Dimethylhydrazine dihydrochloride (DMH) and Bradford reagent were purchased from Sigma-Aldrich (St. Louis, MO, USA). Celecoxib, Etoricoxib and Diclofenac were a generous gift from Ranbaxy Pharmaceuticals Ltd. (Gurgaon, Haryana, India). Primary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and also from BD Biosciences (San Jose, CA, USA). Alkaline phosphatase-conjugated secondary antibodies, Fluorescein isothiocyanate (FITC)-conjugated secondary antibodies and 5-bromo-4-chloro-3-indolyl phosphate disodium/Nitro Blue Tetrazolium (BCIP/NBT) were purchased from Genei (Bangalore, India). All other chemicals and reagents used in the present study were of analytical grade and purchased from HiMedia (Mumbai, India).

2.2. Animal procurement

Female Sprague-Dawley rats of body weight between 150–200 g were obtained from the inbred population of the Central Animal House, Panjab University, Chandigarh. The animals 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 in polypropylene cages with a wire mesh top and a regularly changed husk bed with a maximum of 4–6 animals in each cage. The animal rooms were maintained at the ambient temperature and provided with a room cooler or heater during the summer or winter months, respectively. The animals received food (rodent chow) and water ad libitum, and were exposed to 12 h day/night photoperiod. They were acclimatized for 1 week and then assorted in the different groups.

2.3. Treatment schedule

A six week study was conducted for the present experimental work. Animals were divided into nine different groups having five animals in each group.

Group 1: Control Group: The animals were kept on normal diet and water.

Group 2: Control Group+ vehicle treated: Animals were administered the vehicle, 1 mM EDTA saline, pH 7.0, subcutaneously (s.c.) in a weekly injection and 0.5% carboxymethyl cellulose sodium salt (CMC) per oral (p.o.) daily.

Group 3: DMH treated: Animals were administered with DMH weekly at a dose of 30 mg/kg body weight s.c., as has been established in our laboratory earlier [20]. DMH was freshly prepared in 1 mM EDTA saline (pH 6.5).

Group 4: DMH +Celecoxib: Celecoxib (6 mg/kg body weight) was co-administered per oral daily to the animals along with the weekly dose of DMH. The dose was chosen within the therapeutic anti-inflammatory dose (ED_{50}) for the rats [21].

Group 5: DMH+ Etoricoxib: Etoricoxib (0.6 mg/kg body weight) was co-administered per oral daily to the animals along with the weekly dose of DMH [22].

Group 6: DMH+ Diclofenac: Diclofenac (8 mg/kg body weight) was co-administered per oral daily to the animals along with the weekly dose of DMH [23].

Group 7: Celecoxib: Animals were administered with Celecoxib daily at a dose of 6 mg/kg body weight orally [21].

Group 8: Etoricoxib: Animals were administered with Etoricoxib daily at a dose of 0.6 mg/kg body weight orally [22].

Group 9: Diclofenac: Animals were administered with Diclofenac daily at a dose of 8 mg/kg body weight orally [23].

After 6 week, animals were kept on overnight fasting with drinking water, *ad libitum* and sacrificed the next day under an over anesthesia with ether.

2.4. Aberrant crypt foci (ACFs) analysis

The colons were removed, flushed clean, cut open and divided into different parts such as proximal, middle and distal. 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. The mucosal surface of the colon was evaluated for the number of ACF in the stained colon under 100× magnification using a light microscope. Enlarged and slightly elevated lesions with increased staining were readily identifiable in comparison to the normal adjacent mucosa. ACFs were classified as small (1–3), medium (4–6) or large (>6) by the number of crypts per foci [24].