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Original article

Activation of NF-κB: Bridging the gap between inflammation and cancer in colitis-mediated colon carcinogenesis

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Abbreviations:
ACF, Aberrant Crypt Foci
ANOVA, Analysis of Variance
Cox, Cyclooxygenase
DAI, Disease Activity Index
DMH, 1,2-dimethylbenz(a)anthracene
DSS, Dextran Sulfate Sodium
IFN-γ, Interferon γ
IL, Interleukin
NF-κB, Nuclear Factor κB
NSAID, Non-Steroidal Anti-Inflammatory Drug
TNF-α, Tumor Necrotic Factor α

ABSTRACT

Several studies have shown the anti-neoplastic effects of non-steroidal anti-inflammatory drugs (NSAIDs) on 1,2-dimethylhydrazine (DMH)-induced colon carcinogenesis, but how these drugs act in case of inflammation-augmented tumorigenesis is still not clear. The present study therefore designs an animal model of colitis-associated colon cancer where 3% Dextran sufate sodium (DSS) is used to develop ulcerative colitis and DMH treatment leads to colon carcinogenesis as early as in six weeks. Clinical symptoms for ulcerative colitis were studied using Disease Activity Index (DAI) while myeloperoxidase assay marked the neutrophil infiltration in DSS and DMH treated groups. The present results indicated the upregulation of the activity of inflammatory marker enzyme, cyclooxygenase-2 (cox-2) and pro-inflammatory cytokines such as TNF-α, IL-1β, IL-4 and IFN-γ with the treatment of DSS as well as DMH. The presence of cytokines in the inflammatory milieu might lead to the transformation of cytoplasmic inactive NF-κB (Nuclear Factor κB) to its active nuclear form, thereby leading to tumorigenesis. The administration of celecoxib along with DSS and DMH, revealed its chemopreventive efficacy in colitis as well as colon cancer. The effect of different doses of DMH on mouse colon was also investigated to obtain a minimum dose of DMH which can induce visible lesions in mice colons at a high incidence.

1. Introduction

The procarcinogens such as the hydrazines are known to induce a variety of malignant tumors including skin, mammary and colorectal tissue [1]. To this end, 1,2-dimethylhydrazine (DMH)-induced colon carcinogenesis in rodent model is an effective tool and shares many similarities with human colon carcinoma, including symptomatic similarities and response to promotional or preventive agents [2]. In the present study, we look up to a minimum dose of DMH, sufficient to induce visible lesions in mice colons as early as in 6 weeks in experimental ulcerative colitis and its chemoprevention with the anti-inflammatory drugs.

The pathogenesis of inflammatory bowel disease (IBD) and its association with colorectal cancer remains poorly understood even after many years of research [3]. It is believed that the patients with ulcerative colitis (UC), a form of IBD, are known to be at a higher risk of colorectal cancer [4], and increased epithelial cell proliferation associated with repetitive cycles of inflammation, damage and regeneration in case of ulcerative colitis might lead to carcinogenesis in the long run. In this context, the animal models provide an excellent platform for the study of the mechanisms underlying these diseases [5]. The most widely used model for induction of colitis in mice is found with dextran sulfate sodium (DSS) [6] and its association with colon carcinoma can be studied by the combined administration of the procarcinogen, DMH with DSS [7]. This has been done in the present study where a dose of 30 mg/kg of DMH is given weekly s.c. and two cycles of 3% DSS are given for 7 days, each followed by a 14 day cycle of distilled water to induce carcinogenesis and UC, respectively, and a combination of DMH and DSS to study colitis-associated carcinogenesis.

During inflammation, the formation of prostaglandins such as PGE2 from aracidonic acid is catalyzed by the cyclooxygenase (cox)

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enzyme [8] which has been established as the most important pro-inflammatory mediator and implicated in the process of carcinogenesis. Cox has two isoforms: the housekeeping cox-1 and the inducible cox-2, which is highly upregulated in inflammation, lesions, carcinoma and other disorders [9]. Non-steroidal anti-inflammatory drugs (NSAIDs), which act primarily by the inhibition of the cox enzyme, have been found to be highly effective chemopreventive agents in animal studies [10]. NSAIDs are categorized into traditional NSAIDs (inhibiting cox-1 and cox-2) and coxibs (selectively inhibiting cox-2 while sparing cox-1, thereby creating no hindrance to the homeostatic activities of cox-1). To further elucidate the mechanism of action of the second generation coxibs in the chemoprevention of UC-associated colorectal cancer, we have incorporated celecoxib in the present study.

Chronic inflammation develops through the action of various inflammatory mediators, including TNF-α and IL-1β and IFN-γ, which remove anti-tumor immunity and facilitate tumor progression [11]. IL-2 is an anti-inflammatory cytokine which increases the natural killer cell activity as shown in animals as well as humans [12]. TNF-α has been found to be involved in all the stages of carcinogenesis such as cellular transformation, promotion, survival, proliferation, angiogenesis and metastasis [13]. It acts primarily through the induction of genes encoding nuclear factor-κB (NF-κB)–dependent anti-apoptotic molecules [14].

A missing link between inflammation and cancer could be the activation of NF-κB, a hallmark of inflammatory response, and is frequently detected in malignant tumors [15]. Under normal circumstances, NF-κB dimers are confined to the cytoplasm by the inhibitory κB (IκB). But stimulation from pro-inflammatory cytokines like TNF-α, apart from that in viral and microbial infections, leads to the activation of the canonical NF-κB pathway which activates the Inhibitory κB kinase (IKK) complex [16]. IKK phosphorylates IκBs bind to the NF-κB, leading to the ubiquitination of the former and allowing the released p65 subunits of NF-κB to enter the nucleus where they can initiate the transcription of various cell survival, proliferation and anti-apoptotic genes.

2. Materials and methods

2.1. Animal husbandry

Balb/c mice were procured from the Central Animal House, Panjab University, Chandigarh. Animals were maintained as per the principles and guidelines of the Ethics Committee on the use of experimental animals of Panjab University and with approved protocol. They were housed in polypropylene cages with a wire mesh top and a regularly changed husk bed with a maximum of 6–8 animals in each cage. The animal rooms were maintained at 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.

2.2. Experimental design for DMH dose standardization

The animals were randomly divided into six groups: Group1 (Control), while Group 2 to 6 received a weekly s.c. injection of 1, 10, 20, 30, 50 mg/kg DMH, respectively, for six weeks. The Control group received the vehicle for DMH (1 mM EDTA-saline).

2.3. Morphological analysis

The animals were sacrificed with an excess of diethyl ether anaesthesia. Their colons were removed and flushed clear with ice-cold physiological saline (0.9% NaCl). These were cut longitudinally to examine the presence of any macroscopic lesions, called multiple plaque lesions (MPL). These were divided into proximal, middle and distal segments for subsequent examination and counting.

2.4. Aberrant Crypt Foci (ACF)

Isolated colons were divided into three segments as above and fixed in 10% buffered formalin for 24 h. These were stained with 0.2% methylene blue in Krebs Ringer solution for 5–10 min [17]. The mucosal surface of the colon was evaluated for the number of ACF in the stained colon under 10× magnifications using a light microscope. Enlarged thickening along with the increased staining in the DMH group 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.

2.5. Histological analysis

Colons fixed in formalin (as described above) were further embedded in paraffin wax according to the standard technique for histopathology [18], and five micron thick sections were cut using a hand driven microtome and transferred to egg albumin-coated slides. Sections were then dewaxed in xylene, stained in hematoxylin and eosin, mounted in DPX, and viewed and photographed under a light microscope (40×) (Axioscope A1, Zeiss, Germany), attached with a digital camera (Jenoptik, Thuringia, Germany).

2.6. Colitis-associated colon carcinogenesis and chemoprevention study

The present study consisted of eight groups:

- control, which received vehicle for NSAIDs, (0.5% (w/v) carboxy methyl cellulose per oral daily), vehicle for DSS (distilled water during two weeks of DSS administration in group 2) and DMH (subcutaneous injection of 1 mM EDTA-saline);
- DSS, the animals received two cycles of 3% DSS in distilled water, each for seven days followed by 14 days of tap water, to establish a model of ulcerative colitis [19];
- DMH, 6 weekly injections at 30 mg/kg to develop a model for colon carcinogenesis;
- celecoxib, 6 mg/kg as a chemopreventive agent;
- DMH + DSS;
- DSS + Cel;
- DMH + Cel;
- DMH + DSS + Cel in the above mentioned respective doses.

2.7. Disease Activity Index (DAI)

DAI of the inflamed colon was assessed by the method of Trivedi and Jana [20]. It is referred to as the average combined score of weight loss (0–4), stool consistency (0–4) and rectal bleeding (0–4), and is used to score clinical symptoms in the following manner.

Weight loss: no weight loss (0), 1–5% loss (1), 5–10% (2), 10–20% (3) and >20% (4).

Stool consistency: well formed pellets (0), pasty pellets not sticking to the anus (2) and liquid stool sticking to the anus (4).

Bleeding: no gross bleeding (0) and gross bleeding (4).

Each animal was assessed for these three parameters and average score taken. The animals were scored from 0–4 where 0 indicated normal healthy animals while 4 referred to the maximal diseased condition.

Morphological and histological analyses were performed as explained earlier.