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Asiatic acid abridges pre-neoplastic lesions, inflammation, cell proliferation and induces apoptosis in a rat model of colon carcinogenesis

Aktarul Islam Siddique, Vijay Mani, Senbagarani Renganathan, Rajagopal Ayyanar, Ananthi Nagappan, Nalini Namasivayam*

Department of Biochemistry and Biotechnology, Faculty of Science, Annamalai University, Annamalainagar, 608002, Tamil Nadu, India

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ABSTRACT

The utmost aim of this present study was to investigate the anti-inflammatory, antiproliferative and proapoptotic potential of Asiatic acid (AA) on 1,2-dimethylhydrazine (DMH)-induced colon carcinogenesis in experimental rats. Rats were divided into six groups and received modified pellet diet for 32 weeks. Group 1 served as control rats. Group 2 received AA (4 mg/kg b.w. p.o.). Group 3–6 rats received 15 DMH (20 mg/kg b.w., s.c.) injections once a week starting from the 4th week. Besides DMH, rats received AA (4 mg/kg b.w. p.o.) in group 4 starting 2 weeks before carcinogen treatment till the end of the last DMH; group 5 starting 2 days after last DMH till the end of the experiment; and group 6 throughout the experiment. Pre-neoplastic lesions, xenobiotic metabolizing enzymes, inflammation, cell proliferation and apoptotic markers were analysed in our study. Our results ascertained AA supplementation to DMH-exposed rats significantly decreased the incidence of aberrant crypt foci (ACF) and phase I xenobiotic enzymes; and increased the phase II xenobiotic enzymes and mucin content as compared to DMH-alone-exposed rats. Moreover the increased expressions of mast cells, argyrophilic nucleolar organizer regions (AgNORs), proliferating cell nuclear antigen (PCNA) and cyclin D1 observed in the DMH-alone-exposed rats were reverted and were comparable with those of the control rats, when treated with AA. Concordantly AA also induced apoptosis by downregulating the expression of Bcl-2 and upregulating Bax, cytochrome c, caspase-3 and -9 in the DMH-alone-exposed rats. Thus AA was able to inhibit DMH-induced colon carcinogenesis by detoxifying the carcinogen, decreasing the preneoplastic lesions by virtue of its anti-inflammatory, antiproliferative and proapoptotic effects. Therefore our findings suggest that AA could be used as an effective chemopreventive agent against DMH induced colon carcinogenesis.

1. Introduction

Colorectal cancer (CRC) is one of the most common cancers in both men and women with approximately 694,000 recorded deaths from the disease in 2012 worldwide [1]. Colon cancer arises due to diverse genetic and epigenetic alterations in the colonic epithelium [2]. Amassing evidence from epidemiological studies divulge an interplay between diet and the prevalence of gastrointestinal tract tumors, especially colorectal cancer, which can be promoted by a diet rich in fat and meat [3]. It has been recognized that the development of colon cancer proceeds in sequential stages from polyps to adenocarcinoma [3]. Thus, it is very essential to further empathise the cancer biology and develop effective treatment.

1,2-dimethylhydrazine (DMH), a potent procarcinogen, widely used to induce colon cancer in rodents [4,5], was used in our study. This rat model shows that DMH induces colon carcinogenesis, imitates human colonic epithelial neoplasms [6]. Primarily DMH is converted to azoxymethane (AOM), which is further metabolized to methylazoxymethanol (MAM) and then to methyldiazonium ion which inturn generates carbonium ion that is responsible for the methylation of nucleic acids [7] and acts as a trigger for colon carcinogenesis [8,9].

Aberrant crypt foci (ACF) have been stated as pre-neoplastic lesions in the colon and rectum of rats exposed to the chemical carcinogen DMH [10]. It is reported that DMH is able to stimulate cell division and

Abbreviations: AA, asiatic acid; AB-PAS, alcian blue-periodic acid Schiff; ACF, aberrant crypt foci; AgNORs, argyrophilic nucleolar organizer regions; AOM, azoxymethane; BSA, bovine serum albumin; CDNB, 1-chloro-2,4-dinitrobenzene; CRC, Colorectal cancer; DAB, 3,3′-Diaminobenzidine; DCPIP, 2,6-dichlorophenol indophenol; DMH, 1,2-dimethylhydrazine; DMSO, dimethyl sulfoxide; DTD, DT-diaphorase; GST, glutathione S-transferase; H & E, haematoxylin and eosin; MAM, methylazoxymethanol; PCNA, proliferating cell nuclear antigen; SDS, sodium dodecyl sulphate; TEMED, N,N,N′,N′-tetra methyl ethylene diamine; UDPGT, UDP-glucuronyl transferase; ANOVA, one-way analysis of variance; DMRT, Duncan's multiple range test

* Corresponding author.
E-mail address: nalininam@yahoo.com (N. Namasivayam).

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induce colorectal ACF and tumour formation by interfering with DNA methylation, in a manner similar to that which occurs in humans [11]. In the early stages of colorectal carcinogenesis the ACF evidenced as expansions of the intestinal crypts is due to dysplasia, epithelial hypertrophy and hyperplasia [12,13]. Evaluation of ACF is a short-term bioassay used to assess the role of chemopreventive agents in colorectal carcinogenesis [14,15].

Mucins are highly glycosylated proteins that are the major components of the colonic mucus which lubricate and protect the underlying intestinal epithelium [16]. Mast cells on activation releases synthesized inflammatory mediators which plays an important role in acute inflammation. These mediators are involved in the pathogenesis of gastrointestinal diseases. Silver staining of argyrophilic proteins affiliated with nucleolar organizer regions (AgNORs) are specifically related to loops of DNA containing the sites of ribosomal RNA that plays a role in the synthesis of ribosomes [17]. It has been proposed that the number of AgNOR dots in a nucleus may reflect the condition of cell activation and thus a useful index to assess cell proliferation [18]. Proliferating cell nuclear antigen (PCNA) is a non-histone nuclear acidic protein expressed in the nuclei of proliferating cells during G1and S phase [19,20] and is used as proliferation index of a broader spectrum of cells [21]. Cyclin D1 proto oncogene is an important regulator of G1 to S phase progression in many different cell types, while recent studies have evidenced that it also functions as transcriptional modulator by regulating the activity of several transcription factors such as NF-κB [22], thereby stimulating PCNA and AgNORs.

The pro-apoptotic Bcl-2 family members such as Bax initiates apoptosis with cytochrome c release and activation of the caspase cascade, whereas the anti-apoptotic protein Bcl-2 prevents mitochondrial membrane permeability and inhibits apoptosis, thereby supporting cell survival [23].

Natural products with diverse pharmacological properties have been shown to be excellent and reliable sources for development of anticancer drugs [24]. Triterpenoids which are found widely in nature have recently emerged as a unique group of phytochemicals and have been demonstrated as new promising anticancer drugs [25]. Asiatic acid (AA), a pentacyclic triterpenoid derived from the tropical medicinal plant *Centella asiatica* [26], is reported to have strong cell growth inhibition in hepatoma, breast cancer, melanoma, glioblastoma and gastrointestinal tumour cells [27–31]. Besides anti-tumour properties, AA possesses other pharmacological properties such as anti-inflammatory [32–34], antioxidant [35,36], antidiabetic [37], hepatoprotective [38], neuroprotective [39,40] and wound healing [41,42] effects.

Our previous study revealed that AA exerts inhibitory effects on DMH-induced colon carcinogenesis in a dose dependent manner [43]. Now to investigate and confirm the exact mechanism of its action the present study was formulated to address the chemopreventive efficacy of AA on pre-neoplastic lesions, biotransforming enzymes, mucin cell histochemistry, mast cell inflammation, cell proliferation and proapoptotic responses against DMH-induced colon carcinogenesis in male albino Wistar rats.

2. Materials and methods

2.1. Chemicals

Acrylamide, bovine serum albumin (BSA), Asiatic acid (AA), bromophenol blue, 1,2-dimethylhydrazine (DMH), ethidium bromide, 2-mercaptoethanol, sodium dodecyl sulphate (SDS) and N,N,N′,N′-tetra methyl ethylene diamine (TEMED) were purchased from Sigma Chemicals (St Louis, Missouri, USA). Antibodies for cyclin D1 and PCNA were purchased from Santa Cruz Biotechnology, USA. All other chemicals and reagents were of analytical grade, purchased from HiMedia Private Limited (Mumbai, Maharashtra, India).

2.2. Animals, ethics statement and diet

Male Albino Wistar rats (5 weeks old) weighing 130–150 g were obtained and maintained for the experimental studies at the Central Animal House, Rajah Muthiah Medical College & Hospital, Annamalai University, Annamalainagar, Tamil Nadu, India. The animals were cared in compliance with the principles and guidelines of Ethical Committee for Animal Care of Annamalai University in accordance with the Indian National Law on animal care and use (Reg. No. 160/1999/CPCSEA/1094). They were domiciliated in a hygienic bed of husk in polypropylene cages with wire mesh at top in a specific-pathogen free animal room, under controlled conditions of a 12 h light/12 h dark cycle, with temperature of 22 ± 1°Cand relative humidity of 50 ± 10% till the end of the experiment. Throughout the experimental period, modified pellet diet [43,44] and water were fed ad libitum.

2.3. Treatment schedule

The rats were randomly divided into six experimental groups, each consisting of six rats. Group 1 rats received modified pellet diet for 32 weeks and served as control. Group 2 rats received modified pellet diet along with AA (4 mg/kg b.w.) [43] orally everyday throughout the experimental period of 32 weeks. Groups 3–6 rats received modified pellet diet along with subcutaneous injection of DMH (20 mg/kg b.w.) once a week, for 15 weeks starting from the 4th week of the experiment [45]. In addition, group 4 rats received AA same as group 2 starting 2 weeks before carcinogen treatment and continued till the end of the last DMH injection [initiation (I)], group 5 rats received AA same as group 2 starting 2 days after last injection of carcinogen and continued till the end of the experiment [post initiation (PI)] and group 6 rats received AA throughout the experimental period same as group 2 [entire period (EP)]. The experimental design is schematically depicted in Fig. 1.

2.4. Preparation, administration of Asiatic acid and carcinogen

AA was dissolved in 5% dimethyl sulfoxide (DMSO) [46], just before treatment and was administered orally at the dose of 4 mg/kg b.w., as per the experimental design.

DMH was dissolved in 1 mM EDTA just prior to use and pH was adjusted to 6.5 with 1 mM NaOH, to ensure stability of the carcinogen. It was then administered subcutaneously in the right thigh at 20 mg/kg body weight once a week, for 15 weeks starting from the 4th week of the experiment [45].

2.5. Body weight and growth rate changes

During the experimental period (32 weeks), body weight and growth rate of the control and experimental rats were assessed. The rats were weighed prior to the experiment, subsequently once every week and finally before sacrifice.

$$\text{Growth rate} = \frac{\text{Final body weight} - \text{Initial body weight}}{\text{Total number of experimental days (224)}}$$

2.6. Determination of aberrant crypt foci (ACF)

At the end of the experimental period, colons were excised after laparotomy and flushed with 0.9% saline and opened longitudinally. Each of the fixed colons was cut into proximal and distal portions of equal lengths and lightly sandwiched between strips of filter paper with their luminal surface open and exposed. The colons were then secured and placed in a tray containing 10% buffered formalin overnight [10]. Following that the colons were stained with 0.2% methylene blue solution for 2 min and then rinsed in phosphate buffer to remove excess stain. The segments were then examined using a light microscope (Carl Zeiss, Germany) and analysed according to the method of [10]. The