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Prevention of azoxymethane/dextran sodium sulfate-induced mouse colon carcinogenesis by processed *Aloe vera* gel

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ABSTRACT

The preventive effect of a processed *Aloe vera* gel (PAG) on colon carcinogenesis was examined using an azoxymethane (AOM)-initiated and dextran sodium sulfate (DSS)-promoted mouse colon carcinogenesis model. Oral administration of PAG (200, or 400 mg/kg/day) significantly reduced the multiplicity of colonic adenomas and adenocarcinomas compared with the AOM/DSS only-treated mice. In the mice treated with 400 mg/kg of PAG, adenoma and adenocarcinoma development was reduced to 80% and 60%, respectively, compared to 100% in the PAG-untreated AOM/DSS-treated mice. Western blot analysis using colon extracts showed that PAG reduced the activation of nuclear factor kappa B (NF-κB), resulting in the inhibition of inducible nitric oxide synthase and cyclooxygenase-2 expression. PAG appeared to inhibit the NF-κB activation through the activation of peroxisome proliferator-activated receptor gamma. PAG also inhibited the expression and phosphorylation of signal transducer and activator of transcription 3, which is known to connect inflammation and cancer. In addition, PAG inhibited cell cycle progression-inducing cellular factors, such as extracellular signal-regulated kinases 1/2, cyclin-dependent kinase 4, and cyclin D1. On the other hand, PAG increased the expression of Caudal-related homeobox transcription factor 2, which is known to be a tumor suppressor in colorectal cancer. These findings show that PAG suppresses colitis-related colon carcinogenesis by inhibiting both chronic inflammation and cell cycle progression in the colon.

1. Introduction

Inflammatory bowel disease (IBD), with the most important disorders including ulcerative colitis and Crohn's disease, is a chronic, relapsing, and remitting inflammatory condition resulting from chronic dysregulation of the mucosal immune system in the intestinal tract. Chronic inflammation is believed to promote carcinogenesis, and thus IBD can lead to an increased risk of developing colorectal cancer [1]. Colon cancer is one of the important causes of morbidity and mortality in developed countries [2]. Colitis-associated colon cancers develop in the chronically inflamed mucosa and are believed to develop in a stepwise manner, with the inflamed mucosa giving rise to dysplasia and, ultimately, to cancer [1,3].

A mouse colorectal cancer model induced with azoxymethane (AOM) and dextran sodium sulfate (DSS) has been used most widely in cancer prevention studies [4–7]. AOM is a chemical mutagen, which induces colonic tumors with clinical, histological, and molecular features of sporadic human colon cancer [8]. AOM is a potent genotoxic agent, and the follow-up treatment with the non-genotoxic DSS strongly induces inflammation in the colon, thereby enhancing the colon carcinogenesis in rodents [5,7].

Aloe vera has been shown to exhibit diverse therapeutic properties, including antiviral, antibacterial, antifungal, antidiabetic, analgesic, antitumor, anti-inflammatory, and wound-healing activities [9–15]. The antitumor activity of the gel of *A. vera* was shown in numerous studies, resulting in a reduced tumor burden, tumor shrinkage, tumor necrosis, and prolonged survival rates [13–16]. The active components exerting diverse biological activities are polysaccharides contained in the gel of *A. vera*. The gel of *A. vera* contains a mixture of polymers of varying-length chains of β-(1,4)-linked acetylated mannan, known as acemannan [17]. Acemannan was shown to exhibit potent immunomodulatory and antitumor activities [18–22]. The antitumor activity of acemannan appears to be due to the activation of immune responses. Acemannan has been well documented to activate professional antigen-presenting cells, such as macrophages and dendritic cells [23–26].

A processed *A. vera* gel (PAG) is prepared by cellulase treatment of the gel of *A. vera* and passage of the resulting product through a carbon

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column [18]. The treatment of the viscous *A. vera* gel with cellulase lowers the average molecular weight (MW) of *Aloe* polysaccharides. The average MW of the native polysaccharides in the *A. vera* gel is over 1 MDa. A cellulase-treated gel contains much smaller forms of *Aloe* polysaccharides [18]. For in vivo applications, smaller-MW molecules are usually preferred to higher-MW molecules owing to better bioavailability.

In this study, we examined the preventive effect of PAG on an AOM-initiated and DSS-promoted mouse colon carcinogenesis model. We showed that oral administration of PAG significantly reduced the multiplicity of colonic neoplasms. In addition, we examined the mechanisms of inhibition of colon carcinogenesis by PAG and showed that PAG inhibited both chronic inflammation and cell cycle progression in the colon.

2. Materials and methods

2.1. Preparation of PAG

PAG was prepared from the gel of *A. vera* as described previously [18]. The basic methodology used to prepare PAG involved incubation of the *A. vera* gel with cellulase, termination of the reaction by heating, and filtration through a charcoal column to remove anthraquinones and other colored substances. The molecular size distribution of the polysaccharides and the total polysaccharide content in the PAG were determined as described previously [18]. PAG was dissolved in phosphate-buffered saline (PBS) immediately before oral administration.

2.2. Animals

Six-week-old BALB/c female mice (OrientBio, Inc., Seongnam, Korea) were housed five per cage under specific pathogen-free conditions and kept in an air-conditioned room with controlled temperature (22 ± 1 °C), humidity (50%), and day/night cycle (12 h light, 12 h dark). All animal care and experimental procedures were approved by the Animal Care Committee of Chungbuk National University.

2.3. Induction of colon carcinogenesis, treatment with PAG, and macroscopic observations

To induce colitis-associated colon carcinogenesis, mice were intraperitoneally (i.p.) injected with AOM (10 mg/kg) and maintained on a regular diet and drinking water. After one week, the mice received 2.5% DSS (MW: 40,000, Sigma–Aldrich, St. Louis, MO, USA) in drinking water for one week, followed by two weeks of receiving regular drinking water for recovery, and this cycle was repeated three times. Normal mice, which were not treated with AOM/DSS, served as a negative control group. An AOM/DSS only group which served as a positive control for colitis-associated colon carcinogenesis was orally administered PBS using an oral Zonde needle (Jeungdo Bio&Plant Co., Ltd., Seoul Korea). Two treatment groups were orally administered PAG using an oral Zonde needle at doses of 200 (PAG200) and 400 (PAG400) mg/kg/day, respectively, for 13 consecutive weeks, starting one week before the AOM injection. The overall experimental schedule is depicted in Fig. 1A.

The body weight was measured every week until the termination of the study. The mice were sacrificed and necropsied at week 13, and the colons (from the ileocecal junction to the anal verge) were excised. After measurement of the length, the colons were washed with ice-cold PBS, cut open longitudinally along the main axis, and then macroscopically inspected. All lesions with an elevated growth pattern identified in the whole colon were initially classified as neoplasms, and their sizes were measured using a scaled ruler. After gross examination, the colon was cut longitudinally into two pieces. One half of the colon was fixed in 10% neutral buffered formalin (pH 7.4) for 24 h for further histopathological examination, while the other half was stored in liquid nitrogen for western blot analysis.

2.4. Histopathology

For histopathological examination, hematoxylin and eosin (H&E) staining was performed. After 24 h of fixation with 10% neutral buffered formalin, the tissue was embedded in paraffin, and 4-μm sections were prepared by routine histological methods. The sections were stained with H&E for light microscopic examinations. Based on H&E staining, histological alterations, such as mucosal ulceration, dysplasia, and carcinoma, were verified by a board-certified pathologist. Carcinoma was defined as a high-grade dysplasia of the colonic mucosa that had invaded beyond the muscularis mucosa and into the submucosa.

2.5. Western blot analysis

Frozen colon tissues were crushed in liquid nitrogen and mixed with an equal volume of RIPA buffer [50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 1.0% Igepal CA-630 (NP-40), 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulfate (SDS); Sigma–Aldrich] containing a protease inhibitor cocktail (Roche, Germany). After sonication for 30 s and centrifugation at 15,000 ×*g* at 4 °C for 20 min, the supernatant was collected, and the total protein concentration was determined with a BCA protein assay kit (Thermo Scientific, Rockford, IL, USA). Protein samples (10 μg) were fractionated by 10% SDS–polyacrylamide gel electrophoresis and transferred to pure nitrocellulose membranes (Bio-Rad, Hercules, CA, USA). The membranes were blocked with 5% skim milk in wash buffer (0.1% Tween 20 in PBS) for 1 h and then incubated with a 1:1000 dilution of primary antibodies in wash buffer overnight at 4 °C. Then, the blots were incubated with peroxidase-conjugated secondary antibodies (1:5000) for 1 h. For the assessment of p50 protein levels in the nucleus, nuclear extracts were prepared from the colon tissue lysate using nuclear extract buffer [0.6 M KCl, 20 mM Tris–HCl, 25% glycerol, 1.5 mM $MgCl_2$, 0.2 mM ethylenediaminetetraacetic acid (EDTA), 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol; Sigma–Aldrich] [27]. Antibodies against proliferating cell nuclear antigen (PCNA), cyclin D1, cyclin-dependent kinase 4 (CDK4), extracellular signal-regulated kinases 1/2 (ERK1/2), phospho (p)-ERK1/2, p50 of nuclear factor kappa B (NF-κB), signal transducer and activator of transcription 3 (STAT3), p-STAT3, peroxisome proliferator-activated receptor gamma (PPAR-γ), and Caudal-related homeobox transcription factor 2 (CDX2) were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). Antibodies against inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), and β-actin were purchased from Santa Cruz Biotechnology, Inc. (Dallas, Texas, USA). Anti-isotype antibodies were purchased from Cell Signaling Technology, Inc. Signals were developed using an enhanced chemiluminescence substrate (Santa Cruz Biotechnology, Inc.) and detected and quantified using a Fusion Fx7 chemiluminescence imaging system (Vilber Lourmat, Torcy, France). Western blot bands were quantified using the ImageJ software (National Institutes of Health, Bethesda, MD, USA).

2.6. Flow cytometric analysis of myeloid-derived suppressor cells (MDSCs)

Total spleen cells were prepared from the spleens of the PAG-treated and untreated colon tumor-bearing mice, and single-cell suspensions were prepared by passage through a 70-μm cell strainer (Falcon, Durham, NC, USA). The single-cell suspension was washed with PBS, and then red blood cells were lysed by a treatment with ACK lysis buffer (0.15 M NH_4Cl, 1.0 mM $KHCO_3$, 0.1 mM EDTA) for 3 min. The spleen cells were washed and stained with monoclonal antibodies specific for mouse CD11b and mouse Ly-6G/6C (Gr-1) (BD Biosciences, San Jose, CA, USA), as described earlier [28]. Flow cytometric analysis was performed using a FACSCanto II flow cytometer (BD Biosciences).