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ORIGINAL CONTRIBUTION

Modified apple polysaccharide prevents against tumorigenesis in a mouse model of colitis-associated colon cancer: role of galectin-3 and apoptosis in cancer prevention

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Abstract
Background Colorectal cancer (CRC) is one of the most common and preventable cancers. Regular consumption of apples is conducive to reduction in CRC risk.
Aim of the study To evaluate effects of modified apple polysaccharide (MAP) on tumorigenesis in a mouse model of colitis-associated colon cancer.
Methods One hundred male ICR mice were administered with 1, 2-dimethyl-hydrazine (DMH) and dextran sodium sulfate (DSS). Forty mice were given no further treatment, the rest were fed basal diet blended with three different doses of MAP; 2.5, 5, and 10% (20 mice in each group).
Results MAP significantly protected ICR mice against DMH/DSS-induced tumorigenesis. The incidence of tumor development was 90% (18/20) in the mice treated with DMH/DSS, but that was reduced to 25% (5/20), 15% (3/20), and 5% (1/20), respectively, in the mice treated with basal diets plus 2.5, 5, and 10% of MAP. Study of apoptosis of colonic epithelial cells revealed that MAP moderately increased apoptosis, suggesting that the anti-tumor potency of MAP was probably attributed to its ability to induce apoptosis. Western blot analysis demonstrated that carbohydrate-binding protein galectin-3 changed in both the nucleus and the cytoplasm during the process from colitis to colon cancer in the model. And MAP could inhibit the binding of galectin-3 to its ligand: this is, at least in part, the possible mechanism of MAP by enhancing apoptosis and preventing tumorigenesis.
Conclusions These data suggest that MAP has a potential role in clinical prevention and treatment for colon cancer.

Keywords Modified apple polysaccharide · Galectin-3 · Apoptosis · Colitis-associated colon cancer · Cancer prevention

Yuhua Li and Li Liu are Co-first authors.

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Introduction

Colorectal cancer (CRC) is one of the most common cancers and a leading cause of cancer-related mortality in developed countries, ranked third in both prevalence and lethality [1]. One of the important underlying etiologies of tumorigenesis in the colon is inflammation [2] as evidenced by the increased risk of CRC in patients with inflammatory bowel disease (IBD) [3]. The longer the time of disease lasts, the higher the risk of CRC will be. The incidence of CRC in patients with colitis is nearly 40% at 30 years after

initial onset of disease [4]. Although the anti-inflammatory drugs such as celecoxib have a moderate preventive effect on adenomas in large bowel [5, 6], their long-term use may be limited due to adverse cardiovascular effects [7, 8].

The role of environment and lifestyle in the genesis and progression of colitis and CRC have drawn more and more clinical and scientific attention [9, 10]. It is widely believed that fiber-rich foods (e.g., fruits and vegetables) possess beneficial effects on the intestine, particularly the colon. A diet high in fruits and low in meats decreases the risk of CRC [11].

Preclinical studies in animal models have demonstrated that modified citrus pectin (MCP) can inhibit the growth or/and metastasis of colon, breast, and prostate tumors [12–14]. And evidence has suggested that MCP may inhibit tumor growth and metastasis via its interaction with galectin-3 (gal-3) [13]. Pectin could act as a ligand for gal-3 when it is partially hydrolyzed into smaller linear water-soluble fractions [15].

Gal-3, a member of the family of β-galactoside-binding lectins, has multiple biological functions in the intracellular and extracellular compartments [16–18]. The biological activities of gal-3 are related to the multivalent binding properties, which derive mainly from its carbohydrate recognition domain with a special affinity for glycoconjugates containing galactose [19, 20]. Gal-3 is involved in not only different stages of inflammation, generally viewed as a promoter of inflammatory response [15, 21, 22], but also the processes of tumorigenesis and metastasis [23–25]. The anti-apoptotic effect of gal-3 may play an important role in tumor progression and metastasis [20, 26, 27]. Gal-3 is a prognostic marker and of which the alteration is associated with tumor pathogenesis, progression and/or metastasis in various types of cancers. It is up-regulated in the cancers of colorectal [28, 29], gastric [30], hepatocellular [31], breast [23], uterine [32], head and neck [33], thyroid [34], tongue [35], and melanoma [36], while down-regulated in colorectal [37], breast [38], prostate [39], renal [40], and laryngeal [41] cancers. Therefore, the clinical relevance of its expression is still unclear and controversial and needs further assessment. The different roles of gal-3 in inflammation and/or cancer might be attributed to its pleiotropic functions and presence in multiple subcellular compartments [17, 42].

Epidemiological observations suggest that regular consumption of apples may reduce the risk of CRC [43–45]. It has been shown that several ingredients of apples have anti-tumor potency: apple polyphenols can affect protein kinase C activity and the initiation of apoptosis in human colon carcinoma cells [46]. Apple flavonoids inhibit the growth of human colon cancer cells and regulate the expression of genes involved in the biotransformation of xenobiotics [47]. Cloudy apple juice is observed to decrease DNA damage, hyperproliferation, and aberrant crypt foci development in the distal colon of dimethylhydrazine (DMH)-initiated rats [48]. One component that makes apple juice cloudy is polysaccharides. We prepared modified apple polysaccharide (MAP) by extracting polysaccharides from apple and investigate its anti-CRC effect and the possible mechanisms. The preventive effects of MAP upon colon cancer were evaluated using a mouse model of CACC. The terminal deoxynucleotidyl transferase (Tdt)-mediated nick and labeling (TUNEL) immunohistochemical assay and Caspase-3 Activity Assay Kits were used to observe the effects of MAP on the apoptosis of colonic epithelial cells. Because the MAP was mainly composed of galacturonic acid and galactose, the ability of MAP to bind to gal-3 in SW-1116 cell line was assessed.

Materials and methods

Reagents and antibodies

The colonic carcinogen 1,2-dimethyl-hydrazine (DMH) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Dextran sodium sulfate (DSS) with a molecular weight of 36,000–50,000 was purchased from ICN Biochemicals, Inc. (Aurora, OH, USA). DSS was dissolved in distilled water at a concentration of 2% (w/v). Primary antibodies against the following targets were used: galectin-3 (abcam, UK), β-actin, and histone H3 (Santa Cruz Biotechnology, USA).

Preparation and analysis of modified apple polysaccharide

Red Fuji apples were purchased from Xianyang (Shaanxi, China). Apple polysaccharides were extracted according to the method as previously described [49]. The molecular weight of MAP was 1,000–3,000 Da, as determined by Ohpak SB-804 gelatin column (Shodex Denko K.K., Kawasaki, Japan) (Fig. S1). The sugar content of MAP was >/=85% and the protein content was </=3% as determined by DU 800 Spectrophotometer of Beckman Coulter, Inc. The samples (10 mg) were hydrolyzed with 1 mL of 2 N trifluoroacetic acid at 105 °C for 8 h. Then, the solution was neutralized (pH 7.0) with 3 N NaOH. The major components of MAP were galacturonic acid and galactose (Fig. S2).

Animals and treatments

One hundred and twenty male ICR mice aged 5 weeks were obtained from Slaccas Experimental Animal Inc. (Shanghai, China). The experiment was conducted under institutional guidelines and approved by the Ethical Committee for Animal Care and Use of the Fourth Military Medical University.

The mice were treated as described previously [50]. Briefly, twenty mice were given saline only as control. Forty mice were given DMH/DSS only, and then followed without any further treatment. For MAP treatment, the mice were administered with DMH/DSS and then fed with the pellet basal diets, made by mixing common basal diet powder which was got from the Animal Center of the Fourth Military (Table S1) with three different doses of MAP: 2.5, 5, and 10% (20 mice per group), from the 9th week to the end of experiment. Assessment of general appearance, food uptake, body weight, stool consistency, and rectal bleeding was performed daily after the mice were treated with DMH/DSS.

All mice were killed in the 20th week by ether overdose. The large bowels were flushed with PBS and excised. Their length was measured (from the ileocecal junction to the anal verge). To evaluate tumor development, the colons were inspected under a light microscopy by two independent observers blinded to the mouse genotype and treatment. Then, 4 cm length of colon were excised, weighed, and fixed in 10% of neutral buffered formalin for at least 24 h for histopathological examination. The colonic mucosa was carefully scraped off with a glass slide, snap-frozen in liquid nitrogen, and stored at −80 °C until processed. All procedures were performed in an ice bath and in a blinded manner.

TUNEL immunohistochemical assay and apoptotic index

The DeadEnd^{TM} Colorimetric TUNEL System (Promega, San Luis Obispo, CA, USA) was used for detection of apoptotic cells according to the manufacturer's instructions. The apoptotic index was calculated as the percentage of apoptotic cells among one thousand cells in a randomly selected non-necrotic portion of the tissue within four different fields.

Caspase-3 activity

Caspase-3 activity in colonic mucosa was detected using Caspase-3 Activity Assay Kits (Beyotime Institute of Biotechnology, Jiangsu, China). The assay is based on the hydrolysis of the peptide substrate acetyl-Asp-Glu-Val-Asp p-nitroanilide (Ac-DEVD-pNA) by caspase-3, resulting in the release of a pNA moiety. Absorbance values were measured at 405 nm. Results were adjusted to the total protein content, and activity was expressed as nmol pNA/mg of total protein.

The effect of MAP on binding of SW-1116 cancer cell line in vitro

MAP at different concentrations (0.01, 0.03, 0.1, 0.3, and 1 mg/mL) or a fixed concentration (0.1 mg/mL) was added to 24 well plates. The cells were kept in an atmosphere of 95% oxygen and 5% CO_2 for 30 min at 37 °C or 4 °C. Subsequently, FITC-galactose-BSA at various concentrations (0.00001, 0.0001, 0.001, 0.01, and 0.1 mg/mL) or a fixed concentration (0.01 mg/mL) were added to the plates, and the cells were incubated for another 30 min. After incubation, the cells were washed three times with PBS to remove non-conjugated FITC-galactose-BSA. Fluorescence intensity (FI) was determined by Elisa Reader (TECAN, USA). The assay was done three times in triplicate.

Western blot analysis of gal-3

Proteins were extracted from the cytoplasm and the nucleus of colonic mucosa using the NE-PER® Nuclear and Cytoplasmic Extraction Reagents (Thermo Fisher Scientific Inc., MA, USA). Western blotting analysis was performed as described previously [50]. Briefly, samples (10 mg/lane) were separated by 15% SDS–polyacrylamide gel electrophoresis (SDS–PAGE). Immunoreactive proteins on the membrane were detected using Immobilon Western HRP Substrate (Millipore, USA).

Measurement of gal-3 concentration in blood serum of the mice

The blood collected from the mice was centrifuged and then the serum was got. Gal-3 concentration in the serum was detected by a Gal-3-specific enzyme-linked immunoassay (Boster Biological Technology, LTD., Wuhan, China) according to the manufacturer's instructions.

Statistics

Data were expressed as mean ± standard deviation (SD). Statistical analyses were done using the ANOVA test to compare the different groups. The differences between two groups were examined by the Student's *t*-test. The differences of the ratio between two groups were detected by *chi*-square test. Probability (*p* value) of less than 0.05 was considered to be statistically significant.

Results

MAP protected ICR mice against intestinal toxicity and prevented colon tumor development induced by DMH/DSS

Tables 1 and S2 summarized the ability of MAP at different doses to protect against intestinal toxicity and reduce colon tumor development in the mice treated with DMH

and DSS. After DMH/DSS treatment, piloerection, bowing waist, and loose stool were observed in nearly all the mice (Table 1). The process of carcinogenesis in the colon of the mice treated with DMH/DSS may be described as follows: the mice showed signs of bloody stools (twice, in the 3rd and 9th week), then anal prolapse, and finally tumorigenesis. MAP was initiated in the ninth week after the mice were administered with DMH/DSS, when conspicuous inflammation in the intestine occurred. MAP significantly decreased the incidence of piloerection, bowing waist, and anal prolapse in the mice treated with DMH/DSS (Table 1). And MAP significantly inhibited tumorigenesis in a dose-dependent manner. The incidence of tumor formation was 90% (18/20) in the mice treated with DMH/DSS without MAP, and the ones reduced to 25% (5/20), 15% (3/20), and 5% (1/20) with DMH/DSS plus 2.5, 5, and 10% MAP, respectively. Interestingly, the body weight of the mice treated with DMH/DSS was unchanged compared with that of control group at the end of study, even though the mice had severe intestinal toxicities such as bloody stool, anal prolapse, and colon tumors (Table S1).

The pathological changes and the protective effect of MAP in the mice treated with DMH/DSS

For histopathological studies, the mice were killed in the 20th week and histological examination was carried out in the colons (20 mice for each group) of the mice treated with normal saline (control), DMH/DSS, and DMH/DSS plus MAP. The representative results of pathological examinations were shown in Fig. 1. The normal histomorphology of the colon from the control illustrated integral epithelial cell structure (Fig. 1a). While neoplastic glands (cribriform pattern) developed with marked cytological atypia and mitotic activity of the colon in the 20th week after DMH/DSS treatment (Fig. 1b), and only lymphocytic infiltrate in the mucosa and edema in the submucosa of the colons were observed after the treatment of MAP (Fig. 1c–e). Thus, the results demonstrated that DMH/DSS caused significant colon inflammation, hyperplasia, adenoma, and carcinoma, and MAP was effective in protecting against carcinogenesis in the colon of mice given DMH/DSS.

The effect of MAP on apoptosis in the mice treated with DMH/DSS

To investigate the effect of MAP on preventing tumorigenesis, apoptotic index of colonic epithelial cells in all the mice was evaluated. The results (Fig. 2A) showed that spontaneous apoptotic cells were clearly observed in control mice, and the apoptotic index was 3–7% in the DMH/DSS-treated mice in the 20th week when colon tumors had developed. MAP induced apoptosis of colonic epithelial cells in the mice treated with DMH/DSS in a dose-dependent manner (apoptotic index was 18, 25, and 43% with 2.5, 5, and 10% of MAP, respectively).

The activity of caspase-3 in colonic mucosa was measured. As shown in Fig. 2B, obvious activation of caspase-3 was observed in colonic mucosa of the mice treated with DMH/DSS plus different concentrations of MAP, which seemed to be in a dose-dependent manner whereas low caspase-3 activity was detected in control and DMH/DSS-treated group.

MAP inhibited the binding of FITC-galactose to SW-1116 cells

To evaluate the interaction between MAP and gal-3, FITC-galactose was used as a ligand for gal-3. SW-1116 cell line was selected because it expresses high level of gal-3. Two studies were conducted: (1). FITC-galactose (0.00001–0.1 mg/mL), with or without 0.1 mg/mL MAP, was incubated with SW-1116 cells at 37 and 4 °C; (2). FITC-galactose

Table 1 The incidence of bloody stool, anal prolapse, and colon tumor formation induced by DMH/DSS in mice

Group	Bloody stool		Anal prolapse		Colon tumor
Time	Week 3 ($n = 120$)	Week 9 ($n = 112$)	Week 12 ($n = 108$)	Week 15 ($n = 104$)	Week 20 ($n = 100$)
Control (saline)	0/20 (0%)	0/20 (0%)	0/20 (0%)	0/20 (0%)	0/20 (0%)
DMH/DSS	51/100 (51%)	36/92 (39%)	14/28 (50%)	18/24 (75%)	18/20 (90%)
DMH/DSS + MAP (2.5%)	–	–	5/20 (25%)*	5/20 (25%)*	5/20 (25%)*
DMH/DSS + MAP (5%)	–	–	3/20 (15%)*	4/20 (20%)*	3/20 (15%)*
DMH/DSS + MAP (10%)	–	–	1/20 (5%)*	2/20 (10%)*	1/20 (5%)*

A total of 120 mice were used for the experiment, 20 mice were given saline only as control, and 100 mice were administrated with DMH/DSS and randomly divided into four groups

Forty mice were given no further treatment (group 2) and four mice were killed each time in the 3rd, 5th, 9th, 12th, and 15th week, respectively, the damage of whose intestine were examined

MAP was initiated in the 9th week after the mice treated with DMH/DSS with 20 mice each group. All the mice were killed in week 20

* $p < 0.01$ versus DMH/DSS

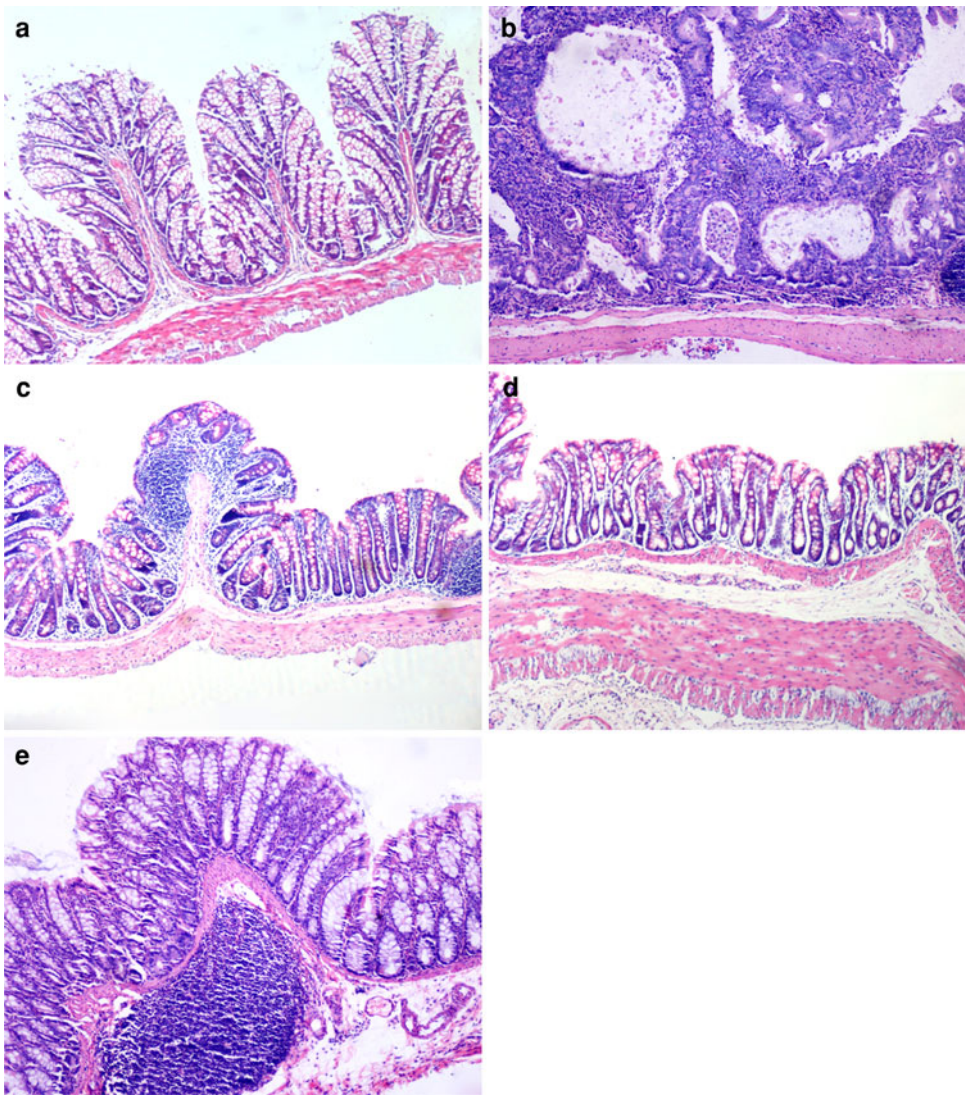

Fig. 1 The pathological changes of colon in mice from the groups of control, DMH/DSS, DMH/DSS + MAP. (100× magnification). **a** Colon of control (mouse treated with saline), **b** colon of the mouse treated with DMH/DSS, **c** colon of the mouse treated with DMH/DSS + 2.5% MAP, **d** colon of the mouse treated with DMH/DSS + 5% MAP, and **e** colon of the mouse treated with DMH/DSS + 10% MAP. MAP treatment was initiated in the 9th week after the mice were treated with DMH/DSS. Twenty mice were used in each group

(0.01 mg/mL), with or without MAP (0.01–1.0 mg/mL), was incubated with SW-1116 cells at 37 and 4 °C. The data in Fig. 3 demonstrated that MAP at 0.1 mg/mL significantly decreased the fluorescence intensity ($p < 0.01$) of FITC-galactose at 37 °C (Fig. 3a) and the inhibitory effect of MAP was dose dependent (Fig. 3b). The results suggested that MAP competitively blocked the binding of FITC-galactose to gal-3.

Effect of MAP on gal-3 protein expression and the variation of gal-3 expression in colonic epithelium during the progression from colitis to colon cancer in the mice treated with DMH/DSS

In order to study the alteration of gal-3 expression during the process from colitis to colorectal cancer (in the week 3–20) and the effect of MAP (in the 20th week), Western blot analysis was used to detect gal-3 expression in the nucleus and in the cytoplasm of colonic mucosa.

The results are shown in Fig. 4a, illustrating the alteration of gal-3 in the nucleus during the process from colitis to colon cancer and the effect of MAP. Compared with that of control, gal-3 in the nucleus decreased in the 3rd week. However, it increased to the level which was significantly higher than that of control ($p < 0.01$) in the 5th week. In the 12th week, gal-3 reached a peak, and then, declined slightly from the peak in the 15th week. It continuously decreased till the 20th week, but was still higher than that of control. In comparison with DMH/DSS alone, MAP treatment increased gal-3 expression in the nucleus ($p < 0.01$) in the 20th week.

In the cytoplasm, the levels of gal-3 were down-regulated from week 3 to week 15 compared with that of control and rose to the same level as that of control in the

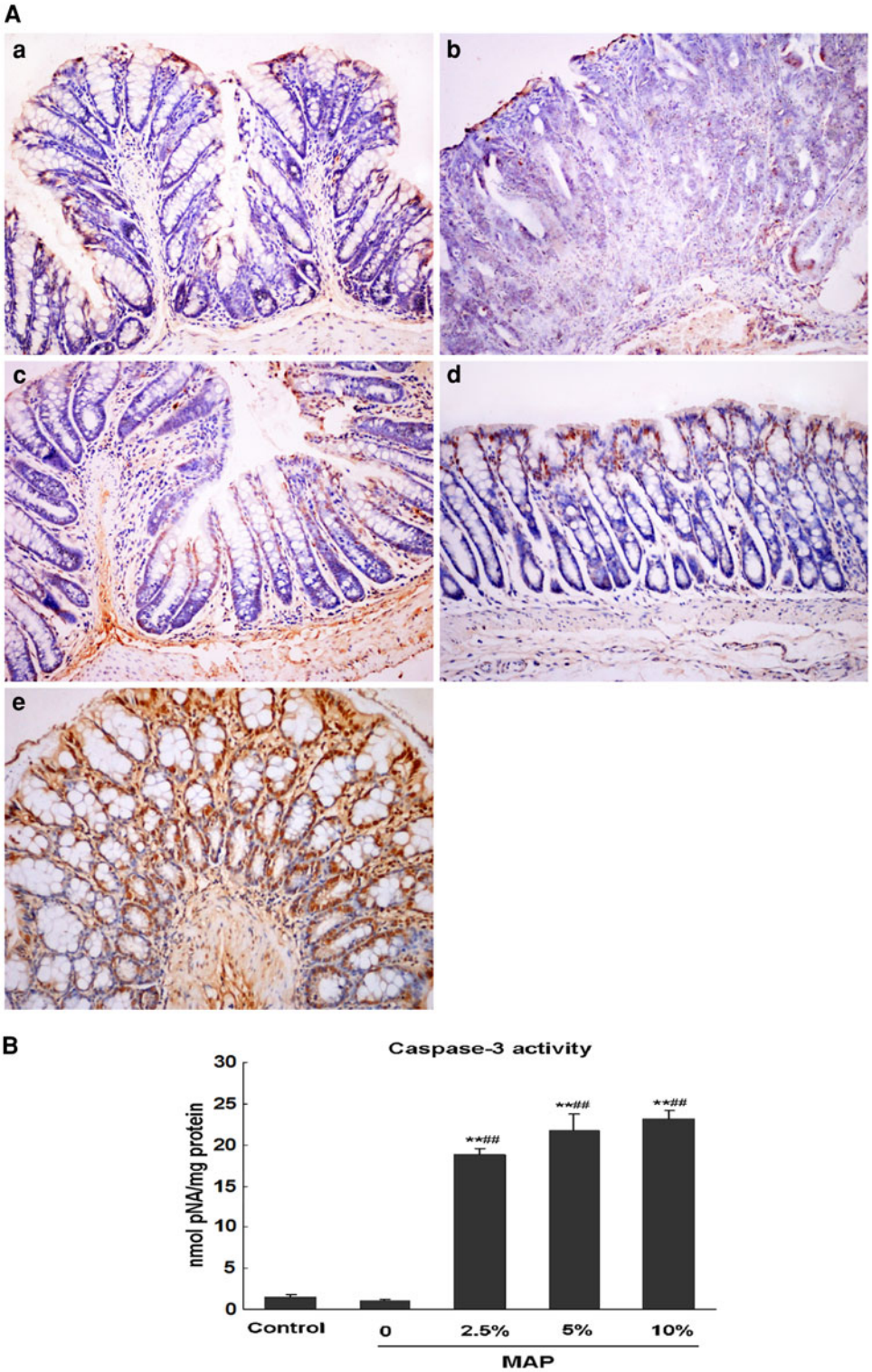

Fig. 2 Apoptosis of colonic epithelial cells of mice from the groups of control, DMH/DSS ± MAP. **A** Apoptotic epithelial cells in the colonic mucosa of mice from the groups of control, DMH/DSS ± MAP, were determined by TUNEL immunohistochemistry method. *Panel a* colon of control (mouse treated with saline); *Panel b* colon of the mouse treated with DMH/DSS, very few apoptotic cells (±); *Panel c* colon of the mouse treated with DMH/DSS + 2.5% MAP, some apoptotic cells (+); *Panel d* colon of the mouse treated with DMH/DSS + 5% MAP, moderate apoptotic cells (++); *Panel e* colon of the mouse treated with DMH/DSS + 10% MAP, numerous apoptotic cells, (peak, ++++). **B** Effect of MAP on caspase-3 activity of colonic mucosa of the mice treated with normal saline (*control*) and DMH/DSS ± MAP. MAP treatment was initiated in the 9th week after the mice were administered with DMH/DSS. Data were the Mean ± SD of three separate experiments. $^{*}p < 0.01$ versus control; $^{\#}p < 0.01$ versus DMH/DSS

20th week when colon cancer had developed. The situation was different to what was seen in the nucleus. MAP significantly increased the levels of gal-3 in a dose-dependent manner (Fig. 4b), which was similar to what was observed in the nucleus.

The data in Fig. 4 showed the ratio of gal-3 in the nucleus versus in the cytoplasm, which was calculated from the values of gal-3 in the nucleus (Fig. 4a), divided by the values of gal-3 in the cytoplasm (Fig. 4b). The results indicated that the ratio decreased at the early phase of

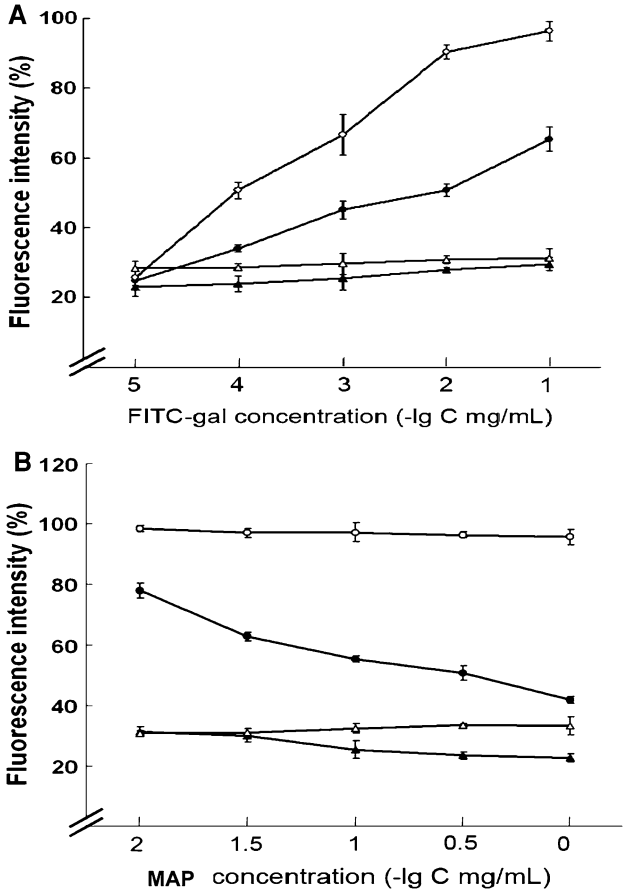

Fig. 3 MAP blocked galectin-3 binding to FITC-Gal in SW-1116 cell line at 37 and 4 °C. **A**: *Filled triangle* FITC standard; *open square* FITC-Gal at 4 °C; *open circle* FITC-Gal at 37 °C; *filled circle* FITC-Gal + 0.1 mg/mL MAP at 37 °C. **B**: *open square* FITC-Gal at 4 °C; *filled triangle* FITC-Gal + MAP (0.01–1.0 mg/mL) at 4 °C; *open circle* FITC-Gal at 37 °C; *filled circle* FITC-Gal + MAP (0.01–1.0 mg/mL) at 37 °C. $p < 0.01$ (FITC-Gal vs. FITC-Gal + MAP at 37 °C). Experiments at each time point were done three times using triplicate samples (mean ± SD). $p < 0.01$ (FITC-Gal vs. FITC-Gal + MAP at 37 °C)

inflammation in the 3rd week compared with that of control, increased from week 5 and reached a peak in the 12th week, then declined from week 15 to week 20, but was still higher than that of control. There was no significant difference between the group of DMH/DSS and the three groups of DMH/DSS plus MAP ($p > 0.05$) in the 20th week.

MAP down-regulated the concentration of gal-3 in the serum of the mice treated with DMH/DSS

It has been found that gal-3 concentrations increased in sera from colorectal cancer patients and were higher in those with metastatic disease than in patients with localized tumors [51]. Then, gal-3 concentrations of mice treated with or without MAP were measured. The concentrations of gal-3 in the serum of the mice treated with DMH/DSS increased significantly compared with those of control mice. MAP effectively decreased the concentrations of gal-3 in a dose-dependent manner in the DMH/DSS-treated mice (Fig. 5).

Discussion

Several studies have indicated that regular consumption of fruits and vegetables decreased the risk of CRC [50, 51] without the potential side effects that anti-inflammatory drugs may have [11, 52–54]. MCP and AP were effective in preventing and treating CRC [12, 13, 53]. Our study showed that MAP significantly protected ICR mice against intestinal toxicity induced by DMH/DSS: Ninety percent of DMH/DSS-treated mice developed colon tumors, while those being given 10% of MAP were almost completely prevented from carcinogenesis in the colon (Table 1). Earlier report suggested that the effect of MCP on tumor growth inhibition was related to gal-3 [13]. In the current study, we demonstrated that MAP competitively blocked the binding of galactose to gal-3 in SW-1116 cell line in a dose-dependent manner (Fig. 3). The study indicated that MAP may bind to gal-3 at the same site of galactose. MAP binding to gal-3 could be one of the possible mechanisms of MAP inhibiting tumorigenesis because many tumor-associated antigens bind to gal-3 through their galactose residues to promote the growth and progression of tumor. MAP on increasing apoptosis of colonic epithelial cells in DMH/DSS-treated mice may also take effect through its interaction with gal-3 [23, 26, 27, 55].

Gal-3 belongs to a family of carbohydrate-binding proteins [18]. By its ability to bind to *N*-lactosamine residue-associated glycoconjugates, gal-3 is involved in diverse biological processes, such as cell growth, adhesion, differentiation, angiogenesis, apoptosis, inflammation, tumorigenesis, and cancer metastasis [11, 23–25, 54–56]. Gal-3 predominantly locates in the cytoplasm after being synthesized and shuts between nucleus and cytoplasm [57]. In the nucleus, gal-3 stimulates cyclin D_1 and c-myc expression to induce cell proliferation and differentiation [58]. In the cytoplasm, gal-3 shows anti-apoptotic activity by the similar feature of Bcl-2 [59]. Gal-3 also inhibits NO-induced apoptosis in human breast cancer BT-549 cell line by suppressing cytochrome c release [27]. In present study, we investigated the alteration of gal-3 protein expressions in both the nucleus and the cytoplasm at different stages from inflammation to tumorigenesis. The effect of MAP on gal-3 expression was also investigated at the phase of tumor formation. Different results were achieved in the nucleus and cytoplasm. In the nucleus, gal-3 was transiently down-regulated in the 3rd week, then up-regulated from week 5 to

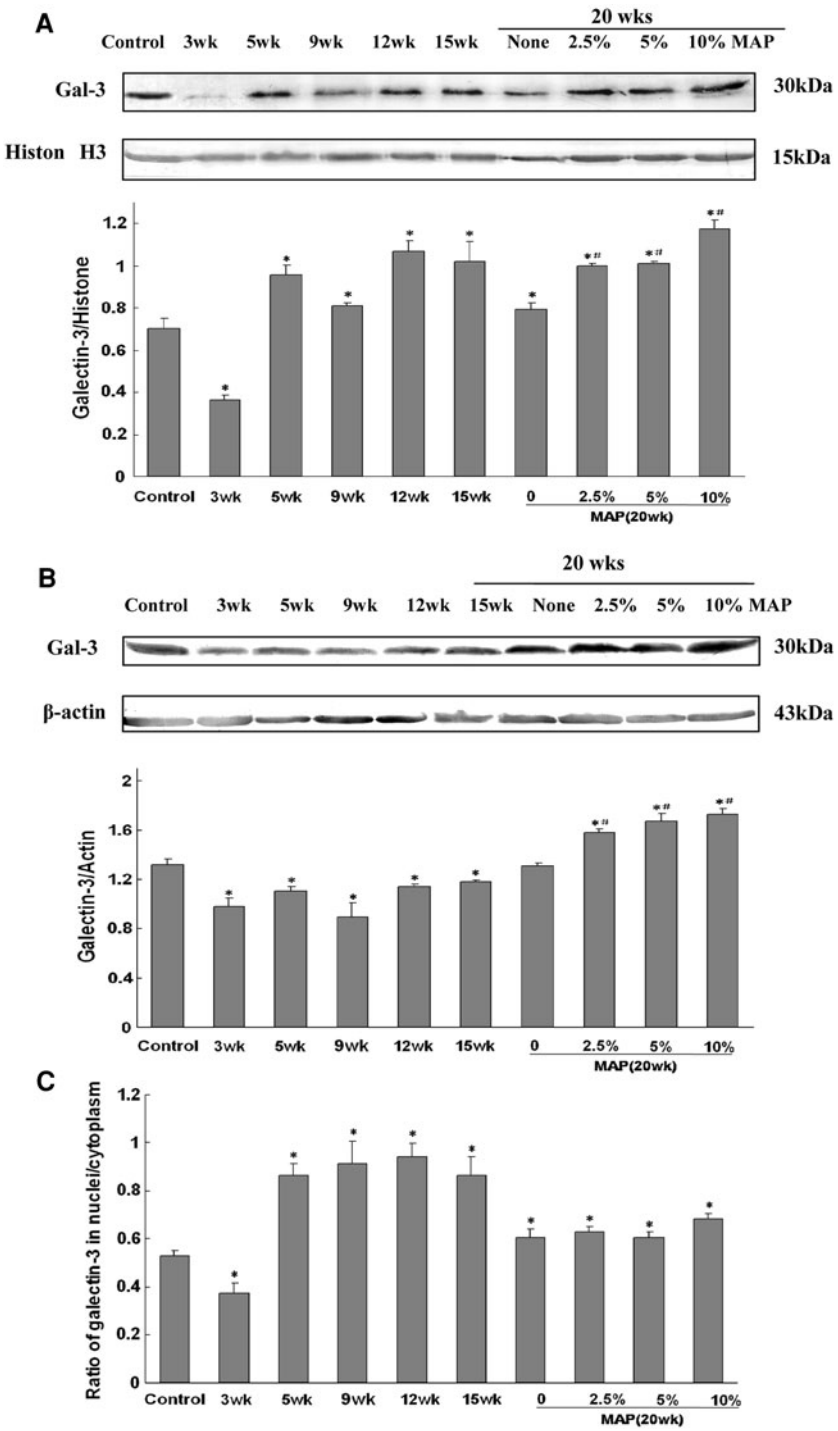

Fig. 4 The protein levels of galectin-3 in the nucleus and the cytoplasm of colonic epithelium at different inflammatory stages (in week 3–20) and the effect of MAP (week 20) in the mice treated with DMH/DSS. Experiments at each time point were done 6 times from 4–20 mice in each group (mean ± SD). **a** The protein levels of galectin-3 in the nucleus; **b** the protein levels of galectin-3 in the cytoplasm; **c** the ratio of galectin-3 in the nucleus to that in the cytoplasm of colonic mucosa. The data were calculated from **a** and **b**. $^{*}p < 0.01$ versus control. $^{\#}p < 0.01$ DMH/DSS +MAP versus DMH/DSS in the 20th week

week 15, and decreased almost to the control level in the 20th week. However, in the cytoplasm, the levels of gal-3 declined from week 3 to week 15 compared with that of control ($p < 0.01$), and gradually rose to the similar level of control in the 20th week when colon cancer developed. DMH-induced colon tumors in rodents are very close to human colon cancer with regard to morphology, pattern of growth, and clinical manifestations [60], while DSS induces colonic inflammation in rodents with clinical and histopathological similarity to human IBD [61]. When Kohno H and his colleagues developed this mouse model, they thus assumed that this model may be a powerful tool for investigating the pathogenesis of IBD-related colon carcinogenesis [62]. Down-regulation of gal-3 in the cytoplasm was concurrent with the argument put forward in Muller's report that gal-3 was down-regulated in the intestinal

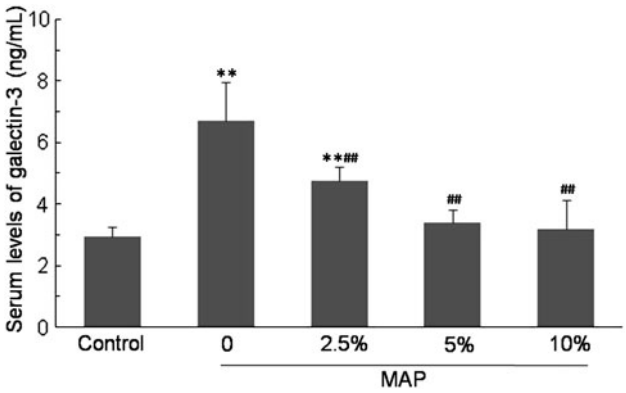

Fig. 5 Galectin-3 levels in the serum of the mice were measured by enzyme immunoassay. Galectin-3 level in the serum of the control mice was 2.94 ± 0.34 ng/mL. $^{**}p < 0.01$ versus control and $^{\#\#}p < 0.01$ versus DMH/DSS treatment. ($n = 5$)

epithelium of patients with Crohns' disease [63], providing another evidence for this assumption. It was interesting to discover that the level of gal-3 in the cytoplasm was the same as that of control in the 20th week after DMH/DSS treatment, when the colon tumor formed, which conformed to the finding in previous reports that the expression of gal-3 did not alter in human primary tumor [64].

MAP increased gal-3 levels in both the nucleus and the cytoplasm compared with those in DMH/DSS alone. The results seemed to conflict with the effects of MAP on enhancing apoptosis, decreasing inflammation, and preventing tumorigeneses. In consideration of the fact that the concentrations of gal-3 in the serum of DMH/DSS-treated mice were decreased in a dose-dependent manner by MAP, we then assumed that long-term use of MAP may competitively block the binding of gal-3 to its target, thus forcing the cells to synthesize more gal-3 to compete with MAP. However, the normal secretion and function of these gal-3 in the extracellular compartment would be inhibited by MAP, such as inducing apoptosis of T cells [65–67]. This may also explain that much higher levels of gal-3 and more obvious dose-dependent effect were found in the cytoplasm than in the nucleus. The highest ratio of gal-3 in the nucleus to that in the cytoplasm (N/C) was observed in the middle phase of inflammation in the week 9–12 after DMH/DSS treatment, and the ratio significantly decreased in the 20th week when tumor formed. MAP did not affect the ratio of gal-3 in N/C too much (Fig. 4).

It has been reported that laminarin with a average molecular weight of 7,700 could be absorbed in a nonlinear way in the intestine [68]. We made MAP with a molecular weight of 1,000–3,000 Da because we hoped that it could be absorbed through intestine. But we cannot deny or exclude the possibility that fermentation plays a role in the metabolism of MAP. MAP was, after all, mainly composed of galacturonic acid and galactose, and the activity of β-D-galactosidase originating mainly from intestine bacteria is very high. We supposed MAP could be absorbed in the intestine and have its effect systemically as well as be fermented and then have its effect in the lumen in the colon [69].

In brief, the results reported herein demonstrated that MAP protected against colitis-associated colorectal cancer induced by DMH/DSS in a mouse model. MAP, which was extracted from apple flesh and residue, showed an anti-cancer property as other apple ingredients do. The possible mechanisms involved that MAP influenced the expression and function of gal-3. In this mouse model, variation trends of gal-3 expression in the nucleus and cytoplasm of epithelial cells were observed in a more direct and incessant way, which may give a clue on the role of gal-3 in the development from colitis to colorectal cancer. Considering that MAP would be got at a comparatively low cost and high yield mainly due to plentiful resource, it will be good for the application of apple in CRC prevention. The role of alteration of gal-3 from inflammation to tumorigenesis, and the relationship between the effect of MAP on gal-3 expression and the prevention of colon cancer remain to be further investigated. Nevertheless, these data suggest MAP has a potential function for clinical prevention and treatment of colon cancer.

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