An apple oligogalactan prevents against inflammation and carcinogenesis by targeting LPS/TLR4/NF-κB pathway in a mouse model of colitis-associated colon cancer

Li Liu1, Yu H.Li1, Yin B.Niu1,2,3, Yang Sun, Zhen J.Guo, Qian Li, Chen Li, Juan Feng1, Shou S.Cao1,2,3 and Qi B.Meit

Key Laboratory of Gastrointestinal Pharmacology of Chinese Materia Medica of the State Administration of Traditional Chinese Medicine, Department of Pharmacology, School of Pharmacy, Fourth Military Medical University, Xian 710032, Shaanxi, People’s Republic of China, 1Department of Cancer Biology, Roswell Park Cancer Institute, Elm and Carlton Streets, Buffalo, NY 14263, USA and 2Visiting Professor of Department of Pharmacology, School of Pharmacy, Fourth Military Medical University, Xian 710032, Shaanxi, People’s Republic of China.

1To whom correspondence should be addressed. Tel: +86 29 84779212; Fax: +86 29 84779212; Email: qbmei@yahoo.com.cn

Evidence strongly supported a link between inflammation and cancer. Patients with colitis have high risk for development of colon cancer. Nuclear factor-kappa B (NF-κB), partially induced by lipopolysaccharide (LPS) binding to Toll-like receptor (TLR) 4, is a vital molecule in supervising the transformation of colitis to colon cancer. It could be a good strategy to prevent colitis carcinogenesis for targeting LPS/TLR4/NF-κB pathway. In the present study, we obtained an oligogalactan composed of five galacturonic acids and evaluated the protective effects on colitis and colon carcinogenesis using a mouse model of colitis-associated colon cancer induced by 1,2-dimethylhydrazine and dextran sodium sulfate (DSS). The apple oligogalactan (AOG) strongly induced the production of TNF-α, a major pro-inflammatory cytokine (9). Gram-negative bacteria are predominant and induce high expression of TLR4 in the gut (16). The immunology of colon mucosa is always abnormal in the patients with inflammatory bowel disease (16). LPS induces excessive TLR4 protein in the colon mucosa and epithelial cells to trigger colon inflammation and impaired intestinal epithelial integrity. It has been demonstrated that TLR4 was positioned at the apical pole where it was poised to monitor the sensitive balance of LPS and responsible for the active level of NF-κB (17). The amino acid mutation at position 712 of murine TLR4 leads to transform TLR4 from cell membrane into cytoplasm to cause hyporesponsiveness to LPS (18). The expression of TLR4 at the cell surface in enterocytes and macrophages could bind and internalize with LPS to trigger the downstream signaling pathway (19). The ligand-specific dynamic regulation affected the location of TLR4 internalization in the cell and prevented extracellular LPS binding to TLR4, thus blocking the downstream response of LPS/TLR4 pathway (20). Therefore, inhibition of TLR4 binding to LPS could decrease NF-κB activation, inhibit inflammatory response and prevent inflammatory carcinogenesis.

The development of colorectal cancer in humans is strongly influenced by both genetic and dietary risk factors. Environment and lifestyle play an important role in the etiology of colorectal cancer (21). The health benefits of fruits and vegetables have been known for many years. Apple has shown significantly health benefits as a famous quote from American ‘An apple a day keeps the doctor away’. A recent study showed that the extraction of apple may help to fight cancer (22). Consumption of apple has been demonstrated to reduce the risk of lung cancer, heart disease and stroke in experimental animals (23). The components with anticancer effect from apple are plant chemicals (phytochemicals), such as flavanoids and polyphenols. These phytochemicals provide the benefits of antioxidant and anticancer (24).

In the present study, we obtained an apple oligogalactan (AOG) composed of five galacturonic acids and evaluated the protective effects on colitis and colon carcinogenesis using a mouse model of colorectal cancer. It could be a good strategy to prevent inflammation and carcinogenesis for targeting LPS/TLR4/NF-κB pathway. Both AOG and LPS are agonists of TLR4 for sharing the same ligand but AOG has a much lower intrinsic activity than that of LPS. AOG may be useful for treatment of colitis and prevention of carcinogenesis in the clinics.

Introduction

Colorectal cancer is one of the most common cancers worldwide and a leading cause of cancer-related mortality (1). One of the important underlying etiologies of carcinogenesis in the colon is inflammation (2). The microenvironment of chronic intestinal inflammation facilitates cell proliferation, migration and angiogenesis, thereby promotes tumor development, growth and progression (3). The incidence of colitis-associated colon cancer (CACC) approaches to ~40% in patients with colitis (4). Anti-inflammatory drugs such as aspirin and celecoxib have demonstrated a moderate preventive effect on adenomas in the large bowel (5,6). However, the risk of gastrointestinal and cardiovascular adverse effects of these drugs limits their long-term use (7). Thus, new preventive strategies are urgently needed.

Nuclear factor-kappa B (NF-κB) is one of the most important molecules involved in innate immunity and inflammation and it has emerged as an important endogenous tumor promoter (8). NF-κB plays the role of supervision in controlling the transformation of inflammation in the context of inflammatory cells and cancer. NF-κB activation in cancer cells is responsible for inflammation-induced tumor growth and inhibition of NF-κB activation could prevent tumor growth (9). Therefore, NF-κB has emerged as an attracting target for drug discovery and development in cancer therapy (10,11). Various studies have demonstrated the pivotal role of NF-κB in tumor initiation and progression in ulcerative CACC. In InB kinase (IKK) knockout mice, the colon carcinogenesis induced by azoxymethane and dextran sodium sulfate (DSS) was significantly decreased (12). The tumorigenesis was also prevented when Toll-like receptor (TLR) 4 and myeloid differentiation primary response gene (88) were knocked out (13,14). These studies suggested that TLR4/NF-κB pathway played a crucial role in tumor development, growth and progression. Lipopolysaccharide (LPS), a glycolipid from the outer membrane of Gram-negative bacteria, is responsible for NF-κB activation in tumors and induces inflammatory mediator release by host cells (15). LPS strongly induced the production of TNF-α, a major pro-inflammatory cytokine (9). Gram-negative bacteria are predominant and induce high expression of TLR4 in the gut (16). The immunology of colon mucosa is always abnormal in the patients with inflammatory bowel disease (16). LPS induces excessive TLR4 protein in the colon mucosa and epithelial cells to trigger colon inflammation and impaired intestinal epithelial integrity. It has been demonstrated that TLR4 was positioned at the apical pole where it was poised to monitor the sensitive balance of LPS and responsible for the active level of NF-κB (17). The amino acid mutation at position 712 of murine TLR4 leads to transform TLR4 from cell membrane into cytoplasm to cause hyporesponsiveness to LPS (18). The expression of TLR4 at the cell surface in enterocytes and macrophages could bind and internalize with LPS to trigger the downstream signaling pathway (19). The ligand-specific dynamic regulation affected the location of TLR4 internalization in the cell and prevented extracellular LPS binding to TLR4, thus blocking the downstream response of LPS/TLR4 pathway (20). Therefore, inhibition of TLR4 binding to LPS could decrease NF-κB activation, inhibit inflammatory response and prevent inflammatory carcinogenesis.

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In the present study, we obtained an apple oligogalactan (AOG) composed of five galacturonic acids and evaluated the protective effects on colitis and colon carcinogenesis using a mouse model of colorectal cancer.
CACC. We studied the effect of AOG on LPS/TLR4/NF-κB signaling pathway by investigating the level and distribution of TLR4 and production of TNF-α at different stages of inflammation induced by 1,2-dimethylhydrazine (DMH)/DSS in vivo and the effect on the expression and localization of TLR4, phosphorylation of IκB and production of TNF-α induced by LPS in vitro.

Materials and methods

Animals

Five-week-old male Institute of Cancer Research mice (body weight 18–20 g) were obtained from Slaccas Experimental Animal (Shanghai, China). All mice were quarantined for 7 days after arriving. They were kept five mice per cage with water and ad libitum in a room with controlled temperature (22 ± 1°C), humidity (50–70%) and 12 h light/dark cycle in the Animal Center of Fourth Military Medical University. All animal experiments were approved by the Ethical Committee for Animal Care and Use of the University according to an approved protocol.

Chemical regents

Apple pectin was purchased from Shaanxi Jihe Phytochem Co., Ltd. (Xian, China). DMH was purchased from Sigma Chemical Co. (St Louis, MO). DSS was purchased from ICN Biochemicals (Aurora, OH). The monoclonal antibodies of anti-TLR4, IκBα and p-IκBα were purchased from Abcam Co. (Cambridge, UK). Kits for extraction of components from cytoplasm and membrane were purchased from Bio-Rad com. ( Hercules, CA). Enzyme-linked immunosorbent assay (ELISA) kit of TNF-α was purchased from Tianyouli Tech Co., Ltd. (Tianjin, China).

Cell culture

HT-29 cell line was obtained from American Type Culture Collection. The cells were cultured in 1640 medium containing 10% fetal bovine serum with a mixture of antibiotics (amphotericin B, penicillin G and streptomycin) and incubated at 37°C in an atmosphere of 5% CO2 in 96-well culture plate (Fisher Scientific, Pittsburgh, PA).

Preparation and primary structure analysis of AOG

Apple pectin was incubated with pectinase at 40–50°C at pH 3.5 for 12 h, then alkaline hydrolysis for 6 h and acid hydrolysis for 2 h and repeated the hydrolysis twice. The suspensions were collected, dialyzed and concentrated. The samples were precipitated three times with 5 vol of absolute ethanol, filtered, dissolved in distilled water and lyophilized. The lyophilized products were dissolved in distilled water at the final concentration of 1% and poured onto a Sephacryl-S400 gel column (5 × 100 cm) and eluted with 0.1 N NaCl at a flow rate of 2 ml/min at room temperature. Ten milliliters of elutes were collected using a Pharmacia LKB Super fraction collector. The molecular weight and the primary structure of AOG were determined by Matrix-Assisted Laser Desorption/Ionization Time of Flight Mass Spectrometry and 1H-nuclear magnetic resonance. For Matrix-Assisted Laser Desorption/Ionization Time of Flight Mass Spectrometry analysis, AOG was dissolved in 0.1% aqueous solution with 70/30 of trifluoroacetic acid and acetonitrile at a ratio of 1:10 of sample/matrix. The product (1 μl) was applied to a MALDI plate and allowed to dry by air. The plate was then inserted into Voyager DE-STR MALDI-TOF MS and spectra acquired between 1000 and 18230 a.m.u.

Induction of experimental CACC

The CACC model was induced by DMH and DSS (25,26). Briefly, male Institute of Cancer Research mice were injected intraperitoneally with a single dose of DMH (15 mg/kg). Seven days later, the mice were given a course of 2% DSS in sterile non-acidified drinking water for 5 days followed by pure drinking water for 3 days in total of three courses. Assessment of DMH/DSS-treated mice was performed daily for general appearance, food uptake, body weight, stool consistency and rectal bleeding. For control group, the same procedure was performed with intraperitoneal normal saline and drinking pure water instead of DMH/DSS treatment.

Treatment of AOG in mice

A total of 240 male Institute of Cancer Research mice were randomly divided into five different groups. Forty mice were given saline and drinking water as control (group 1), and 200 mice were given DMH/DSS with 50 mice in each group fed with regular food (group 2) or basal diets mixed with 2.5% (group 3), 5% (group 4) or 10% AOG (group 5). AOG was initiated at the same time of DMH/DSS treatment.

Assessment of colon damage

The colons were removed from the ileocecal valve to the anal verge and flushed several times with ice-cold phosphate-buffered saline (PBS) after the mice were killed in weeks 3–20. Each colon was weighed and opened longitudinally along the main axis and rinsed with ice-cold PBS, the colon index was calculated as colon weight (milligrams) divided by body weight (grams). The colons were inspected by light microscopy for tumor to document the quantity, size and position of each tumor. Then, a 4 cm length of colon was excised, weighed and fixed in 10% of buffered formalin for at least 24 h for histopathological studies to further assess the damage. The colon 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.

Histopathological study

Samples were fixed in 10% of buffered formalin and then embedded in paraffin for sectioning. Sections were stained with hematoxylin and eosin. Inflammation was assessed according to previous studies (14,26). The severity of mucosal inflammation was evaluated and given a numerical value of 0–4, where the score was determined as normal for 0, slight and moderate edema for 1, severe edema and/or moderate erosions for 2, severe erosions and/or small ulceration for 3 and large ulceration and necrosis for 4. The location and types of tumor (adenoma or adenocarcinoma) were also identified.

Fluorescence imaging of colon tissues and HT-29 cells with confocal laser scanning microscopy

Fluorescence imaging of colon tissues and HT-29 cells were performed with ex vivo and in vitro. Confluent HT-29 cells (105) were incubated with LPS (10 μg/ml) ± AOG (10 mg/ml) on glass coverslips at 37°C for 24 h. After washing with PBS, the cells were fixed in 3% of buffered formalin. Both samples of colon tissues and cells were incubated with anti-TLR4 polyclonal antibody at 4°C overnight and then added goat anti-rabbit TRITC. Cells were counterstained with 4’,6-diamino-2-phenylindole in mounting medium to identify nuclei. The fixed samples were recorded using a FluorView FV1000 Confocal Microscope (Olympus, Tokyo, Japan). TLR4 expression was shown with the red color in the pictures.

Binding of fluorescein isothiocyanate–AOG to HT-29 cells

Confocal laser scanning microscopy and ELISA Reader was used for the binding assay. We use LPS as the sample to compete with AOG in binding to TLR4 because LPS is the main ligand to TLR4. AOG was labeled with fluorescein isothiocyanate (FITC) as described previously (27). The replaceability of FITC–AOG to TLR4 was performed as below (i) FITC–AOG (1 μg/ml) was incubated with HT-29 cells for 60 min at 37°C, then LPS (10 μg/ml) was added and incubated with FITC together for another 60 min; the cells were fixed in 3% buffered formalin after washing three times with PBS and recorded using a FluorView FV1000 Confocal Microscope (Olympus). (ii) FITC–AOG at different concentrations (0.0001, 0.001, 0.01 and 1 mg/ml) was incubated with HT-29 cells in 5% CO2 and 95% air for 60 min at 37°C or 4°C. Then, a fixed concentration of LPS (10 μg/ml) was added to the plates and incubated for another 30 min. After incubation, the cells were washed three times with PBS to remove non-conjugated FITC–AOG. Fluorescence intensity (FI) was determined by ELISA Reader (Tecan, Männedorf, Switzerland). The assay was done three times in triplicate.

Assay of the production of TNF-α

TNF-α was determined from colon tissue secretion ex vivo and HT-29 cells in vitro. To measure TNF-α from colon tissue ex vivo, 100 mg of colon tissues were obtained from normal mice (control) and the mice treated with DMH/DSS ± 5% AOG. The tissues were cultured in 24-well flat-bottom plates in serum-free RPMI 1640 for 24 h. Supernatants were harvested for TNF-α measurement. To measure TNF-α in vitro, the cells were seeded at a density of 104 per well in 96-well plates and incubated with LPS (10 μg/ml) ± AOG (10 mg/ml) for 12 h. The treated cells were divided into two copies. One copy was used to determine directly the level of TNF-α from the harvested supernatants. For another, the cells were centrifuged and the culture medium was carefully removed. Then, the cells were suspended and added LPS (10 μg/ml) again incubated for 30, 60 and 120 min and harvested the supernatants for determining the level of TNF-α.

An ELISA was used with ELISA kits according to the manufacturer’s instruction. The final results were expressed as pg/ml for TNF-α.

Western blot analysis

To determine the TLR4 expression from colonic tissue, colon mucosa was scraped and total proteins were extracted using the Ready Prep Protein.
Extraction Kit (Bio-Rad). The proteins from the cytoplasm or the membrane of HT-29 cells were extracted with the same kit noted above according to the instructions of the manufacturer. The protein solutions were mixed with loading buffer and separated by 15% of sodium dodecyl sulfate–polyacrylamide gel electrophoresis under denaturing conditions and transferred onto nitrocellulose membranes. The membranes were blocked by incubation in Tris buffered saline (10 mmol/l Tris (pH 7.5), 150 mmol/l NaCl and 0.05% Tween-20) containing 5% of a non-fat dry milk for 90 min at room temperature and incubated overnight at 4°C with the specific primary antibodies against TLR4, IκBα or p-IκBα, respectively. Actin or E-cadherin was used as internal loading control. After washing three times with Tween-20–tris buffered saline, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. The signals were detected using the enhanced chemiluminescence kit (Amersham Pharmacia Biotech, Buckinghamshire, UK).

Reverse transcription–polymerase chain reaction for TLR4
RNA was extracted from HT-29 cells using Promega kits. The primers of TLR4 and histone were used for reverse transcription–polymerase chain reaction. Amplification of polymerase chain reaction was performed according to manufacturer’s instruction.

Statistical analysis
Data were expressed as mean ± standard deviation. Statistical analyses were done by using the analysis of variance 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. A P value of <0.05 was considered statistically significant.

Results
The molecular weight and primary structure of AOG
Generally, apple pectin was a high molecular weight galactan with high viscosity. It mainly consists of galacturonic acids with β-1,4 glucosidic bond. Here, AOG was extracted from apple pectin by hydrolysis and enzymolysis. The data in Figure 1 illustrate the...
molecular weight and primary structure of AOG. The data in Figure 1A indicate that the molecular weight of AOG is 900.85 and consists of five repeated galacturonic acids by Matrix-Assisted Laser Desorption/Ionization Time of Flight Mass Spectrometry analysis. The data in Figure 1B identify the β-glucosidic bond of AOG by 1H-nuclear magnetic resonance analysis.

AOG prevents against intestinal toxicities and carcinogenesis induced by DMH/DSS in mice

Table I summarizes the data that showed AOG reduced intestinal toxicities and prevented colon tumor development in mice treated with DMH/DSS. DMH/DSS produced piloerection, bowing waist, loose stool and carcinogenesis. The mice were found in 50% (25/50) and 40% (16/40) of bloody stool in weeks 3 and 9 and 44% (15/35) and 90% (27/30) of anal prolapse in the weeks 12 and 15, respectively. DMH/DSS induced severe colon inflammation in the mice, whereas AOG significantly attenuated but not completely prevent inflammation. The incidence of colon tumor formation was 90% (18/20) in week 20. The tumors appeared as flat nodular polyoid or catarrhalike tumors in the middle and/or distal colon (Figure 2A). No visible poly tum or tumor was observed in week 15 or before, whereas some small tubular polyps were visible in some colon in week 18. AOG significantly decreased the incidence of blood stool, piloerection, bowing waist and anal prolapse in mice treated with DMH/DSS. Surprisingly, AOG decreased the incidence of tumor formation to 20% (4/20), 10% (2/20) and 0% (0/20) with 2.5, 5 or 10% AOG, respectively. Interestingly, the mice treated with DMH/DSS gained some small tubular polyps were visible in some colon in week 18. AOG significantly decreased the incidence of blood stool, piloerection, bowing waist and anal prolapse in mice treated with DMH/DSS.

Table II: The effects of AOG on blood stool, anal prolapse, colon tumor formation, body weight, colon index and inflammation score in mice treated with DMH/DSS

<table>
<thead>
<tr>
<th>Group</th>
<th>Blood stool (%)</th>
<th>Anal prolapse (%)</th>
<th>Colon tumor</th>
<th>Colon weight (mg)</th>
<th>Body weight (g)</th>
<th>Colon index (mg/g)</th>
<th>Inflammation score</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Week 3</td>
<td>Week 9</td>
<td>Week 12</td>
<td>Week 15</td>
<td>Week 20</td>
<td>Week 20</td>
<td>Week 20</td>
</tr>
<tr>
<td>Control</td>
<td>0/0 (0%)</td>
<td>0/0 (0%)</td>
<td>0/0 (0%)</td>
<td>0/0 (0%)</td>
<td>0/0 (0%)</td>
<td>0/0 (0%)</td>
<td>0</td>
</tr>
<tr>
<td>DMH/DSS</td>
<td>25/50 (50%)</td>
<td>16/40 (40%)</td>
<td>15/35 (44%)</td>
<td>27/30 (90%)</td>
<td>18/20 (90%)</td>
<td>1108 ± 85</td>
<td>44.1 ± 3.9</td>
</tr>
<tr>
<td>DMH/DSS + AOG (2.5%)</td>
<td>23/50 (46%)</td>
<td>13/40 (33%)</td>
<td>8/35 (24%)</td>
<td>6/30 (20%)</td>
<td>4/20 (20%)</td>
<td>622 ± 78</td>
<td>43.3 ± 1.7</td>
</tr>
<tr>
<td>DMH/DSS + AOG (5%)</td>
<td>16/50 (32%)</td>
<td>9/40 (23%)</td>
<td>4/35 (12%)</td>
<td>3/30 (10%)</td>
<td>1/20 (5%)</td>
<td>410 ± 65</td>
<td>45.2 ± 3.5</td>
</tr>
<tr>
<td>DMH/DSS + AOG (10%)</td>
<td>12/50 (24%)</td>
<td>7/40 (18%)</td>
<td>2/35 (6%)</td>
<td>1/30 (3%)</td>
<td>0/20 (0%)</td>
<td>379 ± 45</td>
<td>44.1 ± 2.8</td>
</tr>
</tbody>
</table>

A total of 240 mice were used for the experiment, 40 mice were used in control and 50 mice for each group of DMH/DSS ± AOG. AOG was given initially at the same time when the mice were treated with DMH/DSS. Colon index was calculated as the colon weight (milligram) divided by the body weight (gram) of mouse. Inflammation score was determined as normal for 0, slight and moderate edema for 1, severe edema and/or moderate erosions for 2, severe erosions and/or small ulceration for 3 and large ulceration and necrosis for 4.

AOG decreased the elevated levels of TLR4 and TNF-α induced by DMH/DSS in vivo

To study the alteration of TLR4 and TNF-α during the process of colitis to colon cancer and the protective effect of AOG, we investigated the expression of TLR4 and production of TNF-α in colonic mucosa in the mice treated with DMH/DSS from weeks 3 to 20 (Figure 3). The data showed that the elevation of TLR4 level started in week 3 (P < 0.05 versus control), increased gradually in weeks 6 and 9 (P < 0.01 versus control).
control) and peaked in week 12, then declined slightly in week 15 and continuously decreased in weeks 18 and 20 but was still higher than that of control ($P < 0.01$). Five percentage of AOG significantly decreased the TLR4 levels induced by DMH/DSS from weeks 3 to 20 and exhibited the same trend as DMH/DSS alone (Figure 3A and B). The secretion of TNF-$\alpha$ in vivo from colon mucosa showed the same trend as TLR4 in the

Fig. 2. Pathological evaluation of the photograph (A, each colon represents a mouse) and photomicrograph (B, picture shown only one representative colon for each group) changes in the control mice and the mice treated with DMH/DSS ± AOG. The colons were removed at indicated times in conventional formalin–paraffin sections stained with hematoxylin and eosin ($\times 100$ magnification). The photographs and photomicrographs show as a–b: control in weeks 1 and 20; c–i: DMH/DSS alone in weeks 3, 6, 9, 12, 15, 18 and 20; j: DMH/DSS + 2.5% AOG in week 20; k: DMH/DSS + 5% AOG in week 20; l: DMH/DSS + 10% AOG in week 20. Each group had five mice for weeks 1, 3, 6, 9, 12, 15 and 18, whereas 20 mice for week 20.
mice treated with DMH/DSS and AOG also significantly decreased TNF-$\alpha$ level elevated by DMH/DSS (Figure 3C).

**Effects of AOG on location and expression of TLR4 in the colon mucosa of mice in vivo**

We determined the location of TLR4 in the colonic mucosa by confocal image and found TLR4 mainly expressed in colon epithelia and mesenchymocytes. The membranous TLR4 is the position binding to extracellular LPS. TLR4 would lose its response to LPS when it is located in cytoplasm. The data in Figure 4 showed that the FI (red color) was weak in control and increased in weeks 3 and 6, the strongest intensity in weeks 12 and 15, then slightly declined in week 18 and significantly declined in week 20 after DMH/DSS treatment. The variation of FI of TLR4 was similar to that in TLR4 protein expression in the mice treated with DSS/DMH. AOG significantly decreased the FI in a dose-dependent manner in week 20. Interestingly, the fluorescence pattern (red color) of TLR4 was not weak after AOG treatment. The results suggested that TLR4 might locate at the cytoplasm.

**AOG blocked the elevation of TLR4 stimulated by LPS on the cell membrane but not in the cytoplasm in HT-29 cells in vitro**

To study the effect of AOG on variation of TLR4, we determined the subdistribution of TLR in both membrane and cytoplasm of HT-29 cells by western blot analysis. HT-29 cells highly express TLR4 and are responsive to LPS stimulation (28,29). The membrane levels of TLR4 were normalized against E-cadherin, whereas cytoplasmic levels of TLR4 were normalized against cytoplasmic levels of actin. The results showed either LPS or AOG alone increased TLR4 expression on cell membrane and in cytoplasm. Interestingly, when HT-29 cell was incubated with AOG plus LPS, TLR4 level was increased in cytoplasm, whereas significantly decreased even vanished on the membrane (Figure 5A–C). The results indicated that AOG promoted distribution of TLR4 from cell membrane into cytoplasm. Study of the kinetics migration of TLR4 with confocal imaging in the cells showed that the fluorescence concentrated on the cell membrane with AOG or LPS alone, whereas the FI on cell membrane was significantly decreased when AOG was combined with LPS.
Fig. 4. The distribution and level of TLR4 in the colonic mucosa of the mice treated with saline (control), DMH/DSS ± AOG. Nucleoli were counterstained with 4’,6-diamidino-2-phenylindole. In panel (A) (immunofluorescence staining, pictures show only one representative colon from each group): a–b, colons of control (mouse treated with saline and regular food) in weeks 1 and 20; c–i, colons of the mice treated with DMH/DSS in weeks 3, 6, 9, 12, 15, 18 and 20; j–l, colons of the mice treated with DMH/DSS + 2.5%, 5 and 10% AOG in week 20. In panel (B) (FI): *P < 0.05, **P < 0.01 versus Control; ***P < 0.01 versus DMH/DSS. Each time point represents 5–20 mice (mean ± SD).
with LPS. However, the fluorescence pattern in the cytoplasm was the same whether AOG or LPS was used alone or in combination (Figure 5D).

Effects of LPS and AOG alone or in combination on the mRNA level of TLR4, phosphorylation of IκBα and production of TNF-α in HT-29 cells in vitro

To determine whether the decrease of TLR4 distribution on cell membrane was related to the expression of the RNA of TLR4, the mRNA level of TLR4 was measured in HT-29 cells treated with LPS and AOG alone or in combination by reverse transcription–polymerase chain reaction. The results showed that the RNA level of TLR4 was increased when the cells were incubated with LPS or AOG alone compared with the control. The elevation of TLR4 mRNA induced by AOG was much lower than that of LPS. However, the combination of LPS and AOG produced a much lower level of TLR4 mRNA compared with that of LPS alone and similar to that of AOG alone (Figure 6A–B).

IκBα is an inhibitor of NF-κB and known to bind predominantly to p50–p65 heterodimers in the cytoplasm. In response to external stimuli, IκB proteins are phosphorylated and subsequently degraded to initiate transcription of genes regulated by NF-κB. We investigated the IκBα protein level and its phosphorylated form (p-IκBα) to estimate the effects of AOG on the reversed effects on IκBα phosphorylation after LPS stimulation. The data showed that IκBα level was not altered by either LPS or AOG and the combination of LPS and AOG increased the expression of IκBα, whereas p-IκBα level was significantly increased by LPS (P < 0.01) but not AOG (P > 0.05). AOG obviously inhibited the elevation of IκBα phosphorylation induced by LPS and markedly decreased IκBα phosphorylation even below the

Fig. 5. Effects of LPS ± AOG on protein level of TLR4 (A), ratio of TLR4/E-cadherin in cell membrane (B), ratio of TLR4/actin in cytoplasm (C) and cellular distribution of TLR4 by confocal laser scanning microscopy (D) in HT-29 cells. The cells were treated with LPS (10 μg/ml), AOG (10 mg/ml) or LPS plus AOG. Each time point was done three times (mean ± SD). *P < 0.05, **P < 0.01 versus Control; ***P < 0.01 versus LPS or AOG alone.
was determined by ELISA at various time points (30, 60 and 120 min) after the cells were treated with LPS or AOG alone or in combination for 12 h. The data showed that TNF-α product was increased by either LPS or AOG alone; however, the combination of LPS and AOG remarkably decreased the production of TNF-α. Interestingly, TNF-α product started to increase at 30 min and continuously increased at 90 min and reached to the same level of as LPS alone at 120 min after the media were discarded and LPS was added again (Figure 6E).

Effect of AOG on binding of HT-29 cells in vitro

To evaluate the interaction of AOG and TLR4, LPS was used as the sample to compete with AOG in binding to TLR4. The studies were performed with FITC–AOG ± LPS in HT-29 cells (high expression of TLR4 and response to LPS stimulation) by confocal image and ELISA. The data in Figure 7A demonstrated that LPS (10 μg/ml) significantly decreased the FI ($P < 0.01$) of FITC–AOG (0.1 mg/ml) and FITC–AOG showed the similar position to TLR4 on cell membrane. The data in Figure 7B showed the obvious inhibitory effect of LPS on AOG binding to TLR4 at 37°C but not at 4°C. The results suggest that LPS competitively blocks the binding of FITC–AOG to TLR4.

Discussion

In 1863, Rudolf Virchow suggested that inflammation was closely related to cancer (2). Since then, evidence has strongly supported a link between inflammation and cancer, especially in the gut. In general, inflammation is a beneficial host response to tissue injury and helps the tissue to restore its normal structure and function. However, prolonged inflammation results in severe tissue damage (30). Epidemiological investigation showed that up to 40% patients with colitis developed CACC (4). However, the pathological mechanism of CACC is still poorly understood. Many molecules may be involved in the development of cancer from inflammation, such as NF-κB, IkB kinase-β, inducible nitric oxide synthase and cyclooxygenase-II (12,31). Among them, NF-κB may be the most important molecule in inflammation-associated cancer and plays a critical role in inflammatory carcinogenesis (32). In colitis-associated carcinogenesis, the activation of NF-κB is via LPS binding to TLR4. LPS/TLR4/NF-κB pathway plays a pivotal role in the initiation and growth of CACC (12–14). LPS upregulates TLR4 and keeps the persistent inflammatory response, leads to NF-κB activation. NF-κB activation is responsible for the initiation of inflammation-induced tumor growth (9). Inhibition of NF-κB activity blocks the initiation of LPS-induced inflammation-associated tumor (9). Therefore, it might be an excellent choice for prevention of CACC to target LPS/TLR4/NF-κB pathway and keep TLR4 and NF-κB in an appropriate level.

In present study, AOG exhibited amazing effects in attenuation of inflammatory response, protected against intestinal toxicities and colon tumor development induced by DMH/DSS in our model system. While 90% of mice (18/20) developed colon tumors after DMH/DSS treatment, 10% AOG completely prevented the incidence of colon tumor development (0/20) induced by DMH/DSS (Table I). The role of TLR4 in CACC has been demonstrated in knockout mice, colon carcinogenesis was obviously decreased in these mice (14). TLRs were expressed on various cancer cells, such as colon, breast, prostate, melanoma, pancreatic and lung cancers. We have tested three colon cancer cell lines including Caco-2, HT-29 and SW-1116 for TLR4 expression. We found that TLR4 were highly expressed in all the three tested cell lines. However, it was HT-29 cells, but not Caco-2 or SW-1116 cells, responsive to LPS stimulation (Liu et al. unpublished results) so we selected HT-29 cell line in our studies. The result is consistent with the report by Lee et al. (29). In our study, the important molecules of TLR4 and TNF-α in colon mucosa were increased in the mice with colitis and AOG significantly inhibited the elevation of TLR4 and TNF-α by DMH/DSS. The data are consistent with the reports from the literature. TLR4 also had the basic function to maintain the normal function of colonic epithelial cells. Ideally, TLR4 signaling should be significantly diminished but not completely
eliminated (33). Therefore, the best strategy for colon cancer prevention is to keep and maintain suitable levels of TLR4 and TNF-α. The present studies showed that AOG inhibited the elevation of TLR4 and TNF-α induced by DMH/DSS but still kept the levels higher than the control. This may be related to the preventive effects of AOG on carcinogenesis. The inhibitory effects of AOG on colon tumorigenesis may be mainly due to its anti-inflammatory efficacy because we also observed the similar protective effect of AGO on colon carcinogenesis when AGO was initially administrated to the mice at week 5 or week 9 after DMH/DSS treatment (Liu et al. unpublished results).

LPS is the main ligand of TLR4 and plays very important role in carcinogenesis from inflammation (34). Gram-negative germs are dominant and their main product LPS is copious in colon cavity in patients with inflammatory bowel disease. LPS binds to TLR4 and activated NF-κB to initiate colitis. However, TLR4 on the cell membrane is required for LPS binding to activate NF-κB. When TLR4 mutated with the leucines at the positions of 815 and 816, it no longer present on the cell membrane and led to hyporesponsiveness to LPS (18). It appears that AOG affected TLR4 distribution by transferred TLR4 from the cell membrane into the cytoplasm. This may be the reason why AOG could decrease TNF-α production in this model system. In vitro experiments, both LPS and AOG alone increased mRNA level and membrane distribution of TLR4 promoted phosphorylation of IκB and increased TNF-α product. However, the mRNA level of TLR4, phosphorylation of IκB and production of TNF-α were significantly decreased when AOG was combined with LPS to cause redistribution of TLR4 from the cell membrane to the cytoplasm. This may be due to the effect of AOG on inhibition of the response of LPS to TLR4 and the downstream signaling pathway. Interestingly, the response of TLR4 to LPS was recovered within 2 h after AOG was removed. It suggested that AOG was just blocked TLR4 temporarily and it may compete for the same receptor of LPS. The main reason for colitis recurrence is due to excessive LPS contacted persistently with TLR4 to activate NF-κB to increase the production of cytokines. AOG interrupted LPS contacting to TLR4 and prevented colon inflammation and carcinogenesis from colitis by relocated TLR4 into the cytoplasm from the membrane and inhibited upstream signaling of LPS/TLR4/NF-κB pathway. The results in Figure 5 indicated that the combination of AOG and LPS affected the distribution of TLR4 with decreased expression on the cell membrane, whereas increased expression in the cytoplasm.

The important finding of our study is that AOG decreased elevated TLR4 level induced by inflammation and redistributed TLR4 from cell membrane into cytoplasm, sequentially caused hyporesponsiveness to LPS and attenuation of inflammation to prevent carcinogenesis. This is the first report that the decrease of TLR4/NF-κB activation is due to the redistribution of TLR4. The results suggest that AOG and LPS bind at different sites of TLR4. The effect of AOG on TLR4 is different from that of polysaccharides which competitively antagonized LPS binding to TLR4 and inhibited its downstream signaling pathway (35).

Apple is one of the most popular fruits. It has been demonstrated that the beneficial effect of apple to human health and apple exhibited therapeutic effects on some diseases. Studies demonstrated that cloudy apple juice decreased DNA damage, hyperproliferation and aberrant crypt foci development in rats treated with DMH (36). Apple

![Fig. 7.](image_url)
containing different amounts of analyzed procyanidins and pectin showed different cancer-preventive effect and the one with higher amounts of pectin exhibited better preventive effect (36). In our study, AOG exhibited a remarkable ability to reduce intestinal inflammation and prevent carcinogenesis induced by DMH/DSS with no side effect for long-term administration. It is extremely important for cancer prevention because it needs long-term administration and the best choice is originally from food without toxicity. The possible mechanism of AOG may be like other apple products through the phytochemicals to provide the benefits of antioxidant and antiproliferative activity (24). Apple polyphenols could elevate the expression of the phase II gene glutathione S-transferase T2 in colon epithelial cells to provide protection against oxidant-induced DNA damage and prevention of colon cancer (37). We studied and compared the effects of several galactans with different number of galacturonic acids on TLR4 to LPS. We found that five galacturonic acids exhibited the best effect on induction of TLR4 into the cytoplasm from the cell membrane (data not shown).

In conclusion, we obtained an AOG composed of five galacturonic acids with the primary structure of (1→4)-β-D-galacturonic acid from the extraction of apple pectin. The results clearly demonstrated the preventive effects of AOG against DMH/DSS-induced inflammation and carcinogenesis from colitis in this model system. The underlying mechanism of AOG on prevention of carcinogenesis may be related to target LPS/TLR4/NF-κB pathway via affecting TLR4 membrane distribution and interrupting LPS binding to TLR4 to cause hyporesponse to LPS to block the initiation of inflammation. The AOG may be useful for the treatment of patients with inflammatory bowel disease and prevention of carcinogenesis in the clinics.

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References


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