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Anti-inflammatory effects of orally administered glucosamine oligomer in an experimental model of inflammatory bowel disease

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ABSTRACT

Anti-inflammatory effects of oral administration of the glucosamine oligomers (chito-oligosaccharides: COS) were evaluated in an experimental model of inflammatory bowel disease (IBD). Oral administration of COS improved shortening of colon length and tissue injury (as assessed by histology) in mice. Oral administration of COS inhibited inflammation in the colonic mucosa by suppression of myeloperoxidase activation in inflammatory cells, as well as activation of nuclear factor-kappa B, cyclooxygenase-2, and inducible nitric oxide synthase. Oral administration of COS also reduced serum levels of pro-inflammatory cytokines (tumor necrosis factor-α and interleukin-6). Moreover, it prolonged survival time in mice. These data suggest that COS have anti-inflammatory effects in an experimental model of IBD, and could be new functional foods for IBD patients.

1. Introduction

Inflammatory bowel disease (IBD) encompasses ulcerative colitis (UC) and Crohn's disease, and is characterized by chronic inflammation of the gut (Morrison, Headon, & Gibson, 2009). The incidence of IBD has increased steadily in some areas of the world over the past 40 years (Goh & Xiao, 2009), possibly because of changes in dietary habits (particularly consumption of diets low in fiber content) (Rose, DeMeo, Keshavarzian, & Hamaker, 2007). The goal of IBD treatment is to achieve and maintain remission (Pithadia & Jain, 2011). However, drugs not only have beneficial effects but also have adverse effects in IBD patients (Morrison et al., 2009).

Chitooligosaccharides (COS: glucosamine oligomers) have been reported to have anti-tumor (Harish-Prashanth & Tharanathan, 2005; Huang, Mendis, Rajapakse, & Kim, 2006; Shen, Chen, Chan, Jeng, & Wang, 2009; Suzuki et al., 1986; Tokoro, Tatewaki, Mikami, Suzuki, & Suzuki, 1989; Wang et al., 2008) and anti-inflammatory (Li, Liu, Xu, Du, & Xu, 2014; Wei et al., 2012) activities. Moreover, Yousef, Pichyangkura, Soodvilai, Chatsudthipong, and Muanprasat (2012) demonstrated that intraperitoneal injection of COS suppressed inflammation of the colon in an experimental model of IBD (Yousef et al., 2012). However, most reports that describe the bioactivities COS have evaluated intraperitoneal or intravenous administration of COS *in vivo* or *in vitro*.

Recently, it was revealed that oral administration of chitin nanofibrils or glucosamine from chitin led to anti-inflammatory effects in experimental models of IBD (Azuma et al., 2012a; Azuma et al., 2012b; Yomogida et al., 2008). These results indicate chitin derivatives have a potency as an anti-inflammatory agents. However, reports describing the anti-inflammatory effects of COS by oral administration in an experimental model of IBD are lacking; hence, the present study was undertaken.

2. Materials and methods

2.1. Reagents

COS, derived from crab shells, was provided by Koyo Chemical Co. Ltd. (Tokyo, Japan). COS comprise dimers (8.9%), trimers (19.6%),

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tetramers (23.7%), pentamers (25.4%), hexamers (8.4%), heptamers (3.1%), and octamers (1.7%) (Masuda et al., 2014). Dextran sulfate sodium (DSS; molecular mass, 36–50 kDa; reagent grade) was purchased from MP Biomedicals (Solon, OH, USA).

2.2. Animals

Female C57BL/6 mice (5 weeks) were purchased from CLEA Japan (Osaka, Japan). Animals were maintained under standard conditions and used for experimentation after seven days of acclimatization. Use of these mice and the procedures they underwent were approved by the Animal Research Committee of Tottori University (Tottori, Japan).

2.3. Study design

2.3.1. Study of disease severity

Mice ($n = 32$) were randomized into four groups of eight. The NT group was administered tap water and fed a powdered diet (CE-2; CLEA Japan). The DSS group was administered only 3% DSS in tap water and fed a powdered diet. The COS group was administered 3% DSS in tap water and fed a powdered diet containing 2% COS. The GlcN group was administered 3% DSS in tap water and fed a powdered diet containing 2% glucosamine (GlcN). To induce UC, the mice were administered 3% DSS *ad libitum* for 5 days (from day-0 to day-5). The mice in the COS and GlcN groups were also fed a powdered diet with 2% COS or GlcN from day-0 to day-5. Measurements of body weights were undertaken on day-0 and day-5 in all groups. Then, each ratio of the body weights (the percentage of day 5/day 0) was calculated. Colon and blood samplings were done on day-5 in all groups.

2.3.2. Survival study

Mice ($n = 30$) were randomized into three groups of ten. The DSS group was administered only 3% DSS in tap water and fed a powdered diet (CE-2; CLEA Japan). The COS group was administered 3% DSS in tap water and fed a powdered diet containing 2% COS. GlcN group was administered 3% DSS in tap water and fed a powdered diet containing 2% GlcN. To induce UC, the mice were administered 3% DSS *ad libitum* from day 0 to the end of the experiment. Mice in COS and GlcN groups were also fed a powdered diet containing 2% COS or GlcN during the experimental period. The number of days until the mice died was counted. Thereafter, a survival curve was created and median survival time (days) calculated.

2.4. Histopathologic evaluation

The length (cm) and weight (mg) of the colon were measured, and tissue obtained from each colon. Colon tissues were fixed in 10% buffered formalin. Sections (thickness, 3 μm) were made from each sample for histologic observation after staining with hematoxylin and eosin (H&E). Each section was examined microscopically, and histologic scoring was undertaken as described by Ohkawara et al. (2005). In brief, tissue damage was classified using six grades: 0, normal mucosa; 1, infiltration of inflammatory cells; 2, shortening of crypts by less than half of their height; 3, shortening of crypts by more than half of their height; 4, crypt loss; and 5, destruction of epithelial cells. Ten randomly chosen middle-power fields (×100 magnification) for each cross-section were photographed with a digital camera attached to a microscope (Olympus, Tokyo, Japan). Histologic scoring was done in ten fields using three mice in each group. Mean scores for 30 fields were considered as the histologic score for each group.

2.5. Detection of nuclear factor-kappa B (NF-κB) in the colon

Effects of oral administration of COS on NF-κB activation were evaluated in inflamed colons, according to our previous methods (Azuma et al., 2012b). Sections (3 μm) of colon tissue prepared on glass slides were deparaffinized, washed with ethanol and water, and soaked in phosphate-buffered saline (PBS). Then, sections were microwaved with 0.01 M citrate buffer (pH 6.0) for 5 min. Thereafter, they were washed with PBS and incubated with 1% hydrogen peroxide–methanol for 30 min at room temperature. After washing with PBS, sections were incubated with rabbit polyclonal anti-NF-κB p65 antibody (sc-372; 1:500 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 60 min at room temperature, followed by a further wash with PBS. Tissue sections were then visualized by staining with diaminobenzidine tetrahydrochloride (Code No. K3466; Dako, Glostrup, Denmark) and counterstaining with hematoxylin, for 30 min at room temperature.

Then, the extent of NF-κB-positive areas in the colonic epithelium was determined. We used quantitative digital morphometric analyses of NF-κB-positive areas of colonic sections according to a method described previously (Azuma et al., 2012b). In brief, 10 randomly chosen high-power fields (×200 magnification) for each cross-section were photographed with a digital camera attached to a microscope (Olympus). Color wavelengths of the copied image were transformed into digital readings using Lumina Vision software (Mitani, Tokyo, Japan), thereby allowing for quantification of various color wavelengths with pixels as a unit of measure. The original image was used for comparison, and color spectra analyzed; those corresponding to the positive areas of NF-κB were quantified. The percentage of positive areas of NF-κB in the epithelium was calculated by dividing the total pixel area of the positive areas of NF-κB by the total pixel area (which corresponded to the total colonic tissue in the field of view). Colons of three mice were analyzed from each group. The mean score from 30 fields was considered to represent the extent of fibrosis for each group.

2.6. Immunohistochemical (IHC) analyses

Cell staining for myeloperoxidase (MPO; a marker of leukocyte invasion into tissue) (Schindhelm, van der Zwan, Teerlink, & Scheffer, 2009) was undertaken in a routine manner, as described previously (Ohtsuka et al., 2001). Each section was examined microscopically.

For the IHC analyses of cyclooxygenase-2 (COX-2), sections of colon tissue (thickness, 3 μm) prepared on glass slides were deparaffinized, washed with ethanol and water, and soaked in PBS. Then, sections were microwaved with 0.01 M citrate buffer (pH 6.0) for 5 min. Thereafter, they were washed with PBS and incubated with 1% hydrogen peroxide–methanol for 30 min at room temperature. After washing with PBS, blocking was carried out in PBS with 1% bovine serum albumin (BSA) for 30 min at room temperature. Sections were incubated with rabbit monoclonal anti-COX-2 antibody (1:400 dilution; Cell Signaling Technology, Danvers, MA, USA) overnight at 4 °C, followed by a further wash with PBS. Subsequently, the envision method was employed for 30 min at room temperature (Dako). Then, tissue sections were visualized by staining with 3.3′-diaminobenzidine (DAB; Dako) and counterstained with hematoxylin. Each section was examined microscopically.

For the IHC analyses of inducible nitric oxide synthase (iNOS), sections of colon tissue (thickness, 3 μm) prepared on glass slides were deparaffinized, washed with ethanol and water, and soaked with PBS. Then, the sections were microwaved with 0.01 M citrate buffer (pH 6.0) for 5 min. Thereafter, they were washed with PBS and incubated with 1% hydrogen peroxide–methanol for 30 min at room temperature. After washing with PBS, blocking was