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High levels of fish oil enhance neutrophil development and activation and influence colon mucus barrier function in a genetically susceptible mouse model☆,☆☆

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Abstract

Dietary fatty acids influence immunologic homeostasis, but their effect on initiation of colitis, an immune-mediated disease, is not well established. Previously, our laboratory demonstrated that high doses of dietary fish oil (FO) increased colon inflammation and dysplasia in a model of infection-induced colitis. In the current study, we assessed the effects of high-dose dietary FO, 6% by weight, on colon inflammation, neutrophil recruitment and function, and mucus layer integrity in a genetically susceptible, colitis-prone mouse model in the absence of infection. FO-fed SMAD3$^{-/-}$ mice had increased colon inflammation evidenced by increased numbers of systemic and local neutrophils and increased neutrophil chemoattractant and inflammatory cytokine gene expression in the colon. Mucus layer thickness in the cecum and goblet cell numbers in the cecum and colon in FO-fed mice were reduced compared to control. FO consumption affected colitis in male and female mice differently. Compared to female control mice, neutrophils from FO-fed female mice had reduced reactive oxygen species (ROS) upon ex vivo stimulation with phorbol myristate acetate while FO-fed male mice produced increased ROS compared to control-fed male mice. In summary, dietary FO impaired mucus layer integrity and was associated with colon inflammation characterized by increased neutrophil numbers and altered neutrophil function. High-dose FO may have detrimental effects in populations genetically susceptible for inflammatory bowel disease and these effects may differ between males and females.

Keywords: Inflammation; Neutrophil; Fish oil; B lymphocyte; Colon; Mucosal barrier

1. Introduction

Approximately 30 million people in the USA consume fish oil (FO) supplements [1] because they are a rich source of n-3 polyunsaturated fatty acids (n-3 PUFA) including eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). FO is currently prescribed only to treat hypertriglyceridemia, a cardiovascular disease risk factor [2]. However, supplemental and therapeutic use of FO needs more careful consideration because n-3 PUFA can be pro-inflammatory and may exacerbate disease. The risk of prostate cancer increased in humans with elevated serum phospholipid n-3 PUFA [3], and the highest quintile of n-3 PUFA intakes were associated with increased risk of endometrial cancer [4]. Despite evidence suggesting that FO may be beneficial in inflammatory bowel disease (IBD) [5], humans consuming diets high in total fats, PUFAs, omega-6 fatty acids, and meat were at increased risk for developing Crohn's disease (CD) and ulcerative colitis (UC) [6]. Four studies included in this analysis demonstrated an association between high fish and seafood consumption and increased risk of developing UC [6].

Neutrophils play a role in active IBD cases, and regulating neutrophils in active IBD results in disease remission [7]. In general FO is considered anti-inflammatory and affects immunity through various mechanisms [8]. FO consumption, at 3–6 g/day, is associated with reduced reactive oxygen species (ROS) generation by human neutrophils through cyclooxygenase-dependent production of leukotrienes [9–11]. As first responders to infection, neutrophils expand in number and traffic from the bone marrow to the site of infection. Importantly, neutrophil numbers are increased and more activated in UC [12,13]. McCall et al. showed FO was therapeutic in UC patients with a reduction in neutrophil chemotaxis and leukotriene B4 [14]. However, this study was in patients diagnosed with UC, not pre-illness intake and initiation of disease. Reducing neutrophil chemotaxis and

Abbreviations: APRIL, A proliferation inducing ligand; BAFF, B cell activating factor; DCF, Chloromethyl-dichlorofluorescein diacetate; CD, Crohn's disease; Ct, Comparative threshold cycle; CON, Control; DHA, Docosahexaenoic acid; Cdh1, E cadherin; EPA, Eicosapentaenoic acid; FBS, Fetal bovine serum; FITC, Fluorescein isothiocyanate; IBD, Inflammatory bowel disease; IL, Interleukin; MUC, Mucin; n-3 PUFA, n-3 polyunsaturated fatty acid; PNA, Peanut agglutinin; Pen/Strep, Penicillin/Streptomycin; PMA, Phorbol myristate acetate; PBS, Phosphate buffered saline; ROS, Reactive oxygen species; SEM, Standard error of the mean; TGF, Transforming growth factor; TNF, Tumor necrosis factor; UC, Ulcerative colitis.

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function with FO has both advantages and disadvantages, in that it is a benefit to mitigate excessive tissue damage but detrimental during episodes of infection including infection-induced colitis.

Limiting interactions between the microbiota and colon epithelium is critical for health, and breakdown of immune tolerance to the microbiota has been implicated in numerous diseases. Mechanisms of maintaining tolerance to the microbiota include the mucus layer and IgA. Mucosal barriers are altered by diet such as vitamin A deficiency [15–17], pre- and probiotics [18] and fiber [19,20]. There is limited data on the effects of FO on colon mucosal layer integrity, but FO has been shown to regulate mucin expression. In a mouse model of induced cholesterol gallstones, FO attenuated increased mucin 2 (MUC2) gene expression in the gall bladder of mice prone to cholesterol gallstones [21]. Gene expression of MUC4 was reduced in lungs of *Pseudomonas aeruginosa* infected mice consuming FO [22]. In vitro, EPA increased permeability to fluorescein sulphonic acid and reduced transepithelial electrical resistance in CACO-2 cells in a dose dependent manner indicating reduced barrier integrity [23]. Sylvester et al. showed reduced MUC2 expression in poorly differentiated human colorectal tumors demonstrating the importance of mucus barriers in colon cancer [24].

We hypothesized FO would increase neutrophil development and colonic neutrophil infiltration and increase inflammatory cytokines secondary to reduced goblet cell number and mucus layers in a murine model of IBD. Here, we characterize the effects of FO consumption on inflammation and GI health in SMAD3$^{-/-}$ mice in the absence of *Helicobacter hepaticus* infection.

2. Materials and methods

2.1. Animals

SMAD3$^{+/-}$ and *SMAD3*$^{-/-}$ breeder pairs (129-Smad3tm1par/J, 129 background) were generated in-house. Homozygous males and heterozygous females were mated to obtain SMAD3$^{-/-}$ pups started on diet at 6–8 weeks of age. SMAD3$^{-/-}$ pups were confirmed by polymerase chain reaction (PCR). Mice were housed under specific pathogen free conditions in microisolator cages in an Association for Assessment and Accreditation of Laboratory Animal Care-approved facility at Michigan State University. Specific pathogen-free conditions were assured through quarterly serology testing by Charles River Laboratories International (Wilmington, MA, USA) and in-house testing for ectoparasites, endoparasites, and fecal *Helicobacter* species (PCR). All animal protocols were approved by the Michigan State University Institutional Animal Care and Use Committee.

2.2. Diets

SMAD3$^{-/-}$ mice were fed an AIN-93G-based [control (CON)] standard chow or a 6% (wt/wt) FO diet of similar composition to that described previously [25–27]. Experimental FO diets contained 1% (wt/wt) corn oil in addition to the FO. Six-to-eight week old mice were fed experimental diets for 5 weeks. Then, animals were sacrificed at 11–13 weeks of age. FO used was primarily Menhaden oil (Sigma, St. Louis, MO, USA), but DHA-enriched (DSM Nutritional Products, Parsippany, NJ, USA) and EPA-enriched (DSM Nutritional Products, Parsippany, NJ, USA) FOs were also used in some experiments (indicated in figure legends). The effect of different dietary FOs on spleen and bone marrow neutrophils as well as histopathology and fecal IgA were not significantly different when compared to each other (data not shown), and therefore data from all three FOs were combined (CON, n=9–10; FO, n=20–30) and denoted as FO-fed (Figs. 1, 3, and 5C). Flow cytometry experiments (Figs. 4 and 6) were performed as described below in two replicate experiments of four to five mice per sex fed CON and seven to eight mice per sex fed Menhaden oil for a total of 9–10 CON-fed mice per sex and 15 FO-fed mice per sex. Analysis of B cell expression of peanut agglutinin (Fig. 6C) was performed on three CON-fed male, three CON-fed female, seven FO-fed male and eight FO-fed female mice. Bone marrow functional assay (Fig. 2, described below) was performed on five male CON-fed, five female CON-fed, five male FO-fed and five female FO-fed mice. Intestinal permeability was assessed using separate animals in two replicate experiments of five male CON-fed, five female CON-fed, four to five male FO-fed and five female FO-fed mice. Mucus layer analysis was performed on mixed sex mice (10 CON-fed and 10 FO-fed).

2.3. Tissue processing

Mice were euthanized by CO_2 asphyxiation followed by cardiac puncture to acquire peripheral blood for red blood cell fatty acid analysis (data not shown). Hind limbs were excised and transferred into ice cold RPMI-1640 (Sigma). Bone marrow cells were flushed with RPMI-1640 and centrifuged to pellet cells. Red blood cells were lysed using ammonium chloride potassium (ACK) lysis buffer and cells were washed twice with media. An aliquot of single cell suspension was used for hemocytometer counts with trypan blue (Sigma) exclusion dye.

Colon and cecum were processed for a single cell suspension as previously described [28,29]. Briefly, tissue was minced, removed of lumen content by shaking, and incubated with 5 mM ethylenediaminetetraacetic acid (Sigma), 10% fetal bovine serum (FBS: Atlanta Biological, Flowery Branch, GA, USA), 1× penicillin/streptomycin (Pen/Strep: Life Technologies, Grand Island, NY, USA) in Ca/Mg Free Hank's balanced salts solution (Sigma) for 30 min at 25°C. Tissue was filtered through 70 μm mesh and the remaining tissue was further minced and filtered intraepithelial cells were washed and kept on ice. Minced tissue was subsequently incubated with 0.5 mg/ml collagenase D (Roche, Indianapolis, IN, USA), 10% FBS, 1× Pen/Strep in DMEM (Sigma) for 60 min at 37°C. Tissue was filtered through 70 μm mesh and combined with intraepithelial cells, centrifuged at 800×*g* for 8 min at 4°C. Cell pellets were resuspended in 10% FBS in DMEM prior to adding 80% Percoll (Sigma) to a final concentration of 40% and layered over 80% Percoll. Density gradient centrifugation was performed at room temperature for 20 min at 1000×*g*. Mononuclear cells at the interface were removed, washed twice with 10% FBS in DMEM, and subsequently stained for flow cytometry as described below.

Spleen tissue was Dounce homogenized, red blood cells were lysed with ACK lysis buffer, and counted using a hemocytometer as previously described [25].

2.4. Flow cytometry

One to two million aliquoted cells were blocked with 0.5 μg of anti-CD16/anti-CD32 (clone; 2.4 g2) on ice. Subsequently, cells were incubated with optimal concentrations of fluorochrome-conjugated monoclonal antibodies (Supplementary Table 1) in various cocktails. All antibodies and dyes were purchased from BD Biosciences (San Jose, CA, USA), eBioscience (San Diego, CA, USA), Vector Labs (Burlingame, CA, USA), or BMA Biomedicals (Augst, CH, Switzerland). Excess antibody was removed by washing with fluorescence activated cell sorting (FACS) buffer [0.1% sodium azide (Sigma), 1.0% FBS (Atlanta biologicals) in phosphate buffered saline (Sigma)] and centrifugation Data was acquired on a FACS Canto II (BD Biosciences) flow cytometer. Cells were gated (Supplementary Table 1) and analyzed using FlowJo (TreeStar, Ashland, OR, USA). Total cell numbers were determined by multiplying the cell population percentage of live cells by total cell counts obtained by hemocytometer for spleen and bone marrow.

2.5. Dichlorofluorescein assay

Bone marrow cell suspensions were resuspended in DMEM supplemented with 10% FBS and 2 million cells were aliquoted. Chloromethyl-dichlorofluorescein diacetate (DCF, Life Technologies) was reconstituted, diluted, and added to a final concentration of 5 μM. Cells were incubated with DCF at 37°C for 5 min. Phorbol myristate acetate (PMA, Sigma) was added to a final concentration of 100 ng/ml and total stimulation volume of 1 ml. Cells were stimulated for 30 min at 37°C, washed with ice cold FACS buffer, blocked with anti-CD16/anti-CD32, and stained for flow cytometric analysis as described above.

2.6. Histology

Colon and cecum were fixed and stained with hematoxylin and eosin and scored as previously described [30,31]. Briefly, tissue was flushed with phosphate buffered saline (PBS: Sigma), fixed in 10% formalin, embedded in paraffin, sectioned and stained. Sections were graded by a pathologist for inflammation and dysplasia. Colon and cecum were individually scored for inflammation and dysplasia on a scale of 1–4 each for a total score of 4 for healthy control mice and maximum score of 16 as previously described [25]. To assess mucus layer integrity, large intestines were fixed in Carnoy's solution, sectioned and stained with Alcian blue and periodic acid/Schiff to visualize mucus layer and goblet cells in colon tissue as previously described [32,33]. Sections were scored by a pathologist to measure mucus layer thickness and quantify goblet cell numbers. Measurements of mucus layer thickness were acquired from the three sites with thickest mucus layers and averaged for each animal (3 sites/1 slide/animal). Goblet cell counts per animal were measured in random image sites (five 10×10 mm sites/animal) and averaged (5 sites/1 slide/animal). Goblet cell counts were the number of specially stained cells in a 10×10 mm grid area using a 1 cm^2 10×10 grid reticle at 400× magnification.

2.7. RNA extraction and RT-PCR

A segment of the colon was excised and stored in TRIzol (Life Technologies) at −80°C until processing according to manufacturer instructions. Briefly, samples were homogenized, RNA yield and quality were assessed by spectrophotometry (Nanodrop), mRNA was reverse transcribed to cDNA, and qPCR performed on an ABI 7900 HT RT-PCR system. Data was analyzed using the comparative threshold cycle (Ct) method and fold change was calculated according to manufacturer instructions (Life Technologies, Carlsbad, CA). All TaqMan primer/probe sets (Supplementary Table 2) were purchased from Applied Biosystems (Life Technologies).