DHA protects against experimental colitis in IL-10-deficient mice associated with the modulation of intestinal epithelial barrier function

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Abstract
A defect in the intestinal barrier is one of the characteristics of Crohn's disease (CD). The tight junction (TJ) changes and death of epithelial cells caused by intestinal inflammation play an important role in the development of CD. DHA, a long-chain PUFA, has been shown to be helpful in treating inflammatory bowel disease in experimental models by inhibiting the NF-κB pathway. The present study aimed at investigating the specific effect of DHA on the intestinal barrier function in IL-10-deficient mice. IL-10-deficient mice (IL-102/2) at 16 weeks of age with established colitis were treated with DHA (i.g. 35·5 mg/kg per d) for 2 weeks. The severity of their colitis, levels of pro-inflammatory cytokines, epithelial gene expression, the distributions of TJ proteins (occludin and zona occludens (ZO)-1), and epithelial apoptosis in the proximal colon were measured at the end of the experiment. DHA treatment attenuated the established colitis and was associated with reduced infiltration of inflammatory cells in the colonic mucosa, lower mean histological scores and decreased levels of pro-inflammatory cytokines (IL-17, TNF-α and interferon-γ). Moreover, enhanced barrier function was observed in the DHA-treated mice that resulted from attenuated colonic permeability, rescued expression and corrected distributions of occludin and ZO-1. The results of the present study indicate that DHA therapy may ameliorate experimental colitis in IL-102/2 mice by improving the intestinal epithelial barrier function.

Key words: Crohn’s disease; IL-10-deficient mice; DHA; Intestinal barrier function; Epithelial cell apoptosis

Crohn's disease (CD), one of the major forms of inflammatory bowel disease (IBD), is a chronic inflammatory disorder of the bowel that causes segmental lesions in the gastrointestinal tract(1,2). The paracellular permeability of the intestinal epithelium is mediated by tight junctions (TJ), protein complexes composed of transmembrane proteins such as occludin, scaffolding proteins like zona occludens (ZO) and regulatory and signalling molecules(3); these TJ components constitute the major determinant of the intestinal physical barrier(4). A defect in the intestinal barrier is one of the characteristics of IBD(5). An increase in the permeability of the intestinal epithelium leads to mixing of the luminal content, including pathogens, toxins, antigens and immune cells of the lamina propria, which causes and enhances inflammatory response in the intestine(6). The TJ changes and death of epithelial cells caused by intestinal inflammation play an important role in the development of CD(7,8). Therefore, maintenance of the intestinal barrier is imperative for intestinal mucosal homeostasis.

Much of our understanding of the molecular mechanisms involved in IBD has come from transgenic, knockout and chemically induced mouse models(9,10). Studies have shown that IL-10-knockout (IL-10−/−) mice display similar characteristics to that of human CD(11). IL-10 is an important cytokine with anti-inflammatory activity; it is a macrophage deactivator, blocking the induced synthesis of multiple inflammatory

Abbreviations: CD, Crohn’s disease; FITC, fluorescein isothiocyanate; IBD, inflammatory bowel disease; TJ, tight junction; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labelling; WT, wild-type; ZO, zona occludens.

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cytokines (e.g. TNF-α, IL-1 and IL-6) and is a granulocyte/macrophage colony-stimulating factor\(^{12,13}\). The IL-10-deficient mice (generated by gene targeting) mostly suffer from anaemia, growth retardation and chronic colitis under specific pathogen-free conditions\(^{14}\).

In recent years, many data in the literature have suggested a correlation between nutrition and IBD. Exclusive enteral nutrition therapy has been rigorously tested and shown to be a dietary intervention that induces remission of CD\(^{15,16}\) through mucosal healing\(^{16}\), and by affecting the composition of the gut microbiota and modulating of immune function\(^{17}\). Fish oil-derived n-3 PUFA are also known as anti-inflammatory lipids and have beneficial effects in various inflammatory diseases (e.g. psoriasis and active rheumatoid arthritis, etc.)\(^{18}\).

Epidemiologic results from the European Investigation into Cancer and Nutrition and the Nurses’ Health Study have shown that greater consumption of n-3 PUFA and a higher ratio of n-3 to n-6 PUFA appears to protect against the development of IBD\(^{19,20}\). Clinical intervention studies have revealed that nutritional supplementation with n-3 PUFA is considered an alternative or complementary treatment in IBD therapy\(^{21}\). Many studies about the effect of n-3 PUFA have been carried out in human subjects and no certain conclusions have been made so far\(^{22,23}\). Some in vitro studies have also reported that n-3 PUFA treatment can inhibit T-cell proliferation\(^{24}\) and decrease antigen presentation\(^{25}\). The therapeutic effect of n-3 PUFA on animal models of chronic colitis has been widely reported, but few reports have mentioned the effect of DHA, one major component of n-3 PUFA, on experimental colitis in IL-10-deficient mice.

The immunomodulatory action of PUFA on the intestinal mucosa immune cells has been widely studied\(^{26}\), and increasing interest is currently being given to the mechanisms by which PUFA act on intestinal epithelial cells and how they modulate epithelial permeability during inflammatory stress\(^{27}\). It was recently discovered that DHA (22:6n-3), a long-chain PUFA, could modulate the inflammatory response, not merely by decreasing cytokine production and dampening inflammation, but by actively promoting the resolution of inflammation\(^{27}\). DHA helps treat IBD in experimental models by inhibiting the NF-κB pathway\(^{28}\). The study of an in vitro model of the intestinal barrier proved that DHA could partially restore occludin expression in TJ complexes; furthermore, ZO-1 staining and TJ functionality were improved by DHA in a dose-dependent manner\(^{28}\). However, the relationship between epithelial barrier function and DHA treatment has not been studied. The present study aimed to investigate the specific effect of DHA on the intestinal barrier function of IL-10-deficient mice.

Materials and methods

Animals

Wild-type mice and IL-10\(^{-/-}\) (16 weeks old at the beginning of the study) on a C57BL/6 background were obtained from the Jackson Laboratory. Mice were bred and maintained in a specific pathogen-free condition at the Model Animal Research Center of Nanjing University (Nanjing, China). All animal studies were carried out in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of Nanjing University (Nanjing, China).

Drug administration protocol

Mice included in the present study were divided into wild-type group (WT), control group (IL-10 knockout) and treatment group (DHA), containing six mice in each group. IL-10\(^{-/-}\) mice in the treatment group receiving DHA (intragastric administration 55.5 mg/kg per d, Cayman Chemical) treatment for 2 weeks, while the mice in WT and control groups receiving the same volume of vehicle (meaning placebo, normal saline in the present study). Mice were weighed weekly and dosages were adjusted accordingly. Four weeks after the final drug administration, the therapeutic effects of DHA were evaluated. The weight of mice in each group before and after the treatment was recorded for the evaluation of net weight change.

Histology

After mice were euthanised, proximal colons were obtained immediately and fixed in 10% buffer neutral formalin and embedded in paraffin. Thereafter, 6-μm-thick sections were stained with haematoxylin and eosin. Two independent pathologists blinded to the study design gave an inflammation score to samples (one typical proximal colon tissue per mouse and six mice included in each group) taking into account the number of lesions as well as the severity of the disease. Each proximal colon segment was scored from 0 to 4 on the following well-established criteria described by Singh et al\(^{29}\). In brief, grade 0 represented no changes compared with normal tissue; grade 1 represented one or few multi-focal mononuclear cell infiltrates in the lamina propria; grade 2, lesion with several multi-focal cellular infiltrates in lamina propria; grade 3, lesions involved moderate inflammation and epithelial hyperplasia; grade 4, inflammation involved most of the colon sections. The summation of scores per mouse provided a total colonic disease score.

ELISA

For the determination of cytokines in the colonic mucosa, protein extracts were obtained by homogenisation of colonic segments in homogenisation buffer consisting of a protease inhibitor. The measurement of cytokines was according to ELISA in detail according to the manufacturer’s instructions. Cytokines including IL-17 and interferon-γ were measured by ELISA using DuoSet ELISA development kits (R&D Systems). Concentrations of cytokines were established in triplicate supernatants by comparison with standard curves generated using the appropriate recombinant cytokine.

Ussing chamber studies

After the mice were killed, segments of proximal colon were immediately harvested for the assessment of the
intestinal permeability with the method reported by Arrieta et al.\(^{30}\). In brief, the mucosa was mounted in Lucite chambers (Power Integrations) exposing mucosal and serosal surfaces to 10 ml of Ringer’s buffer (115 mm-NaCl, 8 mm-KCl, 1·25 mm-CaCl\(_2\), 1·2 mm-MgCl\(_2\), 2·0 mm-KHPO\(_4\), 25 mm-NaCO\(_3\), pH 7·35–7·37) maintained at 37°C by a heated water jacket and circulated by CO\(_2\). As much as 1 mm of mannitol with 370 KBOH (H3-mannitol) was added to the mucosal side to measure basal mannitol fluxes. The spontaneous transepithelial potential difference (mV) was determined, and the tissue was clamped at zero voltage by continuously introducing an appropriate short circuit current (\(I_{sc}\), \(\mu\)A/cm\(^2\)) with an automatic voltage clamp (DVC 1000; World Precision Instruments). Tissue ion resistance was calculated from the potential difference and \(I_{sc}\) according to Ohm’s law.

**Intestinal permeability assay**

The intestinal permeability assay was performed with fluorescein isothiocyanate (FITC)–dextran (Sigma-Aldrich, 150 \(\mu\)l), as described previously\(^{31}\). A solution containing 25 mg of 4 kDa FITC–dextran, diluted in 0·1 ml of PBS, was injected into the intestinal lumen. Thirty minutes after the injection of FITC–dextran, a blood sample was obtained via cardiac puncture to evaluate the permeability. Blood was then centrifuged at 10000 \(\times\) g for 10 min in ice-cold heparinised tubes. A fluorescence spectrophotometer (F7000; Hitachi) at excitation wavelength (495 nm) and emission wavelength (520 nm) was used to determine the concentration of FITC–dextran in the plasma with a standard curve.

Quantification of epithelial apoptosis by terminal deoxynucleotidyl transferase dUTP nick end labelling assay

Epithelial apoptosis was quantified by terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) technology with the In Situ Cell Death Detection Kit (Roche) according to the manufacturer’s instructions. Sections were permeabilised with 1% Triton X-100, 0·1% sodium citrate, washed and stained for TUNEL according to the manufacturer’s instructions. Sections were counterstained with 4,6-diamidino-2-phenylindole (DAPI).

Finally, after washing with PBS, sections were mounted in 50% glycerol and photographed using confocal microscopy (Olympus).

**Immunofluorescence**

Immunostaining was performed to determine the integrity of the TJ as described previously\(^{52}\). About 6 \(\mu\)m-thick frozen sections of proximal colon were transferred to coated slides, fixed in 1% paraformaldehyde, and washed three times with PBS. Thereafter, non-specific binding was blocked with 5% normal goat serum in PBS. After incubation with monoclonal antibodies against occludin (Abcam) and ZO-1 (Abcam) in PBS with 1% goat serum overnight at 4°C, sections were washed and incubated with Alexa 488-conjugated secondary antibodies for 60 min. Images were visualised using a confocal microscopy (Olympus).

**Western blotting**

Western blotting of TJ protein expressions was performed as described previously\(^ {53}\). The primary antibodies against occludin and ZO-1 were purchased from Abcam. Relative changes in protein expression were estimated from the pixel density using UN-SCAN-IT version 6.1 (Silk Scientific Inc.), normalised to \(\beta\)-actin and calculated as target protein expression/\(\beta\)-actin expression ratios.

**Statistical analysis**

SPSS version 19.0 software (SPSS, Inc.) was used to perform the statistical analyses. The data were expressed as means with their standard errors. Single-factor variance ANOVA analyses were used to evaluate changes in groups. Results were considered statistically significant if \(P\) values were < 0·05.

**Result**

DHA treatment ameliorated chronic colitis and body weight loss in IL-10\(^{-/-}\) mice. First, we assessed the therapeutic efficacy of DHA treatment on colitis severity. As expected, IL-10\(^{-/-}\) mice exhibited more inflammatory cell infiltrations in the colonic mucosa and much higher mean histochemical scores compared with wild-type mice. After DHA administration, the IL-10\(^{-/-}\) mice showed significant reduction in colonic inflammation and inflammatory cell infiltration and much lower mean inflammation scores (Fig. 1). In addition, partially restored glandular and goblet cell architecture was observed in the mice after DHA treatment (Fig. 1). The levels of inflammatory cytokines, such as TNF-\(\alpha\), interferon-\(\gamma\) and IL-17, were significantly suppressed in DHA-treated IL-10\(^{-/-}\) mice compared with the
expression and localisation of TJ proteins, the representative (Fig. 3). To investigate the impact of DHA treatment on the more like wild-type mice in their permeability characteristics effects were prevented in the DHA-treated mice, which were corresponding decrease in electrical resistance. However, these increased in the vehicle-treated IL-10 and increased intestinal permeability to FITC–dextran were CD. In the present study, colonic permeability to mannitol untreated mice (Fig. 2). The DHA-induced improvement in the colonic mucosa resulted in reduced intestinal inflammation. The body weight loss observed in IL-10−/− mice was also attenuated by DHA treatment.

DHA treatment ameliorated colonic and intestinal permeability, epithelial tight junction protein expression and morphology in IL-10−/− mice

Increased intestinal permeability is an important feature of CD. In the present study, colonic permeability to mannitol and increased intestinal permeability to FITC–dextran were increased in the vehicle-treated IL-10−/− mice with a corresponding decrease in electrical resistance. However, these effects were prevented in the DHA-treated mice, which were more like wild-type mice in their permeability characteristics (Fig. 3). To investigate the impact of DHA treatment on the expression and localisation of TJ proteins, the representative TJ-associated proteins occludin and ZO-1 were assessed. The result of Western blotting analysis revealed that the expression of occludin and ZO-1 in vehicle-treated IL-10−/− mice was decreased compared with that in WT mice. However, DHA treatment reversed the changes and up-regulated occludin and ZO-1 expression (Fig. 4(a)). In addition, immunofluorescence analysis showed that occludin and ZO-1 were differentially localised in IL-10−/− mice compared with that in WT mice, especially in regions with inflammatory cell infiltrations, and that TJ density was lower in IL-10−/− mice (Fig. 4(b) and (c)). In contrast, the changes in fluorescence intensity and distribution observed in the IL-10−/− mice were significantly improved by DHA treatment (Fig. 4(b) and (c)). All of these results suggest that DHA treatment promotes normal TJ protein expression and distributions.

Epithelial cell apoptosis in IL-10−/− mice after DHA treatment

To investigate the therapeutic effect of DHA, TUNEL staining was used to identify apoptotic cells in the proximal colon. Vehicle-treated IL-10−/− mice exhibited a remarkable increase in apoptosis compared with WT mice (Fig. 5). However, DHA treatment did not suppress this epithelial cell apoptosis as expected. In contrast, the DHA-treated IL-10−/− mice exhibited similar, or even slightly greater numbers of TUNEL-positive cells as the vehicle-treated IL-10−/− mice (Fig. 5). These data suggest that the therapeutic effect of DHA in IL-10−/− mice is not associated with the modulation of epithelial cell apoptosis.

Discussion

Previously published immunologic and therapeutic evidences suggest that animal models mimicking colitis are relevant to human IBD and that the pathological processes involved are similar(34). Recently, nutrition therapy has become one of the major therapeutic strategies for IBD, especially for CD(35). As immune-modulating nutrient, n-3 PUFA, namely, DHA and EPA, have been shown to exert anti-inflammatory biological actions in IBD(36). Studies of dietary nutrients and mucosal immune function have revealed that the addition of PUFA to the diets of mice can help prevent or treat

Fig. 2. Therapeutic effect of DHA on the level of net weight change and colonic pro-inflammatory cytokines by ELISA analysis in IL-10−/− mice. Values are means (n 6 per group), with their standard errors represented by vertical bars. * Mean value was significantly different from that of IL-10−/− group mice (P<0.05). IFN, interferon; WT, wild-type; KO, knockout.

Fig. 3. The colonic permeability of mice in three groups measured by Ussing chamber and intestinal permeability evaluated by fluorescein isothiocyanate (FITC)–dextran. (a) Mannitol flux; (b) electrical resistance; (c) FITC–dextran. Values are means (n 6 per group), with their standard errors represented by vertical bars. * Mean value was significantly different from that of IL-10−/− group mice (P<0.05). WT, wild-type; KO, knockout.
DHA protects against colitis in IL-10-deficient mice

Experimental colitis in animal models (37). The mechanisms through which n-3 PUFA attenuates intestinal inflammation are associated with its effects on transcription factor regulation (38); the suppression of acute phase reactants; the reduction of inflammatory cytokines (TNF-α, IL-6, C-reactive protein, etc.); and an increase in the three- and five-series eicosanoids, lipoxins, resolvins and protectins that are essentially derived from n-3 PUFA (39). We therefore investigated the therapeutic effect of DHA in a spontaneous mouse model of chronic colitis, using IL-10−/− mice that were previously reported to spontaneously develop chronic colitis characterised by both T helper 1 and T helper 17 polarised inflammation similar to that observed in CD (34, 40). The histopathological changes and reduction in inflammation score shown in Fig. 1 and the decrease in pro-inflammatory cytokine expression (IL-17, TNF-α and interferon-γ) revealed in Fig. 2 demonstrate that DHA obviously reversed the colitis in IL-10−/− mice. n-3 PUFA-rich diets have been reported to significantly ameliorate the inflammation in the terminal ileum in dextran sodium sulphate-induced chronic colitis (18). n-3 PUFA also ameliorated the inflammatory score and reduced NF-κB activation in rats with trinitro-benzene-sulfonic acid (TNBS)-induced colitis (41). The attenuation of morphological changes and the decrease in colonic concentrations of inflammatory mediators were also observed in acetic acid-induced colitis (42). The body weight loss induced by

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**Fig. 4.** The expression and distribution of occludin and zona occludens (ZO)-1 in colon tissues. (a) The expressions of occludin and ZO-1 by Western blot analysis were statistically analysed relative to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression by densitometry. Representative immunofluorescence (green) images of occludin (b) and ZO-1 (c) and nuclei (blue) of proximal colon tissues in three groups (200× magnification). DHA treatment significantly improved the expression and distribution integrity of occludin and ZO-1 in proximal colon tissues. WT, wild-type; KO, knockout; DAPI, 4',6-diamidino-2-phenylindole. (A colour version of this figure can be found online at http://www.journals.cambridge.org/bjn).

**Fig. 5.** Representative images of epithelial apoptosis visualised through the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay (200× magnification). TUNEL-positive cells were stained with green. (a) Wild-type (WT), (b) IL-10−/− and (c) DHA treatment. The epithelial apoptosis in the colon of IL-10−/− mice with DHA treatment remained at the same level with IL-10−/− mice, or even had a slight increase. (A colour version of this figure can be found online at http://www.journals.cambridge.org/bjn).
intestine inflammation in the IL-10−/− mice was also prevented by the DHA treatment.

Intestinal barrier dysfunction is a key feature in IBD, including ulcerative colitis and CD. The intestinal epithelium at the interface between the lymphoid tissue and the intestinal microbiome plays a critical role in the mucosal immune response. Increased intestinal permeability has been linked to a variety of autoimmune and inflammatory disorders, especially CD, and a reduced barrier function is a marker of impending disease re-activation. The enhanced activity of pro-inflammatory cytokines such as IL-17, TNF-α and interferon-γ that highly expressed in chronically inflamed intestine ascribed to the defect in intestinal barrier function. Defects of the intestinal barrier accelerate the onset and enhance the severity of experimental colitis when coupled with disease-inducing stimuli, such as microbes and antigens. The redistribution of TJ proteins around the shedding cell plugs the gap created by the extrusion process and maintains the intestinal barrier; however, TJ organisation has been shown to be disturbed in active CD, suggesting that preservation of the TJ barrier will be beneficial in CD. Several studies have indicated that in active CD, occludin and ZO-1 are down-regulated and delocalised from the TJ.

In the present study, colonic permeability to mannitol was significantly reduced with a corresponding increase in electrical resistance in IL-10−/− mice after DHA treatment based on the Ussing chamber assay shown in Fig. 3(a) and (b). DHA treatment also reduced the intestinal permeability to FITC–dextran in IL-10−/− mice, indicating that DHA could restore the damaged barrier function in IL-10−/− mice. These results suggest that DHA prevents barrier dysfunction and antagonises the distinct effects of inflammation on TJ proteins in intestinal epithelial cells. The expression and localisation of TJ-associated proteins (occludin and ZO-1) were assessed in the proximal colon of mice to investigate the impact of the DHA treatment on the abundance of different TJ proteins. The results of Western blotting analysis revealed a decrease in occludin and ZO-1 expression in vehicle-treated IL-10−/− mice compared with that in WT mice and DHA-treated IL-10−/− mice. The results of immunofluorescence analysis confirmed that the localisation of occludin and ZO-1 was different in IL-10−/− mice compared with that in WT mice, which was most obvious in the regions with inflammatory cell infiltrations and this phenomenon has been reported by Poritz et al. previously. Furthermore, the decreased levels of occludin and ZO-1 observed with immunofluorescence and Western blotting in the present study were significantly rescued by DHA treatment (Fig. 4). Based on the changes observed in both TJ proteins and intestinal barrier function, DHA treatment results in enhanced barrier function that is manifested by the restoration of TJ protein expressions and distributions. Studies of in vitro models have shown that DHA has specific effects on the intestinal barrier and the role of the immune environment of intestinal epithelial cells of occludin and ZO-1 localisation. The mechanisms involved have not yet been verified; however, we suppose that the modulation of gut microbiota might be important. A recent metabolomics study declared that metabolites produced by the gut microbiota closely correlate with CD, and there is a strong correlation between PUFA and the composition of gut bacteria. Dietary PUFA are also able to alter the diversity of faecal bacteria in both mice and IL-10-deficient mice. Previously reported research has noted that n-3 PUFA protect the intestinal barrier by activating the PPARγ pathway and then up-regulating TJ protein expression, indicating that one mechanism of DHA may be through the modulation of TJ proteins. Intestinal inflammation closely correlates with intestinal barrier function and the abundance of TJ; it is associated not only with increased epithelial cell death but also with lower defensin production, suppression of TJ proteins and increased bacterial mucosal invasion. In addition, the activation of the epithelial NF-κB pathway may contribute to fluid loss and diarrhoea in the inflamed intestine. Given the effect of inflammation on intestinal barrier function, we conclude that the observed improvement in epithelial integrity is due to DHA-mediated inhibition of inflammation.

In addition to the observed TJ changes, epithelial apoptosis was also a contributor to the dysfunction of the intestinal barrier. Epithelial apoptosis is significantly elevated in the colons of CD patients compared with that in normal people and the suppression of epithelial apoptosis would be beneficial in CD. However, the effect of DHA on epithelial apoptosis in the present study was not as expected. The level of epithelial apoptosis in the colons of DHA-treated IL-10−/− mice was the same, or even slightly higher, as that in vehicle-treated IL-10−/− mice, suggesting that DHA-induced effect in the IL-10−/− mice was not due to the modulation of epithelial apoptosis.

In summary, the present study provides evidence that DHA treatment can protect against experimental chronic colitis in IL-10−/− mice by improving TJ-dependent barrier function.

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J. Z., P. S. and Y. S. carried out the major part of the biochemical analysis and wrote the manuscript. W.-M. Z. designed this experiment. J. S., J.-N. D., H.-G. W., L.-G. Z. and J.-F. G. contributed to the supervision of the work and the drafting of the manuscript. Y. L., L.-L. G., N. L. and J.-S. L. contributed to the technical support, scientific advice and manuscript revision. The present study is not supported by any industry. The authors have no conflicts of interest to declare.

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