Fucoidan enhances intestinal barrier function by upregulating the expression of claudin-1

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Abstract

AIM: To evaluate the protective effects of fucoidan on oxidative stress-induced barrier disruption in human intestinal epithelial cells.

METHODS: In Caco-2 cell monolayer models, the disruption of barrier function by oxidative stress is mediated by H$_2$O$_2$. The integrity of polarized Caco-2 cell monolayers was determined by measuring the transepithelial resistance (TER) and permeability was estimated by measuring the paracellular transport of FITC-labeled 4-kDa dextran (FD4). The protective effects of fucoidan on epithelial barrier functions on polarized Caco-2 cell monolayers were evaluated by TER and FD4 flux. The expression of tight junction (TJ) proteins was assessed using reverse-transcription polymerase chain reaction (RT-PCR) and immunofluorescence staining.

RESULTS: Without H$_2$O$_2$ treatment fucoidan significantly increased the TER compared to control ($P<0.05$), indicating a direct enhancement of intestinal epithelial barrier function. Next, H$_2$O$_2$ disrupted the epithelial barrier function in a time-dependent manner. Fucoidan prevented the H$_2$O$_2$-induced destruction in a dose-dependent manner. Fucoidan significantly decreased H$_2$O$_2$-induced FD4 flux ($P<0.01$), indicating the prevention of disruption in paracellular permeability. RT-PCR showed that Caco-2 cells endogenously expressed claudin-1 and -2, and occludin and that H$_2$O$_2$ reduced the mRNA expression of these TJ proteins. Treatment with fucoidan attenuated the reduction in the expressions of claudin-1 and claudin-2 but not occludin. Immunofluorescence staining revealed that the expression of claudin-1 was intact and high on the cell surface. H$_2$O$_2$ disrupted the integrity of claudin-1. Treatment with fucoidan dramatically attenuated the expression of claudin-1.

CONCLUSION: Fucoidan enhanced intestinal epithelial barrier function by upregulating the expression of claudin-1. Thus, fucoidan may be an appropriate therapy for the treatment of inflammatory bowel diseases.

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sulfated polysaccharides, display a wide variety of pharmacological anti-inflammatory activities. This study demonstrates that fucoidan protected the epithelial barrier function from oxidative injury of the tight junction as well as barrier disruption by upregulating the expression of claudin-1. Thus, fucoidan may be an appropriate therapy for the treatment of IBD.

INTRODUCTION

Although the gastrointestinal (GI) tract is constantly exposed to bacterial microflora, an excess immune response against the bacterial microflora does not occur in the normal state, as a result of some type of immunological tolerance underlying the GI immune system. However, disruption of this immunological tolerance against intestinal microbial antigens may cause abnormal intestinal inflammation and the development of chronic inflammatory diseases, such as inflammatory bowel diseases (IBD)\(^{[2]}\). IBD can be classified into two distinct diseases, ulcerative colitis (UC) and Crohn’s disease (CD). Although the precise etiology of these diseases remains unclear, several reports have indicated that intestinal microflora is responsible for the pathogenesis of both UC and CD\(^{[4]}\). Intestinal epithelial cells (IECs) play a role as the first line of defense and act as a functional barrier. IECs separate the host’s internal milieu from the external environment. In addition to functioning as a barrier, it has become evident that IECs also play an important role in the maintenance of immune homeostasis\(^{[5]}\). IECs produce anti-microbial peptides, such as defensins, and protect the host from the attachment of luminal bacteria\(^{[6]}\). Not only do IECs function in a direct bacteriocidal role, but IEC-derived factors can also promote the differentiation of anti-inflammatory types of dendritic cells and macrophages to induce mucosal tolerance against luminal bacteria\(^{[7]}\). Furthermore, in intestinal inflammation, IECs can produce several chemokines and pro-inflammatory cytokines in response to luminal bacteria to induce the migration of granulocytes, lymphocytes, and dendritic cells, resulting in the induction of host immunity. Thus, IECs function as a defensive frontline of host mucosal immunity. Accordingly, direct epithelial cell damage, induced by mucosal irritants or cytotoxic agents, results in a marked loss of barrier function\(^{[8]}\). The epithelial barrier consists of several essential elements, including an intact epithelial monolayer and the tight junction (TJ). The TJ consists of four integral membrane proteins: occludins, claudins, tricellulin and the junctional adhesion molecule. A large body of evidence indicates that disruption of the TJ and increased paracellular permeability are critically important in the pathogenesis of IBD\(^{[9]}\). The oxidative stress-induced opening of the TJ barrier is an important mechanism contributing to the TJ barrier defect present in IBD\(^{[10]}\).

Caco-2, a human intestinal epithelial cell line, is the most well-studied cell line for investigations of in vitro intestinal epithelial barrier integrity and function\(^{[11]}\). Hydrogen peroxide (H\(_2\)O\(_2\)), a highly toxic oxidizing agent, is constantly generated within intestinal epithelial cells and must quickly be detoxified by antioxidant enzymes\(^{[12]}\). It has been established that H\(_2\)O\(_2\) is involved in oxidative stress-induced cell injury and disrupts intestinal epithelial barrier function, thus leading to enhanced paracellular permeability and the promotion of marked changes in the expression and/or localization of a number of TJ proteins, including claudins and occludins. In Caco-2 cell monolayer models, the disruption of barrier function by oxidative stress is mediated by H\(_2\)O\(_2\)\(^{[13]}\).

Fucoidan, a dietary substance, represents a class of fucose-enriched sulfated polysaccharides found in the extracellular matrix of brown algae. A growing body of experimental evidence indicates that fucoidans display a wide variety of pharmacological activities, including anti-inflammatory, anti-angiogenic, anti-coagulant, and anti-adhesive effects, in experimental models\(^{[14-16]}\). Thus, great interest has been generated in investigating the potential pharmacological effects of fucoidan on H\(_2\)O\(_2\)-induced TJ destruction in IECs.

In this study, we examined the protective effect of fucoidan on H\(_2\)O\(_2\)-induced TJ destruction in human IECs, which may provide a novel approach for the treatment of IBD.

MATERIALS AND METHODS

Materials

The brown algae Cladosiphon okamuranus Tokida was cultivated in Okinawa, Japan. Purified fucoidan derived from C. a. Tokida was provided by Uruma Bio Co. Ltd., (Okinawa, Japan). Fucoidan was dissolved in Dulbecco’s Vogt modified Eagle’s media (DMEM) (Sigma-Aldrich Co., St. Louis, MO).

Cell culture

A human intestinal epithelial cell line, Caco-2 cells (RBRC-RCB0988 RIKEN Bio Resource Center, Ibaraki, Japan), were cultured in DMEM supplemented with 10% (v/v) heat-inactivated FBS (Nichirei Biosciences Inc., Tokyo, Japan), 100 U/mL penicillin, 100 μg/mL streptomycin (Life Technologies Gibco, France), and 10 ml GlutaMAX\TM (100 ×) (Life Technologies Gibco, France). The cell cultures were incubated on collagen-coated tissue culture plates Transwell\® (Corning, New York, NY) in a humidified atmosphere of 5% CO\(_2\) at 37 °C.
Measurement of transepithelial resistance
The integrity of polarized Caco-2 cell monolayers was determined by measuring the transepithelial resistance (TER), which reflects the tightness of the TJ between epithelial cells. The TER was measured in Ω cm² using a Millicell ERS-2 Epithelial Volt-Ohm Meter (Millicore, Bedford, MA). Caco-2 cells were cultured on 24 mm Transwell® polycarbonate inserts (0.4 µm pore size) for 14 to 21 d. To examine the direct effect of fucoidan on well-polarized Caco-2 cell monolayers, confluent polarized Caco-2 cell monolayers were incubated in the presence or absence of fucoidan (2.5 mg/mL) in apical medium for 24 h. To evaluate the protective effects of fucoidan on epithelial cell injury, serial doses of fucoidan (0, 0.1, 1.0, or 2.5 mg/mL) were added to the apical medium 30 min prior to the administration of H2O2: (500 µmol/L) to the basolateral side of the Transwell®. Changes in the TER during the experimental periods were calculated as the percentage of the corresponding basal values. TER of unseeded inserts was subtracted.

Macromolecular permeability (FITC-dextran flux assay)
Permeability was estimated by measuring the paracellular transport of FITC-labeled 4-kDa dextran (FD4) (Molecular Probes, Netherland). Once the cells were grown to confluence (TER > 350 Ω cm²), sterilized FD4 was added into the apical well at 1 mg/mL. H2O2: (500 µmol/L) was administered to the basolateral side of the Transwell®. Fucoidan (2.5 mg/mL) was added to the apical medium 30 min prior to H2O2: administration. After 6 h of incubation, the basolateral medium was collected, and the fluorescence was measured using a fluorescence spectrometer at an excitation of 485 nm and emission of 535 nm. The permeability was expressed as the percentage of fluorescence of the H2O2-treated group. Flux of unseeded inserts was subtracted.

Analysis of tight junction protein mRNA expression using reverse-transcription polymerase chain reaction
Caco-2 cells were cultured for 14 to 21 d. Once grown to confluence (TER > 350 Ω cm²), H2O2: (500 µmol/L) was administered to the basolateral side of the Transwell®. Fucoidan (2.5 mg/mL) was added to the apical medium 30 min prior to H2O2: administration. After 24 h of incubation, the cells were harvested, and total RNA was isolated using the RNAeasy Mini kit (Qiagen, KJ Venlo, the Netherlands). Isolated RNA was treated with RNase-free DNase I (Qiagen) to prevent any carry-over of genomic DNA. The cDNA was synthesized from 2 µg of total RNA with Quantitect reverse transcriptase (Qiagen). Reverse-transcription polymerase chain reaction (RT-PCR) was performed using a PCR master mix (Takara Biosystems, Foster City, CA). Primers were listed 5'-3' as follows: Claudin-1: F, GGG AAT ATC CAC CTA TCA CTT CAG; R, CAT CAG CAG CAG CCA TGT ACT CTT CAC. Claudin-2: F, CTC CCT GGC CTG CAT TAT CTC; R, ACC TGC TAC CGC CAC TTC GT. Occludin: F, TCA...
Polarized Caco-2 cell monolayers were incubated in the presence or absence of fucoidan (2.5 mg/mL) for 24 h. Changes in intestinal epithelial barrier function were monitored by measuring the trans-epithelial resistance (TER). The data are expressed as the means ± SEM of 5 independent experiments. \( ^* P < 0.05 \) compared with control (Student’s t test).

We further examined the effect of fucoidan on the intestinal epithelial barrier function in a dose-dependent manner. Polarized Caco-2 cell monolayers were incubated in the presence or absence of fucoidan (2.5 mg/mL) for 24 h. Changes in intestinal epithelial barrier function were monitored by measuring the trans-epithelial resistance (TER). The data are expressed as the means ± SEM of 5 independent experiments. \( ^* P < 0.05 \), \( ^{*} P < 0.01 \) compared with cells exposed to H\(_2\)O\(_2\) alone at respective time point (Tukey’s multiple comparison test).

in a time-dependent manner. In contrast, treatment with fucoidan prevented H\(_2\)O\(_2\)-induced intestinal epithelial injury at an early time point \((P < 0.05, P < 0.01\) compared with cells exposed to H\(_2\)O\(_2\) alone at respective time point). However, low dose (0.1 mg/mL) of fucoidan did not protect the intestinal epithelium against H\(_2\)O\(_2\)-induced injury after 4 h of exposure; however, high doses (1 and 2.5 mg/mL) of fucoidan prevented the disruption of the epithelial barrier to some extent even at the late phase. Thus, fucoidan prevented H\(_2\)O\(_2\)-induced destruction of the intestinal epithelial barrier in a dose-dependent manner.

Fucoidan prevented H\(_2\)O\(_2\)-induced destruction of the intestinal epithelial barrier function in a dose-dependent manner.

Figure 1 Fucoidan directly enhanced intestinal epithelial barrier function. Polarized Caco-2 cell monolayers were incubated in the presence or absence of fucoidan (2.5 mg/mL) for 24 h. Changes in intestinal epithelial barrier function were monitored by measuring the trans-epithelial resistance (TER). The data are expressed as the means ± SEM of 5 independent experiments. \( ^* P < 0.05 \) compared with control (Student’s t test).

Figure 2 Fucoidan prevented H\(_2\)O\(_2\)-induced destruction of intestinal epithelial barrier function in a dose-dependent manner. Polarized Caco-2 cell monolayers were incubated in the presence or absence of fucoidan (2.5 mg/mL) for 24 h. Changes in intestinal epithelial barrier function were monitored by measuring the trans-epithelial resistance (TER). The data are expressed as the means ± SEM of 5 independent experiments. \( ^* P < 0.05 \), \( ^{*} P < 0.01 \) compared with cells exposed to H\(_2\)O\(_2\) alone at respective time point (Tukey’s multiple comparison test).

Figure 3 Fucoidan prevented H\(_2\)O\(_2\)-induced increases in paracellular permeability. First, 0.5 mg/mL 4-kDa FITC-labeled dextrans (FD4) were added into the apical well and cultured for 6 h with or without H\(_2\)O\(_2\) (500 \(\mu\)mol/L) and/or fucoidan (2.5 mg/mL). After 6 h of incubation, the basal medium was collected, and the fluorescence was measured as fluxed-FD4. H\(_2\)O\(_2\)-induced FD4 flux was considered 100%. The data are expressed as the means ± SEM of 5 independent experiments. \( ^{*} P < 0.01 \) (Student’s t test).

Fucoidan prevented H\(_2\)O\(_2\)-induced increases in paracellular permeability

Next, we examined whether H\(_2\)O\(_2\) increased the paracellular permeability of Caco-2 cell monolayers following epithelial injury and whether fucoidan could prevent this effect. For this experiment, an FD4 flux assay was performed. H\(_2\)O\(_2\)-markedly increased FD4 flux into the lower well (Figure 3). As expected, pretreatment with fucoidan 30 min prior to H\(_2\)O\(_2\) administration significantly suppressed the increase in FD4 flux into the lower well across the Caco-2 cell monolayers \((P < 0.01)\) (Figure 3). These results suggested that H\(_2\)O\(_2\) functionally injured the Caco-2 cell monolayers and that fucoidan prevented the disruption of intestinal epithelial barrier function.

Fucoidan promoted intestinal epithelial barrier function via direct upregulation of tight junction proteins in IECs

To determine how fucoidan treatment promotes an increase in intestinal epithelial barrier function, we examined the effect of fucoidan on the mRNA expression of major TJ-associated proteins. As shown in Figure 4, Caco-2 cells endogenously expressed claudin-1 and -2, and occludin. H\(_2\)O\(_2\) reduced the mRNA expression of these proteins. In addition, pretreatment with fucoidan attenuated the reduction in the expressions of claudin-1 and claudin-2 mRNA but not occludin mRNA. These results suggested that fucoidan treatment strongly induced the expression of claudin-1 and -2 that promote intestinal epithelial barrier function.

Fucoidan prevented H\(_2\)O\(_2\)-induced destruction of the tight junction protein claudin-1

We further examined the effect of fucoidan on the inter-
cellular localization of claudin-1 using immunofluorescence microscopy. We found that the expression of claudin-1 was intact and high on the cell surface in control cells. H$_2$O$_2$ strongly disrupted the integrity of claudin-1, resulting in lower expression. Furthermore, pretreatment with fucoidan dramatically attenuated the H$_2$O$_2$-induced injury, restoring cell integrity and promoting the expression of claudin-1 (Figure 5).

DISCUSSION

IBD is associated with an epithelial barrier defect characterized by impaired absorptive function and increased mucosal barrier defects, which are caused by impaired TJ complexity, particularly affecting claudins$^{[15-21]}$. Whereas claudin-1, -3, -4, -5 and -8 demonstrate sealing functions, claudin-2, -10b and -15 act as paracellular channels and promote the charge-selective passage of small ions$^{[20]}$. Recent studies have revealed that the expression of barrier-forming claudin-1 and -4 and occludin are downregulated in the intestinal epithelia of patients with UC$^{[25]}$, and downregulation of claudin-3, -5 and occludin have been observed in CD$^{[23]}$. However, the pore-forming protein claudin-2 is upregulated in both UC and CD, resulting in leaky TJ strands$^{[22,23]}$. Amasheh et al$^{[24]}$ recently established an experimental IBD model of native colon in vitro, which showed an impairment of epithelial barrier function via downregulation of claudin -1, -5, and -7 after exposure to tumor necrosis factor (TNF)-α and interferon gamma (IFN)-γ. Because the present study showed the impaired expression of claudin-1 and occludin by oxidative stress, our model mimicked the intestinal inflammation observed in IBD.

Fucoidans represent an intriguing group of naturally occurring polysaccharides that might have promising therapeutic applications in various clinical situations. Algal fucoidans are characterized by a wide variety of biological functions and by a highly complex and heterogeneous structure, which varies within algal species. Fucoidans from various algal species might differentially affect inflammation. Although numerous biological activities of fucoidan have attracted attention, only a few studies have examined the pharmacological activity of fucoidan in intestinal inflammation$^{[23]}$. Matsumoto et al$^{[26]}$ have shown that the oral administration of fucoidan ameliorated murine chronic colitis by downregulating the synthesis of interleukin-6 (IL-6), a key pro-inflammatory cytokine in IBD, in colonic epithelial cells. They concluded that fucoidan derived from C. o. Tokida might be useful as a dietary substance for the treatment of IBD. In addition, Zhang et al$^{[27]}$ revealed that intravenous administration of fucoidan reduced colonic mucosal damage and crypt destruction of dextran sodium sulfate-induced murine chronic colitis by reducing colonic myeloperoxidase activity and abolishing TNF-α-induced venular leukocyte rolling and extravascular recruitment. Moreover, Tanoue et al$^{[28]}$ established an in vitro model of a co-culture system using intestinal epithelial Caco-2 cells and macrophage RAW264.7 cells...
to treat intestinal inflammation by fucoidan. They clearly showed that fucoidan suppressed IL-8 gene expression in epithelial cells via reduction in TNF-α, production from macrophages stimulated with lipopolysaccharide. For gastric inflammation, fucoidan has been found to protect against aspirin-induced gastric ulceration by inhibiting IL-6, TNF-α, and IFN-γ. However, to the best of our knowledge, our study is the first to report that fucoidan protects and strengthens epithelial barrier function, both under physiological and pathological conditions via induction of the expression of claudin-1 in human IECs. The mechanisms how fucoidan regulates the TJ proteins in this study are unknown. We next plan to investigate cytokine studies and signaling pathways which may regulate the expression of claudins and occludin by the treatment of fucoidan with a consistent time course experiments.

Pro-inflammatory cytokines, such as TNF-α, IFN-γ, and IL-13, affect the expression of TJ proteins in IECs and induce epithelial cell apoptosis, resulting in the disruption of intestinal epithelial barrier function. Because IECs function as a defensive frontline of host mucosal immunity in the intestine, disruption of barrier function of IECs causes an excessive immune response to intestinal bacteria. Thus, dysfunction of IECs strongly contributes to the pathogenesis of bacteria-triggered chronic inflammation of the intestine in IBD. However, defects in TJ barrier function are insufficient to cause disease. Increased paracellular permeability can increase mucosal immune activity and enhance disease progression and severity. Thus, restoration of TJ barrier function might be effective, either alone or in combination with other agents, in preventing disease in at-risk individuals or maintaining remission in patients with IBD. Although recent advances in anti-TNF-α antibody therapy can dramatically inhibit intestinal inflammation, strengthening the intestinal epithelial barrier is still challenging and has been eagerly investigated. It is well known that zinc, a trace element, assists with the maintenance of intestinal barrier integrity. Glutamine, an essential amino acid, supports recovery from a loss in TER. Moreover, the expression of claudin-1 and occludin proteins were decreased when Caco-2 cells were deprived of glutamine through inhibition of glutamine synthetase. Furthermore, a direct influence on TJ protein expression has been observed from several plant components, including the flavonoid quercetin and the isoquinoline alkaloid berberine. Quercetin, which is obtained from fruits, enhances barrier function by upregulating claudin-4 expression, whereas berberine, a herbal agent, prevented the barrier impairment induced by TNF-α and IFN-γ. We have demonstrated that fucoidan directly induced the expression of some TJ proteins and might contribute to the enhancement of epithelial barrier functions. Thus, we believe that the activity of fucoidan, which increases the epithelial protective function and promotes epithelial regeneration, might serve as an appropriate therapy for the treatment of IBD.

Although dietary components may regulate TJ permeability by directly targeting the signal transduction pathways involved in TJ regulation, specific dietary components have been identified that influence cytokine signaling, thereby modifying TJ permeability. The intestinal barrier is a complex environment, and the regulation of barrier function cannot be elucidated using in vitro models alone. Interactions between dietary components and microbiota are also crucial in the regulation of barrier integrity. It is important to consider the interactions between different components of the intestinal barrier when establishing strategies to enhance barrier integrity using dietary compounds. The present study may provide insight for the development of novel agents with low toxicity in the treatment of intestinal inflammation. Because the healing of intestinal inflammation is a complex process involving numerous factors, further work is required to elucidate the therapeutic effect of fucoidan.

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Fucoidan enhances intestinal barrier function

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