Fucoidan derived from *Cladosiphon okamuranus Tokida* ameliorates murine chronic colitis through the down-regulation of interleukin-6 production on colonic epithelial cells

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SUMMARY

Our previous study indicated that the interleukin (IL)-6/STAT-3 signal was up-regulated in inflammatory bowel disease (IBD) in both humans and animal models. We also discovered phosphorylated STAT-3 in the nucleus of the colonic epithelial cells in IBD mice. Intestinal epithelial cells (IEC) have been shown to secrete IL-6. Therefore, the secretion of IL-6 from IEC may be one of the mechanisms of STAT-3 phosphorylation in IEC during the pathogenesis of IBD, and inhibition of IL-6 production by IEC may be beneficial in preventing IBD. We examined the preventative effect of various types of fucoidans on IL-6 production in a lipopolysaccahride (LPS)-stimulated murine colonic epithelial cells line, CMT-93, *in vitro*. We also determined *in vivo* the effect of fucoidans on murine chronic colitis induced with dextran sodium sulphate. Among fucoidans, those from *Cladosiphon okamuranus Tokida* and *Kjellmaniella crassifolia* inhibited IL-6 production in CMT-93 cells with the down-regulation of NF-κB nuclear translocation. Analysis of the effect of fucoidan on murine colitis *in vivo* showed that the disease activity index and myeloperoxidase activity decreased in mice fed *Cladosiphon* fucoidan, but not *Fucus* fucoidan. Cytokine profiles in colonic lamina propria indicated that the synthesis of interferon (IFN)-γ and IL-6 decreased and that of IL-10 and transforming growth factor (TGF)-β increased in mice fed *Cladosiphon* fucoidan, compared with mice fed a standard diet or *Fucus* fucoidan. The levels of IL-6 mRNA in colonic epithelial cells was lower in colitis-induced Balb/c mice fed *Cladosiphon* fucoidan than those fed a standard diet. Fucoidan improves murine chronic colitis by down-regulating the synthesis of IL-6 in the colonic epithelial cells. Fucoidan derived from *C. o. Tokida* may be useful as a dietary substance for the patients with inflammatory bowel disease.

Keywords experimental colitis fucoidan IL-6 inflammatory bowel disease intestinal epithelial cell ulcerative colitis

INTRODUCTION

Inflammatory bowel disease (IBD) is a severe intestinal inflammation, the pathogenesis of which is not well understood. It is suspected that the disease is due to complex mucosal immune responses to resident enteric bacteria, because intestinal inflammation was absent from various IBD models reared under germ-free conditions [1–7]. Interleukin (IL)-6 is one of the major cytokines secreted by lamina propria cells in the patients with IBD [8–10]. Strong expression of IL-6 has also been reported in murine acute bowel inflammation [11]. Recent studies using anti-soluble-IL-6 receptor antibodies demonstrated that IL-6 plays a critical role in the development chronic colitis [12,13]. IL-6 has also been shown to play a central role in arthritis [14]. We and other investigators have confirmed that IL-6 gene-disrupted mice are resistant to arthritis and dextran sodium sulphate (DSS)-induced colitis [15,16]. IL-6 is a cytokine which activates the transcription factor signal transducer and activator of transcription (STAT)-3 via intracellular signalling pathways [17,18]. Recently, we investigated the expression of phosphorylated STAT-3 in the intestinal mucosa of patients with IBD and in murine models [16]. Notably, we observed phosphorylated STAT-3 molecules in the nucleus of colonic epithelial cells. Intestinal epithelial cells (IEC) have been shown to produce several cytokines including IL-6 [19]. Therefore, the autocrine secretion of IL-6 by IEC may be one of the mechanisms of spontaneous STAT-3 phosphorylation in IEC

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during the pathogenesis of colitis. It is important to identify the stimuli inducing IL-6 production in the pathogenesis of IBD. Bacterial cell components such as lipopolysaccharide (LPS) are known to induce the expression of IL-6 [20–22]. It is recognized that the total number of bacteroides among intestinal flora increases in IBD patients [23]. Rath *et al*. suggested that overgrowth of bacteroides species induced severe intestinal inflammation in HLA-B27 transgenic rats [24]. Furthermore, Lange *et al*. described that colitis was less severe in mice with a disrupted toll-like receptor 4 (TLR-4) gene than in control mice [25]. Therefore, LPS signals are essential for the development of inflammatory bowel disease. Intestinal epithelial cells (IEC) are the first line of defence against the intestinal luminal environment. One possibility is that bacterial LPS directly interacts at the apical surface and induces responses in IEC, which in turn produce cytokines and other mediators of inflammation. Evidence is mounting that IEC serve a vital function for the mucosal immune system. Cario *et al*. described that intestinal cell lines and intestinal epithelial cells *in vivo* express the TLR family [26,27]. These experimental results led to the hypothesis that LPS-signals delivered through intestinal epithelial cells trigger the production of IL-6 by IEC and initiate colitogenesis. Furthermore, removal of these signals from IEC may contribute to improvement of intestinal inflammation.

Fucoidan is a complex sulphated polysaccharide, derived from marine brown seaweed. There have been many reports on the biological effects of fucoidan on mammalian cells [28,29]. Shibata and colleagues indicated that fucoidan derived from *Cladosiphon okamuranus Tokida* blocked the adhesion of *Helicobacter pylori* to a human gastric cell line [30]. Therefore, fucoidan may be useful as a dietary substance for preventing human disease because its polysaccharide causes no toxicity or irritation. In this study, we examined the effect of fucoidans derived from various brown seaweeds on the production of IL-6 in a LPS-stimulated murine colonic epithelial cell line. Moreover, we determined the improved effect of fucoidan on murine chronic colitis *in vivo*.

MATERIALS AND METHODS

Animals

Female Balb/c mice (8 weeks old) were purchased from Japan Clea Laboratory (Tokyo, Japan). They were maintained under specific pathogen-free (SPF) conditions during the experiments.

Preparation of fucoidans

The brown seaweed *C. o. Tokida* was cultivated in Okinawa, Japan. This seaweed was purchased from the Tropical Technology Centre Co., Ltd (Okinawa, Japan) as salted food. Other brown seaweeds (*H. elongata, S. horneri* and *L. digitata*) were kindly provided by SCETI Co., Ltd (Tokyo, Japan) as dried materials. Fucoidan from *Fucus vesiculosus* was purchased from Sigma (St Louis, MO, USA). Other fucoidans were prepared from brown seaweeds as described in a previous paper [31]. Standard mouse chow (type MF) and fucoidan (*C. o. Tokida* or *F. vesiculosus*)-containing MF chows (0·05% w/w) were tableted and provided by Oriental Yeast Co., Ltd (Tokyo, Japan).

Cell culture

The murine colon carcinoma cell line CMT-93 was purchased from the American Type Culture Collection (ATCC). Cells were cultured in 10% fetal calf serum (FCS)/10 mM Hepes/penicillin–streptomycin/non-essential amino acid/DMEM medium under 5% CO_2 at 37°C. LPS derived from *Escherichia coli* was purchased from Sigma. Before the experiment, various concentrations of LPS were examined for their influence on IL-6 synthesis in the CMT-93 cell line. To assess the effect of the fucoidans on the production of IL-6 synthesis in LPS-stimulated CMT-93 cells, 10 μg/ml of LPS was added to cultured CMT-93 with or without 1 μg/ml of the various fucoidans and the cells were cultured for 72 h. After the culture, the supernatants were collected and stored at −84°C until IL-6 ELISA assay. Toxic effects of fucoidans were detrmined by MTT assay and ^{51}Cr-releasing assay and we could find no ant toxic effect of fucoidans against CMT-93 (data not shown).

ELISA

Murine anti-IL-6 MoAbs (clone: MP5–20F3, MP5–32C11) were purchased from BD PharMingen (LA, CA). ELISA was performed according to the standard recommended by the manufactures.

Nuclear protein extraction and the determination of NF-κB amounts

Nuclear proteins were extracted from LPS-stimulated CMT-93 cells that were treated with or without fucoidan derived from *C. o. Tokida*. The amounts of nuclear-localized NF-κB were determined by using the NF-κB transfactor ELISA kit (CLONTECH Laboratories, Inc.).

Induction of chronic colitis

Chronic colitis was induced in Balb/c mice fed *C. o. Tokida* or *F. vesiculosus*-containing MF chows, or conrol MF chow ($n = 10$, each group) as described by Okayasu *et al*. [32]. Briefly, mice at 10 weeks of age were treated with 4% DSS (molecular mass, 40 kDa; ICN Biomedicals, Aurora, OH, USA) dissolved in drinking water. The colitis was induced by four administration cycles; each cycle was an alternating regimen of 4% DSS for 7 days followed by drinking water without DSS for the next 7 days.

Assessment of disease severity

A disease activity index confirmed to reflect changes in the clinical status of mice with DSS-induced colitis was calculated by scoring from 0 to 4 abnormalities regarding change in body weight, stool consistency and intestinal bleeding and summing the results [33]. In addition, the length of the colon from the caeco–colonic junction to the anal verge was measured as an established inflammatory parameter in DSS-induced colitis.

Lymphocyte preparation and flow cytometry

Lamina propria lymphocytes (LPLs) were prepared from the large intestine of mice, as described previously [34]. Briefly, a longitudinally opened large intestine was cut into 1-cm pieces. The intestinal segments were incubated with Hanks's balanced salt solution (HBSS) containing DTT (0·45 mM) and EDTA (2 mM) twice for 15 min each at 37°C with agitation. Then, after removal of the epithelial layer by decantation, the resultant intestinal segments were incubated with RPMI-1640 containing 2·5% FCS, collagenase (300 μg/ml, Collagenase-Yakult S, Yakult Honsha Co., Ltd) and DNase I (50 μg/ml, Sigma) three times for 45 min each at 37°C with gentle agitation in a CO_2 incubator. Cell pellets were suspended in ice-cold 2·5% FCS/10 mM Hepes/RPMI-1640 and then passed through a nylon column; the lymphocyte population was then isolated from the 44%/100% interface of a Percoll density gradient (Pharmacia, Uppsala, Sweden). Cells were stained

with MoAbs against TCR-β, CD4, CD45RB, CD69 or B220. All the MoAbs were purchased from BD PharMingen. The stained cells were analysed with an EPICS EL cell analyser (Beckman Coulter, Inc., Fullerton, CA, USA).

Cell culture and cytokine assay

LPLs ($1\cdot0 \times 10^6$ cells) were cultured in 24-well tissue culture plates in 10% FCS/10 mM Hepes/2-ME/RPMI-1640 under stimulation with or without immobilized anti-TCR-β MoAb (H57–597, 10 μg/ml); anti-CD28 MoAb (37·51, 1 μg/ml) was added subsequently. After 48 h of culture, supernatants were collected and stored at –84°C until the assays. Cytokine-specific ELISA was performed using the following antibody combinations: anti-interferon (IFN)-γ (clone: XMG1·2, R4–6A2), anti-IL-4 (clone: 11B11, BVD6–24G2); all from BD PharMingen. For the assay of IL-10 and TGF-β1, assay kits were purchased from BioSource International Inc. (Camarillo, CA, USA) and Genzyme Corp. (Cambridge, MA, USA), respectively.

Reverse transcription–polymerase chain reaction (RT-PCR) analysis for IL-6 mRNA on colonic epithelial cells

Colonic epithelial cells were isolated from colitis-uninduced Balb/c mice or from colitis-induced Balb/c mice fed a normal diet, or fucoidan derived from *C. o. Tokida*, and total RNA was prepared from the three groups of mice as described previously [35]. The total RNA (1·0 μg) was reverse-transcribed and then PCR was performed using specific primers for G3PDH, IL-6, tumour necrosis factor (TNF)-α or TLR-4 [36]. After gel electrophoresis, PCR products were visualized with ethidium bromide.

Myeloperoxidase assay

Tissue MPO activity was measured as previously described [37,38]. In brief, the colonic tissue was homogenized with a Polytoron homogenizer in hexadecyltrimethylammonium bromide (Sigma) buffer. The suspension was sonicated on ice and then centrifuged at 15·000 r.p.m. for 30 min. The supernatant was mixed with an enzyme substrate buffer containing 0·167 mg of O-dianisidine hydrochloride (Sigma) per ml and 0·0005% hydrogen peroxide. The changes in the absorbance at 405 nm were measured.

Measurement of immunoglobulin contents in colonic segments

A colonic homogenate sample was prepared as described above. Total IgG-, IgG1- or IgG2a-specific sandwich ELISA assays were performed as described elsewhere [38].

Statistics

All data were expressed as the mean ± s.e. and evaluated by Tukey or Tukey–Kramer test for multiple comparisons. *P*-values of less than 0·05 were considered to be statistically significant.

RESULTS

IL-6 production by CMT-93 cells after stimulation with LPS

CMT-93 cells were cultured in the presence or absence of various concentrations of LPS. Maximum IL-6 production by CMT-93 was observed at 5–10 μg/ml LPS (Fig. 1).

Effect of fucoidans in IL-6 production on CMT-93 stimulated with LPS

To clarify whether fucoidans modulate the production of IL-6 in LPS-stimulated CMT-93 cells, various fucoidans isolated from brown seaweed were tested. Fucoidans derived from *C. o. Tokida* and *K. crassifolia* repressed IL-6 synthesis in LPS-stimulated CMT-93 (Fig. 2a). However, the fucoidans from *F. vesiculosus, H. elongata, S. horneri* and *Lamanaria digitata* did not. The inhibitory effect on IL-6 release in LPS-stimulated CMT 93 cells by the fucoidan derived from *C. o. Tokida* or from *K. crassifolia* was dose-dependent (Fig. 2b). We did not detect any up-regulation in the release of IL-6 from CMT-93 cells cultured in the presence of single fucoidans (data not shown).

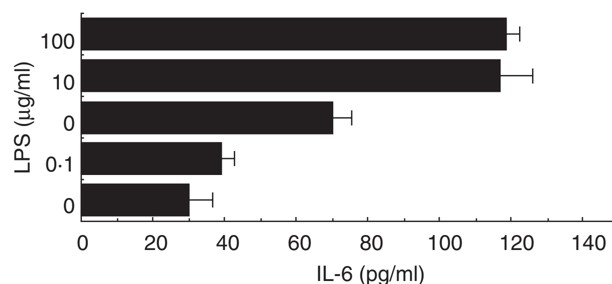

Fig. 1. Effects of various doses of LPS on IL-6 synthesis in the murine colonic epithelial cell line CMT-93. Cells were cultured in the absence or presence of various doses of LPS. After 72 h, the amount of IL-6 in the culture supernatant was determined by sandwich ELISA. Similar results were obtained from five independent experiments. Data are mean ±s.d.

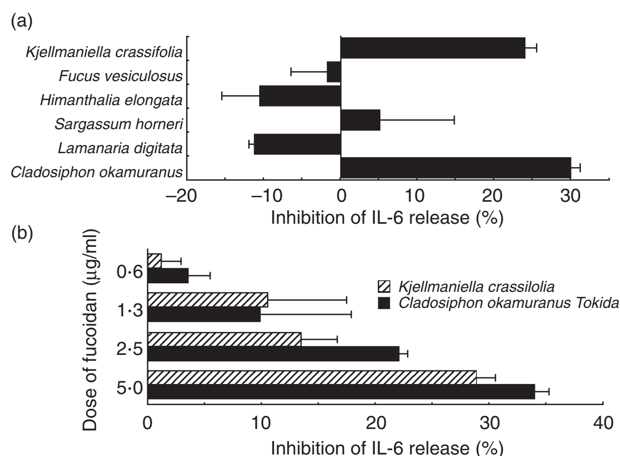

Fig. 2. Effect of various fucoidans on IL-6 synthesis in LPS-stimulated CMT-93 cells (a). Various fucoidans were isolated from marine seaweed as described in Materials and methods. Cells were cultured in the presence of both purified fucoidans (2·5 μg/ml) and LPS (10 μg/ml) for 72 h. Dose-dependent inhibition of IL-6 release by fucoidans in an LPS-stimulated colonic epithelial cell line CMT-93 (b). CMT-93 was cultured with various doses of the fucoidan derived from *C. o. Tokida* or from *K. crassifolia* in the presence of LPS for 72 h. The amount of IL-6 in the culture supernatant was measured by an IL-6-specific sandwich ELISA. Three independent experiments gave similar results. Data are mean ±s.d.

The effect of fucoidan on NF-κB recruitments in LPS-stimulated CMT-93 cells

The amounts of nuclear-localized p65 NF-κB in CMT-93 cells that were treated with or without fucoidan derived from *C. o. Tokida* were determined. The amounts of nuclear-localized p65 NF-κB in LPS-stimulated CMT-93 cells were down-regulated by the treatment of the *Cladpsiphon* fucoidan (Fig. 3).

In vivo *effect of fucoidans on murine chronic colitis*

To examine the effect of fucoidans on murine chronic colitis *in vivo*, colitis was induced in Balb/c mice fed standard mouse chow (MF), MF chow containing fucoidan from *C. o. Tokida* (0·05% w/w) or MF containing fucoidan from *F. vesiculosus* (0·05% w/w) by administering the animals DSS in drinking water and the effect was analysed using various disease parameters. The scores of all the parameters in the disease activity index such as loss of body weight, diarrhoea and occult blood were reduced in Balb/c mice fed *Cladosiphon* fucoidan compared with those fed the standard diet or *Fucus* fucoidan (Fig. 4a). The scores are comparable between mice fed *Fucus* fucoidan and those fed standard diet. Moreover, the length of the colon was longer in colitic mice fed *Cladosiphon* fucoidan than those fed standard diet (Fig. 4b). However, the length of the colon shortened due to severe inflammation in the mice fed the standard diet or *Fucus* fucoidan. To confirm these results, the MPO activity in colonic tissue was compared among the three groups. The activity was lower in colitic Balb/c mice fed *Cladosiphon* fucoidan than those fed standard diet or *Fucus* fucoidan (Fig. 5).

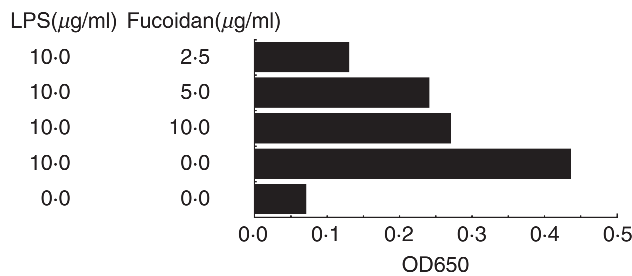

Fig. 3. The effects of fucoidan in NF-κB activities in LPS-stimulated CMT-93 cells. The amounts of nuclear-localized p65 NF-κB in LPS-stimulated CMT-93 were determined by transfactor ELISA system. The amounts of nuclear-localized NF-κB in LPS-stimulated CMT-93 cells were down-regulated by treatment of *Cladosiphon* fucoidan. Two independent experiments gave similar results. Data are mean of duplicate assays.

Immunological characterization

At the beginning of this experiment, we compared LI–LPL phenotypes between control Balb/c mice and mice with colitis induced by DSS. To summarize the results, the total number of B220-positive B cells increased markedly after induction of colitis (data not shown). The increase in the number of TCR-$\alpha\beta$ T cells was small. Among $CD4^+$ TCR-$\alpha\beta$ T cells, the up-regulation of CD69 molecules was pronounced (data not shown). Moreover, cell with the $CD45RB^{high}$ $CD4^+$ TCR-$\alpha\beta$ T cell phenotype decreased in number after the induction of colitis. Based on these results, we compared the phenotypes of LI–LPLs among three groups of colitis-induced Balb/c mice fed standard diet, *Cladosiphon* fucoidan or *Fucus* fucoidan. Total numbers of B220-positive B cells were lower in the mice fed *Cladosiphon* fucoidan than those fed standard diets or *Fucus* fucoidan (Table 1). The numbers of TCR-$\alpha\beta$ T cells were comparable among the three groups. The expression of CD69 and CD45RB antigen was also comparable among the groups (data not shown). Cytokine proflies in TCR-β/CD28-stimulated LI–LPLs indicated that the production of proinflammatory cytokines such as IFN-γ and IL-6 was down-regulated in colitis-induced mice fed *Cladosiphon* fucoidan compared to those fed standard diet or *Fucus* fucoidan (Fig. 6). In contrast, the production of TGF-β was greater in mice fed *Cladosiphon* fucoidan than standard diet or *Fucus* fucoidan. Moreover, more IL-10 was produced in mice fed *Cladosiphon* fucoidan than standard diet. The analysis of IgG contents in the colonic mucosa in three experimental groups showed that the amounts of total IgG,

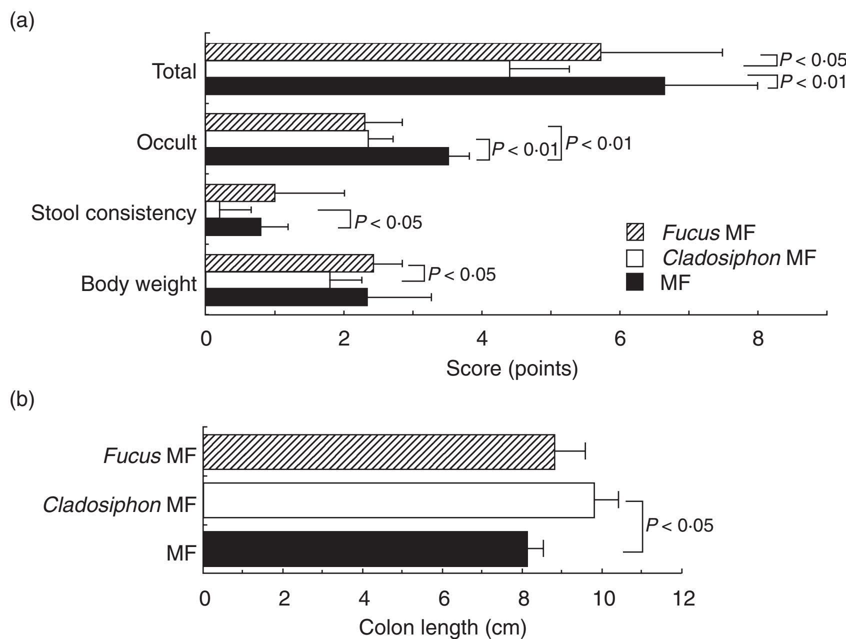

Fig. 4. Disease activity index (a) and colon length (b) in colitis-induced Balb/c mice fed normal diet or fed diets containing fucoidans ($n = 10$, each group). Disease activity index and colon length were determined in colitis-induced Balb/c mice fed normal diet, or fed *Cladosiphon* fucoidan or *Fucus* fucoidan as described in Materials and methods. Similar results were obtained from three independent experiments. Data are mean ± s.d.

Table 1. The cell number of large intestinal lamina propria cells in colitis induced Balb/c mice fed standard diet or fucoidan-containing diets

Diets	No. of TCR-β cells	No. of B220 cells
Control MF	$(5{\cdot}70 \pm 0{\cdot}64) \times 10^6$	$(9{\cdot}77 \pm 1{\cdot}91) \times 10^6$
Chadosiphon MF	$(5{\cdot}01 \pm 0{\cdot}93) \times 10^6$	$(5{\cdot}38 \pm 1{\cdot}23) \times 10^{6a,b}$
Fucus MF	$(4{\cdot}33 \pm 1{\cdot}52) \times 10^6$	$(8{\cdot}53 \pm 1{\cdot}29) \times 10^6$

[a]Significantly different between control MF and *Cladosiphon* MF-treated mice ($P < 0{\cdot}01$). [b]Significantly different between *Cladosiphon* MF and *Fucus* MF treated mice ($P < 0{\cdot}05$).

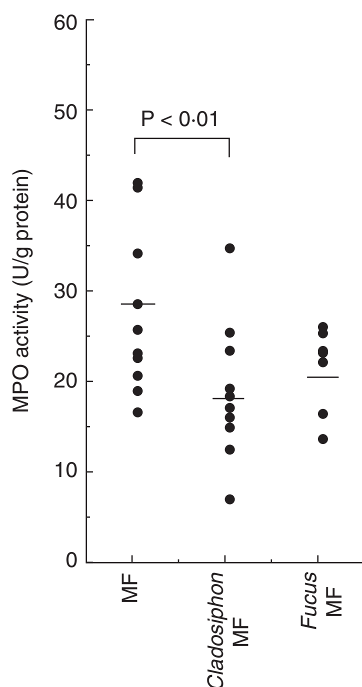

Fig. 5. MPO activity of the colonic tissue in colitis-induced Balb/c mice fed normal diet or fed fucoidan-containing diets ($n = 10$, each group). Colonic tissues were homogenated in HTMB buffer with a polytron-homogenizer. After centrifugation, MPO activity in the supernatants was measured as described in Materials and methods. Down-regulation of MPO activity in the colonic tissue in colitis-induced Balb/c mice fed *Cladosiphon* fucoidan observed in separate experiments is plotted as dots. Bars represent mean values.

IgG1 and IgG2a increased in colitis-induced Balb/c mice fed standard diet and *Fucus* fucoidan, but not those fed *Cladosiphon* fucoidan (Fig. 7).

IL-6 mRNA analysis of colonic epithelial cells

The levels of IL-6 mRNA expression in the colonic epithelial cells that were isolated from colitis-uninduced Balb/c mice, or colitis-induced Balb/c mice fed either a normal diet or *Cladosiphon* fucoidan were determined by RT-PCR. The mRNA of IL-6 increased markedly in colonic epithelial cells of the mice fed standard diet on induction of colitis (Fig. 8). However, the expression of IL-6 mRNA was down-regulated in colonic epithelial cells of Balb/c mice fed fucoidan derived from *C. o. Tokida*. The mRNA expression of TNF-α and TLR-4 were also down-regulated in colonic epithelial cells of mice fed fucoidan derived from *C. o. Tokida* (Fig. 8).

DISCUSSION

In the present study, we examined the inhibitory effects of fucoidan derived from *Cladosiphon okamuranus Tokida,* a traditional food in Japan, on the production of IL-6 in an LPS-stimulated murine colonic epithelial cell line and also examined the effect of these fucoidans on murine chronic experimental colitis *in vivo*. Moreover, we compared the effects with those of other fucoidans both *in vitro* and *in vivo*.

IL-6 is a 21–28-kDa glycoprotein that is secreted by monocytes, macrophages, lymphocytes and epithelial cells, including intestinal epithelial cells [19,39]. IL-6 is important for host defence and excessive production of IL-6 is thought to contribute to inflammatory disorders, including rheumatoid arthritis and inflammatory bowel disease [15,16]. The IL-6 receptor is present in a variety of cells such as monocytes, macrophages and epithelial cells, including intestinal epithelial cells [40]. It is known that high levels of IL-6 in serum are detected in patients with inflammatory bowel disease [40]. IL-6 has been shown to play important roles on the pathogenesis of murine Th-1-mediated colitis [12,13]. Intestinal epithelial cells and lamina propria lymphocytes are a major source of IL-6 in inflammatory bowel disease [16,19]. Sitaraman *et al.* showed that IL-6, released by the cultured intestinal epithelial cell line T84, induced an intracellular (Ca^{++}) flux and degranulation in neutrophils [41]. These results suggested that secretion of IL-6 by intestinal epithelial cells plays a critical role in the pathogenesis of IBD and the prevention of this secretion may be useful in the treatment of inflammatory bowel disease. To test this possibility, we screened for an inhibitory effect of fucoidans isolated from various brown seaweeds on IL-6 secretion in LPS-stimulated CMT-93 and discovered that the fucoidans derived from *C. o. Tokida* and *K. crassifolia* had such an effect. However, fucoidans from other brown seaweeds such as *S. horneri, F. vesiculosus* and *L. digitata* had no effect. Moreover, we discovered the down-regulation of NF-κB signalling in LPS-stimulated CMT-93 cells by treatment of fucoidans derived from *C. o. Tokida*. We also discovered that the fucoidan derived from *C. o. Tokida* prevented the expression of IL-6 mRNA in colonic epithelial cells of colitis-induced Balb/c mice *in vivo*. Next, we examined *in vivo* the effect of the fucoidan-derived form *C. o. Tokida* on murine chronic colitis and compared it with that of the other fucoidans. Administration of *Cladosiphon* fucoidan to Balb/c mice with chronic colitis induced by DSS improved both the disease parameters such as disease activity index and the MPO activity in the colonic tissue that compared with these parameters in the mice fed standard diet or *Fucus* fucoidan. Moreover, the colon of the colitis-induced mice fed *Cladsiphon* fucoidan was the same length as that of normal mice. However, the colon length of mice fed standard diet or *Fucus* fucoidan was reduced due to severe intestinal inflammation. Analysis of the immunological parameters also showed that the production of proinflammatory cytokines in LI–LPLs decreased and that of T regulatory (Tr) and T helper type 3 (Th-3) cytokines, such as IL-10 and TGF-β increased in colitic mice fed *Cladosiphon* fucoidan compared with mice fed standard diet or *Fucus* fucoidan. These results are important. It is well known that proinflammatory cytokines such as IFN-γ and IL-6 are causative factors in the development of DSS-induced colitis [11]. On

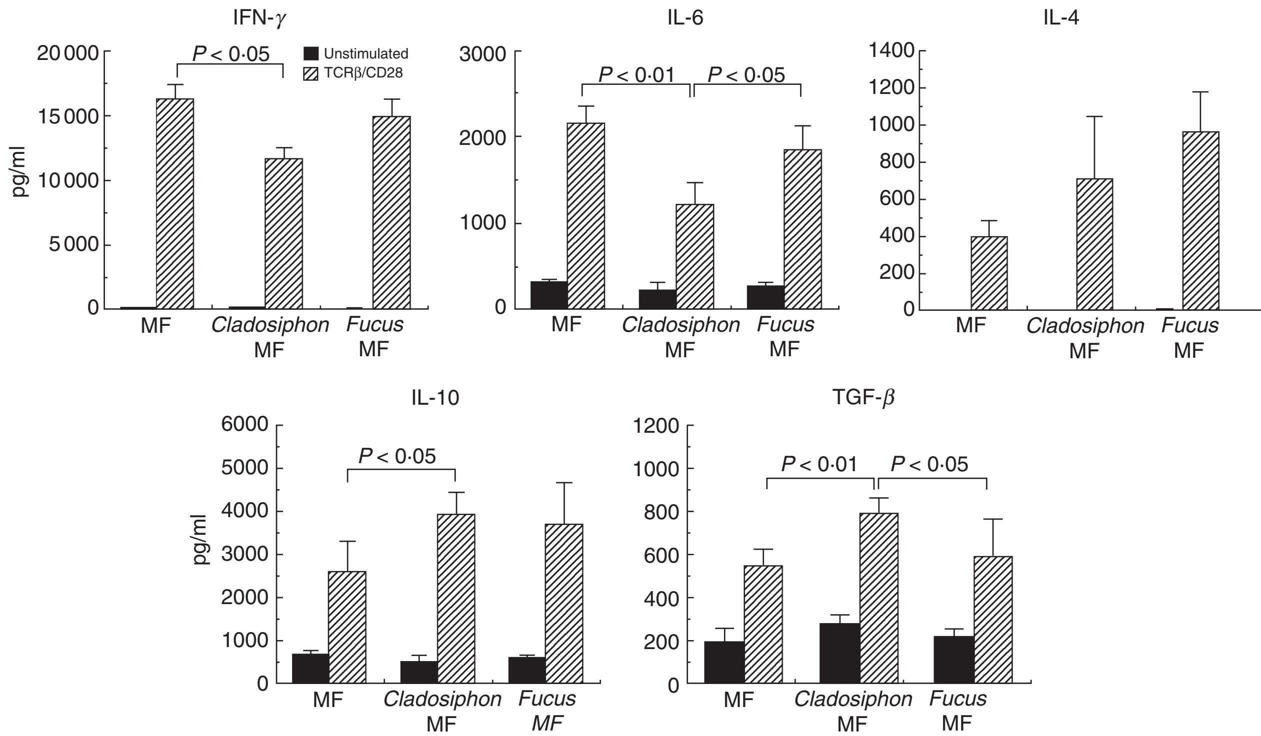

Fig. 6. Cytokine profiles in the culture supernatants of TCR-β/CD28-stimulated LI–LPLs in colitis-induced Balb/c mice fed standard diet or fucoidan-containing diets ($n = 10$, each group). LI–LPLs were stimulated with or without MoAbs TCR-β/CD28. After 48 h culture, amounts of each cytokine were analysed by sandwich ELISA. Similar results were obtained from two independent experiments. Data are mean ± s.d.

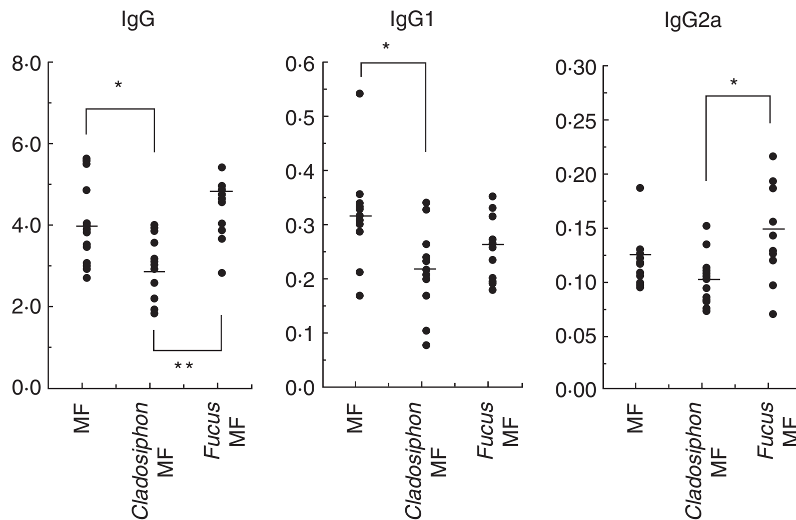

Fig. 7. Immunoglobulin contents of colonic tissue in colitis-induced Balb/c mice fed normal diet, *Cladosiphon* fucoidan or *Fucus* fucoidan ($n = 10$, each group). The IgG, IgG1 and IgG2a contents in the colonic tissue were examined by sandwich ELISA using colonic tissue homogenates isolated from the three groups of mice. The total IgG contents of the mice fed *Cladosiphon* fucoidan was lower than that of the others and the differences were significant. The IgG2a content was significantly lower in the mice fed *Cladosiphon* fucoidan than *Fucus* fucoidan. Each plot shows individual data and bars represent mean values.

the other hand, Tr and Th-3 cytokines improved intestinal inflammation in various models of murine experimental colitis [42–44]. The mechanisms underlying the induction of Tr and Th-3 cytokines in colitis-induced mice fed *Cladosiphon* fucoidan should be investigated further.

The mechanisms of the inhibitory effect of *Cladosiphon* fucoidan on the secretion of IL-6 by LPS-stimulated CMT-93 cells are unknown. It is clear that LPS signals are important for the development of murine colitis [25]. Cario *et al.* reported the overexpression of TLR-4 in the colonic epithelial cells in a patient with

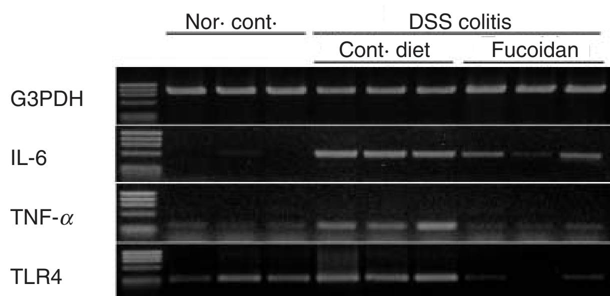

Fig. 8. RT-PCR analysis of cytokines or TLR-4 mRNA in colonic epithelial cells from colitis-uninduced Balb/c mice, or from colitis-induced Balb/c mice fed normal diet or *Cladosiphon* fucoidan. Total RNA was obtained from the colonic epithelial cell fraction of three experimental groups of mice. RT-PCR was performed with each specific primer for G3PDH, IL-6, TNF-α and TLR-4. After electrophoresis, the gel was stained with ethidium bromide and PCR products were visualized by trans-illuminator.

inflammatory bowel disease [27]. In the present study, we observed the up-regulation of TLR-4 mRNA in colonic epithelial cells isolated from colitis-induced Balb/c mice and also the down-regulation in those fed *Cladosiphon* fucoidan. The mechanisms underlying the down-regulation of TLR-4 mRNA in the colonic epithelial cells of mice fed *Cladosiphon* fucoidan were unclear. It was reported that low-dose exposure to LPS induced LPS tolerance in cultured macrophages through a reduction in both the cell surface expression of TLR-4/MD2 complex and TLR-4 mRNA [45]. It is possible that fucoidan interacts with TLR-4 and then induces LPS tolerance. If so, there may be some molecular mimicry between LPS and fucoidan. Further analysis may clarify this point.

Another report suggested that intravenous injection of fucoidan derived from *F. vesiculosus* into mice with DSS-induced colitis prevented colito-genesis through the inhibition of selectin function and lymphocyte rolling [46]. However, we did not detect a clear positive effect of *F. vesiculosus* fucoidan on chronic colitis. Shibata *et al*. also reported that anti-ulcer effects of *Cladosiphon* fucoidan were higher than those of *F. vesiculosus* fucoidan [30]. The difference in effect on colito-genesis between *Cladosiphon* fucoidan and *F. vesiculosus* fucoidan may be due to structural differences. Nagaoka *et al*. has reported that the basic structure of the backbone chains of these fucoidans is the same [31]. However, there were differences in sulphate content and branched sugars. *Cladosiphon* fucoidan has a sulphate group for every 2 mol of fucose, and *F. vesiculosus* fucoidan 2–3 mol of fucose. Moreover, *Cladosiphon* fucoidan has one glucuronic acid residue for every 6 mol of fucose as branched chains, and *F. vesiculosus* fucoidan has fucose branches [31]. These structural differences may influence their anticolitogeneic activity. This possibility is currently being investigated.

Other possibilities for the improved effect of *Cladosiphon* fucoidan should be discussed. *C. o. Tokida* blocked the adhesion of *H. pylori* to a human gastric cell line [30]. It is well known that the numbers of mucosal adherent bacteria was increased in the intestinal mucosa IBD [47]. Fucoidan may inhibit the adhesion of commensal bacteria to the colonic epithelial cells. Moreover, the adhesion of fucoidan to epithelial cells may modulate epithelial barrier functions. Further analysis may clarify this point.

In conclusion, we discovered that fucoidan derived from *C. o. Tokida* inhibited the synthesis of IL-6 in both an LPS-stimulated colonic epithelial cell line *in vitro* and colonic epithelial cells in mice with DSS-induced colitis *in vivo*. Moreover, we observed a positive effect of *Cladosiphon* fucoidan on murine chronic colitis. Fucoidan derived from *C. o. Takida* may be useful as a dietary substance for preventing inflammatory bowel disease in humans.

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