The Differential Effect of High and Low Molecular Weight Fucoidans on the Severity of Collagen-induced Arthritis in Mice

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INTRODUCTION

Rheumatoid arthritis (RA) is an autoimmune disease, resulting in the destruction of cartilage and bone through synovial inflammation that involves lining layer thickening and the infiltration of inflammatory cells into the sublining areas (Vervoordeldonk and Tâk, 2002). The number of macrophages, resident cells covering the synovial layer in normal joints, greatly increases in RA synovium (Kinne et al., 2000), and the degree of increase is strongly correlated with the development of severe cartilage destruction (Mulherin et al., 1996). Consequently, the selective depletion of macrophages from the synovial lining prior to the induction of experimental arthritis prevented both joint inflammation and cartilage destruction (Van Lent et al., 1998).

Fucoidans are high-molecular-weight sulfated, fucose-rich polysaccharides, which are extracted from brown macroalgae (Berteau and Mulloy, 2003) or echinoderms (Mulloy et al., 1994). The effects of fucoidan treatments on the immune system and inflammation have been under intensive investigation. Several in vitro studies reported that fucoidan activated immune cells. In macrophages, fucoidan treatment resulted in the production of tumor necrosis factor (TNF)-α and interleukin (IL)-18 (Hsu et al., 2001). The stimulation of dendritic cells with fucoidan resulted in the secretion of inflammatory cytokines and the up-regulation of co-stimulatory molecules (Jin et al., 2009; Kim and Joo, 2008). Fucoidan enhanced the cytotoxic activity of NK cells and the mitogenic activity of splenic lymphocytes (Choi et al., 2005; Oomizu et al., 2006). Fucoidan also regulates the production of nitric oxide (NO) from macrophages by modulating the transcription and expression of nitric oxide synthase. The promotion of NO production by fucoidan was shown to be mediated by macrophage scavenger receptors which, upon stimulation, induce the activation of p38 MAPK and NF-κB (Nakamura et al., 2006; Yang et al., 2006). In contrast, other studies demonstrated the anti-inflammatory activities of fucoidan. In animal models for inflammatory conditions, fucoidan inhibited leukocyte recruitment and related inflammatory processes (Cumashi et al., 2007). Fucoidan inhibited the P-selectin-mediated interaction of immune cells (Cumashi et al., 2007), induced the expression of an anti-inflammatory cytokine IL-10 (Saito et al., 2006), and had antithrombus activity (Thorlacius et al., 2000). Furthermore, fucoidan has antioxidant properties (Xue et al., 2001). These discrepancies in regard to the role of fucoidan may have been derived from its heterogeneous properties.

Fucoidans have been extensively studied for their various biological activities but the exact role of fucoidans on the inflammatory processes associated with arthritic disease has not been studied. The effect of the treatment of high, medium and low molecular weight fucoidans (HMWF, MMWF and LMWF, respectively) on the progression of collagen-induced arthritis (CIA) was tested. A daily oral administration of HMWF enhanced the severity of arthritis, inflammatory responses in the joint cartilage and the levels of collagen-specific antibodies, while LMWF reduced the severity of arthritis and the levels of Th1-dependent collagen-specific IgG2a. Further in vitro analyses, using macrophage cell lines, revealed that the HMWF induced the expression of various inflammatory mediators, and enhanced the cellular migration of macrophages. These stimulatory effects of fucoidan decreased in fucoidans with lower molecular weights and LMWF did not exhibit any pro-inflammatory effects. Interestingly, the oral administration of HMWF enhanced the production of IFN-γ, one of the Th1 cytokines, in collagen-stimulated spleen cells that had been isolated from CIA mice, while LMWF had the opposite effect. These results indicate that HMWF enhances arthritis through enhancing the inflammatory activation of macrophages while LMWF reduces arthritis through the suppression of Th1-mediated immune reactions. Copyright © 2010 John Wiley & Sons, Ltd.

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nature, and it is necessary to differentiate between the form which activates the inflammatory response and the form which works in reverse.

In order to investigate the effects of fucoidan treatment on the pathogenesis of rheumatoid arthritis, the efficacy of high, medium and low molecular weight fucoidans were compared on a mouse arthritis model. The study also compared their effects on the inflammatory activation of macrophages. The oral administration of mice with different molecular weight fucoidans resulted in different outcomes in the severity of CIA in vivo and corresponding effects on the inflammatory activation of macrophages in vitro.

**MATERIALS AND METHODS**

**Mouse and fucoidan.** Male DBA/1J mice, 8 weeks of age, were used in all experiments. The mice were maintained in a temperature-controlled environment with free access to standard rodent chow and water. Experiments were performed in accordance with the ethical guidelines of Kyungpook National University. The low molecular weight fucoidan (LMWF) and medium molecular weight fucoidan (MMWF), gifts from Bion Co. Ltd, were obtained by means of acid hydrolysis of high molecular weight fucoidan (HMWF) extracts from Undaria pinnatifida, brown seaweed, and corresponding effects on the inflammatory activation of macrophages in vitro.

**Induction of CIA and assessment of arthritis.** Mice were divided into five groups (8 mice in each group) and immunized with type II collagen except the negative control group. One hundred μg of collagen type II (CII) (Sigma-Aldrich Fine Chemicals) was dissolved in 50 μL of 0.05 mM acetic acid and emulsified with an equal volume of complete Freund’s adjuvant (CFA) (Difco Laboratories). One hundred μL of the emulsion was s.c. injected into the base of the tail (day 0). Three weeks later, the mice received boosters with 50 μg of CII in incomplete Freund’s adjuvant by intradermal injection at a hind foot. Collagen immunized mice groups were treated with fucoidans of different molecular weights except the positive control group. Fucoidans, dissolved in distilled water, were orally administered daily at 300 mg/kg at 100 μL volume, from one day after booster immunization until the end of the experiment. The severity of arthritis was represented as the mean of the clinical score on a 0–4 scale basis according to the following criteria (0, normal; 1, erythema or the swelling of one or several digits; 2, erythema and moderate swelling, extending from the ankle to the mid-foot; 3, erythema and severe swelling, extending from the ankle to the metatarsal joints; and 4, complete erythema and swelling, encompassing the ankle, foot and digits, resulting in deformity and/or ankylosis).

**Measurement of antibodies that are specific to collagen.** Sera were obtained on day 49 and stored frozen at −70°C. For the measurement of anti-collagen IgG, IgG2a, and IgG1 antibodies, 96-well flat-bottom microtitre plates were incubated with 100 μL/well of CII (4 μg/mL) at 4°C overnight and washed three times with Tris-buffered saline (TBS) containing 0.05% Tween-20. The wells were then blocked by incubation with 200 μL of TBS, containing 1% bovine serum albumin (BSA) (Sigma), at room temperature (RT) for 60 min. After washing, the plates were incubated with 50 μL of serum diluents (1:50000 for IgG, 1:25000 for IgG1, and 1:10000 for IgG2a) at RT for 60 min. The plates were washed, and incubated 50 μL/well of a 1:1000 dilution of either horseradish peroxidase (HRP)-labeled goat anti-mouse IgG (Sigma) or HRP-labeled goat antimouse IgG/IgG2a (Santa Cruz) at RT for 60 min. After washing, 100 μL per well of tetramethylbenzidine chromogen solution (R&D Systems) was added and the plates were incubated in darkness at RT for 20 min. The reaction was terminated by adding 50 μL/well of 2 N H2SO4. The absorbance was then measured at 450 nm.

**Histological analysis and scoring.** Formalin-fixed limbs were decalcified and paraffin-embedded using standard histotechnical sequences. Serial 3 μm sections were cut and stained with hematoxylin and eosin in order to examine the morphologic features as well as to assess the histological arthritis score. The histological damage was assessed and calculated, using a 0–3 severity grade scoring system (Delgado et al., 2001) (0, normal joint structure; 1, mild to moderate changes, accompanying loss of cartilage, eroding pannus front, and synovial hyperplasia with infiltrating mononuclear cells and polymorphonuclear cells; 2, severe synovitis, cartilage and bone erosion; and 3, total destruction of joint architecture).

**Enzyme-linked immunosorbent assay (ELISA), gelatin zymogram, cell migration and flow cytometry.** For the measurement of cytokines, double-sandwich ELISA was performed. Gelatin zymogram was used for the activity measurement of matrix degrading enzymes in the culture supernatant. The migration potential of macrophage-like cells was measured using Boyden chamber assay. The expression levels of adhesion molecules were measured using flow cytometry. These experiments were performed according to previously described protocols (Chun et al., 2009). For the activation, THP-1 or RAW264.7 cells were seeded (5 × 104/well in a 96-well plate and fucoidan was added in 10 to 100 μg/mL concentrations. Bacterial lipopolysaccharide (LPS) was used to stimulate the cells as a positive control.

**Cell viability assay.** For the measurement of cell viability, a Cell Counting Kit-8 (CCK-8) (Dojindo Laboratories) was used. Briefly, the cells were incubated in the presence or absence of fucoidans for 24 h, and 10 μL of CCK-8 solution was added into each well. After 80 min of incubation at 37°C in 5% CO2, the absorbance at 450 nm was measured using a microplate reader.

**Nitrite quantification.** After RAW264.7 cells were treated with 10–100 ng/mL of LPS in 96-well plates, the concentrations of NO2− in the culture supernatants were
measured in order to assess the NO production. Fifty μL of sample aliquots were mixed with 50 μL of Griess reagent (1% sulfanilamide/0.1% naphthylethylene diamine dihydrochloride/2% phosphoric acid) in 96-well plates and incubated at 25°C for 10 min. The absorbance at 540 nm was measured using a microplate reader. NaNO2 (0 to 145 μM, Sigma) was used as the standard to calculate the NO2− concentrations.

**Spleen cell activation.** For the activation of spleen cells with denatured CII, a stock solution of CII (4 μg/mL in 0.05 mM acetic acid) was diluted in PBS to obtain 1 μg/mL solution. Denaturation was performed by incubating the diluted CII solution at 56°C for 10 min. Denatured CII (40 μg/mL final concentration) was added into the spleen cells (incubated at 2 x 10^6/mL concentration) which had been isolated from CIA mice. Labeling with 2-bromo-5-deoxyuridine (BrdU) was performed using a BrdU labeling kit (Roche) 3 days after the CII treatment by adding 10 μg/mL (final concentration) of BrdU for 2 h and following the manufacturer’s instructions. For the measurement of cytokines, culture supernatants were collected 72 h after activation and cytokine concentrations were measured using ELISA. For the activation of spleen cells with LPS in the presence of LMWF, spleen cells (2 x 10^6 cells/mL) isolated from ICR mice (a mouse strain with H-2q haplotype) were stimulated with 1 μg/mL LPS in the presence or absence of fucoidan (100–300 μg/mL) for 24 h, and then culture supernatants were collected for cytokine measurements using ELISA.

**RESULTS AND DISCUSSION**

**Effect of different molecular weight fucoidans on the severity of CIA as well as the production of collagen-specific antibodies**

Arthritis was induced by immunization and the subsequent booster injection of collagen type II (CII) in DBA/1J mice. As shown in Fig. 1A, the oral administration of HMWF significantly increased the severity of CIA at day 42, while LMWF significantly reduced it from days 37 to 47. The MMWF had a mid-way impact between HMWF and LMWF. The experiment was repeated twice with essentially the same results. Histological studies showed a severe cartilage and bone erosion, accompanied by marked lymphocyte infiltration in the mice that had been immunized with collagen (positive control group) (Fig. 1B). The treatment with HMWF tended to enhance the destruction of the...
cartilage. In contrast, the administration of LMWF reduced the damage in the cartilage and bones, and the number of inflammatory cells was reduced in this group (Fig. 1B). Both clinical and histological analysis indicated that fucoidans with different molecular weights have differential effects on the pathology of CIA: HMWF tended to enhance while LMWF tended to suppress it.

Oral administration of fucoidans is expected to lead to the appearance of fucoidan in peripheral blood. Using the antibody-based detection method, it was demonstrated that daily oral ingestion of 3 g of 75% high-molecular weight fucoidan resulted in the elevation of plasma concentration up to 13 mg/L (Irhimeh et al., 2005). Since the mammalian digestive system, as well as microbes in the gut flora, do not produce enzymes that can break down fucoidans (Michel et al., 1996), the elevation of plasma fucoidan levels indicates that ingested fucoidans can cross the intestinal wall as whole molecules through endocytosis. Furthermore, oral ingestion of fucoidan resulted in increased expression levels of a chemokine receptor, CXCR4, in human CD34 cells (Irhimeh et al., 2007). This indicates that the ingestion of fucoidan can manifest its biological activities.

The balance between cytokines produced by Th1 and Th2 subsets of T helper cells plays an important role in the development of animal models of autoimmune diseases. Studies utilizing the CIA model revealed that Th1 responses are predominant at the time of onset of arthritis, while Th2 responses are suppressed (Mauri et al., 1996). Generally, the production of antibodies with an IgG2a or IgG1 isotype has been used as a marker of Th1 or Th2 predominance, respectively (Hochreiter et al., 2003; Trujillo-Vargas et al., 2005). When the levels of collagen-specific antibodies in the sera were tested in the CIA mice used in this study, the overall levels of collagen-specific IgG had increased in the mice that had been immunized with collagen (positive control group) (Fig. 1C). In the positive control group, the levels of both collagen-specific IgG2a and IgG1 had increased; the optimal detection of IgG2a and IgG1 required 25000 and 10000 fold dilution of sera, respectively. The total levels of collagen-specific total IgG in sera had increased following treatment with HMWF with a statistical significance \( p < 0.0001 \), while LMWF tended to reduce the levels of collagen-specific total IgG \( p = 0.0128 \). The treatment with MMWF, however, failed to show a statistical difference in comparison with the positive control group \( p = 0.0574 \). The treatment with HMWF significantly increased the serum levels of both collagen-specific IgG1 and IgG2a \( p < 0.0001 \) and \( p = 0.000712 \), respectively (Fig. 1C). MMWF-induced reduction of IgG1 was statistically significant \( p = 0.0114 \) while that of IgG2a was not \( p = 0.0659 \). LMWF reduced the serum levels of collagen-specific IgG2a with a statistical significance \( p = 0.00123 \), but the levels of collagen-specific IgG1 had not been affected \( p = 0.462 \). These data indicate that the serum levels of anti-collagen antibodies correspond to the severity of arthritis in the experimental groups, and that HMWF had enhanced the levels of production of both Th1- and Th2-dependent anti-collagen antibodies. In contrast, MMWF and LMWF tended to reduce the production of anti-collagen antibodies. MMWF reduced Th2-dependent production of the antibodies. LMWF had inhibited the production of Th1-dependent anti-collagen antibodies, while the production of Th2-dependent anti-collagen antibodies had been left unaffected.

**HMWF causes inflammatory activation of macrophages in vitro**

The underlying mechanism responsible for the observed difference in the severity of CIA among different fucoidan treatment groups was then investigated in vitro utilizing macrophages. Fucoidan has been reported to affect the inflammatory processes in various cell types. Synovial inflammation, associated with rheumatoid arthritis, is responsible for the cartilage destruction, and macrophages are one of the main sources of various matrix degrading enzymes. In order to investigate whether different molecular weight fucoidans have differential effects on the inflammatory activation of macrophages, HMWF, MMWF and LMWF were treated in cultures containing either the murine macrophage cell line, RAW264.7, or the human macrophage cell line, THP-1. Cellular activities associated with the inflammatory activation of macrophages were then tested. As shown in Fig. 2A and B, treatment with HMWF induced the expression of matrix metalloproteinase (MMP)-9, one of the matrix degrading enzymes. The induction of MMP-9 by MMWF was lower than that of HMWF. LMWF failed to induce an MMP-9 expression. The murine peritoneal macrophages were also treated with different molecular weight fucoidans, and the MMP-9 induction patterns were determined as being similar to that of RAW264.7 and THP-1 cells (data not shown). In agreement with this observation, the expression levels of the major proinflammatory cytokine, TNF-\( \alpha \), was highest in cells treated with HMWF. MMWF induced moderate levels of TNF-\( \alpha \) while LMWF did not (Fig. 2C). The observed difference in both MMP-9 and TNF-\( \alpha \) production may have been caused by an adverse effect of fucoidans on cell viability. In order to test that possibility, the cell viability was tested in cells treated with different molecular weight fucoidans. As shown in Fig. 2D, cell viability was not affected by the treatment with fucoidans.

The induction of NO production accompanies the inflammatory activation of macrophages. The treatment of RAW264.7 cells with different molecular weight fucoidans resulted in the induction of NO production, which had been detected by the presence of nitrite in the culture medium, in a similar manner to both TNF-\( \alpha \) and MMP-9 production (Fig. 2E). The levels of NO that were induced by 100 ng/mL of HMWF treatment were higher than that induced by 10 ng/mL of LPS treatment while lower than that by 100 ng/mL of LPS treatment. When fucoidans were treated in the presence of LPS, NO production was up-regulated by HMWF, but not by LMWF (Fig. 2F). These data indicate that HMWF causes the inflammatory activation of macrophages, leading to the induction of the expression of pro-inflammatory mediators, such as MMP-9, TNF-\( \alpha \) and NO. These pro-inflammatory agents are expected to enhance various processes that are associated with arthritis: MMP-9 can contribute to the degradation of joint cartilage and TNF-\( \alpha \) can enhance the inflammatory activation of various cell types involved in arthritic changes, such as synovial fibroblasts, endothelial cells and the macrophage themselves. These pre-inflammatory activi-
ties of HMWF may account for the enhancement of the severity of CIA by HMWF.

**HMWF induces the up-regulation of adhesion molecules and the migration potential in macrophages**

The macrophage adherence to an extracellular matrix or to the endothelial cell layer in the vessel wall, as well as movement across a chemotactic gradient are required for its efficient performance of functions associated with inflammation. In order to determine whether the difference in the molecular weight of fucoidan affects the expression of adhesion molecules in macrophages, the expression levels of various adhesion molecules were tested after the treatment with fucoidan. The expression levels of CD18, CD49d and CD62L were not affected by the treatment with fucoidans (data not shown). In contrast, the intercellular adhesion molecule (ICAM)-1 and CD11a expression levels were up-regulated by HMWF to an extent that was comparable to that of LPS (Fig. 3A). The extent of the up-regulation decreased as the molecular weight of fucoidan decreased and the treatment with either MMWF or LMWF resulted in only a slight increase in regard to ICAM-1 expression levels and no increase, even a slight decrease, in CD11a expression levels.

The migration potential was also tested in the presence of different molecular weight fucoidans. As a positive control, fibronectin was used as a chemoattractant. Fibronectin fragments are chemotactic to leukocytes and are known to be generated in inflamed tissues (Birdsall et al., 2004). As shown in Fig. 3B, the presence of HMWF and MMWF potentiates the basal transmigration activity. The potentiating effect decreased as the molecular weight of fucoidan decreased, and LMWF did not have any effect on the outcome. The stimulating activities of HMWF with regard to the up-regulation of adhesion molecules and transmigration potential may explain, at least in part, the enhancement of arthritis in the CIA model.

Fucoidans have been known to inhibit the interaction of cell-surface selectins with their corresponding ligands (Cumashi et al., 2007). It is possible that the suppression of the severity of CIA in mice treated with LMWF may have resulted from the suppression of the selectin interaction as well as the resulting inflammatory adhesion of leukocytes into the endothelial layers of the diseased tissue. In order to confirm whether the difference in the molecular weight of fucoidan have any effect on their suppression of selectin interaction, THP-1 cells were treated with fucoidans with different molecular weights and the accessibility of selectin was tested with selectin-specific mAbs. HMWF, but not the MMWF and LMWF, blocked the interaction involving CD62L (L-selectin) (data not shown). This indicates that the observed reduction of the CIA severity in LMWF-treated mice was not caused by the suppression of the interaction between selectins and their corresponding ligands.
HMWF enhances, while LMWF suppresses, the expression of IFN-γ in spleen cells

In CIA models, the response of spleen cells to collagen, which has been added directly into the cell suspension, is often used as an indicator of the lymphocyte response to collagen (Feng et al., 2007). The response to collagen was then tested in spleen cells isolated from CIA mice that had been treated with different molecular weight fucoidans. DBA/1J mice were immunized with CII and boosted after 2 weeks. The mice were orally administered with different molecular weight fucoidans for 14 days before they were killed at day 30 after immunization. The spleen cells were isolated and cultured in the presence of denatured CII, and then the cellular proliferation and the cytokine secretion were analysed. Proliferation, measured by BrdU incorporation 3 days after the collagen treatment, was not affected by fucoidan treatment (data not shown). The culture supernatants were collected at day 2 for the measurement of TNF-α, IL-4, IL-17 and IFN-γ. The induction of TNF-α by the denatured CII treatment was enhanced in spleen cells isolated from mice that had been treated with fucoidan, irrespective of its molecular weight (Fig. 4A). Interestingly, the concentration of IFN-γ induced by the denatured CII treatment was enhanced by HMWF, while spleen cells isolated from CIA mice that had been treated with MMWF or LMWF expressed significantly reduced amounts of IFN-γ. The induction of the expression of IL-4 and IL-17 was not detected (data not shown).
shown). In order to reconfirm the suppressive effects of LMWF in the expression of IFN-γ, spleen cells isolated from normal mice (ICR strain) were stimulated with LPS in the presence of LMWF, and the expression levels of both TNF-α and IFN-γ were measured (Fig. 4B). As expected, LMWF dose-dependently enhanced the LPS-induced TNF-α expression while suppressing the LPS-induced IFN-γ expression. LMWF alone failed to induce the expression of either TNF-α or IFN-γ.

IFN-γ is one of the key cytokines that mediate the Th1 response, and the serum levels of IFN-γ have been shown to be elevated in mice with CIA (Mauri et al., 1996). The suppressive effect of LMWF on the production of IFN-γ corresponds with the observed reduction of collagen-specific IgG₂a in CIA mice that had been treated with LMWF (Fig. 1C). The production of IgG₂a isotype is dependent on the Th1 responses (Hochoerter et al., 2003; Trujillo-Vargas et al., 2005). This indicates that LMWF may have a protective effect on CIA through the blocking Th1 responses, as evidenced by the suppression of IFN-γ expression and collagen-specific IgG₂a production.

Fucoidan is a polyanionic, sulfated polymer of 1-fucose. Its polyanionic structure is expected to allow it to bind to a large number of proteins and, thus, exert its biological activities (O’Leary et al., 2004). Due to its low solubility and multiple activities, acidic hydrolysis or free radical depolymerization (Nardella et al., 1996) has been performed to obtain low-molecular-weight fractions of fucoidans (<30 kDa). The comparison of these different molecular weight fucoidans revealed that molecular weight affects its inflammatory potential. The mild acid hydrolysis method, used in this study for the preparation of MMWF and LMWF, may have contributed to the change in the chemical composition of the fucoidan, which may lead to the different activities of fucoidans according to their molecular weight. In addition, short chain polymers are expected to have a low activating potential toward macrophages, due to their limited interactions with multiple numbers of receptors, such as scavenger receptors.

In the experimental system used in this research, HMWF enhanced the severity of CIA. This enhancing effect appears to be derived from its pro-inflammatory actions. HMWF induced the expression of pro-inflammatory mediators such as MMP-9, NO and TNF-α in conjunction with the up-regulation of both ICAM-1 and CD11a in macrophages. Since macrophages are one of the main cell types found in the synovial tissue of RA, the administration of HMWF is expected to enhance the infiltration of more macrophages into the lesion, as well as to stimulate the synovial macrophages for more arthritic reactions. The inflammatory activation of macrophages by fucoidan treatment has also been reported by other research groups (Nakamura et al., 2006; Oomizu et al., 2006; Yang et al., 2006).

The precise action mechanism of LMWF in the suppression of the severity of CIA is not evident based on the observed results. Interestingly, LMWF inhibited Th1-mediated responses, as evidenced by the reduction of collagen-specific IgG₂a levels in the serum, while Th2-mediated responses were unaffected, as evidenced by the maintenance of collagen-specific IgG₁ levels. In addition, LMWF suppressed the production of IFN-γ, a Th1 cytokine, in spleen cells that had been stimulated with LPS. These observations indicate that LMWF somehow reduced the development of CIA though the modulation of the balance between the Th1 and Th2 immune responses against collagen. Further experiments are required in order to identify the cell type(s) that is affected by LMWF, and to determine its role in IFN-γ production.

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Conflict of Interest

The authors have declared that there is no conflict of interest.

REFERENCES


