Fucoidan exerts protective effects against diabetic nephropathy related to spontaneous diabetes through the NF-κB signaling pathway *in vivo* and *in vitro*

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**Abstract.** Fucoidan, an extract of the seaweed, *Fucus vesiculosus*, has been widely investigated for its antioxidant effects. However, to date and to the best of our knowledge, pathological studies on the effects of fucoidan against diabetic nephropathy (DN) related to spontaneous diabetes have not been carried out. DN is one of the most serious microvascular complications of diabetes. Therefore, in the present study, the effects of fucoidan against DN related to spontaneous diabetes were investigated *in vitro* and *in vivo*. Goto-Kakizaki (GK) rats were allowed free access to standard rat food with or without fucoidan for 13 weeks, and Wistar rats were used as controls. Fucoidan did not show any cytotoxicity on glomerular mesangial cells (GMCs) which were separated from rat kidneys. Fasting blood glucose levels were measured using a blood glucose meter, blood urea nitrogen (BUN) and serum creatinine (Cr) levels were measured using an automatic biochemistry analyzer and urine protein levels were measured using an ELISA kit. Collagen IV levels in the renal cortex were measured using an ELISA kit, and the expression levels of transforming growth factor-β1 (TGF-β1) and fibronectin (FN) in the renal cortex and GMCs, and nuclear factor-κB (NF-κB) in GMCs were determined by western blot analysis. Fasting blood glucose, BUN, serum Cr, urine protein and collagen IV levels, and the expression of TGF-β1 and FN, as well as NF-κB p65 nuclear translocation all significantly increased in the GK rats compared with the control Wistar rats. The increase in the fasting blood glucose, BUN, serum Cr, urine protein and collagen IV levels in the renal cortex was reversed in the GK rats which were orally administered fucoidan. The oral administration of fucoidan also decreased the expression of TGF-β1 and FN in the renal cortex and GMCs, as well as the nuclear translocation of NF-κB p65 in the GMCs. Taken together, the data from our *in vitro* and *in vivo* experiments indicate that fucoidan attenuates hyperglycemia and prevents or impedes the development of DN related to spontaneous diabetes by attenuating the activation of the NF-κB signaling pathway.

**Introduction**

Diabetic nephropathy (DN) is one of the most serious microvascular complications of diabetes and the leading cause of end-stage renal failure (1). The early stage of DN is characterized by renal hypertrophy, glomerular hypertrophy, glomerular hyperfiltration and microalbuminuria (2). One of the characteristic pathological changes in DN is the accumulation of extracellular matrix (ECM) components, including collagens, fibronectin (FN) and laminin in the glomeruli and the interstitium of the kidneys (3). Hyperglycemia is usually considered the main determinant factor of the initiation and progression of DN, which increases the expression of transforming growth factor-β1 (TGF-β1) (4). The increase in TGF-β1 expression levels has been recognized as a marker of DN, and TGF-β1 is the most potent growth factor contributing to ECM accumulation (5). Nuclear factor-κB (NF-κB) is a transcription factor which, under basal conditions, is sequestered as an inactive form in the cytoplasm through its interaction with the inhibitory protein, IκB. Under diabetic conditions, activated NF-κB translocates to the nucleus and triggers the expression of its target genes, including TGF-β1 and FN, further causing ECM accumulation (6).

In recent years, interest in the biological activities of marine organisms has intensified (7,8). Fucoidan, an extract of the seaweed, *Fucus vesiculosus*, whose chemical structure is presented in Fig. 1A, has been widely investigated as an antioxidant, anticancer and anti-inflammatory agent, and has been shown to play an important role in cancer and inflammation (9,10). Thus, in the present study, we sought to determine whether fucoidan prevents or impedes the development of DN related to spontaneous diabetes.

The Goto-Kakizaki (GK) rat is a non-obese rat substrain originally derived by the repeated inbreeding of glucose-intol-
erant Wistar rats (11). It is a model of spontaneous and moderate non-insulin-dependent diabetes. Between 3 and 4 weeks of age, GK rats develop mild hyperglycemia and hyperinsulinemia. Thus, GK rats were selected for use in the present study and Wistar rats were used as the controls.

In the present study, to the best of our knowledge, the protective effects of fucoidan against DN related to spontaneous diabetes were examined in vitro and in vivo for the first time. Our results revealed that fasting blood glucose, blood urea nitrogen (BUN), serum creatinine (Cr), urine protein and collagen IV levels, and the expression of TGF-β1 and FN, as well as the NF-κB p65 nuclear translocation all significantly increased in the GK rats compared with the control Wistar rats. These effects were all reversed in the GK rats which were orally administered fucoidan. Our observations prove that fucoidan prevents or impedes the development of DN related to spontaneous diabetes by inhibiting the NF-κB signaling pathway, suggesting that fucoidan may be a novel therapeutic agent for the treatment of DN.

Materials and methods

Animals. The experiments were carried out at the Animal Experimental Center of Harbin Medical University (Harbin, China). Animal care and the protocols were in accordance with the Animal Experiment Guidelines of Harbin Medical University and ethical approval was obtained from the Harbin Medical University. Male GK rats and Wistar rats were obtained from CLEA Japan, Inc. (Tokyo, Japan). The rats were housed in a controlled environment at a temperature of 24±1°C and under a 12-h light:dark lighting cycle with the lights turned on at 7 a.m. At 6 weeks of age, the rats were allowed free access to standard rat food and water with or without the recommended concentrations of fucoidan (Sigma-Aldrich, Shanghai, China; 50 and 75 mg/kg body weight), as previously described (12) for 13 weeks.

Cell culture. Rats glomerular mesangial cells (GMCs) were separated from the glomeruli of the GK rats and the control Wistar rats and characterized as previously described (13). Briefly, primary cultures were established from freshly isolated glomeruli, which were mechanically sieved and harvested by iterative selection on specific mesh sizes (200, 100 and final 80 μm). Those retained on the sieve were collected and washed by centrifugation at 1,000 rpm for 5 min, and incubated with 250 units/ml collagenase (type I) for 30 min at 37°C. The GMCs were minced and a homogenate was prepared with 10% phosphate-buffered saline (PBS; 0.1 mol/l, pH 7.4) using a homogenizer (UH-50; SMT Co., Ltd., Tokyo, Japan). The kidney homogenate was centrifuged at 15,000 rpm for 15 min at 4°C. The supernatant was collected and the quantity of type IV collagen in the renal cortex was determined using an ELISA kit (R&D Systems, Minneapolis, MN, USA). BUN and serum Cr levels were determined using an automatic biochemistry analyzer (Olympus-2000; Olympus, Tokyo, Japan).

Measurement of fasting blood glucose, BUN, serum Cr and urine protein levels. Fasting blood glucose was measured using a blood glucose meter (OneTouch UltraEasy, Edina, MN, USA). The 24-h urinary albumin levels were measured using an ELISA kit (R&D Systems, Minneapolis, MN, USA). BUN and serum Cr levels were determined using an automatic biochemistry analyzer (Olympus-2000; Olympus, Tokyo, Japan).

Western blot analysis. Electrophoresis was performed using a vertical slab gel with 12% polyacrylamide content according to a previously described method (15). The transfer of proteins from the SDS polyacrylamide gel to a membrane was performed electrophoretically according to a previously described method (16) with certain modifications using a Semi-Dry Electrobaster (Sartorius AG, Goettingen, Germany) for 90 min with an electric current of 15 V. The membrane was treated with Block Ace™ (4%) for 30 min at 22°C. The first reaction was performed using rabbit immunoglobulin (IgG) antibodies against TGF-β1 (SAB4502954), FN (AV41490) and NF-κB (SAB4502610; Sigma-Aldrich) in PBS containing 0.3% Tween-20 for 1 h at 22°C. Following washing in the same buffer, the second reaction was performed using horseradish peroxidase (HRP)-conjugated anti-rabbit goat IgG (20 ng/ml) for 30 min at 22°C. Following washing, the Enhanced chemiluminescence (ECL) reaction was performed on the membrane using the ECL Plus Western Blotting Detection System™ (GE Healthcare Life Sciences, Tokyo, Japan).

Histopathological analysis. For histopathological analysis, the kidneys from the GK and Wistar rats were fixed in 10% neutral-buffered formalin and subsequently embedded in paraffin. Sections (4-μm-thick) of paraffin-embedded tissues were stained with hematoxylin and eosin (H&E) solution for the determination of the histopathological characteristics, as previously described (17). The cross-section yielding the maximum diameter of the glomerulus was photographed and converted into a digital image by an examiner blinded to the tissue source using a light microscope equipped with a camera (Olympus BX-50; Olympus Optical, Tokyo, Japan).

Cytotoxicity assay. The CellTiter 96® AQueous One Solution Cell Proliferation assay (Promega, Madison, WI, USA), which has been reported to be an effective assay for determining cytotoxicity and the number of viable cells (14), was used to determine the cytotoxicity of fucoidan. GMCs from the Wistar rats were inoculated at a concentration of 2x10^5 cells/well and incubated in 96-well plates with conditioned DMEM medium at 37°C under a 5% CO₂ atmosphere for 2 days. The GMCs were treated with various concentrations of fucoidan (0, 10, 50, 100, 500 and 1,000 μg/ml) followed by a further 24 h of incubation. Subsequently, 20 μl of the CellTiter 96 AQueous One Solution Cell Proliferation assay solution was pipetted into all 96-wells and the GMCs were further incubated for 1 h. The absorbance at 490 nm was measured using an MTP-800 microplate reader (Corona Electric Co., Ltd., Ibaraki, Japan).

Measurement of collagen IV levels. The kidney tissue was minced and a homogenate was prepared with 10% phosphate-buffered saline (PBS; 0.1 mol/l, pH 7.4) using a homogenizer (UH-50; SMT Co., Ltd., Tokyo, Japan). The kidney homogenate was centrifuged at 15,000 rpm for 15 min at 4°C. The supernatant was collected and the quantity of type IV collagen in the renal cortex was determined using an ELISA kit (R&D Systems).
4% paraformaldehyde in PBS for 20 min, and permeabilized with 0.1% Triton X-100 for 5 min at room temperature. After further washing, the cells were blocked with 10% goat serum for 30 min at room temperature. The cells were then incubated with anti-rat p65 (dilution 1:100) antibody (sc-8008; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) at room temperature for 2 h followed by incubation with the secondary antibody (Alexa Fluor® 488-conjugated goat anti-mouse IgG, dilution 1:1,000) (A-11029; Invitrogen, Carlsbad, CA, USA) for 1 h. Nucleus were counterstained with Hoechst 33342 solution (5 µg/ml in PBS) for 10 min. The coverslips were mounted on glass lides with anti-fade mounting medium (Invitrogen), and images were acquired using the Carl Zeiss LSM 710 laser confocal fluorescence microscope (Carl Zeiss AG, Jena, Germany).

Statistical analysis. Data are expressed as the means ± standard deviation. Each experiment was repeated at least 3 times. The Student's t-test was used and a value of P<0.05 was considered to indicate a statistically significant difference.

Results

Fucoidan is not cytotoxic to GMCs. GMCs separated from the Wistar rats were inoculated at a concentration of 2x10⁴ cells/well and incubated in 96-well plates with conditioned DMEM medium at 37°C under a 5% CO₂ atmosphere for 2 days. The GMCs were treated with various concentrations of fucoidan (0, 10, 50, 100, 500 and 1,000 µg/ml) for 24 h, and then 20 µl of the CellTiter 96 AQueous One Solution Cell Proliferation assay solution was pipetted into all 96-wells and the GMCs were further incubated for 1 h. Fucoidan did not exert any obvious cytotoxic effects on the GMCs (Fig. 1B).

Fucoidan decreases the high fasting blood glucose, BUN, serum Cr and urine protein levels in the GK rats. Male GK and Wistar rats were housed in a controlled environment at a temperature of 24±1°C and under a 12-h light:dark lighting cycle and allowed free access to standard rat food and water with or without fucoidan for 13 weeks from 6 weeks of age. The fasting blood glucose, BUN, serum Cr and urine protein levels were significantly increased in the GK rats (diabetes) compared with the control Wistar rats (normal; P<0.01). The increased fasting blood glucose, BUN, serum Cr and urine protein levels were significantly decreased in the GK rats which were orally administered fucoidan (50 mg/kg body weight, P<0.05; 75 mg/kg body weight, P<0.01). There were no significant differences observed between the Wistar rats treated with fucoidan and the control (untreated) Wistar rats (data not shown).

Fucoidan decreases the increased collagen IV levels in the renal cortex of GK rats. Male GK and Wistar rats were housed in a controlled environment at a temperature of 24±1°C and under a 12-h light:dark lighting cycle and allowed free access to standard rat food and water with or without fucoidan for 13 weeks from 6 weeks of age. The collagen IV content in the renal cortex was significantly increased in the GK rats (diabetes) compared with the control Wistar rats (normal; P<0.01). The increased collagen IV content was significantly decreased in the GK rats which were orally administered fucoidan (P<0.01).

Oral administration of fucoidan decreases the expression of TGF-β1 and FN in the renal cortex of rats and GMCs. At 6 weeks of age, male GK and Wistar rats were housed in a controlled environment at a temperature of 24±1°C and under a 12-h light:dark lighting cycle with the lights turned on at 7 a.m. and allowed free access to standard rat food and water with or without fucoidan for 13 weeks. The expression levels of TGF-β1 (Fig. 3A) and FN (Fig. 3B) were determined by western blot analysis. The expression levels of TGF-β1 and FN were significantly increased in the GK rats (diabetes)
compared with the control Wistar rats (normal). The oral administration of fucoidan significantly reduced the increased expression levels of TGF-β1 and FN in the GK rats (P<0.01).

Similar effects were observed in our in vitro experiments with the GMCs. The GMCs obtained from the GK rats (diabetes) showed increased levels of TGF-β1 and FN compared with the GMCs obtained from the Wistar rats (normal). However, in the GMCs treated with fucoidan, these levels were decreased (Fig. 3D and E).

**Fucoidan attenuates the histopathological changes in the kidneys of GK rats.** The kidneys from the GK and Wistar rats were fixed in 10% neutral-buffered formalin and subsequently embedded in paraffin. Sections (4-µm-thick) of paraffin-embedded tissues were stained with H&E solution for the determination of the histopathological characteristics. Vacuolation and the regeneration of renal tubular epithelial cells, and inflammatory cell infiltration in the renal interstitium were evident in the kidneys from the GK rats (diabetes) compared with those from the control Wistar rats (normal). Fucoidan significantly attenuated these histopathological changes in the kidneys of GK rats (Fig. 4A).

**Oral administration of fucoidan attenuates the nuclear translocation of NF-κB p65 in GMCs.** At 6 weeks of age, male GK and Wistar rats were allowed free access to standard rat food and water with or without fucoidan for 13 weeks, and the GMCs were then separated from the glomeruli of the GK rats and the control Wistar rats. The results of LSCM (Fig. 4B) and western blot analysis (Fig. 5) revealed that the expression of NF-κB was significantly increased in the GMCs from the GK rats (diabetes) compared with the GMCs from the control Wistar rats (control). The nuclear translocation of NF-κB p65 was significantly attenuated by the oral administration of fucoidan (Fig. 5B; P<0.01).

**Discussion**

To the best of our knowledge, the present study demonstrates in vitro and in vivo, for the first time, that fucoidan reduces hyperglycemia and prevents or impedes the development of DN related to spontaneous diabetes. DN is one of the most serious microvascular complications of diabetes and the leading cause of end-stage renal failure (1). The early stage of DN is characterized by renal hypertrophy, glomerular hyperfiltration, glomerular hyperfiltration and microalbuminuria (2). These changes are related to the subsequent development of glomerular morphological abnormalities and the prognosis of DN (18). Inflammation has emerged as a key pathophysiological mechanism for DN (19). One of the characteristic pathological changes in DN is the accumulation of ECM components, including collagens, FN and laminin in the glomeruli and the interstitium of the kidneys (3). Hyperglycemia is usually considered as the main determinant factor of the
initiation and progression of DN which increases the expression of TGF-β1 (4). The increase in the TGF-β1 expression level is recognized as a marker of DN and TGF-β1 is the most potent growth factor contributing to ECM accumulation (5). In accordance with these data, the present study confirmed that the expression of TGF-β1, FN and collagen IV were significantly reduced in the GK rats which were orally administered fucoidan. β-actin was used for normalization. (A-2) Quantification of (A-1), (B-2) quantification of (B-1), (D-2) quantification of (D-1) and (E-2) quantification of (E-1). Data are expressed as the means ± standard deviation (n=8). *P<0.05 was considered to indicate a statistically significant difference (**P<0.01, GK rats vs. control Wistar rats; ##P<0.01, GK rats treated with fucoidan vs. untreated GK rats).

In recent years, interest in the biological activities of marine organisms has intensified (7,8). Fucoidan, an extract of the seaweed, Fucus vesiculosus, has been widely investigated as an antioxidant, anticancer and anti-inflammatory agent, and has been shown to play an important role in cancer and inflammation (9,10). Fucoidan can suppress various inflammatory cytokines, such as interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), interferon-γ (IFN-γ) and cyclooxygenase-2 (COX-2) (21). In the present study, the effects of fucoidan against DN related to spontaneous diabetes were investigated in vitro and in vivo. Fucoidan did not exert any cytotoxic effects on GMCs separated from Wistar rats, and there were no significant differences observed between the Wistar rats administered fucoidan and the control (untreated) Wistar rats (data not shown). Following the oral administration of fucoidan for 13 weeks, the increased fasting blood glucose, BUN, serum Cr, urine protein and collagen IV levels in the renal cortex, and the expression levels of TGF-β1 and FN in the renal cortex and GMCs from GK rats were all significantly reduced (Figs. 2 and 3). Histopathological analysis also revealed that histopathological changes, such as vacuolation and the regeneration of renal tubular epithelial cells, and inflammatory cell infiltration into the renal interstitium in the kidneys of GK rats were attenuated by the oral administration of fucoidan (Fig. 4).

NF-κB is a transcription factor (6). The nuclear NF-κB family of transcription factors regulates the induction and
resolution of inflammation. NF-κB regulates the expression of numerous genes that play a key role in the inflammatory response during human and experimental kidney injury (22). It is believed that the activation of NF-κB and chronic inflammation play pivotal roles in the pathogenesis of DN (23). Under basal conditions, NF-κB is sequestered as an inactive form in the cytoplasm through its interaction with the inhibitory protein, IκB (6). Under diabetic conditions, following the phosphorylation and degradation of IκB-α, NF-κB p65 translocates to the nucleus and binds to a specific DNA sequence to activate
the transcription of target genes, including TGF-β1 and FN, further causing ECM accumulation (24,25). To better understand the mechanisms of action of fucoidan in the prevention of the development of DN related to spontaneous diabetes, we investigated the nuclear translocation of NF-κB p65. Our results revealed that the increased NF-κB nuclear translocation and NF-κB p65 expression in the GMCs were significantly attenuated in the GK rats orally administered fucoidan (Figs. 4 and 5), suggesting that the NF-κB signaling pathway plays an important role in the development of DN. However, fucoidan attenuated the NF-κB nuclear translocation in the GMCs from the GK rats, but did not completely block it, suggesting that other mechanisms are involved in this process. It has been reported that the NF-κB nuclear translocation is also regulated by reactive oxygen species (ROS) and protein kinase C in GMCs, and phosphoinositide 3-kinase has also reported to be involved in this event (26). The complex process and mechanisms involved require further investigation.

In the present study, to the best of our knowledge, the protective effects of fucoidan against DN related to spontaneous diabetes were determined in vitro and in vivo for the first time. Our observations prove that fucoidan prevents or impedes the development of DN related to spontaneous diabetes by inhibiting the activation of the NF-κB signaling pathway; thus, fucoidan may be considered as a novel therapeutic agent for the treatment of DN.

References


