Attenuation of obesity-induced inflammation in mice orally administered with salmon cartilage proteoglycan, a prophylactic agent

（サケ鼻軟骨抽出プロテオグリカンの経口投与は肥満由来の炎症を軽減する）

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Abstract

Obesity is associated with chronic inflammation of adipose tissue and causes development of type 2 diabetes. M1 macrophage population was increased in adipose tissue of obese mouse. M1 macrophages induce insulin resistance through the secretion of proinflammatory cytokines. Our previous studies demonstrated that salmon cartilage proteoglycan (PG) suppresses excess inflammation in various mouse inflammatory diseases. In this study, we examined the effect of PG on type 2 diabetes using high-fat-diet (HFD) induced obese mouse model. Oral PG administration enhanced the population of small adipocytes (area less than 1000 μm^2) without body and tissue weight gain. In addition, PG administration suppressed mRNA expression of TNF-α, IL-6 and CXCL2 in adipose tissue. The proportion of M1 macrophages was decreased by PG administration. In addition, PG administration suppressed hyperglycemia after intraperitoneal glucose injection. Fasted serum insulin level was decreased in PG-administered mice. Moreover, insulin-stimulated phosphorylation of Akt was enhanced in the liver and gastrocnemius skeletal muscle of PG-administered mice. These data suggested that PG administration improves hyperglycemia and insulin sensitivity in obese mice by modulation of M1 macrophages which secrete proinflammatory cytokines in adipose tissue and activation of

Akt in liver and skeletal muscle.

Introduction

In modern society, high consumption of energy-dense foods has led to a substantial increase in overweight and obese persons. It is increasingly accepted that obesity is characterized by chronic inflammation of adipose tissues, which causes development of insulin resistance and type-2 diabetes [1]. In adipose tissues of obese persons, adipose tissue macrophages (ATMs) play important roles in inflammation [2-5]. While the population of ATMs in lean mice is around 5%, it increases up to 50% in obese mice [4]. Besides increasing number, localization and phenotype of ATMs are changed during development of obesity. Macrophages can be classified into two phenotypes as classically activated (M1) and alternatively activated (M2) macrophages. M1 macrophages activate microbicidal activity, whereas M2 macrophages participate in allergic and antiparasitic responses [6]. M1 macrophages infiltrate into obese adipose tissues and secrete excessive pro-inflammatory cytokines, such as tumor necrosis factor-α (TNF-α), which then induce the additional accumulation of macrophages in adipose tissues [7]. Moreover, in the context of insulin action, M1 macrophages induce insulin resistance through the secretion of pro-

inflammatory cytokines [8]. Therefore, to improve insulin resistance due to obesity-related

inflammation, it is important to regulate the proportion of M1 macrophages and to reduce the

production of pro-inflammatory cytokines in obese adipose tissue.

Proteoglycan (PG) consists of core protein with one or more covalently attached

glycosaminoglycan chain(s). It is a compound of extracellular matrix materials that exists in

connecting tissue such as skin, bone, cartilage and vascular wall by forming a complex with

collagen, fibronectin, laminin, hyaluronic acid and other glycoproteins. In corporation with

collagen, fibronectin and laminin, PG has been shown to be involved in cellular proliferation and

adhesion [9]. We have previously demonstrated that PG extracted from salmon nasal cartilage has

a potent effect on suppression of inflammatory responses induced by heat-killed *Escherichia coli*

in mouse macrophages [10]. In addition, daily oral administration of PG attenuates the severity

of experimental inflammatory colitis [11], autoimmune encephalomyelitis [12] and collagen-

induced arthritis [13]. Our previous study also suggested that ingested PG may contribute to

homeostasis of host immunity mediated through the balance in composition of gut microbiota

[14].

Here, we report that PG has a prophylactic effect on obesity-related inflammation and blood

glucose level in C57BL/6J mice fed a high-fat diet (HFD). We demonstrated that PG reduced the production of pro-inflammatory cytokines and the proportion of M1 macrophages in obese adipose tissue. In addition, PG was able to improve hyperglycemia in diet-induced obese mice. These results suggest that PG may have a beneficial effect on obesity-induced inflammation.

Materials and methods

Animals and diets

Male C57BL/6 mice, 4 weeks old, were purchased from Clea Japan, Tokyo, Japan, and maintained in a temperature-controlled room (22℃) on a 12-h light-dark cycle at Institute for Animal Experimentation, Hirosaki University Graduate School of Medicine. Mice were either fed HFD (60% energy from fat) or received continuous feeding of a normal diet (ND) (5.6% energy from fat). Both diets were purchased from Clea Japan. All animal experiments were carried out in accordance with the Guidelines for Animal Experimentation of Hirosaki University. All mouse experiments were approved by the Committee on the Ethics of Animal Experimentation of Hirosaki University (permission number M10003).

PG administration

PG extracted from salmon nasal cartilage was purchased from Kakuhiro Co. Ltd, Aomori, Japan. Mice from HFD-fed group and ND-fed group were administered with 0.2 mg/ml PG through drinking distilled water (DW) for up to 16 weeks. DW only was used as a negative control. Body weight and food intake of mice were measured once two weeks and once a week, respectively. Adipose tissue and liver were isolated and their weights were measured from 16 week-fed mice.

Measurement of adipocyte area

After 16 weeks of feeding, epididymal adipose tissue was collected and fixed in 4% (w/v) paraformaldehyde buffer at 4°C for overnight. Tissues were then embedded in paraffin and cut into 5-μm thick sections. Deparaffinized sections were stained with hematoxylin and eosin. The tissues were observed under BZ-X700 microscope (Keyence, Tokyo, Japan) and adipocyte area were measured from randomly selected cells (n=400).

Real-time quantitative reverse transcription-PCR (qRT-PCR).

Total RNAs from adipose tissues were isolated using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instruction. First-strand cDNAs were synthesized from 1 μg total RNA using random primers (Takara, Shiga, Japan) and Moloney murine leukemia virus reverse transcriptase (Invitrogen). The primers used to amplify target genes from cDNA are listed in Table 1. PCRs were run at the following conditions: initial activation of Taq DNA polymerase at 95°C for 5 min, 40 cycles of 30 sec at 95°C for denaturing, 30 sec at 60°C for annealing, and 30 sec at 72°C for elongation. Gene expression levels were determined by real-time qPCR analysis using the SYBR green Supermix (Bio-Rad Laboratories, San Jose, CA). Dissociation curves were used to detect primer-dimer conformation and nonspecific amplification. The detection threshold was set to the log linear range of the amplification curve and kept constant (0.05) for all data analysis. The threshold cycle (C_T) of each target product was determined and set in relation to the amplification plot of glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The difference between the C_T values (ΔC_T) of two genes was used to calculate the relative expression; i.e., relative expression $= 2^{-(CT\ of\ target\ gene\ -\ CT\ of\ GAPDH)} = 2^{-\Delta CT}$.

Preparation of adipose-derived cells in the stromal vascular fraction

Adipose tissue was placed into RPMI1640 medium (Nissui Pharmaceutical Co., Tokyo, Japan) and digested with 0.3 units/ml collagenase IA (Roche Applied Science, Indianapolis, IN) at 37℃ for 40 min. The cells obtained were passed through a sterile 100 µm strainer (Fischer Scientific, Franklin, MA), and collected by centrifugation (500×g) at 4℃ for 10 min. After hemolysis, cells in the stromal vascular fraction (SVF) were resuspended and kept in staining buffer [phosphate-buffered saline (PBS) containing 1 mM EDTA, 25 mM HEPES, and 1% (w/v) fatty acid-free bovine serum albumin (BSA, fraction V, Sigma-Aldrich Japan Inc., Tokyo, Japan)] until immunolabeling.

Flow cytometry

Macrophages in the SVF were stained with fluorescein isothiocyanate (FITC)-conjugated rat anti-mouse F4/80 monoclonal antibody (mAb, eBioscience, San Diego, CA), phycoerythrin (PE)-conjugated rat anti-mouse CD11c mAb (Biolegend, San Diego, CA), and Alexa647-conjugated rat anti-mouse CD206 mAb (Biolegend) or isotype controls (BD Biosciences, San Jose, CA). Fluorescent intensity was analyzed by FACSCantoTM II flow cytometer (BD Biosciences).

Immunohistochemical analysis

After 8 weeks of feeding, epididymal adipose tissue was collected and fixed in 4% (w/v) paraformaldehyde buffer at 4°C for overnight. Tissues were soaked in PBS containing 30% sucrose and they were frozen in optimal cutting temperature medium (Sakura Finetek Japan, Tokyo, Japan) at -80°C. Frozen tissues were sliced into 10 μm, washed with PBS and incubated with 2% (v/v) normal rat serum in PBST [0.05% (v/v) Tween 20 in PBS] for 30 min at room temperature. Then, the sections were rinsed with PBST and double stained with FITC-conjugated anti-mouse F4/80 mAb (0.2 μg/ml) and PE-conjugated anti-mouse CD11c mAb (0.2 μg/ml) or Alexa647-conjugated anti-mouse CD206 mAb (0.2 μg/ml) at 4°C for 16 h. After rinse with PBST, the sections were counterstained with 4',6-diamidino-2-phenylindole (DAPI; Wako Pure Chemical Industries, Osaka, Japan) and coverslipped with Fluoromount (Life Technologies, Carlsbad, CA), and then they were observed under BZ-X700 microscope. Crown-like structures (CLSs) were randomly counted from seven histological sections.

Serum insulin measurement and intraperitoneal glucose tolerance test

Blood samples were obtained from 6 h-fasted mice at 12 week-feeding. Serum insulin level

was determined using mouse insulin ELISA kit (Morinaga Inst. Biol. Sci. Inc, Yokohama, Japan).

For intraperitoneal glucose tolerance test (IPGTT), 14 week-fed mice were fasted for 6 h and

injected intraperitoneally with 20% glucose in PBS at a dose of 2 mg/g body weight. Then, blood

samples were collected at 0, 15, 30, 60 and 120 min for glucose measurement using a Nipro stat

strip XP (Nipro, Osaka, Japan).

Western blotting

8 week-fed mice were fasted for 6 h and injected intraperitoneally with insulin (2.0 U/kg body

weight). Liver and gastrocnemius skeletal muscle were collected 10 min later and homogenized.

The proteins were separated by SDS-PAGE and transferred to PVDF membrane. After blocking

with 5% skim milk (Morinaga Milk Co., Tokyo, Japan) in PBST, Akt and phosphorylated Akt

were detected using anti-Akt and anti-phospho-Akt (Ser473) antibodies (Cell Signaling

Technology Inc., Beverly, MA), respectively. After incubation with horseradish peroxidase

(HRP)-conjugated protein A (Bio-Rad Laboratories), chemiluminescence signals were visualized

using ECL western blotting detection reagents (GE Healthcare Biosciences, Piscataway, NJ).

Immunoblotted band intensity was quantified using Image Lab software (Bio-Rad Laboratories).

Statistical analysis

Data were expressed as means ± standard deviations, and *P*<0.05 from student's *t* test analysis

was used to determine the significance of the differences.

Results

PG showed no significant effect on body weight, food intake or tissue weight.

To determine whether PG affects body weight and food intake, we measured these parameters

during HFD or ND feeding with or without PG administration. Although body weight of HFD-

fed mice was significantly higher than that of ND-fed mice, there was no significant difference in

body weight or food intake between the PG-administered and the control mice (Fig. S1A). Tissues

were then collected and weighed at 16 weeks after feeding. As expected, weights of the adipose

tissue and liver of HFD-fed mice were significantly higher than those of ND-fed mice. In addition,

there was no significant difference in tissue weights between the PG-administered group and the

control group (Fig. S1B). However, the number of small adipose cells with the area less than 1000

μm^2 was increased in the PG administered HFD-fed mice when compared with the control HFD-fed mice (Fig. S1C).

PG suppressed mRNA expression of TNF-α, IL-6 and CXCL2 in adipose tissue.

To determine the anti-inflammatory effect of PG in adipose tissue, mRNA expression of proinflammatory cytokines was evaluated by qRT-PCR. We firstly assessed mRNA expression levels of TNF-α, IL-6 and CXCL2 at 4 weeks after feeding (Fig. 1A). PG showed no effect on mRNA expression levels of these cytokines in ND-fed mice. In contrast, TNF-α mRNA expression in the adipose tissue was significantly decreased by PG administration in HFD-fed mice. No significant difference was observed in mRNA expression of IL-6 or CXCL2 between the PG-administered group and the control group. We next assessed mRNA expression levels at 8 weeks after feeding (Fig. 1B). In HFD-fed mice, mRNA expression levels of TNF-α, IL-6 and CXCL2 in the PG-administered mice were significantly decreased compared to the control group (Fig. 1B). In ND-fed mice, TNF-α mRNA expression of the PG-administered mice was significantly decreased compared with the control mice.

The proportion of M1 macrophages was decreased by PG administration.

We further examined the population of M1 and M2 macrophages in adipose tissue. After the gating of the F4/80-positive cells from the SVF cells of epididymal adipose   tissue, the cells were then analyzed using anti-CD11c and anti-CD206 antibodies as markers of M1 and M2 macrophages, respectively. The population of CD11c$^+$F4/80$^+$ cells was increased by HFD feeding compared with ND feeding (Fig. 2A). The proportion of CD11c$^+$F4/80$^+$ cells was significantly decreased by PG administration in HFD-fed mice. In contrast, the proportion of CD206$^+$F4/80$^+$ cells was not significantly altered by PG administration (Fig. 2B).

M1 macrophages and CLS number were decreased in adipose tissue of PG-administered mice.

Type of ATMs was assessed by immunostaining using anti-F4/80, anti-CD11c and anti-CD206 antibodies. In HFD-fed mice, CD11c$^+$F4/80$^+$ cells were observed less frequently in the PG-administered mice compared with the control group (Fig. S2A), but frequency of CD206$^+$F4/80$^+$ cells was not changed (Fig. S2B). As previously reported [15], CD11c$^+$F4/80$^+$ cells formed CLSs in the epididymal adipose tissue of HFD-fed mice (Fig. S2A), which were rarely observed in that

of ND-fed mice (data not shown). To analyze population of CLSs, we randomly counted CLSs in the immunostained adipose tissue sections. In HFD-fed mice, the CLS number of the PG-administered mice was significantly decreased compared with the control group (Fig. S2C). On the other hand, the $CD206^+F4/80^+$ cell number was not altered by PG administration (Fig. S2D).

PG improved glucose tolerance and serum insulin level.

To assess glucose homeostasis in HFD-fed mice administered with PG, we next performed glucose tolerance test. In HFD-fed mice with PG administration, a significant reduction in blood glucose levels was found at 15 and 30 min after glucose injection (Fig. 3A). Next, we measured fasting insulin levels in serum. As previously described [16], hyperinsulinemia in the HFD-fed mice was shown compared with the ND-fed mice (Fig. 3B). The HFD-fed mice with PG administration exhibited the significantly lower fasting insulin level compared with the control group.

Phosphorylation of Akt stimulated by insulin was enhanced in the liver and gastrocnemius skeletal muscle of HFD-fed mice with PG administration.

As reported previously, phosphorylated Akt is required for activation of insulin signaling [17]. Therefore, we investigated the effect of PG administration on phosphorylation of Akt stimulated with insulin in liver and gastrocnemius skeletal muscle. The band intensity of Akt in HFD-fed mice was comparable to those in ND-fed mice and PG did not alter the band intensity of Akt (Fig. 3A, B). In contrast, enhancement of phosphorylated Akt at Ser473 was observed in the PG-administered group of HFD-fed mice (Fig. 4A, B). After normalization with the band intensity of Akt, the amount of phosphorylated Akt in HFD-fed mice with DW administration was significantly lower than that of ND-fed mice. In addition, the amount of phosphorylated Akt in HFD-fed mice with PG-administered group was significantly higher than that in the control group (Fig. 4C, D).

Discussion

Previous studies have indicated that chronic inflammation is intensively involved in obesity and diabetes [1]. We have reported that PG has an anti-inflammatory effect on mouse models of various inflammatory diseases [11-13]. In addition, we reported that composition of gut microbiota was altered by oral PG administration [14]. In this study, we presented that PG

administration reduced adipocyte size in HFD-induced obesity mice. Gut microbiota is associated with inflammation and changes of adipocyte cell size in adipose tissue [18]. Reduction of adipocyte size may occurr by alteration of gut microbiota via PG administration. Additionally, we observed that oral PG administration suppressed mRNA expression of pro-inflammatory cytokines, TNF-α and IL-6, and chemokine such as CXCL2 in obese adipose tissue, suggesting that PG is able to prevent obesity-related inflammation. HFD-induced obesity is associated with increase in M1 macrophages which secrete pro-inflammatory mediators in ATMs [19]. In particular, M1 macrophages, which infiltrate into the adipose tissue of obese rodents and humans, are a major source for proinflammatory cytokines, such as TNF-α and IL-6 [20]. It has been reported that accumulation of M1 macrophages is involved in induction of insulin resistance and that pro-inflammatory cytokines secreted from M1 macrophages directly impair insulin action [21]. Indeed, treatment with omega-3 fatty acids or taurine decreases the ratio of M1 macrophages coincident with increased insulin sensitivity in obese mice [22,23]. Therefore, we analyzed the cells in SVF of epididymal adipose tissues from mice by flow cytometry and found that PG administration reduced the proportion of M1 macrophages in obese adipose tissue. Although significant differences of the body and tissue weights between the control and PG-treated mice

were not found, size of adipocytes was reduced in PG-administered HFD-fed mice. It has been

shown that decease of adipocyte size relates with reduction of inflammatory cytokine production

and M1 macrophage proportion [24]. To confirm the effect of PG on proportion of M1

macrophage proportion, we employed immunohistochemical analysis in obese adipose tissue.

The number of M1 macrophages and CLSs in obese adipose tissue was decreased by PG

administration. These results suggest that PG might decrease or inhibit differentiation or

infiltration of M1 macrophages in adipose tissue of HFD-fed mice. For insulin resistance, Akt is

a key mediator of glucose metabolism. Akt is clearly involved in glycogen synthesis and

suppression of gluconeogenesis in liver and skeletal muscle [25]. We demonstrated that PG

administration enhances insulin-stimulated phosphorylation of Akt in the liver and gastrocnemius

skeletal muscle. Moreover, PG administration improved glucose tolerance and decreased serum

insulin level in HFD-fed mice. These results suggest that PG administration improves the insulin

sensitivity and has potency to attenuate type 2 diabetes in obese mice.

In conclusion, we demonstrated that administration with PG reduces the production of pro-

inflammatory mediators and the number of M1 macrophages. In addition, PG is able to improve

hyperglycemia and insulin sensitivity in obese mice. Our study suggests that PG is a beneficial

material for prevention of obesity-induced disorders.

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Fig. 1. Effect of PG on mRNA expression of proinflammatory cytokines in HFD-fed mice.

Adipose tissue was obtained from ND-fed mice with DW (ND-DW), ND-fed mice with PG (ND-PG), HFD-fed mice with DW (HFD-DW) and HFD-fed mice with PG (HFD-PG) at (A) 4 weeks and (B) 8 weeks after starting of HFD feeding and PG administration. The mRNA expression of TNF-α, IL-6 and CXCL2 was estimated by qRT-PCR (n=7). Asterisks * and ** indicate that P value was less than 0.05 and 0.01, respectively.

Fig. 2. Effect of PG on macrophage phenotypes in the adipose tissues of HFD-fed mice. Cells in SVF were prepared from each group of mice (n=4) at 8 weeks after starting of HFD feeding and PG administration. The cells were stained with FITC-conjugated anti-mouse F4/80 mAb, PE-conjugated anti-mouse CD11c mAb and Alexa647-conjugated anti-mouse CD206 mAb. (A) F4/80$^+$CD11c$^+$ cells and (B) F4/80$^+$CD206$^+$ cells were assessed by flow cytometry. An asterisk *

indicates that *P* value was less than 0.05.

Fig. 3. Effect of PG on glucose tolerance and serum insulin levels in HFD-fed mice. (A) IPGTT was carried out at 14 weeks after starting of HFD feeding and PG administration (n= 6). (B) Serum insulin levels were determined at 12 weeks after starting of HFD feeding and PG administration (n=6). Asterisks * and ** indicate that *P* value was less than 0.05 and 0.01, respectively.

Fig. 4. Effect of PG on insulin-stimulated Akt phosphorylation in HFD-fed mice. Phosphorylated Akt in (A) the liver and (B) gastrocnemius skeletal muscle of HFD-fed and ND-fed mice at 12 weeks after starting of HFD feeding and PG administration. Intensity of bands was normalized and expressed as a ratio of pAkt/Akt in (C) the liver and (D) gastrocnemius skeletal muscle. Asterisks * and ** indicate that *P* value was less than 0.05 and 0.01, respectively.

Fig. S1. Effect of PG on HFD-induced obesity. Mice were fed HFD or ND from 4 weeks of age and they were administered with DW containing 0 or 0.2 mg/ml PG. (A) Body weight and food intake were monitored. (B) Mice were sacrificed and weights of adipose tissue and liver were

measured at 16 weeks after starting of HFD feeding and PG administration. (C) Adipocyte size distribution (%) in ND-fed mice received DW (ND-DW) or PG (ND-PG) and HFD-fed mice received DW (HFD-DW) or PG (HFD-PG) was shown (n=400).

Fig. S2. Immunohistochemical analysis of macrophage phenotypes in the adipose tissue of HFD-fed mice with or without PG. The adipose tissue of HFD-fed mice was obtained at 8 weeks after starting of HFD feeding and PG administration. The frozen sections of the tissue were stained with (A) FITC-conjugated anti-mouse F4/80 mAb and PE-conjugated anti-mouse CD11c mAb or (B) FITC-conjugated anti-mouse F4/80 mAb and Alexa647-conjugated anti-mouse CD206 mAb. These cells were counterstained with DAPI (blue). (C) CLSs were randomly counted from seven sections. (D) F4/80+CD206+ cells were randomly counted form 7 sections. P value less than 0.01 indicates a significant difference between the PG administered mice and DW control mice.

Table 1. Primers used to amplify target genes from cDNA

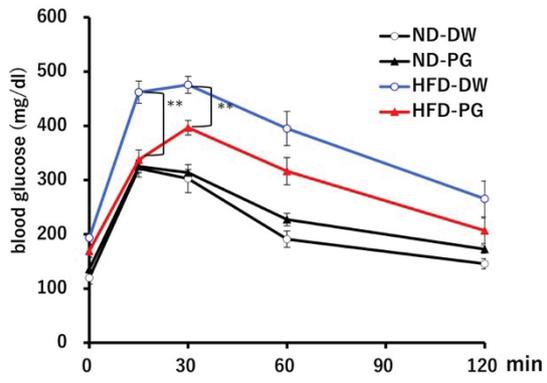| Gene	Primer	Sequence
TNF-α	Forward	5'-GGCAGGTCTACTTTGGAGTCATTGC-3'
	Reverse	5'-ACATTCGAGGCTCCAGTGAATTCGG-3'
IL-6	Forward	5'-TGGAGTCACAGAAGGAGTGGCTAAG-3'
	Reverse	5'-TCTGACCACAGTGAGGAATGTCCAC-3'
CXCL2	Forward	5'-GAACAAAGGCAAGGCTAACTGA-3'
	Reverse	5'-AACATAACAACATCTGGGCAAT-3'
GAPDH	Forward	5'-TGAAGGTCGGTGTGAACGGATTTGG-3'
	Reverse	5'-ACGACATACTCAGCACCAGCATCAC-3'

Fig. 1

Fig. 2

A

CD11c+ Fluorescence intensity

CD11c+ Macrophage

B

CD206+ Fluorescence intensity

CD206+ Macrophage

Fig. 3

A

B

Fig. 4

A   Liver

B   Muscle

C   Liver

D   Muscle

Fig. S1

Fig. S2

A

B

C

D