Anto-Obezity Effects of Granulocyte-Colony Stimulating Factor in Otsuka-Long-Evans-Tokushima Fatty Rats

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Abstract
Granulocyte-colony stimulating factor (G-CSF) has molecular structures and intracellular signaling pathways that are similar to those of leptin and ciliary neurotropic factor (CNTF). It also has immune-modulatory properties. Given that leptin and CNTF play important roles in energy homeostasis and that obesity is an inflammatory condition in adipose tissue, we hypothesized that G-CSF could also play a role in energy homeostasis. We treated 12 38-week-old male Otsuka-Long-Evans-Tokushima fatty rats (OLETF, diabetic) and 12 age-matched male Long-Evans-Tokushima rats (LETO, healthy) with 200 μg/day G-CSF or saline for 5 consecutive days. Body weight reduction was greater in G-CSF-treated OLETF (G-CSF/OLETF) than saline-treated OLETF (saline/OLETF) following 8 weeks of treatment (−6.9 ± 1.6% vs. −3.1 ± 2.2%, p < 0.05). G-CSF treatment had no effect on body weight in LETO or on food intake in either OLETF or LETO. Body fat in G-CSF/OLETF was more reduced than in saline/OLETF (−32.2 ± 3.1% vs. −20.8 ± 6.2%, p < 0.05). Energy expenditure was higher in G-CSF/OLETF from 4 weeks after the treatments than in saline/OLETF. Serum levels of cholesterol, triglyceride, interleukin-6 and tumor necrosis factor-α were lower in G-CSF/OLETF than in saline/OLETF. Uncoupling protein-1 (UCP-1) expression in brown adipose tissue (BAT) was higher in G-CSF/OLETF than in saline/OLETF, but was unaffected in LETO. Immunofluorescence staining and PCR results revealed that G-CSF receptors were expressed in BAT. In vitro experiments using brown adipocyte primary culture revealed that G-CSF enhanced UCP-1 expression from mature brown adipocytes via p38 mitogen-activated protein kinase pathway. In conclusion, G-CSF treatment reduced body weight and increased energy expenditure in a diabetic model, and enhanced UCP-1 expression and decreased inflammatory cytokine levels may be associated with the effects of G-CSF treatment.

Introduction
Granulocyte-colony stimulating factor (G-CSF) is a cytokine found in vertebrates that primarily acts as a regulator in the formation and development of neutrophils [1]. G-CSF is also a member of the class-1 cytokine superfamily. Many members of this superfamily have anti-obesity properties that take effect through changing appetite, energy expenditure or adipose tissue development. Such cytokines include granulocyte monocyte colony-stimulating factor (GM-CSF), ciliary neurotrophic factor (CNTF), interleukin-6 (IL-6), leukemia inhibitory factor (LIF) and leptin [2–9]. The molecular structure and intracellular signaling pathway of G-CSF receptors (G-CSFR) are similar to those of leptin, CNTF, LIF and IL-6 receptors [2,10]. Their intracellular signaling pathway commonly includes Janus kinase/signal transducer and activator of transcription (JAK/STAT) and mitogen-activated protein kinase (MAPK), which are also associated with appetite, adipose tissue development and energy homeostasis [2,11–13]. Obesity accompanies chronic systemic inflammation, characterized by pro-inflammatory cytokines including IL-6 and tumor necrosis factor-α (TNFα), which are also known to accelerate insulin resistance and other metabolic abnormalities of obesity [14–16]. G-CSF blunts the activation of major inflammatory cytokines, including TNFα and attenuates the harmful responses of inflammatory reactions [17,18]. Moreover, in our previous experiments on the effects of G-CSF on diabetic neuropathy, we found unexpected weight loss in Otsuka-Long-Evans-Tokushima fatty (OLETF) rats treated with G-CSF. For these reasons, we hypothesized that G-CSF might also play a role in regulating energy homeostasis and body weight.

Obesity is a disorder associated with an imbalance between food intake and energy expenditure. Appetite and energy expenditure are primarily governed by hypothalamic mediators such as neuropeptide Y (NPY), agouti-related peptide (AgRP), pro-opiomelanocortin (POMC) and cocaine-amphetamine regulated transcript (CART) [19]. Leptin and many other class-1 cytokines influence these hypothalamic mediators and reduce appetite or enhance energy expenditure [5,8,19–22]. The best-known mech-
anism of the hypothalamus to control energy expenditure via the 
anatomic nerve system is BAT non-shivering thermogenesis, in 
which uncoupling protein-1 (UCP-1) plays a key role [23]. Leptin 
and CNTF also enhance the thermogenesis of BAT [23,24]. 
Therefore, we investigated whether an alteration in body weight 
occurred after G-CSF treatment and whether it resulted from 
alterations in food intake or energy expenditure. We also 
investigated the effects of G-CSF treatment on appetite mediator 
expressions in the hypothalamus and UCP-1 expression in BAT to 
gain additional insight into alterations of food intake or energy 
expenditure.

Materials and Methods

Animals

This study was performed according to the ARRIVE guidelines 
for animal research [25]. The Hanyang University Institutional 
Animal Care and Use Committee approved all protocols. Male 
OLETF rats, characterized by hyperphagia due to a mutated 
choleystokinin-1 receptor that results in an obese diabetic 
phenotype, were used as the animal model for obesity. Male 
Long-Evans Tokushima (LETO) rats were used as healthy non-
diabetic controls of similar body size. The rats were supplied by 
the Tokushima Research Institute, Otsuka Pharmaceutical 
(Tokushima, Japan). OLETF rats are well-established animal 
models of obesity [26] and type 2 diabetes mellitus [27]. The 
avimals were maintained in the Hanyang University Medical 
School Animal Experiment Center in a specific, pathogen-free 
facility at a controlled temperature (23±2°C) and humidity 
(55±5%) with a 12-hour artificial light and dark cycle. All animals 
were provided ad libitum with standard rodent chow (20.14% 
protein, 13.12% moisture, 5.9% fat and 5.02% fiber; Lab Rodent 
Chow; 38057; Purina Korea Inc., Seoul, South Korea).

Animal models and experimental design

The experimental protocol is shown schematically in Figure 1. 
We obtained 12 OLETF and 12 LETO rats at 4 weeks of age and 
measured body weight and blood glucose every 4 weeks from 
10 weeks of age to 38 weeks of age. At 38 weeks, when the body 
weights of the OLETF and LETO rats approached a similar point, 
the OLETF and LETO groups were each randomly divided into 
two subgroups: G-CSF-treated OLETF rats (G-CSF/OLETF), 
saline-treated OLETF rats (saline/OLETF), G-CSF-treated 
LETO rats (G-CSF/LETO) and saline-treated LETO rats 
(saline/LETO). We then administered 200 μg/kg of human 
recombinant G-CSF (Leucostim; Dong-A Pharmaceutical, South 
Korea) intra-peritoneally for 5 days to the G-CSF-treated groups. 
The same volume of saline was administered to the saline-treated 
groups in the same manner. All animals were followed for 8 weeks 
after treatment. Body weight and food intake were measured daily. 
Energy expenditure was measured with an indirect calorimeter at 
the end of the week before the treatments and at the end of every 
week for 7 weeks after the treatments. Dual energy X-ray 
absorptiometry (DEXA) was measured in all animals at the week 
before treatment and 7 weeks after treatment. The animals were 
euthanized 8 weeks after the treatments and the expression of 
UCP-1 in BAT and of the neurotransmitters for appetite in the 
hypothalamus were analyzed. Blood samples for biochemical 
analyses were taken just before the animals were euthanized.

Analysis of biochemical profiles and inflammatory 
markers

Blood samples were taken from the tail vein after 8 hours of 
fasting. Sera were obtained by centrifugation and stored at −

Figure 1. Schematic description of the experimental protocol. 
Twelve OLETF and 12 LETO rats were prepared at 10 weeks of age and 
randomized into either a G-CSF or saline treatment group. All animals 
were treated with G-CSF or saline for 5 consecutive days and were 
followed for 8 weeks. Body composition was measured using dual 
energy X-ray absorptiometry, and energy expenditure was measured 
with an indirect calorimeter. OLETF, Otsuka Long-Evans Tokushima 
Fatty rats; LETO, Long-Evans Tokushima Otsuka rats; G-CSF, granulocyte-
colony stimulating factor; UCP-1, uncoupling protein-1; BAT, brown 
adipose tissue. 
70°C. Serum glucose, total cholesterol, triglyceride and free fatty 
acid (FFA) levels were measured using an Olympus AU400 auto 
analyzer (Olympus GmbH, Germany). Insulin, IL-1β, IL-6 and 
TNF-α were measured using a Milliplex Analyzer Luminex 200 
System (Millipore, USA) with a Rat Cardiovascular Disease Panel 
kit.

Body composition

The lean body and fat composition of the animals was measured 
by DEXA using a Hologic QDR 4500 device (Hologic Inc., USA) 
with an internal adapted collimator for small animal measure-
ments (Hologic QDR Software for Windows XP version). The 
scan field was adjustable to a maximum of 36 cm×18 cm and 
spatial resolution was approximately 1 mm. All animals were 
anesthetized with Ketalar® (75 mg/kg) and Rompun® (5–10 mg/
kg) before the measurement. After anesthesia, the animals were 
positioned ventrally on a reference film with all limbs extended.
Energy expenditure

VO₂ and VCO₂ were measured in all animals for 6 hours at the end of every week using an indirect calorimeter (Oxymax; Columbus Instruments, USA). Energy expenditure was calculated from the following formula provided by the manufacturer:

Energy expenditure (kcal) = 3.815 + 1.239 × (VCO₂/VO₂) × VO₂

The results were transformed into values for 24 hours and normalized by total body weight [kg].

Brown adipocyte isolation, culture and differentiation

Brown preadipocytes were obtained from the interscapular BAT of postnatal (1–2 days after birth) Sprague-Dawley rats. Isolation and culture of brown adipocytes were performed, as described previously [28]. The obtained tissues were dispersed in isolation buffer containing 100 μg collagenase (Sigma-Aldrich, South Korea) and 4% bovine serum albumin (BSA) (GenDEPOT, USA). The isolation buffer was composed of 0.123 M NaCl, 5 mM KCl, 1.3 mM CaCl₂, 5 mM glucose and 100 mM hydroxyethyl piperazineethanesulfonic acid (all Sigma-Aldrich, South Korea), and maintained at 4°C. After 45 minute incubation with shaking at 37°C, isolated cells were suspended briefly in a 1:3 mixture of erythrocyte lysis buffer (Lonza, USA) and maintenance media (Delbecco’s Modified Eagle’s Medium [DMEM] containing 1% penicillin and 20% fetal bovine serum [FBS] [all Sigma-Aldrich, South Korea]). After centrifuged at 1000 rpm for 5 minutes, isolated cells were cultured in the maintenance media at 37°C in a humidified atmosphere with 5% CO₂.

Brown preadipocytes were induced to differentiate to mature brown adipocytes 2 days after confluence (day 0), as described previously [29]. The cells were briefly exposed to media containing DMEM with 10% FBS and a differentiation cocktail previously [29]. The cells were briefly exposed to media containing 0.5 mM isobutylmethylxanthine, 0.5 mM 3-isobutyl-1-methylxanthine, 1 μM dexamethasone, 100 nM insulin, 125 μM indomethacin and 1 nM 3,3′,5-triodo-L-thyronine [T³] (Sigma-Aldrich, South Korea) and then placed in a maintenance medium composed of DMEM, 10% FBS, 1 mM T³ and 20 mM insulin. Maintenance media was replenished every 2 days.

Oil red-O staining for cultured brown adipocytes

Oil red-O (Sigma-Aldrich, South Korea) staining was used to identify the lipid droplets of mature brown adipocytes on day 0 and 4 after differentiation (day 4), as described previously [30].

Immunofluorescence stain for G-CSFR

Immunofluorescence staining for G-CSFR was performed to locate G-CSFR in BAT and on the surface of brown adipocytes. The interscapular BAT was obtained from a 20 week-old male OLETF rat. The BAT sections were blocked with 0.1% BSA containing 10% normal goat serum (NGS) (all Sigma-Aldrich, South Korea) for 1 hour, and were incubated for 90 minutes with mouse monoclonal anti-G-CSFR primary antibodies (1:100 dilution; Santa Cruz Biotechnology Inc., USA). The sections were washed and then incubated with fluorescein isothiocyanate-conjugated secondary antibody (1:500 dilution; Abcam, USA) for 60 minutes. Brown adipocytes were fixed with 4% paraformaldehyde. After washed, the cells were exposed to 0.3% TritonX-100 for 10 minutes at room temperature to be permeable. Then, the cells were blocked with 0.1% BSA containing 10% NGS (all Sigma-Aldrich, South Korea) for 1 hour. The cell were then, incubated for 2 hours with a monoclonal anti-G-CSFR antibody as the primary antibody (1:50 dilution; Santa Cruz Biotechnology Inc., USA). After washed again, the

cells were incubated with fluorescein isothiocyanate-conjugated secondary antibody (1:500; Abcam, USA) for 60 minutes. Images were obtained on an ECLIPSE 80i microscope equipped with an iAi progressive scan camera (Nikon, Tokyo, Japan) and CytoVision system software (Applied Imaging, UK).

G-CSF and p38 MAPK inhibitor treatment on brown adipocytes

Mature brown adipocytes were exposed to a maintenance media containing G-CSF for 30 minutes on day 4. The optimal G-CSF concentration was determined over multiple attempts by elevating the G-CSF concentration in log scale. In some experiments, mature brown adipocytes were pretreated with 10 μM p38 MAPK inhibitor (SB203580; Santa Cruz Biotechnology, USA) for 1 hour before G-CSF treatment.

PCR for appetite mediator, G-CSFR, brown adipocyte differentiation marker and UCP-1

Hypothalamis were obtained to perform quantitative PCR (Q-PCR) for NPY, POMC, CART and AgRP. RT-PCR for G-CSFR and Q-PCR for UCP-1 were performed in BAT, and cultured brown adipocytes were obtained on day 0 and day 4 for RT-PCR of G-CSFR, UCP-1, PR domain containing 16 (PRDM16), peroxisome proliferator-activated receptor-γ (PPARγ) and PPAR-γ co-activator 1-α (PGC-1α). RNA was extracted using Qiazol reagent (Qiagen, USA). The primers in the uses are shown in Table 1.

RT-PCR for G-CSFR, UCP-1, PRDM16, PPAR and PGC-1α.

RNA samples (1 μg) were reverse-transcribed with Moloney murine leukemia virus reverse transcriptase (Invitrogen Co, USA). PCR mixtures contained 1 μl cDNA, 2 μl 10X PCR buffer, 2 μl dNTP, 0.2 μl Taq DNA polymerase (iNtRON, South Korea) and 0.4 μl 25 pmol/μl of sense and anti-sense primers (Bioneer, South Korea). We performed PCR using a C1000TM Thermal Cycler (Bio-Rad, USA) under the following conditions: initial denaturation at 95°C for 5 minutes, 30 amplification cycles (denaturation at 95°C for 30 sec, annealing at 60°C for 30 sec, extension at 72°C for 1 minute) and final extension at 72°C for 3 minutes. The products were visualized on 2% agarose gels stained with ethidium bromide using Gel-Doc 2000 (Bio-Rad, USA). For quantification of UCP-1 expression levels in brown adipocytes, gels were scanned, and pixel intensity for each band was determined using Image J software (NIH Image, USA) and normalized to the intensity of GAPDH.

Q-PCR for NPY, POMC, CART, AgRP and UCP-1.

RNA samples (3 μg) were reverse-transcribed as above. Expression was quantified using a Light Cycler® 480 System (Roche, Switzerland) with a FastStart DNA Master SYBR Green 1 kit (Roche Diagnostics, USA). We performed PCR using the following steps: incubation for 10 minutes at 95°C followed by 45 cycles of 10 sec at 95°C, 10 sec at 60°C and 5 s at 72°C and a final dissociation curve step at 65°C for 15 sec. The crossing point of each PCR was automatically determined by the LightCycler® program. PCR for all samples was run in duplicate. The transcript levels were normalized against those of GAPDH.

Western blotting for UCP-1 and p38 MAPK activity

BAT was obtained from all of the animals for Western blotting for UCP-1. Mature brown adipocytes on day 4 were used to assess p38 MAPK activity by Western blotting. The samples were homogenized in ice-cold homogenization buffer (Pro-preb; iNtRON, South Korea). We transferred 50 μg proteins into a sample buffer that was then separated by 10% sodium dodecyl
sulfate polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes (0.45 μm pore size, Immobion-P; Bio-Rad, USA). After blocked in 5% skim milk solution for 60 minutes, the membrane was incubated with primary antibody for UCP-1 (1:500; Calbiochem, USA), followed by incubation with HRP-conjugated anti-rabbit antibody (1:2000; Jackson Immunoresearch, USA), as the secondary antibody. For p38 MAPK, we transferred 80 μg proteins. After the cells were blocked with 5% BSA for 60 minutes, the membrane was incubated with primary antibody for phosphorylated and total p38 MAPK (1:1000; Cell Signaling Technology, USA), followed by incubation with the secondary antibody. GAPDH was used as a protein loading control. Positive protein bands were visualized using an ECL kit (Amersham Pharmacia Biotech, Piscataway, NJ, USA). The results were quantified with an image analyzer (Image lab 3.0, Bio-Rad, Hercules, CA, USA).

### Statistical analysis

The results are presented as the mean ± standard error of the mean. Mann-Whitney U-tests were performed to compare the changes in body weights and food intakes of the G-CSF-treated groups and the saline-treated groups each week after the treatments and to compare the changes in body composition, biochemical measurements and UCP-1 expression of the G-CSF-treated groups and the saline-treated groups. Kruskal-Wallis tests were used to compare body weights pre-treatment and at 8 weeks after G-CSF or saline treatment and the expression of hypothalamic appetite mediators in all 4 groups. A *p*-value <0.05 was considered statistically significant. All statistical analyses were performed using SPSS statistics 20.0 for Windows (IBM, New York, USA).

### Results

**Body weight and food intake changes**

The body weight of the OLETF rats peaked at 22 weeks of age, then gradually decreased and reached a similar value to that of the LETO rats at 34 weeks of age. The body weight of the LETO rats peaked at 34 weeks of age and then decreased at a rate similar to that of the OLETF rats (Figure 2A, Table S1). Pre-treatment body weights were not significantly different in the 4 groups (Figure 2B).

**Table 1. Primers for PCR.**

| Primer | Sequences (5’ to 3’) | Size (bp) |
|-------|----------------------|-----------|
| UCP-1 | Forward CCG GTG CAT GTG GTA AAA AC | 242 |
| Reverse CTC CAA GTC GCC TAT GTG GT | |
| PRDM16 | Forward ACC ATG CAT TTT TAG ATG AGA AGG A | 526 |
| Reverse ATC ATT GCA TAT GCC TGG TTC TTA G | |
| PGC-1α | Forward CCT TAT TTG CTC AAA GAC CCC AAA G | 516 |
| Reverse CTT CAT TTT ATA TCA CCA TGA T | |
| PPARγ | Forward ACT CCC ATT CCT TTT AGA ACA TC | 265 |
| Reverse TCC CCA CAG ACT GGG CAC AC | |
| G-CSFR | Forward CCA TGG TCC ATC TTT GGG ATC | 234 |
| Reverse CCT GGA AGC TGT TGG TGT ATG | |
| GAPDH | Forward GAC CAT TTT GTT GGA GTG GA | 133 |
| Reverse AGT CAG GGA TGA TGT TCT CTT | |

UCP-1, uncoupling protein-1; PRDM16, PR domain containing 16; PPARγ, Peroxisome proliferator-activated receptor γ; PGC-1α, PPAR γ co-activator 1-α; G-CSFR, granulocyte-colony stimulating factor receptor; POMC, pro-opiomelanocortin; CART, cocaine amphetamine regulated transcript; NPY, neuropeptide Y; AgRP, agouti-related peptide; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

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greater body weight reductions were observed in the G-CSF/OLETF than in the saline/OLETF 4 weeks after treatment. On the contrary, body weight reductions were not significantly different between the G-CSF/LETO and saline/LETO groups in any week after treatment. The body weight reductions in the saline/OLETF were similar with those in the LETO rats (Figures 3A, Table S3). The percentage of body weight reduction from the pre-treatment body weight 8 weeks after treatment was higher in the G-CSF/OLETF than in the other groups, but was not significantly different in the G-CSF/OLETF, saline/LETO and G-CSF/LETO (Figures 3B). Food intakes in the OLETF rats were approximately twice those of the LETO rats; however, food intakes in all 4 groups were unaffected by treatment with G-CSF or saline (Figures 3C, Table S4).

Body composition changes
The differences in the percentage of body fat mass in the G-CSF/OLETF were greater than those in the saline/OLETF. In contrast, the differences in lean body mass percentages were not significantly different between the G-CSF-treated groups and the saline-treated groups in both OLETF and LETO rats (Figure 4, Table S5).

Energy expenditure changes
Energy expenditures in the OLETF rats did not change significantly for 3 weeks after the treatments. However, 4, 5 and 7 weeks after the treatments, energy expenditures were significantly higher in the G-CSF/OLETF than in the saline/OLETF. Energy expenditures in the G-CSF/LETO and saline/LETO were not different during the experimental period (Figure 5, Table S6).

Differences in biochemical and inflammatory markers
Biochemical and inflammatory markers of the G-CSF-treated group and the saline-treated group of both OLETF and LETO rats were compared at 8 weeks after treatment. Serum levels of total cholesterol and triglyceride in the G-CSF/OLETF were lower than the saline/OLETF, whereas serum levels of glucose, insulin and free fatty acids were not significantly different in the G-CSF/OLETF and saline/OLETF. None of the biochemical test results were different in the G-CSF/LETO and saline/LETO rats (Table 2). Serum levels of IL-1β, IL-6 and TNFα were higher in the saline/OLETF than those in the saline/LETO (IL-1β 32.6±13.5 pg/ml vs. 6.7±2.0 pg/ml, p = 0.037; IL-6 64.3±8.7 pg/ml vs. 3.9±2.5 pg/ml, p = 0.003; TNFα 5.1±1.0 pg/ml vs. 0.0±0.0 pg/ml, p = 0.002). Serum levels of IL-6 and TNFα in the G-CSF/OLETF rats were lower than those in the saline/OLETF, whereas serum levels of IL-1β were not different in the G-CSF/OLETF and saline/OLETF. There was no difference between serum levels of the inflammatory cytokines in the G-CSF/OLETF and those in the saline/LETO (Figure 6, Table S7).

mRNA expression of hypothalamic appetite mediators
Hypothalamic NPY mRNA levels were not different between the two OLETF groups, but were higher in the G-CSF/LETO than those in the saline/LETO. The mRNA levels of POMC were higher in the G-CSF-treated groups than that in the saline-treated groups in both OLETF and LETO rats. There were no significant differences in hypothalamic mRNA levels of AgRP and CART between the two groups of OLETF and LETO rats (Figure 7, Table S8).

Expression of UCP-1 and G-CSFR in BAT
Values in the G-CSF/OLETF were higher than those of the saline/OLETF 8 weeks after the treatments; however, there was no difference between the UCP-1 mRNA levels in BAT in the G-CSF/LETO and those in saline/LETO. UCP-1 protein levels in the G-CSF/OLETF were also higher than those in the saline/OLETF rats, whereas no difference was found in the G-CSF/LETO and the saline/LETO (Figures 8A and B, Table S9). G-CSFR mRNA was expressed in BAT (Figure 8C). The immunofluorescence staining for G-CSFR revealed that G-CSFR was mainly present in the islands of brown adipocytes in the interscapular BAT of a 20 week-old male OLETF rat (Figure 8D, E and F).

UCP-1 expression in brown adipocytes by G-CSF via p38 MAPK pathway
Because G-CSF treatment increased energy expenditure and UCP-1 expression in BAT in OLETF and G-CSFRs were expressed in BAT, we performed additional experiments using brown adipocyte primary culture whether G-CSF could directly enhance UCP-1 expression in brown adipocytes. After exposure to differentiation cocktail, brown preadipocytes differentiated into mature brown adipocytes containing lipid droplets and expressed differentiation indicators including PRDM16, PGC-1α, PPARγ and UCP-1 2 days after differentiation. UCP-1 expression intensities were decreased at 4 days after differentiation (day 4) and not observed at 6 days after differentiation (Figure 9A and B).

Figure 2. Body weight and blood glucose levels before G-CSF or saline treatment. (A) Body weights of the OLETF rats peaked at 22 weeks of age, then gradually decreased and were not significantly different with those of the LETO rats around 34 weeks of age; (B) Pre-treatment body weights at 38 weeks of age were not significantly different in all animal groups; (C) Blood glucose levels after 8 hours of fasting indicate the OLETF rats showed an overt diabetic phenotype after 30 weeks of age. - The data was presented as the mean ± S.E.M. OLETF, Otsuka Long-Evans Tokushima Fatty rats; LETO, Long-Evans Tokushima Otsuka rats; NS, not significant.
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G-CSFR mRNA was expressed in brown preadipocytes (day 0) and in mature brown adipocytes (day 4), and immunofluorescence staining demonstrated that G-CSFRs were present on brown preadipocytes and mature brown adipocytes (Figure 9C). UCP-1 expression was observed at 1 hour and 6 hours after brown adipocytes were exposed to 3 μg/mL (6.5 μM) G-CSF. The relative quantities of UCP-1 expression mRNA also significantly increased after 1 hour from G-CSF treatment and the UCP-1 protein levels were also increased after 1 hour from G-CSF treatment (Figure 9D). However, UCP-1 mRNA and protein expressions were not enhanced after G-CSF treatment when brown adipocytes were pretreated with p38 MAPK inhibitor (SB203580; Sigma-Aldrich, USA) (Figure 9E). p38 MAPK phosphorylation which was enhanced after G-CSF treatment, was repressed after G-CSF treatment, when the brown adipocytes were pretreated with SB203580 (Figure 9F).

Discussion

G-CSF is a member of the class-1 cytokine superfamily, many other members of which are known to play a role in energy homeostasis, and its intracellular signal pathways are similar to many cytokines that have known anti-obesity effects [1,2,11]. For example, IL-6, CNTF, LIF, GM-CSF and leptin are known to alter appetite, glucose and lipid metabolism, and energy expenditure [3–9]. We found, for the first time, that G-CSF treatment reduced body weight without altering appetite in OLETF rats. Unlike many inflammatory cytokines (such as IL-1 and IL-6) that have been identified as causing cachectic effects [31], the effect of G-CSF results not from cachectic effects, but from anti-obesity effects. Body weight reduction after G-CSF treatment was mainly due to a reduction in fat components. The muscle mass represented by the lean body components was not affected by G-CSF treatment. The body weight reduction was accompanied by some beneficial effects, including lower blood levels of total cholesterol and triglyceride and reduced levels of cytokines closely associated with fat inflammation, such as TNFα and IL-6 [14–16]. Increases in energy expenditure and UCP-1 expression in BAT...
were observed after G-CSF treatment in OLETF rats. It was also first found that brown preadipocytes, mature brown adipocytes and BAT expressed G-CAFR and that G-CSF enhanced UCP-1 expression in brown adipocytes via p38 MAPK pathway. This could be one of mechanisms to explain increased energy expenditure and UCP-1 expression in OLETF rats after G-CSF treatment.

It has been reported that the body weights of OLETF rats peak approximately 30 weeks of age and then gradually decrease [32]. In our data, the body weights of OLETF rats began to decrease at 26 weeks of age. Decreasing body weights of the OLETF rats at the time of treatment may cause concerns about the effects of G-CSF on the body weights of OLETF rats. We started the G-CSF and saline treatment at 38 weeks of age, because the body weights of both animals had approached at a similar level at 34 weeks of age and then decreased at a similar rate. The blood glucose levels in the OLETF rats also reached overt diabetic levels after 34 weeks of age. Because we had observed previously that G-CSF treatment does not affect the body weight of LETO rats, the healthy animal model without diabetes, it was important to start the treatments after the OLETF rats developed overt diabetes and to compare the effect of G-CSF on body weight in OLETF rats with that in LETO rats. The results show that the body weight reduction in G-CSF/OLETF was greater than that in saline/OLETF 4 weeks after the treatments, and the rates of body weight reduction were similar among the other animal groups.

The effect of G-CSF treatment was not observed in the LETO rats. Appetites were not affected by G-CSF treatment in the OLETF or LETO rats; however, the reductions of body weight and fat mass, the decreases in serum levels of cholesterol and triglyceride, the improvement in systemic inflammation and increases in energy expenditure and UCP-1 expression all appeared only in the G-CSF/OLETF rats. Although the body weights of both the OLETF and LETO rats were similar at the

Table 2. Biochemical test results after treatment with G-CSF or saline.

|                | OLETF            | Saline            | LETO             | G-CSF             | Saline            |
|----------------|------------------|-------------------|------------------|-------------------|-------------------|
| Glucose (mg/dl)| 208.8±20.6       | 198.5±21.8        | 109.5±2.4        | 107.8±2.5         |
| Total cholesterol (mg/dl) | 212.5±7.5*       | 254.8±10.9        | 110.3±2.9        | 115.2±6.9         |
| Triglyceride (mg/dl)        | 102.3±27.6*      | 161.7±39.4        | 360±9.4          | 323±1.8           |
| Free fatty acid (µEq/l)    | 675.8±38.1       | 698.5±24.7        | 577.5±46.4       | 554.7±31.8        |
| Insulin (mIU/l)            | 12.0±0.6         | 13.6±1.3          | 12.7±3.3         | 10.3±2.3          |
| HOMA-IR                   | 6.2±2.0          | 6.6±0.6           | 3.4±0.8          | 2.8±0.7           |

* p<0.05 compared to saline-treated groups.

- Data was shown as the mean ± S.E.M.
- G-CSF; granulocyte-colony stimulating factor; OLETF, Otsuka Long-Evans Tokushima Fatty rats; LETO, Long-Evans Tokushima rats.

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The potential mechanisms for G-CSF treatment effects could be associated with enhanced UCP-1 expression in BAT. It is well-known that the hypothalamus regulates BAT non-shivering thermogenesis via sympathetic innervations [23]. Norepinephrine signals from the sympathetic nerve enhance thermogenesis by multiple pathways, including the stimulation of lipolysis to fuel thermogenesis, the enhancement of UCP-1 expression and the decrease in brown adipocytes apoptosis [23,39]. Leptin stimulates sympathetic nerve signals to BAT via POMC expression in the hypothalamus [19,23]. Leptin receptors share many parts of intracellular domain structures and signaling pathways with G-CSFR [11,12]. However, unlike leptin treatment, G-CSF treatment does not affect appetite or hypothalamic appetite mediators other than POMC in OLETF rats. And, hypothalamic POMC levels are higher in the G-CSF-treated rats of both OLETF and LETO groups. Moreover, no data for the geographical distribution of hypothalamic G-CSFR are included in our study. Therefore, it is difficult to suggest that increased POMC are associated with the effect of G-CSF treatment and enhanced UCP-1 expression in BAT. Further experiments are necessary to illuminate whether the increased POMC expression was due to the G-CSF treatments.

We demonstrated that the BAT of OLETF rats expressed G-CSFRs. The intracellular signaling pathway of G-CSF includes JAK/STAT and p38 MAPK [11]. A recent study showed that BNP stimulates UCP-1 expression in BAT via the p38 MAPK pathway [40]. Norepinephrine stimuli also activate p38 MAPK to increase UCP-1 expression in BAT [39]. CNTF has its receptors on BAT, which are structurally similar to G-CSFR and directly activates UCP-1 expression in BAT via p42/44 MAPK [24]. Our in vitro experiments also revealed that brown adipocytes express G-CSFRs and that G-CSF-treated mature brown adipocytes expressed UCP-1 mRNA via p38 MAPK activation. Therefore, enhanced UCP-1 expression in BAT could be associated with G-CSF-mediated p38 MAPK activation.

Obesity is a low-grade systemic inflammatory condition mediated by cytokines such as TNF-α and IL-6 [14]. TNF-α has been reported to induce brown adipocytes apoptosis in primary culture, inhibit UCP-1 expression and mediate BAT atrophy in insulin resistance syndromes such as obesity and diabetes [37,41,42]. G-CSF attenuates the TNF-α response to systemic inflammation [17,18]. Similarly, we observed lower serum levels of IL-6 and TNF-α in G-CSF/OLETF than in saline/OLETF. Therefore, this immune-modulatory effect of G-CSF might also have ameliorated BAT atrophy and contributed to body weight reductions in the diabetic model.

The major limitation of our study is that the detailed mechanisms of the body weight reduction effect of G-CSF were not sufficiently explored. In vitro experiments with brown adipocytes may partly resolve the question of the enhanced UCP-1 expression in BAT. However, the influence of the immune-modulatory function of G-CSF on BAT still requires further histological and molecular investigation. Long-lasting effects of G-CSF on body weight and energy expenditure are also difficult to explain from our data. G-CSF serum levels significantly decreased only several hours after intra-peritoneal G-CSF injection [43], it is difficult to suggest that the effects of G-CSF treatment are associated with metabolic effects of circulating G-CSF. However, we reported that diabetes-related conditions,
diabetic nephropathy, steatohepatitis and diabetic cardiomyopathy were improved in OLETF rats 4 weeks after G-CSF treatment in the same manner [44–46], and the long-lasting effects of G-CSF in this study were also similar to those in our previous reports. Further investigations are necessary to determine the long-lasting effects of G-CSF. Genetic lack of cholecystokinin-1 receptor in OLETF rats induced the animal into a hyperphagic state [47]. The OLETF rat model is similar to the model of type 2 diabetes with mild obesity [48]. However, the lack of cholecystokinin receptors affects hypothalamic NPY and POMC expressions in OLETF rats [49]. Since UCP-1 expression is influenced by these hypothalamic appetite mediators, the effect of G-CSF on UCP-1 expression and energy expenditure could be different in other diabetic animal models. In addition, G-CSF action on the feeding behavior could be also affected in OLETF rats. Therefore, the effects of G-CSF on body weight should be also tested in other diabetic animal models.

In conclusion, G-CSF has anti-obesity effects in an animal model with diabetes and obesity. Treatment with G-CSF reduces body weight and fat components, improves lipid abnormalities and ameliorates systemic inflammation without altering appetite. The anti-obesity effect of G-CSF is not associated with improved insulin resistance or glycemic control but is associated with increased energy expenditure and enhanced UCP-1 expression in BAT in a diabetic animal model. The effects of G-CSF treatment on UCP-1 expression in BAT could be mediated by G-CSFR and p38 MAPK pathway or immune-modulatory function of G-CSF. Further experiments are needed to illuminate in detail the mechanisms of the effects of G-CSF on body weight reduction.

Supporting Information

Table S1 Body weight before the treatments of G-CSF or saline. (XLSX)
Table S2 Blood glucose level before the treatments of G-CSF or saline. (XLSX)
Table S3 Body weight changes after the treatments of G-CSF and saline. (XLSX)
Table S4 Weekly average food intakes after the treatments of G-CSF or saline. (XLSX)
Table S5 Changes in body composition after the treatments of G-CSF and saline. (XLSX)
Table S6 Energy expenditure changes after the treatments of G-CSF or saline. (XLSX)
Table S7 Serum inflammatory cytokines after the treatments of G-CSF and saline. (XLSX)
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Author Contributions

Conceived and designed the experiments: YGL KSK. Performed the experiments: YSS CHF BIS [JPY HWJ] HGP GYS. Analyzed the data: YGL YSS JHS HK KSK. Contributed reagents/materials/analysis tools: HK YHA KSK. Wrote the paper: YGL. Helped to make a major draft for the manuscript: JHS AYH KHK.

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