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Expression Profile-Dependent Improvement of Insulin Sensitivity by Gene Delivery of Interleukin-6 in a Mouse Model of Type II Diabetes

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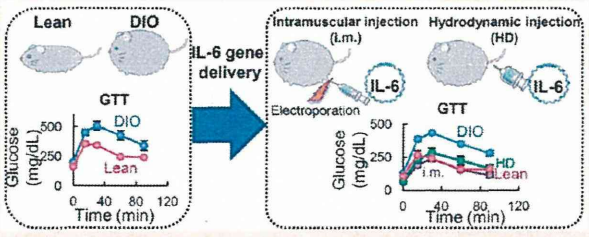
ABSTRACT: Type II diabetes is one of the most problematic metabolic disorders and is associated with secondary conditions such as heart disease and eye complications. Interleukin-6 (IL-6), a multifunctional cytokine, could influence conditions of altered glucose metabolism such as insulin resistance in diabetic patients. However, a consensus about the role of IL-6 on glucose metabolism has not been reached. The aim of the present study is to investigate whether the expression of IL-6 affects glucose metabolism in diet-induced obesity (DIO) mice, a model of type II diabetes and obesity, using gene delivery of IL-6. DIO mice received hydrodynamic or intramuscular injection of IL-6-expressing plasmid to investigate the importance of the site of IL-6 expression. DIO mice that received a sustained IL-6 gene transfer showed similar glucose levels to lean mice in a glucose tolerance test. DIO mice exhibited reduced food intake and low body and epididymal fat weights after IL-6 gene transfer. IL-6 gene delivery reduced the mRNA expression of metabolism-related genes in the liver, skeletal muscle, and adipose tissue of DIO mice. The metabolic status of DIO mice receiving intramuscular injections was moderately better than that of DIO mice receiving hydrodynamic injections. The infiltration of inflammatory cells into the sites where the IL-6-expressing plasmid DNA was delivered was observed. Transient expression of IL-6 had limited effects on all parameters examined. These results indicate that the expression of IL-6 has an effect on obesity and the metabolism of glucose and lipid in diabetic mice and that the expression site of IL-6 is not an important factor.



KEYWORDS: *IL-6, insulin resistance, plasmid DNA, skeletal muscle, liver*

INTRODUCTION

Type II diabetes is one of the most problematic metabolic disorders because of its associated conditions, such as heart diseases and eye complications. Physical exercise and diet therapy are the first choices to treat type II diabetes patients.[1] However, the progression of the disease results in an increase in the number of patients who have to take oral medications. Even at this stage, exercise therapy is effective in increasing the therapeutic effect of oral medications such as acarbose, rosiglitazone, and losartan.[1−3] In addition to increasing energy expenditure, which is effective in losing weight, physical exercise also produces various changes in the body. Recently, it has been found that the concentrations of several cytokines in the serum increase during physical exercise.[4,5]

Of the cytokines that are reported to increase during exercise, interleukin (IL)-6 is produced in working muscles, which results in an increase in the blood concentration of IL-6 up to 100-fold.[6] Considering the fact that exercise improves glucose metabolism, such as insulin sensitivity, and that IL-6 is a typical cytokine induced by exercise, a close relationship between IL-6 and glucose metabolism has been suggested.[7−10] The beneficial effects of IL-6 in diabetes, such as an increase in insulin sensitivity, improvement in the obese state, and an increase in central leptin action, have been demonstrated by several studies.[11−14] However, Klover et al. reported that chronic exposure to IL-6 reduced insulin sensitivity in the liver.[15] Because previous in vitro studies reported that IL-6 increased the insulin sensitivity of muscle cells but reduced the sensitivity of hepatocytes,[16−21] cell-type-specific responses to IL-6 may be a reason for the controversy regarding the effect of IL-6 on insulin sensitivity. In addition, the different periods of IL-6 exposure (i.e., the different IL-6 pharmacokinetic profiles between the different studies) may also account for the controversy. However, the relationship between the time profile of IL-6 exposure and its effect on glucose metabolism has not been investigated in detail in whole animal models.

In the present study, we investigated the relationship between the expression profile of IL-6 and the effect of IL-6 in DIO mice. To obtain different pharmacokinetics of IL-6, we used two types of IL-6-expressing plasmid vectors, each of which shows transient or sustained transgene expression. The effects of IL-6 gene transfer on glucose and lipid homeostasis in the whole body as well as the metabolic status in skeletal muscle, liver, and adipose tissue were investigated to evaluate the pharmacodynamics of IL-6. In addition, to investigate

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whether the production site of IL-6 is important for its effect on insulin sensitivity, the IL-6 transgene was delivered to the liver or skeletal muscle.

EXPERIMENTAL SECTION

Animals. Four-week-old male C57BL/6J mice were purchased from Japan SLC, Inc. (Shizuoka, Japan). Mice were fed a high-fat diet (D12492-RodentDiet with 60 kcal % fat, Research Diets, New Brunswick, Canada) or a normal diet (D12450B-RodentDiet with 10 kcal % fat, Research Diets) for 12 weeks to obtain DIO and lean mice, respectively. All protocols for the animal experiments were approved by the Animal Experimentation Committee of the Graduate School of Pharmaceutical Science of Kyoto University.

Plasmid DNA Construction. mRNA was extracted from RAW264.7 cells using Sepasol RNA I super (Nacalai Tesque, Kyoto, Japan), and a cDNA sample was prepared using SuperScript II (Invitrogen, Carsbad, CA, USA). An IL-6 cDNA fragment was amplified from the RAW264.7 cDNA sample and subcloned into the EcoR V/Xba I sites of the pcDNA 3.1 vector (Invitrogen) to construct pCMV-IL-6. pCpG-IL-6 was constructed by inserting the murine IL-6 cDNA fragment from pCMV-IL-6 into the Nhe I/Nco I sites of the pCpG-mcs vector (Invivogen, San Diego, CA, USA). pcDNA3.1 and pCpG-mcs, which encode no cDNA, were used as mock vectors for pCMV-IL-6 and pCpG-IL-6, respectively.

Plasmid DNA Administration. Mice received an intramuscular or hydrodynamic injection of naked plasmid DNA (pDNA) for the delivery of the pDNA to the skeletal muscle or liver, respectively. For intramuscular gene transfer, mice received an intramuscular injection of 10 units of hyaluronidase (Sigma-Aldrich, St. Louis, MO, USA) into the gastrocnemius muscle of both hindlegs 2 h before pDNA administration to increase the delivery efficiency of pDNA.[22] Next, the indicated dose of pDNA dissolved in saline was injected into the muscle of both hindlegs. Immediately after the injection, electroporation was applied to each injection site (175 V/cm, 20 ms, 2 Hz, 10 pulses) using a pair of 1 cm^2 forceps-type electrodes connected to a rectangular direct-current generator (CUY-21, Nepagene, Chiba, Japan). Because our preliminary study showed that the intramuscular delivery of the pCMV vector could obtain a higher level of transgene expression than that of the pCpG vector and that the duration of transgene expression was comparable between these two vectors, the pCMV vector was used for the intramuscular gene delivery. For the hepatic gene transfer, the indicated dose of pDNA was dissolved in saline and injected into the tail vein of mice over a period of 5 s.[23] To obtain a transient or continuous transgene expression profile after hepatic gene transfer, the pCMV vector or pCpG vector was utilized, respectively.[24,25]

Measurement of the Serum Concentrations of IL-6, Insulin, Nonesterified Fatty Acids (NEFA), Triglyceride (TG), Cholesterol, and Amyloid A. Blood samples were obtained from the tail vein of mice at the indicated times after gene transfer, kept at 4 °C for 2 h to allow clotting, and centrifuged at 8000*g* for 20 min to obtain serum. The concentration of IL-6 in the serum was measured by an enzyme-linked immunosorbent assay (ELISA) using OptEIA sets (BD Biosciences, San Diego, CA, USA). The concentration of insulin was measured using an ELISA kit (Mercodia, Uppsala, Sweden). The concentration of NEFA, TG, and cholesterol was measured using a kit (Wako Pure Chemical Industries, Osaka, Japan). The concentration of serum amyloid A (SAA) was measured using an SAA Mouse ELISA Kit (Invitrogen).

mRNA Quantification. Total RNA was extracted from approximately 100 mg tissue samples using Sepasol RNA I Super (Nakalai Tesque). After the removal of contaminating DNA by DNase I (Takara Bio, Shiga, Japan), reverse transcription was performed using a ReverTra Ace qPCR RT kit (TOYOBO, Osaka, Japan) followed by ribonuclease H treatment (Takara Bio). For a quantitative analysis of mRNA expression, real-time polymerase chain reaction (PCR) was carried out with total cDNA using the Kapa SYBR FAST ABI Prism 2× qPCR Master Mix (Kapa Biosystems, Boston, MA, USA). The oligonucleotide primers used for amplification were IL-6 (forward) 5′-GTTCTCTGGGAAATCGTGGA-3′ and (reverse) 5′-TGTACTCCAGGTAGCTATGG-3′; suppressors of cytokine signaling 3 (SOCS-3) (forward) 5′-AAGGGAGGCAGATCAACAGA-3′ and (reverse) 5′-TGGGACAGAGGGCATTTAAG-3′; pyruvate kinase 1 (PK-1) (forward) 5′-AAGACAGTGTGGGTGGACTACCA-3′ and (reverse) 5′-CGTCAATGTAGATGCGGCC-3′; pyruvate kinase 3 (PK-3) (forward) 5′-GCCGCCTGGACATTGACTC-3′ and (reverse) 5′-CATGAGAGAAATTCAGCCGAG-3′; glucose-6-phosphatase (G6Pase) (forward) 5′-TCGGAGACTGGTTCAACCTC-3′ and (reverse) 5′-AGGTGACAGGGAACTGCTTTAT-3′; phosphoenolpyruvate carboxykinase (PEPCK) (forward) 5′-GGTGTTTACTGGGAAGGCATC-3′ and (reverse) 5′-CAATAATGGGGCACTGGCTG-3′; glycogen synthase 1 (GYS-1) (forward) 5′-GAACGCAGTGCTTTTCGAGG-3′ and (reverse) 5′-CCAGATAGTAGTTGTCACCCCAT-3′; glycogen synthase 2 (GYS-2) (forward) 5′-ACCAAGGCCAAAACGACAG-3′ and (reverse) 5′-GGGCTCACATTGTTCTACTTGA-3′; long chain acyl-CoA dehydrogenase (LCAD) (forward) 5′-TCTTTTCCTCGGAGCATGACA-3′ and (reverse) 5′-GACCTCTCTACTCACTTCTCCAG-3′; peroxisome proliferator-activated receptor α (PPAR-α) (forward) 5′-TGTCGAATATGTGGGGACAA-3′ and (reverse) 5′-AATCTTGCAGCTCCGATCAC-3′; peroxisome proliferator-activated receptor γ (PPAR-γ) (forward) 5′-CCCACCAACTTCGGAATCA-3′ and (reverse) 5′-TGCGAGTGGTCTTCCATCAC-3′; acetyl-CoA carboxylase 1 (ACC-1) (forward) 5′-GCCTCTTCCTGACAAACGAG-3′ and (reverse) 5′-TGACTGCCGAAACATCTCTG-3′; sterol regulatory element-binding protein 1c (SREBP-1c) (forward) 5′-CCCTGTGTGTACTGGCCTTT-3′ and (reverse) 5′-TTGCGATGTCTCCAGAAGTG-3′; uncoupling protein 2 (UCP-2) (forward) 5′-GCCTCTGGAAAGGGACTTCTC-3′ and (reverse) 5′-ACCAGCTCAGCACAGTTGACA-3′; and cyclophilin (forward) 5′-GGAGATGGCACAGGAGGAA-3′ and (reverse) 5′-GCCCGTAGTGCTTCAGCTT-3′. Amplified products were detected via the intercalation of the fluorescent dye using a StepOnePlus Real Time PCR System (Applied Biosystems, Foster City, CA, USA). The mRNA expression of target genes was normalized against the mRNA level of cyclophilin.

Glucose Tolerance Test. At the indicated time points after IL-6 gene transfer, glucose tolerance tests (GTTs) were performed. In brief, mice received an intraperitoneal injection of glucose (1 g/kg body weight) after a 12 h period of starvation. The glucose concentration in the blood was then measured using an automatic glucose analyzer (Accucheck, Roche Diagnostics, Indianapolis, IN, USA).

Histological Analysis. Mice were killed on day 14 after gene transfer, and the gastrocnemius muscle, liver, and epididymal fat samples were collected, fixed in 4% paraformal-

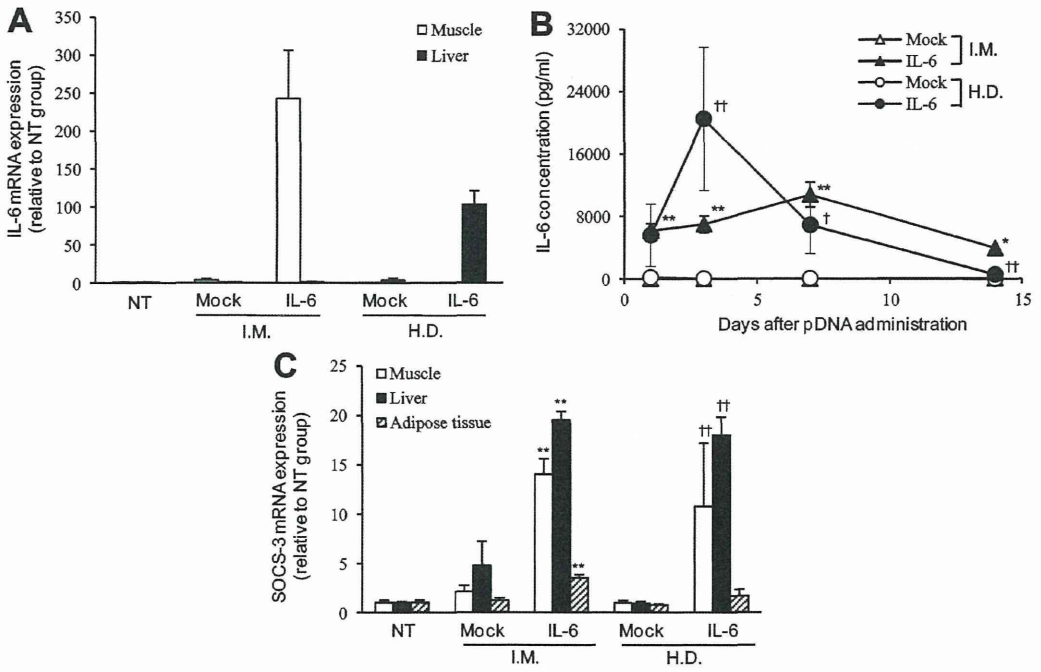

Figure 1. IL-6 and SOCS-3 expression after gene delivery into muscle and liver. DIO mice received an intramuscular injection (I.M.) of pcDNA3.1 (mock) (200 μg/mouse) or pCMV-IL-6 (200 μg/mouse) or a hydrodynamic injection (H.D.) of pCpG-mcs (mock) (0.75 μg/g bodyweight) or pCpG-IL-6 (0.75 μg/g bodyweight). (A) mRNA expression of IL-6 in the muscle and liver on day 14. The results are expressed as the mean + SE of five mice except for the H.D. injection in the pCpG-IL-6 group (n = 3). (B) Time course of the concentration of IL-6 in the serum after gene delivery. The results are expressed as the mean ± SE of five mice except for the H.D. injection in the pCpG-IL-6 group (n = 3). (C) mRNA expression of SOCS-3, an IL-6-induced gene, in the muscle, liver, and adipose tissue on day 14. The results are expressed as the mean + SE of five mice except for the H.D. injection in the pCpG-IL-6 group (n = 3). $^{**}P < 0.01$ compared with the mock intramuscular injection group and $^{\dagger}P < 0.05$ and $^{\dagger\dagger}P < 0.01$ compared with the mock hydrodynamic injection group.

dehyde, embedded in paraffin, sectioned, and stained with hematoxylin and eosin (HE). The stained samples were viewed under a microscope (Biozero BZ-8000, KEYENCE) for histological evaluation.

Statistical Analysis. Differences were statistically evaluated by student's t test. A P value of less than 0.05 was considered to be statistically significant. The area under the serum concentration–time curve (AUC) and the mean retention time (MRT) were calculated using a moment analysis method.[26] These parameters were calculated for each animal.

RESULTS

IL-6 Expression and IL-6-Induced SOCS-3 Expression in DIO Mice after IL-6 Gene Delivery. To obtain sustained IL-6 expression in the skeletal muscle (gastrocnemius muscle) and in the liver, mice received an intramuscular injection of pCMV-IL-6 or a hydrodynamic injection of pCpG-IL-6, respectively. IL-6 mRNA expression was increased in the skeletal muscle or liver of mice that received an intramuscular or a hydrodynamic injection of IL-6-expressing pDNA, respectively (Figure 1A). IL-6 mRNA expression in the liver was hardly increased in the mice that received an intramuscular injection of IL-6-expressing pDNA. On the contrary, the mRNA expression in the muscle was hardly increased in the mice that received a hydrodynamic injection of IL-6 expressing pDNA. These mice showed a sustained level of IL-6 in the serum for at least 2 weeks after gene delivery (Figure 1B). The AUC values of the IL-6 concentration in the serum were 100 ± 20 and 83 ± 15 ng/(mL·day) in mice that received an intramuscular or a hydrodynamic injection of IL-6-expressing pDNA, respectively. The MRT values were 6.3 ± 0.4 and 3.8 ± 0.6 days for the intramuscular or hydrodynamic group, respectively. As an indicator of the local concentration of IL-6 in the skeletal muscle, liver, and epididymal adipose tissue, we measured the mRNA expression of SOCS-3 in these tissues because SOCS-3 is a typical IL-6-induced gene. IL-6 gene delivery significantly increased the SOCS-3 mRNA expression in skeletal muscle and liver to a similar extent in both cases (Figure 1C). However, the SOCS-3 mRNA expression in adipose tissue was greatly increased after intramuscular injection of IL-6-expressing pDNA, whereas it was slightly, but not significantly, increased after hydrodynamic injection of IL-6-expressing pDNA.

Effect of IL-6 Gene Delivery on GTTs and Serum Insulin Concentration. GTTs were performed on days 7 and 13 after gene transfer (Figure 2). After an intraperitoneal injection of glucose, the untreated and mock-treated DIO mice showed higher glucose levels over the time periods studied than the lean mice. In contrast, the DIO mice that received a hydrodynamic and intramuscular injection of IL-6-expressing pDNA showed similar glucose levels to those of the lean mice, which implies the improvement of insulin sensitivity by IL-6 gene delivery. The serum concentration of insulin in the untreated DIO mice and mock-treated DIO mice was higher than that in the lean mice. IL-6 gene delivery reduced the serum insulin concentration in DIO mice, which suggests the improvement in their metabolic status by IL-6 gene delivery.

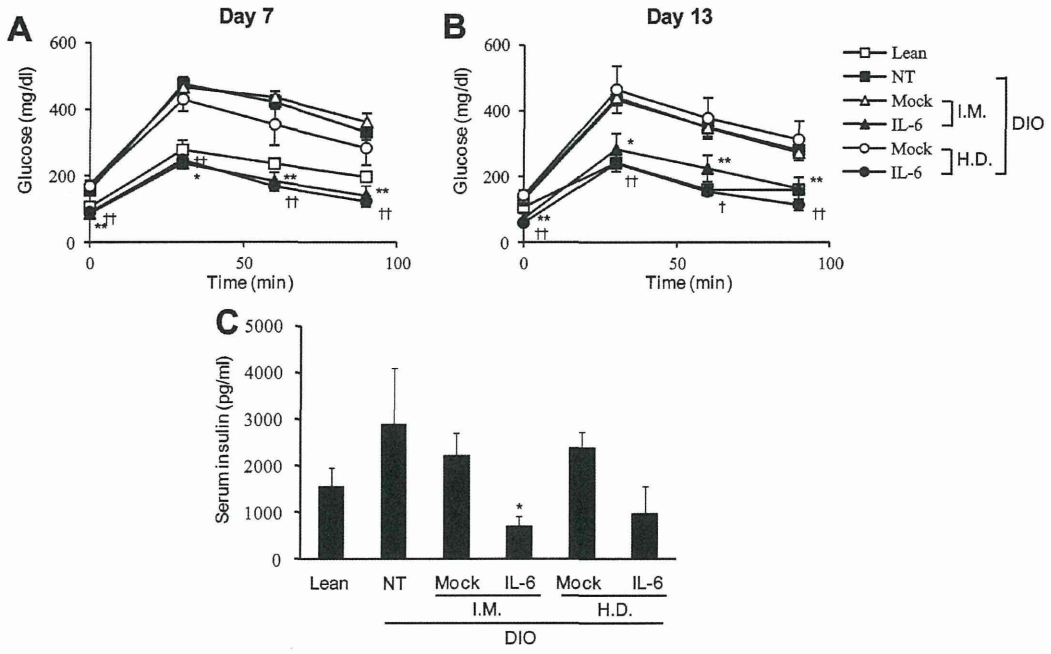

Figure 2. Effect of IL-6 gene delivery into the muscle and liver on the glucose tolerance test. DIO mice received an intramuscular injection (I.M.) of pcDNA3.1 (mock) (200 μg/mouse) or pCMV-IL-6 (200 μg/mouse) or a hydrodynamic injection (H.D.) of pCpG-mcs (mock) (0.75 μg/g bodyweight) or pCpG-IL-6 (0.75 μg/g bodyweight). (A, B) On (A) day 7 and (B) day 13, glucose tolerance tests were performed. After a 12 h fast, the mice received an intraperitoneal injection of glucose (1 g/kg), and the glucose concentration was determined in tail blood samples. The results are expressed as the mean ± SE of five mice except for the H.D. injection in the pCpG-IL-6 group ($n = 3$). (C) On day 14, the serum concentration of insulin was measured by ELISA. The results are expressed as the mean + SE of five mice except for the I.M. injection of pCMV-IL-6 group ($n = 4$) and the H.D. injection in the pCpG-IL-6 group ($n = 3$). *$P < 0.05$ and **$P < 0.01$ compared with the mock intramuscular injection group. †$P < 0.05$ and ††$P < 0.01$ compared with the mock hydrodynamic injection group.

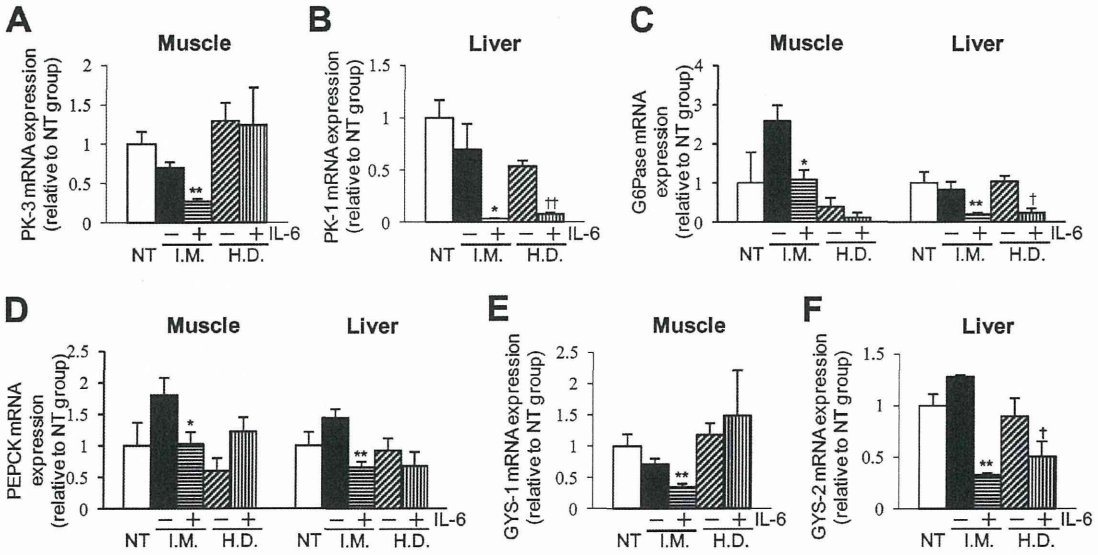

Figure 3. Effect of IL-6 gene delivery on the mRNA expression of glucose metabolism-related genes in the muscle and liver. DIO mice received an intramuscular injection (I.M.) of pcDNA3.1 (mock) (200 μg/mouse) or pCMV-IL-6 (200 μg/mouse) or a hydrodynamic injection (H.D.) of pCpG-mcs (mock) (0.75 μg/g bodyweight) or pGpG-IL-6 (0.75 μg/g bodyweight). (A, B) mRNA expression of (A) PK-3 and (B) PK-1 in the muscle and liver on day 14, respectively. (C, D) mRNA expression of (C) G6Pase and (D) PEPCK in the muscle and liver on day 14. (E, F) mRNA expression of (E) GYS-1 in the muscle and (F) GYS-2 in the liver on day 14. The results are expressed as the mean + SE of five mice except for the H.D. injection in the pCpG-IL-6 group ($n = 3$). *$P < 0.05$ and **$P < 0.01$ compared with the mock intramuscular injection group. †$P < 0.05$ and ††$P < 0.01$ compared with the mock hydrodynamic injection group.

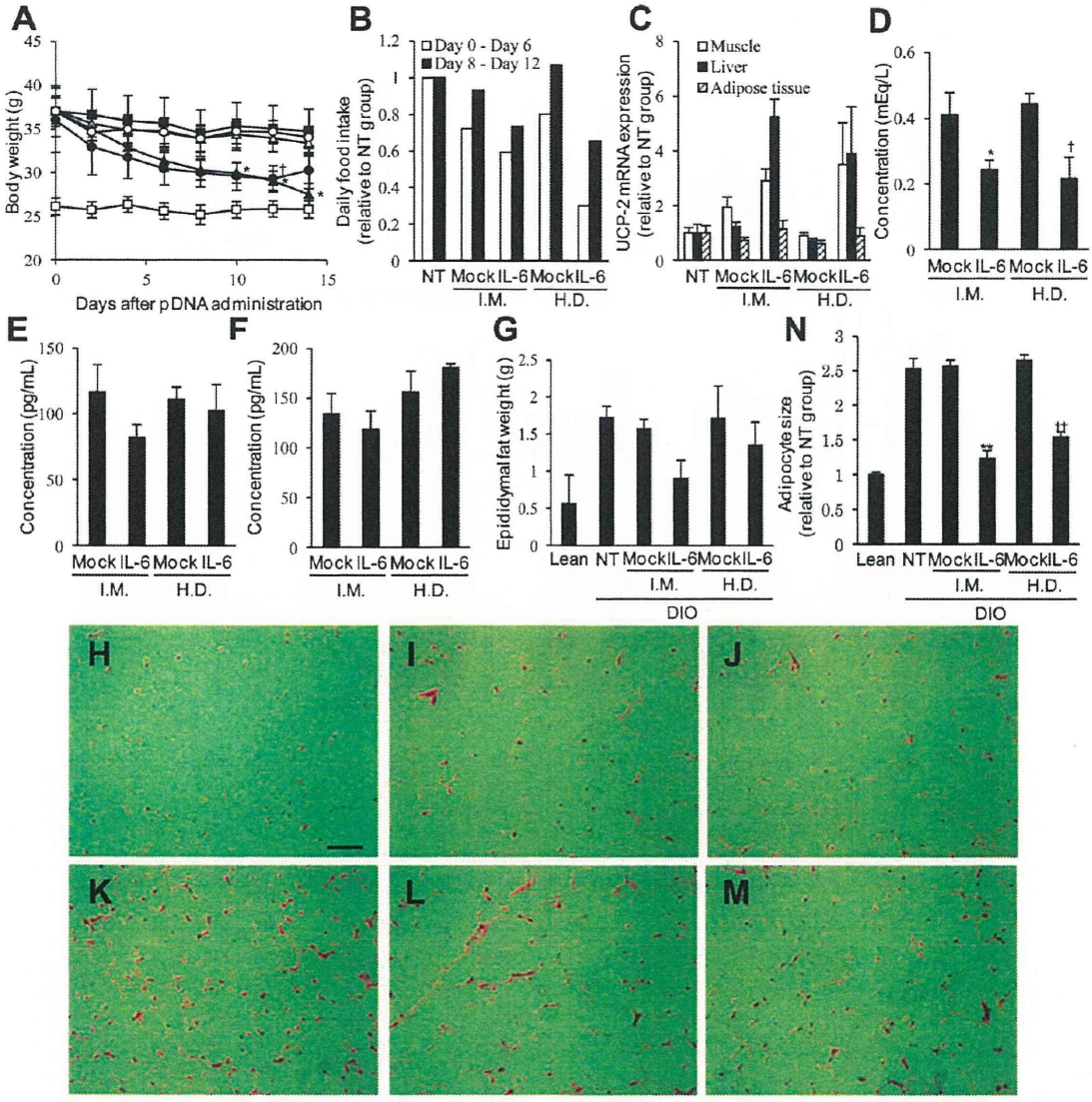

Figure 4. Effect of IL-6 gene delivery on the obesity of DIO mice. DIO mice received an intramuscular injection (I.M.) of pcDNA3.1 (mock) (200 μg/mouse) or pCMV-IL-6 (200 μg/mouse) or a hydrodynamic injection (H.D.) of pCpG-mcs (mock) (0.75 μg/g bodyweight) or pGpG-IL-6 (0.75 μg/g bodyweight). (A) Body weight change of lean mice (open square), untreated DIO mice (closed square), and DIO mice treated with an intramuscular injection of mock vector (open triangle), an intramuscular injection of pCMV-IL-6 (closed triangle), a hydrodynamic injection of mock vector (open circle), or a hydrodynamic injection of pCpG-IL-6 (closed circle). The results are expressed as the mean ± SE of five mice except for the H.D. injection in the pCpG-IL-6 group (n = 3). (B) Relative food intake from days 0–6 and 8–12. The results are expressed as the mean relative value to the no-treatment group. (C) mRNA expression of UCP-2 in the muscle, liver, and adipose tissue on day 14. The results are expressed as the mean + SE of five mice except for the H.D. injection in the pCpG-IL-6 group (n = 3). (D) Serum concentration of NEFA on day 14. The results are expressed as the mean + SE of five mice except for the H.D. injection in the pCpG-IL-6 group (n = 3). (E) Serum concentration of TG and cholesterol on day 14. The results are expressed as the mean + SE of four mice except for the H.D injection in the mock groups (n = 5). (F) Serum concentration of cholesterol on day 14. The results are expressed as the mean + SE of four mice except for the H.D injection in the mock groups (n = 5). (G) Epididymal fat weight on day 14. The results are expressed as the mean + SE of five mice except for the H.D. injection in the pCpG-IL-6 group (n = 3). (H–M) Representative images of HE-stained adipose tissue sections of (H) lean mice and DIO mice that were (I) untreated or (J) treated with an intramuscular injection of mock vector, (K) an intramuscular injection of pCMV-IL-6, (L) a hydrodynamic injection of mock vector, or (M) a hydrodynamic injection of pCpG-IL-6. The scale bar is 100 μm. (N) Relative adipocyte size calculated from the adipocyte numbers per field on day 14. The results are expressed as the mean + SE of five images. *P < 0.05 and **P < 0.01 compared with the mock intramuscular injection group. †P < 0.05 and ††P < 0.01 compared with the mock hydrodynamic injection group.

Effect of IL-6 Gene Delivery on the mRNA Expression of Glucose Metabolism-Related Genes in the Skeletal Muscle and Liver. To investigate whether IL-6 improves glucose metabolism via the regulation of glucose metabolism-related genes at molecular level, we examined their mRNA expression. The mRNA expression level of all of the genes investigated significantly decreased in the skeletal muscle and liver following the intramuscular injection of IL-6-expressing

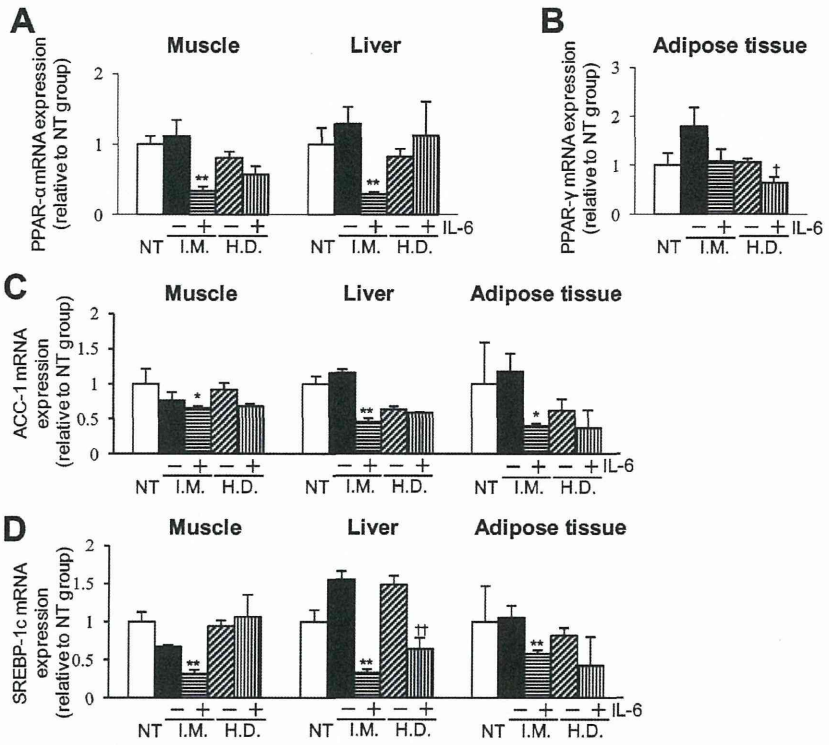

Figure 5. Effect of IL-6 gene delivery on the mRNA expression of lipid metabolism-related genes in the muscle, liver, and adipose tissue. DIO mice received an intramuscular injection (I.M.) of pcDNA3.1 (mock) (200 μg/mouse) or pCMV-IL-6 (200 μg/mouse) or a hydrodynamic injection (H.D.) of pCpG-mcs (mock) (0.75 μg/g bodyweight) or pGpG-IL-6 (0.75 μg/g bodyweight) and were sacrificed on day 14. (A) mRNA expression of PPAR-α in the muscle and liver. (B) mRNA expression of PPAR-γ in the adipose tissue. (C, D) mRNA expression of (C) ACC-1 and (D) SREBP-1c in the muscle, liver, and adipose tissue. The results are expressed as the mean + SE of five mice except for the H.D. injection in the pCpG-IL-6 group (n = 3). *P < 0.05 and **P < 0.01 compared with the mock intramuscular injection group. †P < 0.05 and ††P < 0.01 compared with the mock hydrodynamic injection group.

pDNA (Figure 3). However, the hydrodynamic injection of IL-6-expressing pDNA significantly reduced the mRNA expression level of PK1, G6Pase, and GYS in the liver (Figure 3C,F), but it did not significantly change the mRNA expression level of the other genes.

Effect of IL-6 Gene Transfer on the Obese State of DIO Mice. IL-6 gene delivery to the skeletal muscle and liver gradually reduced the body weight of DIO mice (Figure 4A). Gene transfer also reduced the food intake for the first week but only moderately reduced it for the second week (Figure 4B). To evaluate the changes in energy expenditure produced by IL-6 at the molecular level, we measured the mRNA expression level of UCP-2, a key enzyme involved in converting chemical energy into thermal energy. The UCP-2 mRNA expression level was significantly increased in the liver by IL-6 gene delivery (Figure 4C). The UCP-2 mRNA expression level in the muscle and adipose tissue was not significantly changed.

Next, we measured the serum concentrations of NEFA, TG, and cholesterol to evaluate further the lipid-metabolism status in DIO mice. The NEFA level was significantly reduced by IL-6 gene delivery (Figure 4D). The TG level was significantly reduced by the intramuscular gene delivery of IL-6 but not by hydrodynamic delivery (Figure 4E). The cholesterol level, however, was hardly changed by IL-6 gene delivery (Figure 4F).

We then measured the weight of the epididymal fat pad (Figure 4G). The fat pad weight of mice that received IL-6 gene delivery was moderately smaller than that of the corresponding control groups, although the difference was not significant. Examination of the HE-stained adipose tissue sections revealed that the adipocyte size was reduced by IL-6 gene delivery (Figure 4H–M). To evaluate quantitatively the size of the adipocytes, the number of adipocytes per field was counted (Figure 4N). The average size of the adipocytes in mice receiving IL-6 gene delivery was significantly smaller than in the mock-treated groups.

Effect of IL-6 Gene Delivery on the mRNA Expression of Lipid Metabolism-Related Genes in the Skeletal Muscle, Liver, and Adipose Tissue. To evaluate the effect of IL-6 gene delivery on lipid metabolism, the mRNA expression level of genes related to lipogenesis in the skeletal muscle, liver, and adipose tissue was measured (Figure 5). The function of ACC-1 is to provide the malonyl-CoA substrate for the biosynthesis of fatty acids, and SREBP-1 is a lipogenic transcription factor. IL-6 gene transfer showed a trend toward reduced mRNA expression of lipogenic genes compared with that in the mock-treated group. A greater reduction was observed in the mice that received an intramuscular injection of IL-6-expressing pDNA compared with those that received a hydrodynamic injection of IL-6-expressing pDNA.

Effect of IL-6 Gene Transfer on Inflammation. Next, we evaluated the inflammatory status after IL-6 gene delivery. To evaluate the degree of systemic inflammation induced by IL-6, the serum concentration of SAA, a representative pro-inflammatory protein, was measured on day 14. As shown in

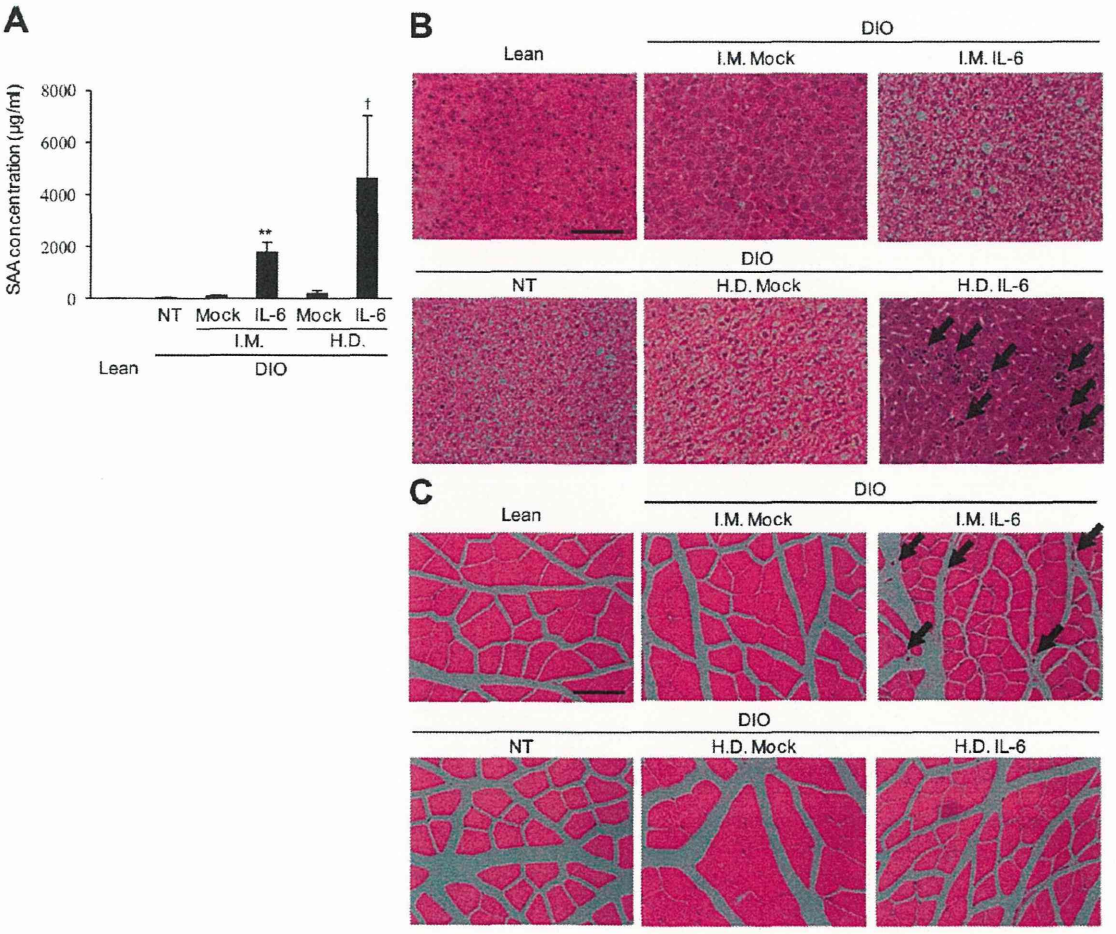

Figure 6. Effect of IL-6 gene delivery on inflammation. DIO mice received an intramuscular injection (I.M.) of pcDNA3.1 (mock) (200 μg/mouse) or pCMV-IL-6 (200 μg/mouse) or a hydrodynamic injection (H.D.) of pCpG-mcs (mock) (0.75 μg/g bodyweight) or pGpG-IL-6 (0.75 μg/g bodyweight). (A) SAA concentration in the serum on day 14. The results are expressed as the mean +SE of five mice except for the H.D. injection in the pCpG-IL-6 group ($n = 3$). $^{**}P < 0.01$ compared with the mock intramuscular injection group. $^{\dagger}P < 0.05$ compared with the mock hydrodynamic injection group. (B, C) Representative images of HE-stained (B) liver and (C) muscle sections. The arrows indicate the infiltration of inflammatory cells. The scale bar is 100 μm.

Figure 6A, serum levels of SAA were increased by IL-6 gene transfer.

The HE-stained liver sections revealed that there were a large number of glycogen vacuoles in the liver of the untreated DIO mice and mock-treated DIO mice (Figure 6B). In the liver sections of DIO mice that received a hydrodynamic injection of IL-6-expressing pDNA, an infiltration of inflammatory cells was observed. No infiltration of inflammatory cells was observed in the liver of DIO mice that received an intramuscular injection of IL-6-expressing pDNA. In the HE-stained muscle sections, an infiltration of inflammatory cells was observed in the injected muscle of the mice that were given IL-6-expressing pDNA by intramuscular injection (Figure 6C). No infiltration of inflammatory cells was observed in the muscle of the mice that received a hydrodynamic injection of IL-6-expressing pDNA.

Effect of Transient IL-6-Expression on GTT and Body Weight in DIO Mice. To confirm whether the sustained expression of IL-6 is required for the improvement of glucose homeostasis, DIO mice received a hydrodynamic injection of pCMV-IL-6 instead of pCpG-IL-6. In our previous studies, we demonstrated that hydrodynamic injection of the pCMV vector produces a transient transgene expression.[24] As expected, the mice showed a high IL-6 concentration on day 1 and then the concentration fell quickly (Figure 7A). The AUC and MRT values were 150 ± 60 ng/(mL·day) and 0.26 ± 0.02 days, respectively. On day 2, mice given pCMV-IL-6 showed similar glucose levels to those of the lean mice in the GTT (Figure 7B). On 7 and 14 days after pDNA administration, mice given pCMV-IL-6 showed higher glucose concentrations in the GTT (Figure 7B), although the glucose level was lower than the level before the administration of pCMV-IL-6. The changes in the body weight of the pCMV-IL-6-treated DIO mice were similar to those of the untreated DIO mice.

■ DISCUSSION

The involvement of IL-6 in glucose metabolism and insulin signaling has been suggested by previous studies. The present study has demonstrated that IL-6 exhibits positive effects on glucose metabolism in DIO mice after IL-6 gene transfer with both hydrodynamic delivery to the liver and intramuscular injection, corresponding well to the results obtained in most of

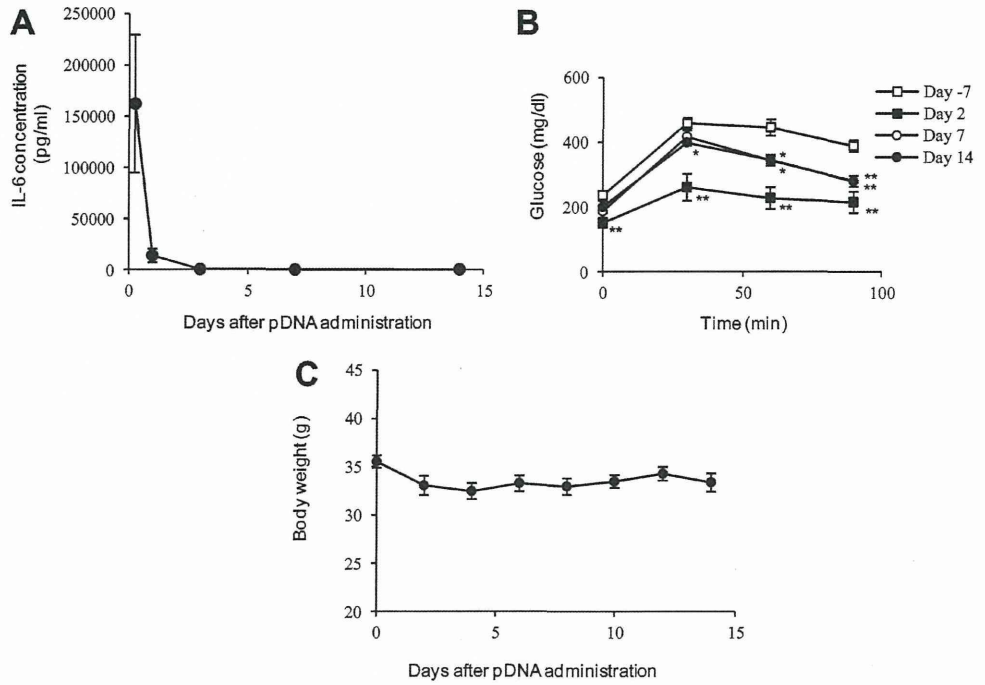

Figure 7. Effects of transient IL-6 expression on glucose homeostasis and obesity. DIO mice received a hydrodynamic injection of pCMV-IL-6 (0.25 μg/g bodyweight). (A) Time course of the concentration of IL-6 in the serum after gene delivery. (B) GTT test performed on days −7, 2, 7, and 14 after the hydrodynamic injection of pCMV-IL-6. The results are expressed as the mean ± SE of four mice.*$P < 0.05$ and **$P < 0.01$ compared with the day −7 group. (C) Body weight change after a 2-day interval.

the previous studies. Moreover, the pharmacokinetic profile of IL-6 has been shown to be an important factor. Significant effects were observed in the DIO mice that received a sustained IL-6 gene transfer, whereas transient expression of IL-6 had limited effects. The AUC value of each group was similar, whereas the MRT value in the mice with transient gene expression (0.26 ± 0.02 day) was significantly ($p < 0.01$) smaller than those (6.3 ± 0.4 and 3.8 ± 0.6 day) in the mice with sustained gene expression after intramuscular and hydrodynamic injection. These results suggest that a prolonged concentration in the circulation would be required for the positive pharmacodynamic actions of IL-6 in the DIO mice. With regard to the expression site of IL-6, the metabolic status, such as the mRNA expression level of metabolism-related genes and the serum insulin concentration, was improved more by the intramuscular injection than by the hydrodynamic injection. This observation suggests that the therapeutic effect of IL-6 in DIO mice is exerted via its action in the skeletal muscle rather than that in the liver, which is in agreement with previous studies.[14,20−22]

The decrease in body weight induced by IL-6 appears to be at least partly a result of the decrease in fat weight because we found that there was a reduction in the epididymal fat weight and adipocyte size (Figure 3). One possible reason for the fat reduction is the reduced food intake and increase in the expression level of energy-expenditure-related genes (Figure 4). Wallenius et al. showed that intracerebroventricular IL-6 treatment reduced body fat in DIO rats,[27] and this was caused by an increase in energy expenditure and a decrease in food intake. A study by Matthews et al. also demonstrated that genes associated with oxidative phosphorylation, the electron transport chain, and the tricarboxylic acid cycle were uniformly decreased in IL-6-deficient mice, which suggests that IL-6 has an increasing effect on energy expenditure.[28] Their results are consistent with our results that show that IL-6 gene delivery reduced food intake and increased energy expenditure, which implies that IL-6 expressed in the liver or skeletal muscle reached the brain and had an effect there. Moreover, the decrease in the mRNA expression level of lipid metabolism-related genes in the adipose tissue might be another reason for the reduction in the fat weight (Figure 5). We found that IL-6 gene delivery reduced the serum concentration of NEFA, which also suggests an improvement in the status of lipid metabolism by IL-6 gene delivery.

Regarding the relationship between the insulin sensitivity of muscle or liver and IL-6, some previous studies have reported that IL-6 enhanced insulin sensitivity in the muscle and increased insulin resistance in the liver.[20−22] The results of the current study showed that IL-6 gene delivery to both the liver and the muscle improved glucose metabolism (Figure 2). In addition, IL-6 gene delivery reduced the mRNA expression level of glucose metabolism-related genes in the liver and skeletal muscle (Figure 3). Moreover, IL-6 gene delivery reduced the serum insulin concentration in DIO mice (Figure 2). These results suggest that IL-6 gene delivery increased insulin sensitivity. Because SOCS-3 mRNA induction was observed in the muscle, liver, and adipose tissue after IL-6 gene delivery irrespective of the tissues transduced (Figure 1), IL-6 was expressed at the delivery site and transferred to distant tissues via the blood circulation. In fact, we observed high IL-6 concentrations in the blood circulation in both cases (Figure 1B). Recently, it was reported that IL-6 improved glycemia by stimulating glucagon-like peptide-1 (GLP-1) secretion from intestinal L cells and pancreatic alpha cells.[29] Therefore, IL-6 in

systemic circulation might stimulate GLP-1 secretion from those types of cells, although the GLP-1 concentration was not determined in the present study. The changes in insulin sensitivity on days 2 and 14 after the hydrodynamic injection of pCMV-IL-6 (Figure 7) indicate that the IL-6 concentration in the serum plays an important role in the overall improvement of insulin sensitivity after IL-6 gene delivery and that the IL-6-mediated improvement in insulin resistance is partially retained even after the level of IL-6 expression returned to normal.

In the present study, we have demonstrated that sustained supplementation of IL-6 improves the obese state and the metabolism of glucose and lipid in a mouse model of type II diabetes, suggesting that IL-6 gene delivery could be a therapeutic option for diabetes. However, great attention should be paid when using this approach because IL-6 is a typical inflammatory cytokine, which might cause serious side effects. In addition, long-term IL-6 therapy possesses a risk of inducing unexpected problems. In fact, a significant inflammation was observed in the site of IL-6 gene delivery in the present study, which is probably due to a high local concentration of IL-6. As chronic inflammation may accompany various types of diseases,[30] side effects including inflammation and inflammation-associated effects should be carefully monitored in performing IL-6 gene therapy.

We have demonstrated that IL-6 gene delivery improves systemic insulin sensitivity and obesity in a type II diabetes model. Our results clearly indicates that the time profile of IL-6 exposure is important for the effect of IL-6 in improving glucose and lipid metabolism.

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The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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