P-Selectin Glycoprotein Ligand-1 Deficiency Is Protective Against Obesity-Related Insulin Resistance

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OBJECTIVE—An inflammatory process is involved in the mechanism of obesity-related insulin resistance. Recent studies indicate that monocyte chemoattractant protein-1 (MCP-1) is a major chemokine that promotes monocyte infiltration into adipose tissue; however, the adhesion pathway in adipose tissue remains unclear. We aimed to clarify the adhesion molecules that mediate monocyte infiltration into adipose tissue.

RESEARCH DESIGN AND METHODS—We used a DNA microarray to compare the gene expression profiles in epididymal white adipose tissue (eWAT) between db/db mice and WT mice each fed a high-fat diet (HFD) or a low-fat diet (LFD). We investigated the change of insulin resistance and inflammation in eWAT in P-selectin glycoprotein ligand-1 (PSGL-1) homozygous knockout (PSGL-1−/−) mice compared with wild-type (WT) mice fed HFD.

RESULTS—DNA microarray analysis revealed that PSGL-1, a major ligand for selectins, is upregulated in eWAT from both db/db mice and WT mice fed HFD. Quantitative real-time RT-PCR and immunohistochemistry showed that PSGL-1 is expressed on both endothelial cells and macrophages in eWAT of obese mice. PSGL-1−/− mice fed HFD showed a remarkable reduction of macrophage accumulation and expression of proinflammatory genes, including MCP-1 in eWAT. Moreover, adipocyte hypertrophy, insulin resistance, lipid metabolism, and hepatic fatty change were improved in PSGL-1−/− mice compared with WT mice fed HFD.

CONCLUSIONS—These results indicate that PSGL-1 is a crucial adhesion molecule for the recruitment of monocytes into adipose tissue in obese mice, making it a candidate for a novel therapeutic target for the prevention of obesity-related insulin resistance. Diabetes 60:189–199, 2011

Obesity is correlated closely with chronic low-grade inflammation in adipose tissue and insulin resistance, which causes systemic metabolic disorders (1). Accumulation of macrophages in adipose tissue is positively correlated with body weight and insulin resistance in both humans and rodents (2,3). Adipose tissue macrophages (ATMs) secrete a variety of proinflammatory cytokines and chemokines, including tumor necrosis factor (TNF)-α (4), interleukin (IL)-6, and monocyte chemoattractant protein (MCP)-1 (5), which enhance insulin resistance. ATM accumulation and insulin resistance are ameliorated in MCP-1−/− mice (6) and C-C chemokine receptor 2 (CCR2)-deficient mice (7) fed a high-fat diet (HFD). Conversely, overexpression of MCP-1 resulted in increased numbers of ATMs along with the development of insulin resistance (6,8). These findings indicate that ATMs enhance obesity-related insulin resistance.

Monocyte infiltration into inflamed tissues is promoted by chemokines and adhesion molecules that are expressed on endothelial cells and monocytes (9). Selectin molecules and those ligands mediate leukocytes rolling along the activated endothelium, which is the first step of leukocyte recruitment into inflamed tissues. The second step is monocyte adhesion on endothelial cells mediated by intercellular adhesion molecule-1 (ICAM-1) or vascular cell adhesion molecule-1 (VCAM-1). Earlier, we reported that an inflammatory process is involved in the pathogenesis of diabetic nephropathy and that ICAM-1 deficiency is protective against the development of renal injury in diabetic mice without change of blood glucose (10–13). Several studies in humans have shown that serum levels of soluble ICAM-1 are elevated in obesity and positively correlate with central adiposity (14,15) and insulin resistance (16). Other studies have shown that serum levels of soluble E-selectin are associated with BMI or insulin resistance (17,18).

The predominant adhesion pathway of monocyte infiltration into adipose tissue is unclear. To clarify the adhesion molecules that promote monocyte infiltration into obese adipose tissue, we screened the gene expression profiles of adhesion molecules in adipose tissues from two different types of obese model mice and evaluated the functions of the candidate gene using knockout mice.

RESEARCH DESIGN AND METHODS

Animals and animal care. Six-week-old C57/BL6 (BL6) mice were purchased from CLEA Japan (Tokyo, Japan). The db/db mice (C57BL/KsJ-db/db) and P-selectin glycoprotein ligand-1 (PSGL-1) homozygous knockout (PSGL-1−/−) mice on the C57BL6J background (19,20) were purchased as 6-week-old animals from The Jackson Laboratory (Bar Harbor, ME). All mice used in this study were males and were maintained under a 12-h light/12-h dark cycle with access to food and tap water ad libitum. The animal care and all procedures were done according to the Guidelines for Animal Experimentation at Okayama University Medical School, the Japanese Government Animal Protection and Management Law (number 105), and the Japanese Government Notification on Feeding and Safekeeping of Animals (number 6).

Experimental protocol

Protocol 1. The db/db mice and the WT (C57/BL6) mice were fed a normal diet (Oriental Yeast, Osaka, Japan). All mice were killed at 8 weeks old, and

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epididymal white adipose tissue (eWAT) was harvested, weighed, and fixed in 10% (vol/vol) formalin. The remaining tissue was stored at −80°C.

Protocol 2. BL6 mice were fed HFD consisting of 60% kcal fat or a low-fat diet (LFD) consisting of 10% kcal fat (D12492 and D12450B, respectively; Research Diets, New Brunswick, NJ) from 7 to 19 weeks old. Intraperitoneal glucose and insulin tolerance tests were done at 15 or 16 weeks old. All mice were killed at 19 weeks old.

Protocol 3. PSGL-1+/− and PSGL-1−/− (WT; C57/BL6) mice were fed HFD from 7 to 17 weeks old. Intraperitoneal glucose and insulin tolerance tests were done at 15 or 16 weeks old. All mice with <40 g body weight were killed at 17 weeks old. PSGL-1−/− mice were healthy and showed delayed neutrophil recruitment and moderate neutrophilia.

Analysis of metabolic parameters. Body weight and food intake were monitored weekly. For the glucose tolerance test, the mice were injected with glucose (1.2 g/kg body mass i.p.) after fasting for 12–16 h. For the insulin tolerance test, mice allowed access to food ad libitum were injected with human regular insulin (0.7 units/kg body mass i.p., Eli Lilly, Indianapolis, IN). We measured the concentration of glucose with a blood glucose meter (Glutest Pro; Sanwa Kagaku Kenkyusho, Nagoya, Japan), plasma insulin and leptin with an assay kit (Morinaga Institute of Biological Science, Kanagawa, Japan), and plasma adiponectin with an ELISA kit (Otsuka Pharmaceutical, Tokyo, Japan).

RNA preparation from eWAT and liver. Total RNA was extracted from each specimen of eWAT and liver using the RNeasy Lipid Tissue Mini Kit or RNeasy Plus Mini Kit following the instructions provided by the manufacturer (Qiagen, Valencia, CA).

DNA microarray analysis. We used five RNA samples from each group (db/db versus WT mice and BL6 mice fed HFD versus LFD) for DNA microarray analysis. Preparation of cRNA and hybridization of probe arrays (Mouse Genome 430 2.0) were performed according to the manufacturer's
TABLE 1
DNA microarray analysis

| Gene                                 | Description                                           |
|--------------------------------------|-------------------------------------------------------|
| Fibulin 2                            |                                                       |
| CD97 antigen                         |                                                       |
| Parvin, gamma                        |                                                       |
| C-type lectin domain family 4, member e |                                                       |
| A disintegrin and metalloproteinase domain 8 |                                                       |
| Integrin alpha m                     |                                                       |
| Killer cell lectin-like receptor, subfamily a, member 2 |                                                       |
| Procollagen, type I, alpha 1          |                                                       |
| Selectin, platelet (p-selectin) ligand |                                                       |
| Integrin alpha 7                     |                                                       |
| Expressed sequence c79673            |                                                       |
| Procollagen, type v, alpha 3          |                                                       |
| Plakophilin 2                        |                                                       |
| Elastin microfilbr interfilbr 2      |                                                       |
| Cell adhesion molecule with homology to 11cam |                                                       |
| Integrin beta 2                      |                                                       |
| Milk fat globule-EGF factor 8 protein |                                                       |
| Pleckstrin homology, sec7 and coll-oid domains, binding protein |                                                       |
| CD44 antigen                         |                                                       |
| Calcyntenin 2                        |                                                       |
| Carboxypeptidase x 1 (m14 family)    |                                                       |
| A disintegrin and metalloproteinase domain 23 |                                                       |
| Oxidized low density lipoprotein (lectin-like) receptor 1 |                                                       |
| Procollagen, type v, alpha 2          |                                                       |

Gene ontology of cell adhesion category of more than twice upregulated genes db/db versus wild-type mice is shown (total, 47 genes).

instructions (Affymetrix, Santa Clara, CA). These arrays contain probe sets for >45,000 transcripts. The criteria for selecting genes were as follows: 1) genes whose flags were “present” and 2) ratio of expression level of >2.0-fold increase in db/db compared with WT mice or in BL6 mice fed HFD compared with LFD. Gene Ontology Biological Process classification of the >2.0-fold upregulated genes from each group was done with the DAVID Bioinformatics Database functional-annotation tools (http://niaid.abc.ncifcrf.gov) (21).

**Quantitative real-time RT-PCR analysis for eWAT and liver.** The mRNA expression of each gene was measured by quantitative real-time RT-PCR as described previously (22). The amounts of PCR products were normalized with a housekeeping gene was measured by calculating the relative expression ratios. Each experiment was done in triplicate. The primers were as follows: MCP-1: 5'-AAGCTGGTGAATCTTGGCA-3' (forward), 5'-GGCA GATCGGATTTTATAA-3' (reverse); PSGL-1 (Selp): 5'-TTGTGCTGCTGAC CATCCT-3' (forward), 5'-TCTCCTAATTCATCATT-3' (reverse); P-selectin (Selp): 5'-CAGTGGCTTCTACACAGGCC-3' (forward), 5'-TTGCTCATG CAGCGTTA-3' (reverse); E-selectin (Sele): 5'-CAGGTCGATCATCGATTCAAACCT-3' (forward), 5'-GCAGTCTGAGTCCATCTT-3' (reverse); CD68: 5'-GGCTGTT GAATCAATTGTC-3' (forward), 5'-AGAAGAGCGCGCCAGTCAGGT-3' (reverse); \( \beta \)-actin: 5'-CCTGTATGCCTCTGGTCGTA-3' (forward), 5'-GCGGTG CATACTTCTGCTC-3' (reverse).

These primers were purchased from Nihon Gene Research Labs (Sendai, Japan). ICAM-1 (GenBank accession code X52264, cat. no. 4651782) and NOS2 (inOS) (GenBank accession code NM_010927, cat. no. 5026474) were in the Light Cycler-Primer Set (Roche Diagnostics, Rotkreuz, Switzerland). F4/80 (Mm01269099_m1), CD11c (Mm00498695_m1), IL-10 (Mm01288386_m1), IL-6 (Mm0046190_m1), LPL (Mm00434770_m1), leptin (Mm00434759_m1), fatty acid synthesis (FAS) (Mm00627559_m1), sterol regulatory element binding protein-1c (SREBP-1c) (Mm0138344_m1), acetyl-CoA carboxylase-1 (ACC-1) (Mm0134289_m1), and p62phos-proliferator-activated receptor-\( \alpha \) (PPAR-\( \alpha \)) (Mm00627559_m1) were TaqMan gene expression assays (Applied Biosystems, Tokyo, Japan).

**Isolation of adipocytes and stromal-vascular fractions.** Stromal vascular fraction (SVF) and peripheral blood mononuclear cells (PBMCs) were isolated from db/db mice or BL6 mice at 10 weeks of age. SVF cells were isolated as described (23,24). PBMCs were separated by density gradient centrifugation using a Lymphocyte Separation Medium (MP Biomedicals, Tokyo, Japan). Isolation of eWAT was performed from mice, fixed in 10% formalin, and embedded in paraffin. The area was measured in six high-power fields from each of five mice. Immunohistochemical staining. Immunoperoxidase and immunofluorescent staining were done as described (11,12,25). Paraffin sections (4 \( \mu \)m thick) were deparaffinated and rehydrated and then stained with periodic acid Schiff stain. The adipocyte acid was traced manually and analyzed with Lumina Vision OL V2.4.4 software (Mitani, Tokyo, Japan). The area was measured in six high-power fields from each of five mice. Immunohistochemical staining. Immunoperoxidase and immunofluorescent staining were done as described (11,12,25). Paraffin sections were deparaffinated and rehydrated before antigen unmasking by boiling in R-Buffer U at a dilution of 1:10 (PickCell Laboratories, Amsterdam, the Netherlands) for 10 min.

We used immunoperoxidase staining for macrophage and PSGL-1 in eWAT of db/db mice and WT mice on normal food. Rat anti-mouse monocot/macrophase (Mac-3) monoclonal antibody (mAb) at a dilution of 1:50 (Santa Cruz Biotechnology) and rat anti-mouse PSGL-1 (CD162) mAb at a dilution of 1:50 ( Fitzgerald Industries International, Concord, MA) were applied to the sections as the primary reaction, followed by a second reaction with biotin-labeled donkey anti-rat IgG antibody (Jackson Immunoresearch Laboratories, West Grove, PA) at a dilution of 1:50. The avidin-biotin coupling reaction was done with the Vectastain Elite kit (Vector Laboratories, Burlingame, CA).

We used double immunofluorescence staining to clarify the expression and localization of PSGL-1, leukocyte/macrophage, and endothelial cells in eWAT of db/db mice. PSGL-1 mAb followed by Alexa Fluor 488 donkey anti-IgG (A-21208; Molecular Probes, Eugene, OR) and goat anti-mouse leukocyte/macrophage (CD45) mAb (sc-1121; Santa Cruz Biotechnology) followed by
Alexa Fluor 546 rabbit anti-goat IgG (A-21085; Molecular Probes) were applied to the sections. Similarly, PSGL-1 mAb followed by Alexa Fluor 488 donkey anti-rat IgG and goat anti-mouse endothelial cell (PECAM-1/CD31) mAb (sc-1506; Santa Cruz Biotechnology) followed by Alexa Fluor 546 rabbit anti-goat IgG were applied to the sections.

**Measurement of hepatic triglyceride content.** Measurement of the hepatic triglyceride content in WT mice and PSGL-1/H11002 mice fed HFD was done by the Folch technique (26) at Skylight Biotech (Akita, Japan), and the triglyceride concentration was measured with Cholestest TG (Sekisui Medical, Tokyo, Japan). The tissue triglyceride concentrations were corrected for liver weight.

**Statistical analysis.** All data are expressed as mean ± SE and were analyzed by the Mann-Whitney U test with the level of statistical significance set at \( P < 0.05 \).

**RESULTS**

**Analysis of eWAT from db/db mice.** The db/db mice showed significantly increased body weight, weight of eWAT, levels of serum LDL cholesterol, postprandial plasma glucose, and plasma insulin compared with WT mice, but A1C was not different between the two groups (Fig. 1A).

DNA microarray analysis detected 1,080 genes that were more than twofold upregulated in db/db mice compared with WT mice. Gene ontology analysis indicated that 47 cell adhesion-related genes, including L-selectin (2.0-fold change, db/db versus wild-type) and PSGL-1 (2.0-fold change, db/db versus wild-type) were upregulated in db/db mice compared with WT mice (supplementary Fig. 1, available in an online-only appendix at http://diabetes.diabetesjournals.org/cgi/content/full/db09-1894/DC1; Table 1).

We focused on PSGL-1 because it is expressed on both PSGL-1/MAC-3 wild type db/db

**FIG. 2.** A: Immunohistochemical localization of PSGL-1, macrophages, and endothelial cells in adipose tissue. Epididymal fat pads from 8-week-old db/db mice and WT mice were stained with anti–MAC-3 (left-hand panels) and anti–PSGL-1 antibodies (right-hand panels). Macrophages and PSGL-1 expressed around the small vessels in the interstitium of adipose tissue in db/db mice are shown. The scale bars represent 50 μm. B: Double immunofluorescence staining of adipose tissue from db/db mice with the antibodies against PSGL-1 (green) and leukocyte (CD45, red). PSGL-1 and CD45 were stained in the interstitium of adipose tissue and are colocalized in the merged picture. C: Double immunofluorescence staining of adipose tissue from db/db mice with the antibodies against PSGL-1 (green) and endothelial cell (CD31, red). PSGL-1 and CD31 were stained along small vessels of adipose tissue and are colocalized in the merged picture. D–F: The expression of PSGL-1 on cells in WT mice and db/db mice was analyzed using flow cytometry. D: The expression of PSGL-1 in PBMCs. E: The expression of PSGL-1 in F4/80+ macrophages in the SVF from adipose tissue. F: The expression of PSGL-1 in CD31+ endothelial cells in the SVF from adipose tissue. (A high-quality digital representation of this figure is available in the online issue.)
leukocytes and endothelium and has a wide range of binding capacity to all three types of selectin (27–29). Real-time RT-PCR demonstrated that PSGL-1 mRNA expression was significantly higher in eWAT of db/db mice compared with that in WT mice. The transcriptional levels of other proinflammatory genes, including F4/80, MCP-1, and P-selectin mRNA, were also increased significantly in db/db mice (Fig. 1B), whereas the mRNA expression of other adhesion molecule genes, such as E-selectin and ICAM-1, in db/db mice was not different from that in WT mice.

Immunoperoxidase staining was used for MAC-3, a macrophage marker, and PSGL-1 in eWAT from db/db mice and WT mice fed normal diet. The expression of macrophage and PSGL-1 increased around the small vessels in the interstitium of eWAT in db/db mice (Fig. 2A).

Furthermore, to estimate the distribution of PSGL-1, macrophage, and endothelial cells in eWAT, we used double

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**FIG. 3.** A: Metabolic characteristics of C57/BL6 mice fed LFD or HFD from 7 to 19 weeks old. Metabolic parameters of mice fed LFD diet (○, □) or HFD (■, ●) are shown (n = 9 [LF]: n = 11 [HF]). B: Blood glucose level (left-hand panel) and plasma insulin levels (right-hand panel) during the glucose tolerance test (1.2 g/kg body mass) (n = 9 [LF], ○; n = 11 [HF], ■). C: Blood glucose level during the insulin tolerance test (0.7 units/kg body mass) (n = 9 [LF], ○; n = 11 [HF], ●). D: Gene expression in epididymal fat from C57/BL6 mice fed a LFD (○) or HFD (■) from 7 to 19 weeks old analyzed by quantitative real-time RT-PCR (n = 9 [LF], n = 10 [HF]). Data are means ± SE. *P < 0.05 vs. LFD, **P < 0.005 vs. LFD.
imunofluorescence staining in eWAT from db/db mice. PSGL-1 (green) and leukocyte/macrophage (CD45, red) were detected in the interstitium of eWAT. They were mostly coexpressed in a merged picture (Fig. 2B) in addition, PSGL-1 (green) and endothelial cells (CD31, red) were mostly detected in the interstitium of eWAT and mostly coexpressed in a merged picture (Fig. 2C).

We examined PBMCs from WT mice or db/db mice. F4/80^+^ PSGL-1^+^ cells were similarly contained (WT mice 82.3 ± 1.2% versus db/db mice 84.6 ± 2.5% of F4/80^+^ cells, P = 0.827) between the two groups by flow cytometry analysis. We isolated SVF cells from epididymal fat pads excised from WT mice or db/db mice fed a normal diet and analyzed cells by flow cytometry. F4/80^+^PSGL-1^+^ cells were not different (WT mice 23.2 ± 2.2% versus db/db mice 34.3 ± 4.3% of F4/80^+^ cells, P = 0.149) between WT mice and db/db mice. CD31^+^PSGL-1^+^ cells significantly increased (WT mice 36.6 ± 2.5% versus db/db mice 53.9 ± 3.5% of CD31^+^ cells, P = 0.021) in db/db mice compared with WT mice (Fig. 2D–F). These results indicate that CD31^+^PSGL-1^+^ cells were increased in adipose tissue of db/db mice.

**Upregulation of PSGL-1 expression in BL6 mice fed HFD.** We next examined eWAT in BL6 mice fed LFD or HFD to determine whether PSGL-1 expression increased in HFD-induced obese mice. BL6 mice fed HFD showed significantly increased body weight, weight of eWAT, serum LDL cholesterol, fasting plasma glucose, plasma insulin, and A1C compared with BL6 mice fed LFD (Fig. 3A). Plasma glucose and insulin levels during a glucose tolerance test were markedly higher in BL6 mice fed HFD compared with mice fed LFD (Fig. 3B). Similarly, BL6 mice fed HFD showed impaired insulin sensitivity as measured by the insulin tolerance test (Fig. 3C).

DNA microarray profiling indicated that 572 genes were upregulated more than twofold in BL6 mice fed HFD compared with those fed LFD. Analysis by gene ontology categories showed that 41 cell adhesion–related genes, including PSGL-1, were upregulated in BL6 mice fed HFD (twofold change, HFD/LFD) (supplementary Fig. 2, Table 2). Quantitative real-time RT-PCR showed that transcriptional levels of CD68, MCP-1, PSGL-1, and P-selectin mRNA were increased significantly in BL6 mice fed HFD. E-selectin and ICAM-1 mRNA expression were not different between the two groups (Fig. 3D).

**PSGL-1 deficiency improved insulin sensitivity.** As described above, PSGL-1 was upregulated in eWAT of two different rodent models for obesity-related insulin resistance. We further examined the role of PSGL-1 in eWAT of diet-induced obese mice using PSGL-1–deficient (PSGL-1^−/−^) mice. The PSGL-1^−/−^ and PSGL-1^+/+^ WT mice were fed HFD for 10 weeks.

Body weight, the weight of each adipose tissue per unit body weight, and food intake were not different between the two groups (Fig. 4A). There was no difference in fasting plasma glucose and A1C between the two groups, although fasting plasma insulin level was significantly lower in PSGL-1^−/−^ mice than it was in WT mice fed HFD (Fig. 4B). These data indicated that PSGL-1 deficiency improved insulin resistance without a change of body weight or the amount of eWAT.

Intraporterineal glucose and insulin tolerance tests were used to further confirm that PSGL-1 deficiency improves insulin sensitivity. Blood glucose levels during the glucose tolerance test were similar in the two groups, although plasma insulin levels were lower in PSGL-1^−/−^ mice than those in WT mice fed HFD (Fig. 4C). The glucose-lowering effect of insulin was significantly greater in PSGL-1^−/−^ mice than it was in WT mice, as measured by the insulin tolerance test (Fig. 4D). These data confirmed that insulin sensitivity was improved in PSGL-1^−/−^ mice fed HFD.

To further investigate insulin sensitivity in PSGL-1^−/−^ mice, we examined insulin-stimulated phosphorylation of Akt in liver and muscle. Akt phosphorylation in liver was not different between the two groups. However, Akt phosphorylation in muscle was significantly increased in PSGL-1^−/−^ mice compared with WT mice fed HFD (Fig. 4E).
**FIG. 4.** A: Metabolic characteristics of WT mice and PSGL-1−/− (KO) mice fed HFD from 7 to 17 weeks old. Body composition and food intake in WT mice (□) and PSGL-1−/− mice (■) fed HFD (n = 7 [WT-HF]; n = 8 [KO-HF]) is shown. B: Metabolic parameters of WT mice (□) and PSGL-1−/− mice (■) fed HFD (n = 7 [WT-HF]; n = 8 [KO-HF]). C: Blood glucose level (upper panel) and plasma insulin levels (lower panel) during the glucose tolerance test (1.2 g/kg body mass) (n = 9 [WT-HF], ◦ n = 8 [KO-HF]). D: Blood glucose level during the insulin tolerance test (0.7 units/kg body mass) (n = 9 [WT-HF], ◦ n = 8 [KO-HF]). Data are means ± SE. *P < 0.05 vs. WT-HFD. **P < 0.005 vs. WT-HFD. E: Equal amounts of protein in total lysates of liver and muscle were immunoblotted with anti–phospho-Akt (pAkt) and anti-Akt antibodies. The relative ratio of Akt phosphorylation was calculated after normalization with the Akt signal (n = 5 [WT-HF], ◦ n = 5 [KO-HF]). Data are means ± SE. *P < 0.05 vs. WT-HFD. FFA, free fatty acid.
PSGL-1 deficiency decreased macrophage infiltration and inflammation in obese adipose tissue. Morphometric analysis demonstrated that adipocytes in eWAT were smaller in PSGL-1−/− mice than in WT mice fed HFD (Fig. 5A and B). Immunohistochemistry showed a decrease of MAC-3–positive cells in eWAT from PSGL-1−/− mice fed HFD (Fig. 5C). The mRNA expression of F4/80, MCP-1, IL-6, iNOS, and leptin was decreased in eWAT from PSGL-1−/− mice compared with that in WT mice fed HFD (Fig. 5D). The levels of TNF-α and adiponectin mRNA were not statistically different between the two groups (data not shown). IL-10 mRNA levels were significantly increased, whereas CD11c mRNA levels tended to be decreased without significant difference in PSGL-1−/− mice fed HFD compared with WT mice fed HFD (Fig. 5D).

PSGL-1 deficiency improved lipid metabolism and hepatic steatosis. In this study, the weight of liver and the level of serum triglycerides were reduced significantly, and the hepatic triglyceride content tended to be decreased in PSGL-1−/− mice compared with WT mice fed HFD (Figs. 4B and 5A). The mRNA expression of CD68 was not different between the two groups (Fig. 6B). A few lipid metabolism–related genes in liver were not different between the two groups as follows: FAS (WT-HF 9.37 ± 3.07 vs. KO-HF 5.37 ± 1.38, P = 0.223), SREBP-1c (WT-HF 3.29 ± 0.48 vs. KO-HF 3.77 ± 0.64, P = 0.685), ACC-1.
WT-HF 20.25 ± 2.63 vs. KO-HF 19.59 ± 3.09, *P = 0.935),
PPAR-α (WT-HF 24.45 ± 5.25 vs. KO-HF 22.93 ± 4.03, *P = 0.685),
and LPL (WT-HF 1.17 ± 0.15 vs. KO-HF 1.95 ± 0.79,
*P = 0.685). The amounts of PCR products were normalized with a housekeeping gene [GAPDH] to calculate the relative expression ratios. On the other hand, the mRNA expression of LPL in adipose tissue improved in PSGL-1<sup>-/-</sup> mice compared with WT mice fed HFD (Fig. 5D). Histologically, the liver of WT mice fed HFD showed massive hepatocyte ballooning around the central veins; however, the hepatic steatosis was improved in PSGL-1<sup>-/-</sup> mice fed HFD (Fig. 6C). Furthermore, the levels of serum total cholesterol, LDL, free fatty acids, and leptin were lower in PSGL-1<sup>-/-</sup> mice than in WT mice fed HFD (Fig. 4B), but aspartate aminotransferase and alanine aminotransferase were not different between the two groups (data not shown).

**DISCUSSION**
To investigate the mechanism of monocytes/macrophages infiltration into adipose tissue, we used a DNA microarray
for obese adipose tissue in db/db mice or WT mice fed HFD. Both groups were in a state of intensive insulin resistance but were not diabetic. Expression of the adhesion molecule PSGL-1 was increased in obese adipose tissue from db/db mice and from WT mice fed HFD, as determined by DNA microarray analysis. In addition, an increase in PSGL-1 mRNA expression was observed with real-time RT-PCR and immunohistochemistry in obese adipose tissue. Furthermore, PSGL-1–deficient mice had reduced macrophage accumulation, insulin resistance, lipid metabolism, and steatohepatic change associated with obesity.

PSGL-1 was originally identified by expression cloning of a functional ligand for P-selectin (30). PSGL-1 is a mucin-like cell adhesion molecule expressed on the surface of leukocytes and endothelial cells and then involved in platelet-leukocyte and endothelium-leukocyte interactions. PSGL-1 mRNA was expressed in a variety of tissues, including bone marrow, brain, adipose tissue, heart, kidney, and liver. PSGL-1 is highly expressed in hematopoietic cells and in nonhematopoietic tissues, including adipose tissue and brain. The domain structure of PSGL-1 and amino acids are highly conserved between humans and rodents (31). PSGL-1 interacts with all three selectins: L-selectin (32), E-selectin (19, 22), and P-selectin (29).

Earlier, it was reported that mice deficient in ICAM-1, or other leukocyte adhesion molecules, increased body weight and white fat pad weight (% of body weight) on normal food and on HFD. Mac-1 (αMβ2, CD11b/CD18) is a counter-receptor for ICAM-1, and Mac-1–deficient mice showed a similar obesity phenotype (33). AIC was not different in ICAM-1–deficient db/db mice, streptozocin-induced ICAM-1–deficient mice, db/db mice (13), and streptozocin-induced WT mice (12). Furthermore, the number of leukocytes in the adipose tissue of ICAM-1–deficient mice and Mac-1–deficient mice fed HFD were the same as in the WT mice fed HFD. Consequently, these adhesion receptors are not required for leukocyte migration into adipose tissue (34).

In this study, PSGL-1 expression on peripheral blood monocyte was not increased in db/db mice compared with WT mice. Moreover, PSGL-1 expression on ATMs was also similar between db/db mice and WT mice by flow cytometry analysis. On the other hand, CD31+PSGL-1+ cell content was significantly increased in the SVF of eWAT in db/db mice compared with WT mice. These results indicate that increased expression of PSGL-1 in endothelial cells in adipose tissue is involved in infiltration of macrophage and inflammation in adipose tissue of obese mice.

The accumulation of macrophages in adipose tissue is correlated with increased body weight and insulin resistance in both humans and rodents (2, 3). MCP-1 contributed to macrophage infiltration into adipose tissue and insulin resistance in mice (6, 8). Those reports indicated that the ATMs might play an important role in the development of insulin resistance. Moreover, there are recent reports that the polarization of macrophages is changed from M2 to M1 in obese inflamed adipose tissue (35). M1 macrophages are induced by proinflammatory mediators such as lipopolysaccharide and γ-interferon (IFN-γ) and produce proinflammatory cytokines TNF-α and IL-6. Instead, M2 macrophages generate high levels of anti-inflammatory cytokines IL-10 and IL-1. Our data showed that MCP-1, IL-6, and iNOS mRNA levels were reduced, and IL-10 mRNA, an M2 macrophage marker, was significantly increased in eWAT from PSGL-1−/− mice compared with WT mice fed HFD. Whereas CD11c mRNA as an M1 macrophage marker tended to be decreased in PSGL-1−/− mice compared with WT mice fed HFD. These results demonstrated that PSGL-1 deficiency reduced the number of ATMs and changed the phenotype of macrophages from M1 to M2 and then affected inhibition of inflammation in eWAT. Furthermore, PSGL-1 deficiency improved insulin signaling in the muscle, as evidenced by an increase in Akt phosphorylation in animals fed HFD. As a result, PSGL-1−/− mice fed HFD ameliorated systemic glucose tolerance and insulin sensitivity.

Several studies reported that blockade of PSGL-1 reduced inflammatory reactions. In the model of cold ischemia/reperfusion, hepatic endothelial neutrophil infiltration and hepatocyte injury were diminished, and the expression of TNF-α, IL-6, iNOS, IL-2, and IFN-γ mRNA was decreased in livers pretreated with recombinant PSGL-Ig (36). Other reports showed that blockade of PSGL-1 attenuated macrophage recruitment in intestinal mucosa and ameliorated ileitis in a mouse model of Crohn’s disease (37, 38).

In this study, serum triglyceride and hepatic steatosis improved in PSGL-1−/− mice fed HFD. The mRNA expression of LPL was improved in adipose tissue of PSGL-1−/− mice compared with WT mice fed HFD. These results might be explained if the amelioration of insulin resistance increased LPL in adipose tissue. Consequently, lipid metabolism, including serum free fatty acids, triglyceride, cholesterol, and hepatic triglycerides, might be improved in PSGL-1−/− mice compared with WT mice fed HFD.

We found that plasma leptin levels and leptin mRNA expression in eWAT were decreased in PSGL-1−/− mice fed HFD, despite no difference of body weight or weight of fat. The plasma leptin concentration is positively correlated with BMI and weight of body fat in humans (39). Obese individuals are generally in a state of leptin resistance, although the pathophysiology of leptin resistance has not been clarified. Our data suggested that improvement of leptin sensitivity resulted in lower levels of plasma leptin in PSGL-1−/− mice than that in WT mice fed HFD. However, further studies are needed to determine whether PSGL-1 deficiency improves leptin sensitivity in obese animals.

In conclusion, our results indicate that PSGL-1 is a crucial adhesion molecule for recruitment of monocytes into adipose tissues in obese mice. PSGL-1 is a candidate for a novel therapeutic target for prevention of obesity-related insulin resistance.

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