Impaired Adipose Tissue Development in Mice With Inactivation of Placental Growth Factor Function

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Placental growth factor (PlGF)-deficient (PlGF^{-/-}) and wild-type mice were kept on a standard-fat or high-fat diet for 15 weeks. With the standard-fat diet, the body weights of PlGF^{-/-} and wild-type mice were comparable, whereas the combined weight of subcutaneous and gonadal adipose tissues was lower in PlGF^{-/-} mice ($P = 0.02$). With the high-fat diet, PlGF^{-/-} mice had a lower body weight ($P < 0.05$) and less total subcutaneous plus gonadal adipose tissue ($P < 0.0001$). Blood vessel size was lower in gonadal adipose tissue of PlGF^{-/-} mice with both the standard-fat and high-fat diet ($P < 0.05$). Blood vessel density, normalized to adipocyte number, was significantly lower in subcutaneous adipose tissue of PlGF^{-/-} mice fed the high-fat diet ($P < 0.01$). De novo adipose tissue development in nude mice injected with 3T3-F442A preadipocytes was reduced ($P < 0.005$) by administration of a PlGF-neutralizing antibody. Bone marrow transplantation from wild-type or PlGF^{-/-} mice to wild-type or PlGF^{-/-} recipient mice revealed significantly lower blood vessel density in PlGF^{-/-} recipient mice without an effect on adipose tissue growth. Thus, in murine models of diet-induced obesity, inactivation of PlGF impairs adipose tissue development, at least in part as a result of reduced angiogenesis. *Diabetes* 55:2698–2704, 2006

Approximately 50–60% of the adult population in most industrialized countries are overweight or obese (1,2). Overweight and obesity are considered to be major risk factors for development of the metabolic syndrome and associated cardiovascular disease (2–4).

Adipose tissue, unlike most other organs, grows and develops continuously throughout life. Its development involves extracellular matrix proteolysis, adipogenesis, and angiogenesis (5). Several observations suggested that adipogenesis may be regulated by factors that drive angio-

genesis (5–9). Studies in mice suggested that control of the vasculature through inhibition of angiogenesis may impair adipose tissue accretion (6–8).

It is widely accepted that vascular endothelial growth factor (VEGF) accounts for much of the angiogenic activity of adipose tissue (9). VEGF regulates angiogenesis by interacting with two tyrosine kinase receptors, VEGF receptor 1 (VEGFR-1 or Flt-1) and VEGF receptor 2 (VEGFR-2 or Flk-1) (10). A homologue of VEGF was discovered in human placenta and called placental growth factor (PlGF) (11). PlGF is, in fact, produced by many cell types, including adipocytes (12). Different isoforms of PlGF have been identified, of which only PlGF-2 is present in the mouse. PlGF specifically binds Flt-1 and not Flk-1 (13), but its binding to Flt-1 stimulates a cross-talk between both receptors, thereby amplifying Flk-1-driven angiogenesis (14,15).

Because of its expression in adipose tissue, its functional homology with VEGF, and its interaction with VEGFRs, we investigated in the present study whether PlGF may play a role in adipose tissue-related angiogenesis and in control of fat mass.

RESEARCH DESIGN AND METHODS

Male wild-type or PlGF-deficient (PlGF^{-/-}) mice (both with genetic background 50% Swiss and 50% 129SV) were obtained as described elsewhere (16). Wild-type and *ob/ob* mice (both 100% C57BL/6) were generated in the animal facility of the Molecular Cardiovascular Medicine Group (KU Leuven, Leuven, Belgium). Nude (BALB/c) mice were purchased from Charles River Laboratories (Wilmington, MA). Mice were kept in microisolation cages on a 12-h day-night cycle and fed water and a standard-fat diet (KM-04-k12, containing 13% kcal as fat, with a caloric value of 10.9 kJ/g; Muracon; Carfil) or a high-fat diet (TD88137, containing 42% kcal as fat with a caloric value of 20.1 kJ/g; Harlan Teklad, Madison, WI). Mice were weighed every week, and food intake was measured for 24-h periods throughout the experimental period. Physical activity was evaluated using cages equipped with a turning wheel linked to a computer to register full turning cycles.

At the end of the experiments, after overnight fasting, total body fat was determined by noninvasive densitometry (PIXImus densitometer; Lunar, Madison, WI). The interassay coefficients of variation for the determination of body fat and bone weight were 2.6 and 0.40%, respectively. Lean body mass is given as the difference between body weight and weight of bones and fat tissues. The mice were killed by intraperitoneal injection of 60 mg/kg sodium pentobarbital (Nembutal; Abbott Laboratories, North Chicago, IL), and blood was collected from the retroorbital sinus on trisodium citrate (final concentration 0.01 mol/l), and plasma was stored at -20°C . Intra-abdominal (gonadal) and inguinal subcutaneous fat pads were removed and weighed; portions were immediately frozen at -80°C for protein or RNA extraction and other portions were used to prepare 10- μm paraffin sections for histological analysis. Other organs, including pancreas, spleen, kidneys, liver, heart, and lungs, were also removed and weighed. All animal experiments were approved by the local ethical committee (Project P03112) and performed in accordance with the guiding principles of the American Physiological Society and the International Society on Thrombosis and Hemostasis (17).

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BMT, bone marrow transplantation; ELISA, enzyme-linked immunosorbent assay; mAb, monoclonal antibody; PlGF, placental growth factor; VEGF, vascular endothelial growth factor; VEGFR, VEGF receptor.

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TABLE 1

Effect of PIGF deficiency on body and tissue weights after 15 weeks of a standard-fat diet or a high-fat diet

	Standard-fat diet		High-fat diet	
	Wild type	PIGF ^{-/-}	Wild type	PIGF ^{-/-}
<i>n</i>	21	19	10	10
Body (g)*	38 ± 1.4	38 ± 1.0	57 ± 1.4	49 ± 3.1†
Subcutaneous fat (mg)	910 ± 80	460 ± 61‡	2,850 ± 110	1,550 ± 230‡
Gonadal fat (mg)	1,050 ± 120	860 ± 120	1,740 ± 68	1,350 ± 180
Liver (mg)	1,470 ± 46	1,390 ± 49	3,430 ± 380	2,790 ± 360
Pancreas (mg)	250 ± 14	270 ± 20	290 ± 18	320 ± 29
Spleen (mg)	96 ± 5.8	120 ± 7.2	160 ± 8.0	180 ± 22

Data are means ± SE. *Body weight after overnight fasting. † $P < 0.05$ and ‡ $P < 0.0005$ vs. wild type.

Assays. The mean adipocyte size was determined by computer-assisted image analysis of adipose tissue sections stained with hematoxylin and eosin or with biotinylated *Bandeiraea (Griffonia) simplicifolia* BSI lectin (Sigma-Aldrich, Bornem, Belgium) followed by signal amplification with the Tyramide Signal Amplification Cyanine system (Perkin Elmer, Boston, MA). For each animal, three to five areas in four different sections each were analyzed; the data were first averaged per section and then per animal. The lectin staining also visualizes blood vessels (18); average blood vessel size is calculated by dividing the total stained area by the number of vessels, and blood vessel density is expressed as the number of vessels per measured surface area (9–12 sections were analyzed per animal and results were then averaged). Immunostaining for PIGF was performed using rat anti-mouse PIGF-2 mAb465 (R&D Systems, Lille, France).

Extraction of adipose tissues was performed as described (19), and the protein concentration of the supernatants was determined using the BCA protein assay. Insulin (Mercodia, Uppsala, Sweden), leptin (R&D Systems), PIGF (R&D Systems), and endoglin (CD105, a marker of neovascularization [20]) (R&D Systems) antigen levels were determined with commercially available enzyme-linked immunosorbent assays (ELISAs). Blood glucose concentrations were measured using Glucocard strips (Menarini Diagnostics, Florence, Italy); triglyceride and total, HDL, and LDL cholesterol levels were evaluated using routine clinical assays.

For mRNA determination, subcutaneous and gonadal fat pads were homogenized using lysing matrix tubes (Qbiogene, Carlsbad, CA) in a Hybrid Ribolysar (Thermo, Waltham, MA). Total DNA-free RNA was extracted using an RNeasy kit (Qiagen, Valencia, CA), and the concentration was determined using a RiboGreen RNA quantification kit (Molecular Probes, Eugene, OR). PIGF, VEGF-A121, VEGF-A165, VEGF-A189, Flt-1, and leptin mRNA levels were determined by quantitative RT-PCR and normalized to 18 S rRNA, as described previously (12).

In vivo experiments. To study the effect of PIGF deficiency on adipose tissue development, 5-week-old male wild-type or PIGF^{-/-} mice were fed a standard-fat or high-fat diet for 15 weeks and analyzed as described above. In all four groups, mice of at least four different breeding couples were used.

To evaluate the effect of PIGF neutralization on adipose tissue development, mice were injected with the PIGF neutralizing monoclonal antibody PL5D11D4, which blocks binding to Flt-1 (21) or with the control monoclonal antibody 1C8, which is directed against human tissue plasminogen activator. The monoclonal antibodies (mAbs) were screened for the absence of endotoxins and were injected three times per week intraperitoneally at a dose of 1 mg per mouse. Three different models were studied: 1) 5-week-old male wild-type (C57BL/6) mice were fed a high-fat diet for 7 weeks and then were injected with the mAbs during 6 weeks; 2) 5-week-old male *ob/ob* and corresponding wild-type (C57BL/6) mice were fed a high-fat diet and injected with the mAbs during 5 weeks; 3) 5-week-old male nude mice (BALB/c) were injected subcutaneous in the back with 1×10^7 3T3-F442A preadipocytes, cultured, and characterized as described elsewhere (22,23), to induce de novo adipose tissue formation (24,25). These mice were then fed a high-fat diet, and mAbs were injected during 5 weeks. In all three studies, the mice were monitored, killed, and analyzed as described above.

To monitor the potential recruitment of bone marrow-derived precursor cells to adipose tissue, bone marrow transplantation (BMT) was performed using 5-week-old male wild-type and PIGF^{-/-} mice. The recipient mice were lethally irradiated with an X-ray source at a single dose rate of 9.5 Gy (26). Bone marrow was aseptically removed from the femoral bone of wild-type or PIGF^{-/-} mice by flushing with medium (RPMI-1640 medium, 10 units/ml heparin and 2% fetal bovine serum) and washing with the RPMI-1640 medium. About 5×10^6 cells suspended in 200 μ l of RPMI-1640 medium were injected into the tail vein of the recipient mice. The recipient mice were divided into four groups; wild-type and PIGF^{-/-} mice received either bone marrow from a

wild-type littermate or from a PIGF^{-/-} littermate. After the transplantation, the mice were kept in sterile cages, fed water and a high-fat diet for 16 weeks, and analyzed as described above.

Statistical analysis. Data are expressed as means ± SE. Statistical significance for differences between groups was analyzed by nonparametric *t* testing. Correlations between groups are evaluated by the Spearman rank test. Progress curves of body weight or weight gain versus time were analyzed by repeated-measures ANOVA. Significance is set at $P < 0.05$.

RESULTS

Effect of PIGF deficiency on adipose tissue development

PIGF^{-/-} and wild-type mice fed a standard-fat diet. At birth, the body weight of PIGF^{-/-} pups (two litters) ($n = 10$) was already significantly lower than that of wild-type mice ($n = 8$) (1.53 ± 0.06 vs. 1.79 ± 0.05 g) ($P = 0.008$). The placenta weights after 18.5 days of pregnancy (from three mice) were 0.12 ± 0.009 g ($n = 17$) for wild-type mice compared with 0.096 ± 0.008 g ($n = 20$) for PIGF^{-/-} mice ($P = 0.04$).

When kept fed a standard-fat diet for 15 weeks, PIGF^{-/-} mice ($n = 19$) had body weights comparable to those of the wild-type controls ($n = 21$) (42 ± 1.4 vs. 41 ± 1.2 g). After overnight fasting, body weights were still comparable for both genotypes, whereas the subcutaneous ($P < 0.0005$) and gonadal ($P = \text{NS}$) adipose tissue weight was lower for PIGF^{-/-} mice (Table 1). The combined weight of the subcutaneous and gonadal fat pads was significantly lower for PIGF^{-/-} mice ($1,315 \pm 166$ vs. $1,954 \pm 200$ mg, $P = 0.025$). Furthermore, total body fat determined by noninvasive densitometry was significantly lower in PIGF^{-/-} mice ($18 \pm 1.5\%$; $n = 6$; body weight 40 ± 0.9 g) than in wild-type mice ($34 \pm 3.1\%$; $n = 6$; body weight 43 ± 2.9 g) ($P = 0.004$), whereas the lean body mass was higher (30 ± 0.94 vs. 25 ± 0.82 g; $P = 0.004$). The weights of other organs, including liver, spleen, and pancreas, were comparable (Table 1).

Food intake was 5.9 ± 0.16 g · mouse⁻¹ · day⁻¹ for wild-type and 6.4 ± 0.16 g/day for PIGF^{-/-} mice ($P = 0.04$). Physical activity at night was comparable ($23,400 \pm 360$ cycles/12 h for wild-type vs. $23,000 \pm 1,080$ for PIGF^{-/-} mice).

Adipocyte size was somewhat, but not significantly, lower in subcutaneous and gonadal adipose tissues of PIGF^{-/-} compared with wild-type mice. Blood vessel size was significantly lower in gonadal but not in subcutaneous adipose tissue of PIGF^{-/-} mice. Blood vessel density was somewhat higher, but this difference largely disappeared after normalization to the adipocyte number (ratio of 0.97 or 0.83 for subcutaneous adipose tissue of PIGF^{-/-} or wild-type mice, respectively, with corresponding values of 0.77 for gonadal adipose tissue of both genotypes) (Table

TABLE 2

Effect of PIGF deficiency on adipocyte and blood vessel size and density in adipose tissue after 15 weeks of a standard-fat or high-fat diet

	Standard-fat diet		High-fat diet	
	Wild type	PIGF ^{-/-}	Wild type	PIGF ^{-/-}
<i>n</i>	15	11	10	10
Adipocyte size (μm ²)				
Subcutaneous fat	1,260 ± 130	1,050 ± 280	2,900 ± 120	2,240 ± 280
Gonadal fat	1,590 ± 180	1,280 ± 280	3,400 ± 190	2,590 ± 360
Adipocyte density (× 10 ⁻⁶ /μm ²)				
Subcutaneous fat	670 ± 57	740 ± 97	264 ± 9.2	470 ± 92*
Gonadal fat	510 ± 66	580 ± 75	240 ± 16	400 ± 66†
Blood vessel size (μm ²)				
Subcutaneous fat	17 ± 1.0	19 ± 1.0	28 ± 2.4	25 ± 1.7
Gonadal fat	27 ± 1.7	20 ± 2.2†	41 ± 3.1	30 ± 2.2†
Blood vessel density (× 10 ⁻⁶ /μm ²)				
Subcutaneous fat	540 ± 42	700 ± 97	320 ± 15	430 ± 57
Gonadal fat	370 ± 37	410 ± 53	290 ± 23	350 ± 44
Normalized blood vessel density				
Subcutaneous fat	0.83 ± 0.053	0.97 ± 0.073	1.22 ± 0.045	0.96 ± 0.036*
Gonadal fat	0.77 ± 0.035	0.77 ± 0.056	1.21 ± 0.096	0.96 ± 0.087

Data are means ± SE. **P* = 0.01 and †*P* < 0.05 vs. wild type.

2). Endoglin levels in adipose tissue extracts were comparable for wild-type and PIGF^{-/-} mice (140 ± 37 vs. 130 ± 42 ng/g subcutaneous adipose tissue and 150 ± 35 vs. 160 ± 36 ng/g gonadal adipose tissue).

Metabolic parameters, including glucose, insulin, total cholesterol, HDL cholesterol, LDL cholesterol, and triglycerides, were also comparable for PIGF^{-/-} and wild-type mice fed a standard-fat diet (not shown). However, leptin levels were significantly lower in the PIGF^{-/-} mice (6.3 ± 2.9 ng/ml [*n* = 11] vs. 15 ± 2.5 ng/ml [*n* = 15]; *P* < 0.005). Leptin levels correlated with subcutaneous adipose tissue mass in both wild-type (*r* = 0.77, *P* < 0.0001) and PIGF^{-/-} (*r* = 0.80, *P* < 0.0001) mice; a correlation with gonadal adipose tissue mass was observed only in the wild-type mice (*r* = 0.77, *P* < 0.0001).

After 15 weeks of a standard-fat diet, expression of PIGF mRNA in gonadal adipose tissue was three- to fourfold higher than in subcutaneous adipose tissue in wild-type mice. PIGF antigen levels in adipose tissue extracts were, however, below the detection limit of the ELISA (<6 ng/mg protein). Expression of isoforms VEGF-A121, VEGF-A165, and VEGF-A189 was comparable in subcutaneous adipose tissue of both genotypes but was higher in gonadal adipose tissue of PIGF^{-/-} mice. Flt-1 expression levels were not different (Table 3).

PIGF^{-/-} and wild-type mice fed a high-fat diet. At the start of the diet (5 weeks of age), the body weights of the

PIGF^{-/-} and wild-type mice were comparable (29 ± 0.9 vs. 28 ± 0.8 g; *n* = 10). When fed a high-fat diet for 15 weeks, PIGF^{-/-} mice gained less weight than wild-type controls (24 ± 2.9 vs. 34 ± 1.2 g; *P* < 0.005), resulting in significantly lower body weight (53 ± 3.0 vs. 62 ± 1.5 g; *P* < 0.05) (Fig. 1). Repeated-measures ANOVA confirmed that both genotypes react significantly differently to the high-fat diet (*P* < 0.000001). After overnight fasting, the weight difference remained (*P* < 0.05), and the weights of the isolated subcutaneous (*P* < 0.0005) or gonadal (*P* = NS) fat pads were lower in the PIGF^{-/-} group (Table 1). The combined weights of the subcutaneous and gonadal fat pads were significantly lower for PIGF^{-/-} mice (2,901 ± 371 vs. 4,588 ± 158 mg, *P* < 0.0001).

Food intake was comparable for both genotypes (4.8 ± 0.15 or 4.5 ± 0.13 g · mouse⁻¹ · day⁻¹ for wild-type or PIGF^{-/-} mice, corresponding to 86 ± 4.6 or 82 ± 3.2 mg · day⁻¹ · g body wt⁻¹, respectively) and was relatively constant during the diet period. Physical activity at night was comparable for wild-type and PIGF^{-/-} mice fed a high-fat diet (20,100 ± 260 vs. 19,600 ± 1,200 cycles/12 h).

Adipocytes and blood vessels were somewhat smaller in PIGF^{-/-} mice (Fig. 2 and Table 2). When the blood vessel density was normalized to the adipocyte number in the tissue, it was lower in the adipose tissues of PIGF^{-/-} compared with wild-type mice (ratio of 0.96 vs. 1.22 [*P* = 0.001] for subcutaneous and 0.96 vs. 1.21 [*P* = 0.09] for

TABLE 3

mRNA expression levels in subcutaneous and gonadal adipose tissue of mice kept on a standard-fat or high-fat diet for 15 weeks

	Standard-fat diet				High-fat diet			
	Wild type		PIGF ^{-/-}		Wild type		PIGF ^{-/-}	
	Subcutaneous	Gonadal	Subcutaneous	Gonadal	Subcutaneous	Gonadal	Subcutaneous	Gonadal
PIGF	4.6	18	ND	ND	8.9	15	ND	ND
VEGF-A121	12	13	12	25	5.2	5.9	7.3	11
VEGF-A165	15	13	17	30	3.9	5.6	6.6	11
VEGF-A189	3.6	2.0	3.4	10	1.9	3.4	2.7	5.1
Flt-1	10	12	10	12	9.2	10	9.2	10
Leptin	1.8	1.6	2.4	5.5	4.9	8.0	3.9	6.1

Data are normalized to 18 S rRNA and represent pools of 10 samples measured in duplicate in two determinations. ND, not detected.

FIG. 1. Body weight (A) and weight gain (B) of wild-type (●) or $PIGF^{-/-}$ (■) mice fed a high-fat diet for 15 weeks. Data are means $\pm$ SE of 10 experiments.

gonadal adipose tissue). Endoglin levels were also comparable in adipose tissue extracts of wild-type or $PIGF^{-/-}$ mice (54 ± 4.4 vs. 74 ± 22 ng/g subcutaneous adipose tissue and 79 ± 9.6 vs. 68 ± 18 ng/g gonadal adipose tissue).

After 15 weeks of high-fat diet, plasma levels of glucose, insulin, total cholesterol, HDL and LDL cholesterol, and triglycerides were not significantly different for wild-type and $PIGF^{-/-}$ mice (not shown). Plasma leptin levels were 54 ± 13 ng/ml for $PIGF^{-/-}$ mice compared with 83 ± 7.6 ng/ml for wild-type mice ($P = 0.10$). Leptin mRNA expression was somewhat higher in gonadal than in subcutaneous adipose tissue but was comparable for wild-type and $PIGF^{-/-}$ mice (Table 3).

$PIGF$ mRNA was detected in both subcutaneous and gonadal adipose tissue of wild-type mice fed the high-fat diet for 15 weeks (at an approximate twofold higher level

in gonadal tissue) (Table 3). Immunostaining with a rat anti-mouse $PIGF$ -2 mAb confirmed expression of $PIGF$ in wild-type adipose tissues (not shown). $PIGF$ antigen levels in adipose tissue extracts were, however, undetectable by ELISA. Expression of the isoforms VEGF-A121 and VEGF-A165 was approximately twofold higher in gonadal adipose tissue of $PIGF^{-/-}$ mice compared with wild-type controls. Expression of Flt-1 was comparable in subcutaneous and gonadal adipose tissue of both genotypes (Table 3).

Effect of $PIGF$ neutralization on adipose tissue development. Administration of the $PIGF$ -neutralizing mAb PL5D11D4 or the control mAb 1C8 to 12-week-old wild-type mice ($n = 10$ each) fed a high-fat diet for 6 weeks resulted in very similar body weights (38 ± 0.9 vs. 38 ± 1.5 g) and weights of the isolated subcutaneous ($1,510 \pm 80$ vs. $1,500 \pm 57$ mg) or gonadal ($2,100 \pm 60$ vs. $2,160 \pm 88$ mg) adipose tissues after 6 weeks of administration. Leptin levels in adipose tissue extracts were comparable for mAb PL5D11D4- and mAb 1C8-treated mice, both in subcutaneous (80 ± 8.5 vs. 83 ± 10 ng/g tissue) and in gonadal (65 ± 15 vs. 66 ± 9.6 ng/g tissue) adipose tissue.

Similarly, administration of mAb PL5D11D4 or mAb 1C8 during 5 weeks to 5-week-old *ob/ob* mice ($n = 8$ or 7) or to corresponding wild-type mice ($n = 9$ or 8) resulted in comparable body weights (46 ± 1.4 vs. 47 ± 1.7 g for *ob/ob* and 26 ± 0.80 vs. 26 ± 0.75 g for wild type), and weights of isolated subcutaneous ($2,990 \pm 120$ vs. $3,270 \pm 220$ mg for *ob/ob* and 510 ± 120 vs. 430 ± 75 mg for wild type) and gonadal ($3,040 \pm 200$ vs. $3,030 \pm 160$ mg for *ob/ob* and 610 ± 75 vs. 610 ± 99 mg for wild type) adipose tissues after 5 weeks of administration. In both models, food intake was not different for the two mAbs; adipocyte and blood vessel size and density in the adipose tissues were not significantly different, and weights of other organs including liver, kidney, spleen, pancreas, lung, and heart were comparable (data not shown).

Administration of mAb PL5D11D4 during 5 weeks to nude mice previously injected with 3T3-F442A preadipocytes, in contrast, caused a significant reduction of the de novo fat pad formation compared with mice receiving the control mAb 1C8 (14 ± 2.5 mg [$n = 10$] vs. 27 ± 3.6 mg [$n = 6$], $P = 0.0047$). In the mAb PL5D11D4- and mAb 1C8-treated groups, total body weights (25 ± 0.45 vs. 24 ± 0.25 g) and weights of the isolated subcutaneous (267 ± 33 vs. 283 ± 17 mg) or gonadal (276 ± 58 vs. 314 ± 17 mg) adipose tissues were indistinguishable. Quantitative analysis of the fat pads (Table 4) revealed comparable adipocyte size and density for both mAbs, whereas the blood vessel size was somewhat smaller in de novo, subcutaneous, and gonadal adipose tissues of the mAb PL5D11D4-treated mice. Blood vessel densities, normalized to adipocyte number, were, however, not different. The weight of other organs was also very similar.

Effect of BMT on adipose tissue development. High-fat diet feeding for 16 weeks after BMT of wild-type or $PIGF^{-/-}$ donor to wild-type or $PIGF^{-/-}$ recipient mice produced $\sim 25\%$ lower weight gain in the $PIGF^{-/-}$ recipient mice (Table 5). Weights of the isolated subcutaneous and gonadal adipose tissues, as well as the adipocyte and blood vessel size, were comparable. However, blood vessel densities before and after normalization to adipocyte number were significantly lower in the $PIGF^{-/-}$ recipient mice, both for BMT (wild type $\rightarrow$ $PIGF^{-/-}$) and for BMT ($PIGF^{-/-}$ $\rightarrow$ $PIGF^{-/-}$). For BMT ($PIGF^{-/-}$ $\rightarrow$ wild type),

FIG. 2. Staining with the *B. simplicifolia* lectin of subcutaneous adipose tissue from wild-type (A and B) or $PIGF^{-/-}$ (C and D) mice fed a standard-fat diet (A and C) or a high-fat diet (B and D) for 15 weeks. The yellow structures are blood vessels.

TABLE 4

Effect of PIGF neutralization on adipocyte and blood vessel size and density in adipose tissues after injection of 3T3-F442A preadipocytes into nude mice

	mAb PL5D11D4			mAb 1C8		
	De novo	Subcutaneous	Gonadal	De novo	Subcutaneous	Gonadal
Adipocyte size (μm^2)	1,200 $\pm$ 110	720 $\pm$ 110	1,140 $\pm$ 93	1,440 $\pm$ 93	730 $\pm$ 35	1,260 $\pm$ 88
Adipocyte density ($\times 10^{-6}/\mu\text{m}^2$)	620 $\pm$ 34	1,090 $\pm$ 120	710 $\pm$ 56	550 $\pm$ 23	1,000 $\pm$ 20	620 $\pm$ 27
Blood vessel size (μm^2)	27 $\pm$ 3.9	27 $\pm$ 5.5	19 $\pm$ 2.5	33 $\pm$ 4.6	30 $\pm$ 2.3	24 $\pm$ 3.2
Blood vessel density ($\times 10^{-6}/\mu\text{m}^2$)	1,160 $\pm$ 68	1,260 $\pm$ 130	880 $\pm$ 64	1,010 $\pm$ 120	1,160 $\pm$ 72	670 $\pm$ 75
Normalized blood vessel density	1.90 $\pm$ 0.11	1.18 $\pm$ 0.05	1.25 $\pm$ 0.07	1.85 $\pm$ 0.19	1.19 $\pm$ 0.05	1.09 $\pm$ 0.11

Data are means $\pm$ SE.

these data were comparable to those for BMT (wild type $\rightarrow$ wild type) (Table 5).

Successful BMT in this model is confirmed by the fact that the mice survived, whereas control mice without BMT died within 1–2 weeks of the irradiation. Furthermore, blood cell counts were normalized within 4 weeks of the BMT (data not shown).

PIGF mRNA levels, normalized to 28 S RNA, were comparable in subcutaneous and in gonadal adipose tissues after BMT (wild type $\rightarrow$ wild type) and BMT (PIGF^{-/-} $\rightarrow$ wild type). No PIGF expression was detected in adipose tissues after BMT (wild type $\rightarrow$ PIGF^{-/-}) or BMT (PIGF^{-/-} $\rightarrow$ PIGF^{-/-}) (data not shown).

DISCUSSION

PIGF is expressed in murine adipose tissue, both in adipocytes and in the stromal-vascular cell fraction (12), but no information on a potential functional role is available. In the present study, we have investigated whether PIGF inactivation affects adipose tissue development in murine models of obesity.

With a standard-fat diet, the total body weights of

PIGF^{-/-} and wild-type mice were comparable; although total body fat content was lower in PIGF^{-/-} mice, their lean body mass was higher. With a high-fat diet, PIGF^{-/-} mice had significantly reduced body weights and subcutaneous and gonadal fat pad weights. Blood vessel density normalized to the adipocyte number (27) is 1.5- or 1.6-fold higher in subcutaneous or gonadal adipose tissue of obese wild-type mice compared with that of lean controls. In contrast, there is little difference between PIGF^{-/-} lean or obese mice (1.0- or 1.2-fold for subcutaneous or gonadal tissue, respectively). Thus, in obese (15 weeks of a high-fat diet) PIGF^{-/-} mice, subcutaneous and gonadal adipose tissue blood vessel densities are lower than those in wild-type mice (Table 2), indicating that development of new blood vessels is impaired in the PIGF^{-/-} mice fed a high-fat diet. PIGF^{-/-} mice also showed significantly lower blood vessel size in gonadal adipose tissues, with both a standard-fat and high-fat diet. These findings suggest that impaired angiogenesis in PIGF^{-/-} mice fed a high-fat diet, possibly associated with reduced adipose tissue perfusion, contributes to the reduced adipose tissue growth. In mice fed a standard-fat diet, however, the lower

TABLE 5

Effect of BMT on adipose tissue development

	BMT			
	(Wild type $\rightarrow$ wild type)	(PIGF ^{-/-} $\rightarrow$ wild type)	(Wild type $\rightarrow$ PIGF ^{-/-})	(PIGF ^{-/-} $\rightarrow$ PIGF ^{-/-})
<i>n</i>	7	7	10	9
Weight gain (g)	13 $\pm$ 1.4	13 $\pm$ 2.1	9.2 $\pm$ 2.5	9.9 $\pm$ 1.4
Weight (mg)				
Subcutaneous fat	1,270 $\pm$ 150	1,200 $\pm$ 170	990 $\pm$ 110	840 $\pm$ 130
Gonadal fat	1,630 $\pm$ 220	1,390 $\pm$ 230	1,210 $\pm$ 150	1,420 $\pm$ 200
Adipocyte size (μm^2)				
Subcutaneous fat	1,560 $\pm$ 110	1,790 $\pm$ 250	1,870 $\pm$ 130	1,560 $\pm$ 130
Gonadal fat	3,460 $\pm$ 500	3,320 $\pm$ 420	3,150 $\pm$ 310	3,090 $\pm$ 250
Adipocyte density ($\times 10^{-6}/\mu\text{m}^2$)				
Subcutaneous fat	540 $\pm$ 30	510 $\pm$ 59	470 $\pm$ 29	540 $\pm$ 38
Gonadal fat	270 $\pm$ 29	290 $\pm$ 38	310 $\pm$ 32	300 $\pm$ 27
Blood vessel size (μm^2)				
Subcutaneous fat	16 $\pm$ 1.3	16 $\pm$ 1.9	15 $\pm$ 0.69	16 $\pm$ 0.69
Gonadal fat	26 $\pm$ 3.6	27 $\pm$ 3.2	21 $\pm$ 2.0	21 $\pm$ 0.99
Blood vessel density ($\times 10^{-6}/\mu\text{m}^2$)				
Subcutaneous fat	370 $\pm$ 16	310 $\pm$ 40	260 $\pm$ 18*	270 $\pm$ 22†
Gonadal fat	240 $\pm$ 22	240 $\pm$ 35	160 $\pm$ 9.0*	180 $\pm$ 16‡
Normalized blood vessel density				
Subcutaneous fat	0.73 $\pm$ 0.07	0.63 $\pm$ 0.07	0.55 $\pm$ 0.03	0.52 $\pm$ 0.04‡
Gonadal fat	0.96 $\pm$ 0.11	0.83 $\pm$ 0.05§	0.59 $\pm$ 0.07†	0.65 $\pm$ 0.08‡

Data are means $\pm$ SE. * $P < 0.005$, † $P < 0.01$, and ‡ $P < 0.05$ vs. BMT (wild type $\rightarrow$ wild type); § $P < 0.05$ vs. BMT (wild type $\rightarrow$ PIGF^{-/-}).

subcutaneous adipose tissue weight does not seem to be angiogenesis dependent.

Plasma leptin levels were lower in PIGF^{-/-} than in wild-type mice and correlated with adipose tissue mass, as reported previously (28). This may be due to differences in secretion from adipose tissues or in clearance of leptin. Our finding that leptin levels in subcutaneous and gonadal adipose tissues were not affected by treatment with a PIGF-neutralizing antibody do not support a direct interaction between leptin and PIGF.

De novo fat pad formation in nude mice could be impaired with mAb PL5D11D4. This mAb blocks binding of PIGF to its receptor Flt-1 and was previously successfully used at the same dose in murine models of basal adhesion formation after laparoscopy (21) and in several tumor models (C. Fischer, P.C., unpublished data). In contrast, attempts to reduce adipose tissue development by administration of this mAb to wild-type mice fed a high-fat diet or to *ob/ob* mice fed a standard-fat diet failed, indicating that direct interactions of PIGF with Flt-1 do not promote ongoing adipose tissue development. Thus, neutralization of PIGF function does not affect ongoing adipose tissue formation but has the capacity to prevent or impair de novo fat pad formation. This finding is in agreement with the observed impaired adipose tissue formation in mice with a genetic deficiency of PIGF. It was previously shown in the same diet model that expression of PIGF, primarily in subcutaneous adipose tissue, decreases with time (between 2 and 15 weeks of diet) (12). It is conceivable that PIGF is mainly important during the first stages of adipose tissue formation, whereas in later phases other angiogenic factors may become more important.

In our model, we did not observe significant effects on adipose tissue formation (during 16 weeks of a high-fat diet) of BMT from wild-type or PIGF^{-/-} donor to wild-type or PIGF^{-/-} recipient mice, although weight gain was consistently lower in PIGF^{-/-} recipient mice. We did observe a significantly lower blood vessel density (Table 5) in adipose tissues of PIGF^{-/-} recipient mice, which is in agreement with the findings in the diet study (Table 2). After BMT, PIGF mRNA was detected only in adipose tissues of mice with BMT (wild type → wild type) and (PIGF^{-/-} → wild type) but not in mice with BMT (wild type → PIGF^{-/-}), indicating that recruitment of precursor cells from bone marrow to adipose tissue does not occur in this model. It is thus conceivable that PIGF derived from adipocytes and/or stromal-vascular cells in the adipose tissue contributes to local angiogenesis.

Our observations are in line with the emerging concept that PIGF deficiency has little or no effect on angiogenesis under normal conditions (as with a standard-fat diet) but is associated with impaired angiogenesis under stress conditions (29–33) (as with a high-fat diet). Thus, reduced angiogenesis during the early stages of adipose tissue development in mice with PIGF deficiency may contribute to impaired obesity.

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