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Bone Marrow Plasminogen Activator Inhibitor-1 Influences the Development of Obesity*

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Plasma levels of plasminogen activator inhibitor-1 (PAI-1) are elevated in obesity and correlate with body mass index. The increase in PAI-1 associated with obesity likely contributes to increased cardiovascular risk and may predict the development of type 2 diabetes mellitus. Although adipocytes are capable of synthesizing PAI-1, the bulk of evidence indicates that cells residing in the stromal fraction of visceral fat are the primary source of PAI-1. We hypothesized that bone marrow-derived PAI-1, *e.g.* derived from macrophages located in visceral fat, contributes to the development of diet-induced obesity. To test this hypothesis, male C57BL/6 wild-type mice and C57BL/6 PAI-1 deficient mice were transplanted with either PAI-1$^{-/-}$, PAI-1$^{+/-}$, or PAI-1$^{+/+}$ bone marrow. The transplanted animals were subsequently fed a high fat diet for 24 weeks. Our findings show that only the complete absence of PAI-1 protects from the development of diet-induced obesity, whereas the absence of bone marrow-derived PAI-1 protects against expansion of the visceral fat mass. Remarkably, there is a link between the PAI-1 levels, the degree of inflammation in adipose tissue, and the development of obesity. Based on these findings we suggest that bone marrow-derived PAI-1 has an effect on the development of obesity through its effect on inflammation.

Plasminogen activator inhibitor type-1 (PAI-1)[2] is the principal inhibitor of tissue-type plasminogen activator and urokinase-type plasminogen activator and, therefore, is a key regulatory protein of the plasminogen/pro-matrix metalloproteinase system (for review, see Ref. 1). As such, PAI-1 is involved in many overlapping processes as fibrinolysis, activation of pro-matrix metalloproteinases, cell migration, angiogenesis, and tissue remodeling (for review, see Ref. 2). Plasma PAI-1 likely represents synthesis in a number of tissues, including vascular endothelial cells (3), the liver (4), smooth muscle cells (5), and adipocytes (6).

There is substantial evidence linking PAI-1 with obesity and insulin resistance. In humans, the visceral fat mass correlates with plasma PAI-1. Conversely, weight loss promotes a decrease in plasma PAI-1 levels, which predictably rebound when weight is regained (7, 8). PAI-1 levels fall and fibrinolytic activity improves in patients undergoing weight reduction surgery (9, 10). Additional evidence supporting the relationship between PAI-1 and obesity has been observed in experimental studies using genetically modified mice. PAI-1 deficiency reduces adiposity and improves the metabolic profile in genetically obese mice (11). Similarly, PAI-1 deficiency attenuates nutritionally induced obesity and insulin resistance in C57BL/6 mice (12). However, the relationship between PAI-1 and obesity is not strictly linear, and the site of PAI-1 expression and the genetic background of the mice influence the response to high caloric intake. For example, transgenic mice overexpressing PAI-1 in fat gain less weight than their wild-type counterparts (13), and PAI-1 deficient mice on a mixed genetic background have been reported to gain weight faster than WT animals (14). In *ob/ob* mice, PAI-1 levels are increased 5-fold compared with their lean littermates (15). Adipose tissue from *ob/ob* mice also exhibits increased PAI-1 gene expression (16). In addition to influencing the development of obesity, it appears that PAI-1 likely contributes to the complications of obesity (17). Plasma PAI-1 predicts the development of type 2 diabetes independent of insulin resistance (18) and ischemic cardiovascular events.

Although PAI-1 is known to be synthesized in vascular tissues (including the endothelium), the liver, and adipose tissue, the source and mechanisms of increased PAI-1 in obesity are incompletely understood (19, 20). Adipose tissue is a significant source of PAI-1 with the bulk of production derived specifically from visceral fat (8, 21, 22). However, the exact source of PAI-1 in adipose tissue is still debatable. The increased plasma PAI-1 in obesity may be derived directly from the cellular constituents of fat (adipocytes, stromal cells, or vascular tissue) and/or indirectly by the effects of other adipose-derived factors (peptides, cytokines, hormones, or lipids) that stimulate local and systemic PAI-1 production (23, 24). Recent studies indicate that stromal cells may be the primary source of PAI-1 in visceral adipose tissue (25–27), which is supported by the finding that PAI-1 mRNA is localized within the cytosol of small cells free of lipids (25). However, immunohistochemical analysis indicates that PAI-1 antigen is also localized to lipid-laden adipocytes (6, 23, 25), which may reflect the attachment of PAI-1 protein to the surface of the adipocytes. Alternatively, this observation may reflect a phagocytic activity of these cells (28).

In this study we addressed the questions regarding the sources of PAI-1 in visceral fat as well as the effect of PAI-1 on

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[2] The abbreviations used are: PAI-1, plasminogen activator inhibitor-1; WT and KO, PAI-1 wild-type and PAI-1-deficient hosts, respectively; PAI-1$^{+/+}$, PAI-1$^{+/-}$, PAI-1$^{-/-}$ are used to indicate bone marrow derived from wild-type, heterozygous, or knock-out animals respectively; HFD, high fat diet; RT-QPCR, real-time quantitative PCR; EGFP, enhanced green fluorescent protein; ANOVA, analysis of variance.

the development of obesity using bone marrow transplantation. Marrow-derived cells not only impact upon PAI-1 production in fat, but they also influence the development of obesity.

EXPERIMENTAL PROCEDURES

Animal Procedures—Adult (6–8 week old) male PAI-1-deficient mice (PAI-$1^{-/-}$, KO) on a C57BL/6J background and wild-type (PAI-$1^{+/+}$, WT) C57BL/6J mice were purchased from The Jackson Laboratory (Bar Harbor, ME). PAI-$1^{+/-}$ mice were generated by in-house breeding. Mice were housed in a pathogen-free barrier facility (12-h light/12-h dark cycle). A week before and 2 weeks after bone marrow transplantation, 100 mg/liter neomycin and 10 mg/liter polymyxin B sulfate (both from Sigma-Aldrich) were added to the acidified water (pH 2.6). Bone marrow was collected from donor mice by flushing femurs and tibia with RPMI 1640 media (Invitrogen) containing 2% fetal bovine serum and 5 units/ml heparin (Sigma-Aldrich). Bone marrow cells were washed, resuspended in fresh media, and counted. Recipient mice were lethally irradiated (9 gray), and 4 h later 2–5 $\times$ 10^6 marrow cells in 0.2 ml were transplanted by retro-orbital venous plexus injection. In practice, male PAI-1 KO mice and WT mice on a C57BL/6J background received either PAI-$1^{-/-}$, PAI-$1^{+/-}$, or PAI-$1^{+/+}$ C57BL/6J bone marrow (n = 5–10). The success of the deletion of the original bone marrow and the uptake of the received donor bone marrow was assessed by quantifying mRNA for PAI-1 in bone marrow of the recipient mice after sacrifice using real-time quantitative PCR (RT-QPCR). In addition, B6D2F1/J WT mice (The Jackson Laboratory) were lethally irradiated and transplanted with bone marrow from a transgenic mouse expressing enhanced green fluorescent protein (EGFP) driven by the proximal −2.9-kilobase of the human PAI-1 promoter on the same background (29). After a recovery period of 2 weeks, mice were placed on a high fat diet (HFD) for 24 weeks (Diet TD 88137; Harlan Teklad, Madison, WI) in which 42% of total calories were derived from fat. This diet has been shown to induce obesity, hyperglycemia, and insulin resistance in C57BL/6J mice (14, 30). Food intake was determined 3 times at different days during the 24 weeks on HFD, each time over a 24-h period, and expressed as g/mouse/h. One day before sacrifice the body composition of unanaesthetized mice was determined using a Minispec Model mq 7.5 (7.5 mHz) (Bruker Optics, Billerica, MA). Groups of mice were sacrificed under anesthesia with isoflurane after a 4-h fasting period after 24 weeks on HFD. White adipose tissue, heart, liver, spleen, kidneys, brain, pancreas, aorta, lungs, and bone marrow were harvested. Livers, kidneys, spleens, and white adipose tissue were weighed. One portion of each organ was snap-frozen in liquid nitrogen and stored at −80 °C for RNA extraction; other portions were used for immunohistochemistry. Body weights were measured every 2 weeks. Metabolic studies were performed as indicated below. All animal protocols were approved by the Vanderbilt University Institutional Animal Care and Use Committee.

Blood and Plasma Measurements— 4 days before irradiation, and again after the recovery period (before switching to the HFD), and after 12 and 24 weeks on the HFD, venous blood was obtained from the mice by retro-orbital sinus puncture using a tube with anticoagulant (ammonium heparin). Each time mice were fasted for 4 h. The plasma was isolated by centrifugation at 3000 $\times$ g for 15 min and stored at −80 °C. Fasting glucose levels were determined using a HemoCue glucose monitor (HemoCue AB, Ángelholm, Sweden). Mouse leptin and resistin were quantified using murine specific enzyme-linked immunosorbent assays (ELISAs) (Linco, St. Charles, MO); mouse adiponectin and insulin were measured using a mouse adiponectin and ultrasensitive insulin ELISA (Alpco, Salem, NH); PAI-1 antigen levels were measured using a murine PAI-1 antigen ELISA (Molecular Innovations, Southfield, MI).

TABLE 1
Primers for quantitative real-time PCR

Target	Primer pair sequence 5′→3′
PAI-1	ACGCCTGGTGCTGGTGAATGC
	ACGGTGCTGCCATCAGACTTGTG
CD68	CTTCCCACAGGCAGCACAG
	AATGATGAGAGGCAGCAAGAGG
Resistin	AGCTGTGGGACAGGAGCTAA
	AGCAGCTCAAGACTGCTGTG
Adiponectin	AGAGGACTGACTTTTGCTGC
	GCCAGAAGAGGGTGTTGTAT
Leptin	CCCCTCAGATCCTCCAAAAT
	AACCCTGCTTGCAGTCTATT
18 S	GGGTCGGGAGTGGGTAATT
	GAGAGGGAGCCTGAGAAAC

Glucose Challenge—Two weeks before sacrifice glucose tolerance was tested after a 4-h fast by intraperitoneal injection of glucose (Sigma-Aldrich; 1.8 mg/g body weight). Blood glucose levels were determined from tail vein samples taken immediately before (t = 0) as well as at 15, 30, 60, 90, and 120 min after the glucose administration using a HemoCue glucose analyzer.

Indirect Calorimetry—Oxygen consumption (VO_2) and the respiratory exchange ratio were measured by an Oxymax indirect calorimeter (Columbus Instruments, Columbus, OH) with an air flow of 0.6 liters/min. VO_2 is expressed as the volume of O_2 consumed/kg of body weight/h. After a 1-h adaptation period in the metabolic chamber, VO_2 was measured starting at 10:00 a.m. in individual mice for 1 min at 25-min intervals for a total of 24 h under a consistent environmental temperature (22 °C). The respiratory exchange ratio is the ratio of the volume of CO_2 produced to the volume of O_2 consumed. Energy expenditure (EE) was calculated as EE = (3.815 + 1.232 $\times$ VCO_2/VO_2) $\times$ VO_2. Mice ambulatory activity was simultaneously estimated by the number of laser beams broken in both x and y directions.

Real-time Quantitative PCR—Total RNA was isolated using Trizol reagent (Invitrogen) following manufacturer's instructions. The RNA was reverse-transcribed using random hexamer primers and oligo-dT with the iScript cDNA synthesis kit (Bio-Rad) following the recommended conditions from the manufacturer. Subsequently, specific cDNA targets (primer sequences, Table 1) were amplified using iQ SYBR Green Supermix (Bio-Rad) on an *i*-Cycler instrument (Bio-Rad). 18 S cDNA was used as an endogenous reference. Samples without cDNA were included to exclude nonspecific reactions due to primer interactions. Furthermore, a positive control containing cDNA for the analyzed target was included. The relative levels of mRNA were quantified using the $2^{-\Delta\Delta CT}$ method (31). Prim-

ers for the specific targets were designed using Beacon Designer 2.0.

Histology and Immunohistochemistry—Tissues for immunohistochemistry were fixed in 10% neutral, phosphate-buffered formalin for 24–48 h and paraffin-embedded. Subsequently the paraffin embedded tissues were processed in 4-μm sections. Histological sections of epididymal fat pads were stained with hematoxylin and eosin and studied under 20-fold magnification to compare adipocyte size. On five randomly selected fields from three different sections, the number of stroma cell nuclei and the number of adipocytes, based on morphological criteria, were determined, and the results were expressed as a ratio. Mature adipocytes are defined as differentiated cells distended with lipid material. They contain peripherally inconspicuous nuclei. Blood vessels were stained using a rabbit anti-human polyclonal antibody against von Willebrand factor (vWF, 1:500) that has been shown to bind mouse vWF (Chemicon, Temecula, CA). PAI-1 antigen was localized using biotin labeled rabbit anti-mouse PAI-1 IgG fraction (1:500) (Molecular Innovations). Finally, macrophages were localized using a rat anti-mouse F4/80 monoclonal antibody (1:250) (Abcam, Cambridge, MA) and a rat anti-mouse Mac3 monoclonal antibody (1:50) (BD Pharmingen). For detection we used ready-to-use biotinylated secondary antibodies against the appropriate IgGs in combination with ready-to-use streptavidin-horseradish peroxidase and AEC for detection (all from BioGenex, San Ramon, CA). For fluorescence detection we used a secondary goat anti-rat IgG coupled to Texas Red (Invitrogen) followed by Vectashield Mounting Medium with 4′,6-diamidino-2-phenylindole (H-1200) (Vector Laboratories, Burlingame, CA).

Statistical Analysis—Data are expressed as the mean ± S.E. Statistical significance for the difference between groups was evaluated using nonparametric ANOVA or Student's t test. Values of $p < 0.05$ are considered statistically significant.

RESULTS

PAI-1 Production in Bone Marrow-derived Cells Contributes to Plasma and Adipose Tissue PAI-1 Production—The success of the bone marrow transplantation was confirmed after sacrificing the animals using RT-QPCR for PAI-1 mRNA on total RNA isolated from bone marrow cells (Fig. 1*A*). In the WT hosts, the bone marrow PAI-1 mRNA levels confirmed eradication of the host bone marrow. Transplantation of WT mice with either heterozygous (PAI-$1^{+/-}$→WT) or knock-out (PAI-$1^{-/-}$→WT) marrow led to dose-dependent reductions in marrow mRNA levels (24- and 38-fold reduction, respectively, $p < 0.05$). In the KO hosts, the PAI-1 mRNA levels corresponded with the genotype of the bone marrow donor (PAI-$1^{+/+}$→KO 0.8 ± 0.3, PAI-$1^{+/-}$→KO 0.1 ± 0.04, and PAI-$1^{-/-}$→KO not detected relative to WT hosts receiving PAI-$1^{+/+}$ marrow). PAI-1 mRNA levels were not significantly different when comparing the WT and KO host combination that received the same bone marrow (*i.e.* PAI-$1^{+/+}$→WT *versus* PAI-$1^{+/+}$→KO; PAI-$1^{+/-}$→WT *versus* PAI-$1^{+/-}$→KO; PAI-$1^{-/-}$→WT *versus* PAI-$1^{-/-}$→KO) ($p > 0.05$).

Plasma PAI-1 antigen levels generally corresponded with bone marrow PAI-1 mRNA expression levels in the KO hosts (Fig. 1*B* and Table 2). After 24 weeks on a HFD, transplantation

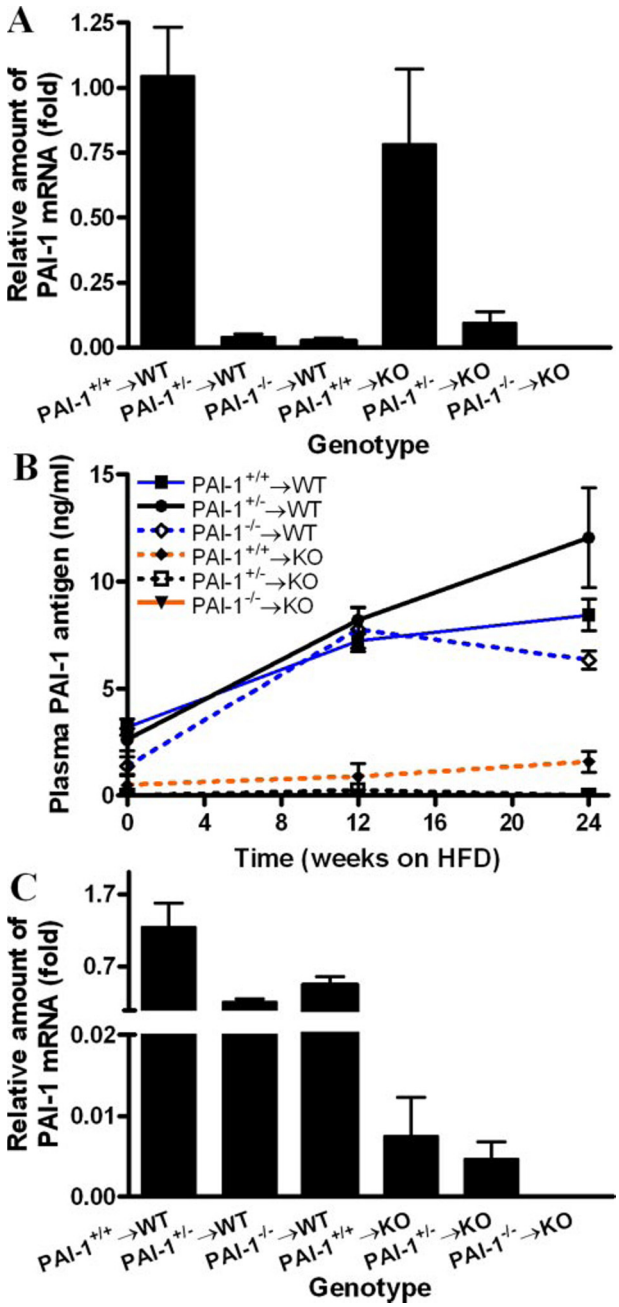

FIGURE 1. **Bone marrow-derived PAI-1 contributes to total PAI-1 expression.** *A*, the transplantation of WT and KO hosts with either PAI-$1^{+/+}$, PAI-$1^{+/-}$, or PAI-$1^{-/-}$ bone marrow was analyzed using RT-QPCR for PAI-1 mRNA isolated from the bone marrow of the transplanted animals. The mRNA levels are expressed relative to those in PAI-$1^{+/+}$→WT mice (=1). *B*, plasma PAI-1 antigen levels show a contribution of bone-marrow derived PAI-1 production. *C*, PAI-1 mRNA levels in the epididymal fat pad suggest that the majority of PAI-1 production is derived from the host adipose tissue but confirms an important contribution for cells originating from the bone marrow (n = 5–10 for all analyses).

of WT hosts with PAI-$1^{-/-}$, PAI-$1^{+/-}$, or PAI-$1^{+/+}$ bone marrow produced plasma PAI-1 levels of 6.3 ± 0.4, 12.1 ± 2.3, and 8.5 ± 0.7 ng/ml, respectively ($p < 0.01$). Transplantation of the KO hosts with PAI-$1^{-/-}$, PAI-$1^{+/-}$, or PAI-$1^{+/+}$ bone marrow produced plasma PAI-1 levels of 0, 0.004 ± 0.004, and 1.6 ± 0.5 ng/ml, respectively ($p < 0.05$).

RT-QPCR was also used to quantify PAI-1 mRNA expression in adipose tissue (Fig. 1*C*). WT hosts expressed significantly

TABLE 2

Biochemical markers of obesity development

Base, before start of the HFD (0 weeks); sack, after 24 weeks on the HFD ($n \geq 5$). Numbers represent plasma levels except for glucose where whole blood is analyzed.

Marker	PAI-1$^{+/+}$→WT		PAI-1$^{+/-}$→WT		PAI-1$^{-/-}$→WT		PAI-1$^{+/+}$→KO		PAI-1$^{+/-}$→KO		PAI-1$^{-/-}$→KO	
	Base	Sack	Base	Sack	Base	Sack	Base	Sack	Base	Sack	Base	Sack
Glucose (mg/dl)[a]	122 ± 10	206 ± 8	173 ± 12	187 ± 18	105 ± 11	239 ± 10	144 ± 11	211 ± 10	164 ± 8	185 ± 4	137 ± 7	179 ± 15
Insulin (ng/ml)	0.5 ± 0.1	1.5 ± 0.3	0.8 ± 0.1	1.9 ± 0.4	0.7 ± 0.3	1.1 ± 0.3	0.7 ± 0.5	1.1 ± 0.2	1.1 ± 0.2	2.2 ± 0.4	0.4 ± 0.1	1.2 ± 0.2
PAI-1 (ng/ml)[a,b]	3.2 ± 0.4	8.5 ± 0.7	2.6 ± 0.5	12.1 ± 2.3	1.5 ± 0.5	6.3 ± 0.4	0.5 ± 0.5	1.6 ± 0.5	0.0 ± 0.0	0.004 ± 0	0.0 ± 0.0	0.0 ± 0.0
Leptin (ng/ml)[a,b]	0.2 ± 0.1	8.8 ± 2.8	0.5 ± 0.1	23.5 ± 4.1	0.2 ± 0.1	7.5 ± 1.4	0.8 ± 0.2	10.0 ± 1.9	0.5 ± 0.0	20.9 ± 2.4	0.3 ± 0.1	3.6 ± 0.8
Resistin (ng/ml)[a,b]	4.7 ± 0.1	14.8 ± 1.3	6.4 ± 0.4	14.1 ± 1.4	6.0 ± 1.0	13.8 ± 0.8	5.2 ± 1.0	19.1 ± 0.7	8.1 ± 1.2	13.1 ± 1.3	6.3 ± 0.5	13.0 ± 0.8

[a] Sacrifice values are different among the various groups; $p < 0.05$ (ANOVA).
[b] Base-line values are different among the various groups; $p < 0.05$ (ANOVA).

higher PAI-1 mRNA levels than the different transplantation combinations in the KO hosts ($p < 0.0001$). In KO mice receiving PAI-1$^{+/+}$ or PAI-1$^{+/-}$ marrow, PAI-1 mRNA levels were a fraction of those seen in WT mice (0.007 ± 0.005-fold; $p < 0.01$ and 0.005 ± 0.002-fold; $p < 0.01$, respectively, both relative to PAI-1$^{+/+}$→WT). In WT mice receiving PAI-1$^{+/+}$ bone marrow, PAI-1 mRNA levels were 5.8- and 2.8-fold higher compared with those receiving PAI-1$^{+/-}$ and PAI-1$^{-/-}$ marrow, respectively (1.25 ± 0.33-fold (reference) *versus* 0.22 ± 0.04 and 0.45 ± 11-fold; $p < 0.05$). PAI-1 mRNA levels were not significantly different between the WT hosts receiving PAI-1$^{+/-}$ and PAI-1$^{-/-}$ marrow ($p > 0.05$).

PAI-1 Production in Bone Marrow-derived Cells Influences the Development of Obesity—KO hosts receiving PAI-1$^{-/-}$ bone marrow gained less weight than all other host/donor combinations ($p < 0.05$) (Fig. 2*A*). The difference in weight gain observed between WT hosts receiving PAI-1$^{+/+}$ bone marrow and KO hosts receiving PAI-1$^{-/-}$ bone marrow was similar to the differences observed between C57BL/6J WT mice and C57BL/6J KO mice after 20 weeks on a HFD (Fig. 2*B*). The weight difference observed for the transplanted animals was confirmed by the body composition (body composition of mice receiving PAI-1$^{+/-}$ marrow was not determined), showing a lower fat content in the KO mice receiving PAI-1$^{-/-}$ bone marrow in comparison with the other groups ($p < 0.05$) (Fig. 2*C*). No difference was observed for lean mass ($p = 0.99$, data not shown). Determination of the epididymal fat pad weight was consistent with a protective effect of the total absence of PAI-1 for the development of obesity (Fig. 3*A* and Table 3). Remarkably, the PAI-1$^{-/-}$→WT mice also developed less epididymal fat. The epididymal fat pad weight was reduced by 25% ($p < 0.05$) in the WT host receiving PAI-1$^{-/-}$ bone marrow and by 58% in KO hosts receiving PAI-1$^{-/-}$ bone marrow. The ratio of stromal cells/adipocytes was significantly different between the host/donor groups ($p < 0.01$), with fewest stromal cells present in the PAI-1$^{-/-}$→WT and PAI-1$^{-/-}$→KO combinations (79 and 67% of the ratio of the PAI-1$^{+/+}$→WT group, respectively) (Fig. 3*B* and Table 3). For the KO hosts, a bone marrow-derived PAI-1 concentration effect appeared with a higher number of stromal cells corresponding with higher levels of bone marrow PAI-1 (Fig. 4). A similar pattern was observed with respect to epididymal fat pad weight. The average adipocyte size also differed between the various host/donor combinations ($p < 0.05$) (Figs. 3*C* and 4 and Table 3). Adipocytes in the PAI-1$^{-/-}$→WT group were 30% smaller, and whole adipocytes in the PAI-1$^{-/-}$→KO were 50% smaller than those in the other groups. No differences were seen in food consumption, oxygen consumption, energy expenditure, or mobility between the different groups. Quantification of leptin mRNA in the epididymal fat pad showed a pattern similar to that observed in epididymal fat pad weight, number of stromal cells, and adipocyte cell size ($p < 0.05$) (Fig. 3*D*). Leptin expression levels in PAI-1$^{-/-}$→WT, PAI-1$^{-/-}$→KO, and PAI-1$^{+/-}$→KO were 42, 20, and 57% that of those seen in PAI-1$^{+/+}$→WT mice, respectively.

The Degree of Inflammation in Adipose Tissue Is Associated with the Morphological Parameters of the Epididymal Fat Pad and PAI-1 Levels Therein—To quantify the number of macrophages present in the epididymal fat pad, we analyzed the relative expression of CD68 mRNA using RT-QPCR. The number of macrophages in the fat pad differed between the groups ($p < 0.05$) (Fig. 3*E*). KO hosts receiving PAI-1$^{-/-}$ bone marrow have 9-fold lower CD68 mRNA levels than WT hosts receiving PAI-1$^{+/+}$ bone marrow ($p < 0.001$). Analysis of CD68 mRNA levels in the epididymal fat pad of WT animals compared with KO animals fed a high fat diet for 20 weeks yielded 5-fold higher CD68 mRNA levels for the WT animals ($p < 0.005$) (Fig. 5*A*). Although both KO and WT mice exhibited increased CD68 expression in response to HFD, genotype strongly influenced the magnitude of CD68 expression (Fig. 5, *B* and *C*). In WT mice CD68 levels increased by more than 30-fold ($p < 0.05$), whereas CD68 levels in fat increased 6.5-fold in KO mice fed the HFD ($p < 0.05$). When comparing the different KO host/donor combinations, a "bone-marrow derived PAI-1" concentration effect on CD68 mRNA levels appears (Fig. 3*E*). In agreement with previous observations, CD68 mRNA levels in the epididymal fat pad of WT mice receiving PAI-1$^{-/-}$ marrow and KO mice with PAI-1$^{+/-}$ marrow were 2.9- and 2.6-fold lower than those in PAI-1$^{+/+}$→WT mice, respectively ($p < 0.05$). When all host/donor combinations are considered, different correlations between inflammatory markers, morphological parameters of the fat pad, and PAI-1 levels become apparent (Table 4). Plasma PAI-1 levels are related to CD68 mRNA levels in the epididymal fat pad ($r = 0.49$, $p < 0.05$) and adipocyte size ($r = 0.44$, $p < 0.05$). CD68 mRNA levels are strongly correlated with the epididymal fat pad weight ($r = 0.61$, $p < 0.05$) and body weight at sacrifice ($r = 0.57$, $p < 0.05$). Finally, plasma leptin levels, marker of both obesity and inflammation, are related with all the variables analyzed (Table 4).

Macrophages Are a Source of PAI-1 in Adipose Tissue—In addition to the quantification of CD68 mRNA levels, an immunohistochemical analysis of F4/80, a macrophage marker, was performed. Macrophages were detected throughout the entire fat section, with the majority of localized around fat cells as previously reported (32, 33) (Fig. 6*A*). Staining for PAI-1 anti-

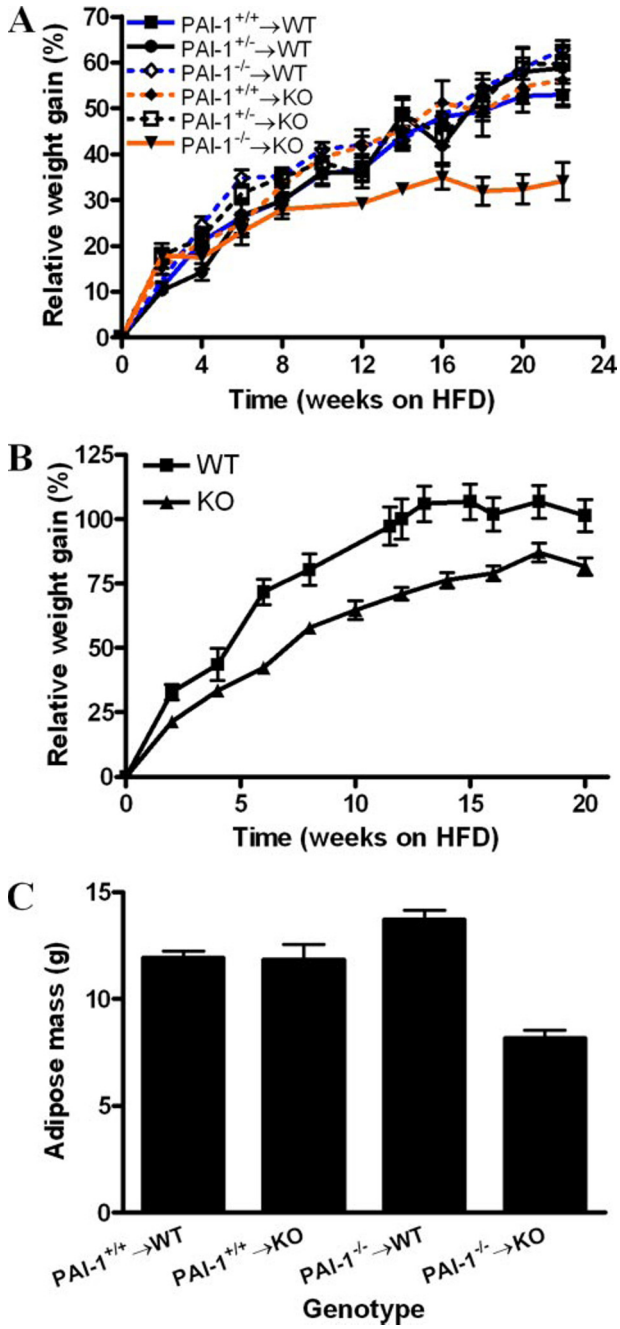

FIGURE 2. **Importance of bone marrow-derived PAI-1 for the development of obesity.** *A*, changes in relative weight gain (% relative to weight at start of HFD) caused by a HFD are plotted *versus* time. Time is shown as weeks on the diet. Data indicate that only the PAI-$1^{-/-}$→KO transplant is protected from the development of obesity ($p < 0.05$; $n = 5$–10). *B*, a similar partial protection from the development of obesity is seen for non-transplanted WT and KO ($p < 0.05$; $n = 5$). *C*, analysis of the body composition shows that the PAI-$1^{-/-}$→KO mice have a lower amount of adipose tissue in comparison with the other groups ($p < 0.05$; $n = 5$).

gen revealed that these cells are an important source of PAI-1 expression in fat (Fig. 6*B*). The clustering of PAI-1-producing macrophages was observed in the proximity of blood vessels as shown after staining for von Willebrand factor (Fig. 6*C*). Finally, we used a mouse model to verify which bone-marrow derived cells contribute to PAI-1 production in adipose tissue. B6D2F1/J WT mice were lethally irradiated and transplanted with bone marrow from a transgenic mouse expressing EGFP driven by the proximal −2.9 kilobase of the human PAI-1 promoter on the same background (29). Even though this model uses the human PAI-1 promoter, the model is useful since many of the key regulatory elements in the human and murine PAI-1 promoters are highly conserved. EGFP was detected in fat tissue and localized to cells co-staining for Mac3, a macrophage marker, indicating that bone marrow-derived macrophages are an important source of PAI-1 in visceral fat (Fig. 7).

Effect of Bone Marrow-derived PAI-1 on Markers of Obesity—Results from the analysis of plasma samples for hormone and biomarkers of obesity are shown in Table 2. Although there were no significant differences for adiponectin plasma levels (data not shown), the plasma leptin levels after 24 weeks on a HFD differed significantly among the various groups ($p < 0.001$) with the lowest leptin levels seen in the PAI-$1^{-/-}$→KO group (3.6 ± 0.8 and 8.8 ± 2.8 ng/ml for PAI-$1^{-/-}$→KO and $PAI^{+/+}$→WT, respectively). WT hosts receiving PAI-$1^{-/-}$ marrow have slightly lower leptin levels than PAI-$1^{+/+}$→WT mice, but this was not statistically significant. Plasma resistin levels were significantly different after 24 weeks on the HFD, mainly due to the higher value for PAI-$1^{+/+}$→KO. The corresponding levels of adipokine mRNA expression from isolated epididymal fat revealed similar patterns.

Insulin Resistance and Bone Marrow-derived PAI-1—In the transplanted mice fed a HFD for 24 weeks, there was a statistically significant difference in the plasma fasting glucose levels between the different groups ($p < 0.05$) (Table 2). Even though the difference in plasma glucose levels for PAI-$1^{+/+}$→WT *versus* PAI-$1^{-/-}$→KO was marginal (206 ± 7 *versus* 167 ± 13 mg/dl; $p = 0.06$), it was comparable with the difference seen for the non-transplanted WT and KO animals fed a high fat diet for 20 weeks (222 ± 11 *versus* 167 ± 14 mg/dl; $p < 0.05$). Plasma insulin levels did not vary among the different host/bone marrow combinations ($p = 0.07$). However, fasting insulin levels differed in non-transplanted WT *versus* KO animals after 20 weeks on a HFD (2.1 ± 0.3 *versus* 1.1 ± 0.2 ng/ml; $p < 0.05$). WT mice that were transplanted with PAI-$1^{-/-}$ marrow showed a tendency toward lower plasma insulin levels than the PAI-$1^{+/+}$→WT group (1.1 ± 0.3 ng/ml *versus* 1.5 ± 0.3 ng/ml, $p = 0.3$). Even though the glucose tolerance for the different groups also showed no difference ($p = 0.36$), there was a tendency for an improved glucose tolerance for the KO hosts receiving PAI-$1^{-/-}$ bone marrow (data not shown).

DISCUSSION

The plasminogen activator/plasmin system and PAI-1, specifically, are known to play a key role in cell migration and tissue remodeling. Morange *et al.* (14) observed that PAI-1 deficiency did not protect against the development of obesity. However, PAI-1-deficient mice on a mixed genetic background (81% C57BL/6J and 19% 129SV) showed milder hyperinsulinemia than the corresponding WT mice after 17 weeks on a HFD (14). Schafer *et al.* (11) demonstrated that the absence of PAI-1 reduced adiposity and improved the metabolic profiles in genetically obese mice, which were on a C57BL/6J background. This is in agreement with the results of Ma *et al.* (12) who reported that PAI-1 deficiency protected against obesity and insulin resistance in C57BL/6J mice. However, targeted overex-

pression of PAI-1 using a fat-specific promoter surprisingly attenuated nutritionally induced obesity (13). Besides the impact of different levels of PAI-1 expression on the development of obesity, the primary source of PAI-1 in adipose tissue remains a point of discussion. It has been established that adipose tissue is a significant source of PAI-1, with the bulk of production derived specifically from visceral fat (8, 21, 22, 34). More recently, it has been supported that stromal cells are the primary source of PAI-1 production in visceral adipose tissue (25, 26).

To clarify (i) the influence of different PAI-1 levels on the development of obesity and (ii) the importance of adipocytes and/or stromal cells during the development of obesity, we transplanted both PAI-1 KO and WT hosts with either PAI-$1^{-/-}$, PAI-$1^{+/-}$, or PAI-$1^{+/+}$ bone marrow. PAI-1 mRNA levels in bone marrow cells confirmed the success of the transplantation. Because irradiation of mice with 9 gray will eradicate rapidly replicating cells, we hypothesized that PAI-1-producing cells in the marrow of WT hosts receiving PAI-$1^{-/-}$ marrow could not be completely eradicated. Because we were interested in the potential effects of PAI-1 derived from fast replicating cells such as *e.g.* macrophages but also in the general effects of PAI-1 originating from bone marrow, we used RT-QPCR on the total bone marrow RNA rather than just characterizing circulating blood cells as is frequently used in studies looking more specifically at macrophage-derived markers. Plasma levels of PAI-1 are in agreement with the combinations host/donor. Although it is clear that the majority of PAI-1 in plasma is not derived from bone marrow cells, PAI-1 plasma levels in PAI-1 KO hosts transplanted with PAI-$1^{+/+}$ indicate that they make a significant contribution to PAI-1 antigen plasma levels. Furthermore, plasma PAI-1 antigen levels in the KO hosts show that bone marrow-derived PAI-1 leads to plasma PAI-1 levels that are "genotype-concentration"-dependent. A similar conclusion can be made for PAI-1 mRNA levels in the epididymal fat pad. Whereas PAI-$1^{+/+}$→WT has 166-fold higher PAI-1 mRNA levels in the epididymal fat pad in comparison with PAI-$1^{+/+}$→KO, the presence of PAI-1 mRNA in the fat pad of KO animals receiving either PAI-$1^{+/+}$ or PAI-$1^{+/-}$ marrow confirms a contribution of bone marrow-derived PAI-1 that is again genotype-dependent. The fact that the PAI-1 mRNA levels are not significantly different between PAI-$1^{+/-}$→WT

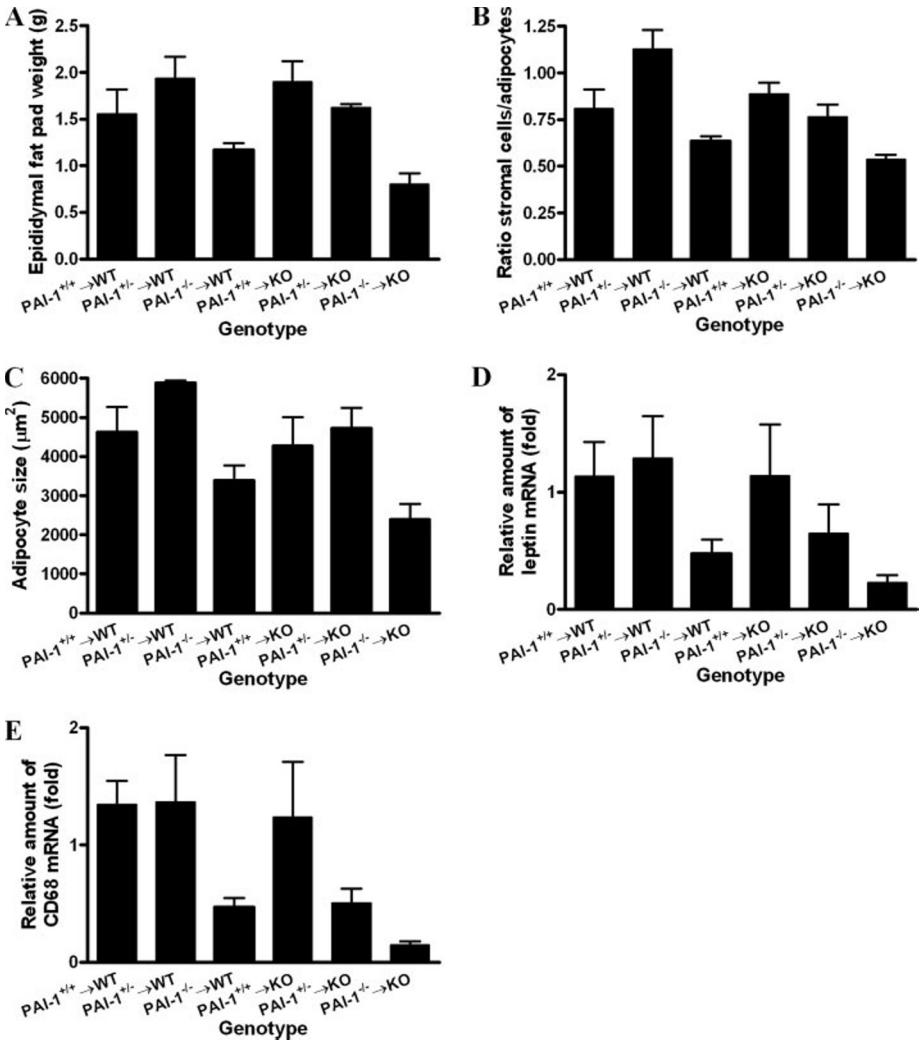

FIGURE 3. **Host/donor PAI-1 genotypes are correlated with markers of obesity/inflammation.** *A*, epididymal fat pad weight. *B*, ratio stromal cells/adipocytes. *C*, adipocyte size. *D*, leptin mRNA levels in the epididymal fat pad. *E*, CD68 mRNA levels in the epididymal fat pad show a similar profile that indicates that the absence of bone marrow PAI-1 protects from visceral fat accumulation and inflammation. A concentration dependence can be seen for the KO hosts transplanted with PAI-$1^{+/+}$, PAI-$1^{+/-}$, and PAI-$1^{-/-}$ marrow. mRNA levels are expressed relative to the PAI-$1^{+/+}$→WT levels (=1).

TABLE 3
Morphological parameters of the epididymal fat pad

Marker	PAI-$1^{+/+}$→WT	PAI-$1^{+/-}$→WT	PAI-$1^{-/-}$→WT	PAI-$1^{+/+}$→KO	PAI-$1^{+/-}$→KO	PAI-$1^{-/-}$→KO
Fat pad weight (g)[a]	1.6 ± 0.3	1.9 ± 0.2	1.2 ± 0.1	1.9 ± 0.2	1.6 ± 0.0	0.8 ± 0.1
Stromal cells/adipocytes (ratio)[b]	0.81 ± 0.10	1.13 ± 0.10	0.64 ± 0.02	0.89 ± 0.06	0.77 ± 0.06	0.54 ± 0.02
Adipocyte size (μm^2)[c]	4628 ± 3028	5879 ± 3654	3389 ± 2366	4277 ± 2845	4719 ± 2975	2391 ± 1543

[a] $p = 0.02$ (ANOVA; $n \geq 5$).
[b] $p = 0.0008$ (ANOVA; $n \geq 5$).
[c] $p = 0.03$ (ANOVA; $n \geq 5$).

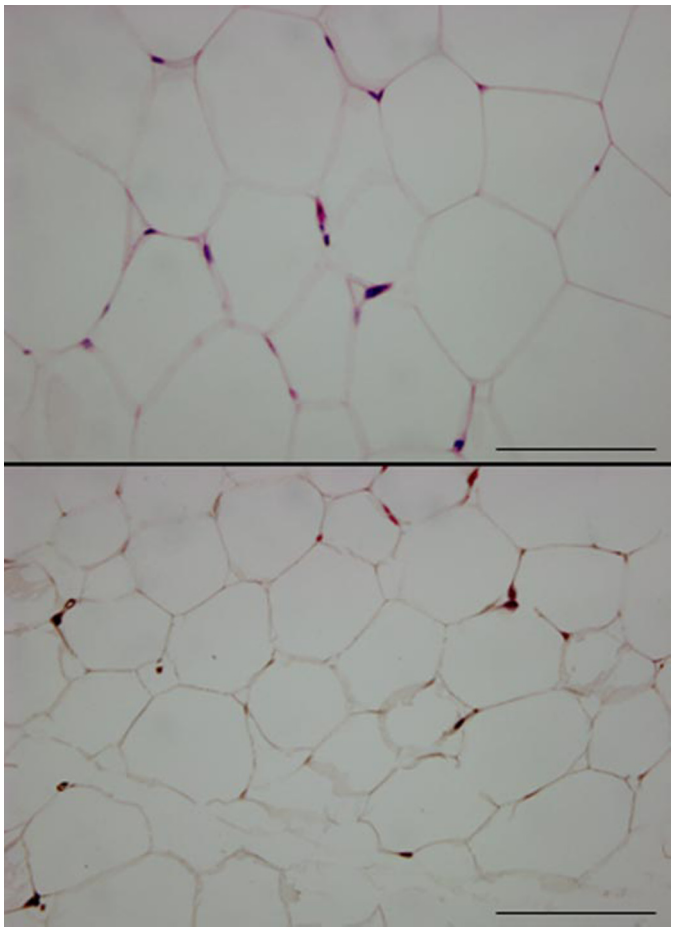

FIGURE 4. **PAI-1 influences the adipose tissue morphology.** Hematoxylin and eosin staining shows that adipocytes in the epididymal fat pad of PAI-$1^{+/+}$→WT mice (*upper panel*) are on average larger than those seen in PAI-$1^{-/-}$→KO mice (*lower panel*) due to (i) their larger size and (ii) a lower number of small adipocytes. The higher ratio of stromal cells/adipocytes in the PAI-$1^{+/+}$→WT is visible by the number of large nuclei (*dark purple*), which are more abundant in the PAI-$1^{+/+}$→WT (*upper panel*) in comparison with the PAI-$1^{-/-}$→KO mice (*lower panel*). *Bar*, 100 μm.

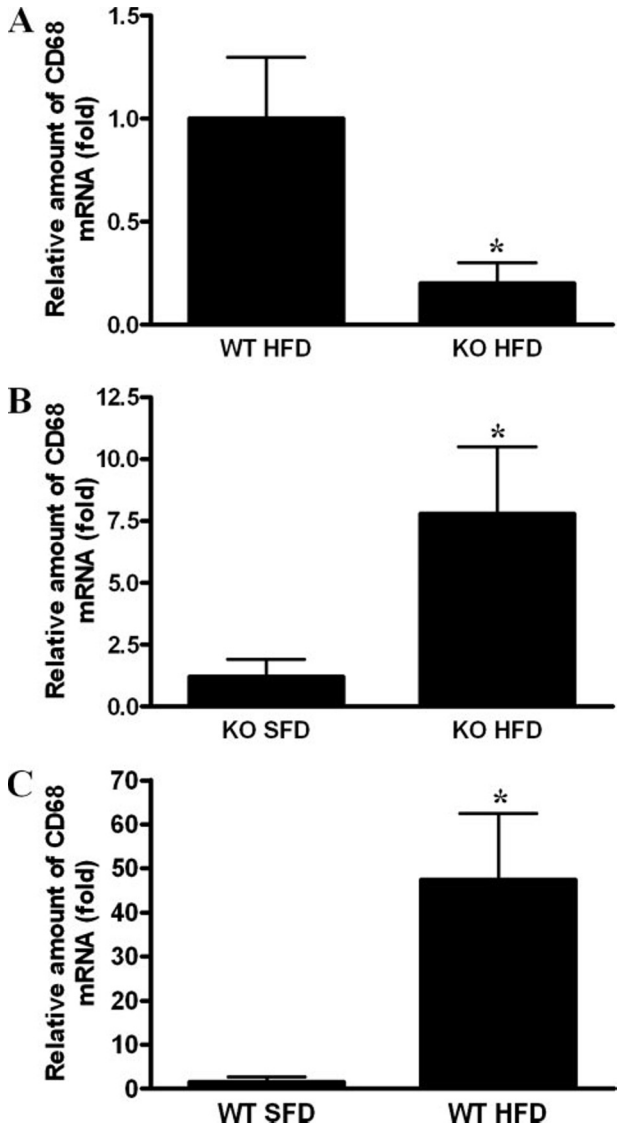

FIGURE 5. **Infiltration of macrophages during obesity development is attenuated in the epididymal fat pad of PAI-1 deficient animals.** *A*, the epididymal fat pad of WT animals contains 5-fold more CD68 mRNA than that of KO animals after feeding all animals a HFD for 20 weeks ($p < 0.005$; $n = 5$). *B* and *C*, the fact that obesity corresponds to a chronic low grade inflammatory state is confirmed by the increase in the levels of CD68 mRNA in the epididymal fat pad of both WT (*B*) and KO animals (*C*) when comparing HFD with standard fat diet (SFD) after 20 weeks (32-fold ($p < 0.005$ and 6.5-fold ($p < 0.05$) increase for WT and KO, respectively; $n = 5$). Levels of CD68 mRNA are expressed relative to WT HFD (*A*), WT standard fat diet (*B*) and KO standard fat diet (*C*), respectively.

and PAI-$1^{-/-}$→WT is probably due to the higher expression levels in the host tissue in comparison with those in the donor cells.

Even though plasma and tissue PAI-1 levels vary depending upon the combination of host/donor, only the mice lacking PAI-1 (PAI-$1^{-/-}$→KO) are protected from the development of obesity. The observed difference in weight gain between the WT host receiving PAI-$1^{+/+}$ bone marrow and the KO host receiving PAI-$1^{-/-}$ bone marrow is similar to the difference we observed between WT C57BL/6J mice and PAI-1 KO C57BL/6J mice after receiving a HFD for 20 weeks. The discrepancy between the relative weight gain data (35–60% and 75–100% for the transplanted and non-transplanted animals, respectively) may reflect nonspecific effects on animals undergoing the radiation-transplantation procedure. The observed partial protection against the development of obesity differs from the report that PAI-1 deficiency provides complete protection from diet-induced obesity (12). The reasons for this are unclear and may include experimental design, diet differences, and other unidentified environmental effects. Remarkably, even though the PAI-$1^{-/-}$→WT mice were not protected from weight gain, they accumulated a significantly smaller epididymal fat pad than the PAI-$1^{+/+}$→WT animals. Importantly, this effect on fat pad size occurred in the absence of a reduction of total body fat by body composition analysis, suggesting that PAI-1 influences body fat distribution and/or energy expenditure. However, there were no differences in metabolic rate/oxygen consumption between the different host/donor combinations. Because plasma leptin levels do not differ for PAI-$1^{+/+}$→WT and PAI-$1^{-/-}$→WT animals whereas the levels of leptin mRNA expression in epididymal fat pad and the mass of the fat pad itself did indeed differ, this indicates that adipose tissue must accumulate at another site in the WT hosts receiving PAI-$1^{-/-}$ marrow.

TABLE 4
Pearson correlation coefficients

Marker	Plasma PAI-1 at sack	Body weight at sack	Epididymal fat pad weight	CD68 mRNA levels in fat	Adipocyte size	Ratio stromal cells/adipocytes	Plasma leptin at sack	PAI-1 mRNA levels in fat
Plasma PAI-1 at sack	1[a]							
Body weight at sack	0.25	1[a]						
Epididymal fat pad weight	0.26	0.90[a]	1[a]					
CD68 mRNA levels in fat	0.49[a]	0.57[a]	0.61[a]	1[a]				
Adipocyte size	0.44[a]	0.75[a]	0.74[a]	0.52[a]	1[a]			
Ratio stromal cells/adipocytes	0.43[a]	0.59[a]	0.64[a]	0.38[a]	0.55[a]	1[a]		
Plasma leptin at sack	0.40[a]	0.72[a]	0.85[a]	0.56[a]	0.82[a]	0.66[a]	1[a]	
PAI-1 mRNA levels in fat	0.49[a]	0.19	0.26	0.13	0.30	0.11	−0.16	1[a]

[a] Correlation is statistically significant ($p < 0.05$).

The difference in the mass and morphology of the epididymal fat pad indicates less fat accumulation or energy storage for the mice receiving PAI-$1^{-/-}$ marrow. These mice have significantly smaller fat cells and less visceral fat inflammation as indicated by the ratio stromal cells/adipocytes, CD68 mRNA levels, and leptin mRNA levels. The latter adipokine is not merely a marker of adipocyte differentiation but one of inflammation as well (35). As expected, when we consider all host/donor combinations there is a correlation between the plasma PAI-1 levels and the fat cell size as has been reported before. Interestingly, there is also a correlation between plasma PAI-1 levels and the CD68 mRNA levels in the epididymal fat pad. Furthermore, CD68 mRNA levels in epididymal fat are related to the PAI-1 genotype. In agreement with the recent insights indicating that obesity is characterized by low grade chronic inflammation, there is a significant correlation between the epididymal fat pad size and the CD68 mRNA levels in the fat pad. This increase in inflammation with increasing fat pad size is also suggested by a higher cellularity leading to a higher ratio of stromal cells/adipocytes. Because mice lacking PAI-1 in their bone marrow are consistently protected from visceral obesity (reflected by smaller epididymal fat pad size, smaller adipocytes, lower epididymal leptin mRNA levels, and lower CD68 mRNA levels) we infer that PAI-1 influences visceral fat accumulation and the associated inflammatory amplification. Because no female mice were included in the current study we can only assume that our findings are, like inflammatory mechanisms, mainly gender-independent.

Previous studies have emphasized the importance of macrophages in the development of obesity (32, 36). It has been suggested by Weisberg *et al.* (21) that the increased accumulation of macrophages in adipose tissue of the obese is due to an influx of bone marrow-derived precursors in the adipose tissue. Several studies using supraphysiological levels of PAI-1 have shown that PAI-1 inhibits the migration of several cell types, *i.e.* endothelial cells, smooth muscle cells, human monocytes, etc. (37, 38). However, other studies have reported that low levels of PAI-1 expression or immobilized PAI-1 enhances cell adhesion and migration (39–43). Interestingly, PAI-1-deficient mice exhibit reduced angiogenesis, which is partially explained by reduced cell migration (44, 45). These apparently conflicting data on the effects of PAI-1 on cell migration and related processes were rationalized by identifying a bell-shaped dose dependence (46–48). Physiological concentrations of PAI-1 (0.1–100 ng/ml) are angiogenic through the antiproteolytic activity (44). In contrast, a complete lack of PAI-1 and supraphysiological concentrations of PAI-1 inhibit angiogenesis (44, 45, 49). Taken together, this indicates that PAI-1 might either promote or inhibit cell migration depending on its local concentration. Based on these findings together with the data reported in the current study, we infer that varying PAI-1 concentrations in fat and plasma are predicated upon marrow PAI-1 status. Furthermore, the intensity of marrow PAI-1 expression determines the extent of cell migration, either promoting or retarding macrophage migration to the epididymal fat pad. As reported by Cinti *et al.* (33), the initial adipose tissue-residing macrophages will surround and scavenge apoptotic adipocytes, thereby releasing adipokines which trigger an even more pronounced inflammatory reaction. Because PAI-$1^{-/-}$→KO is the only combination resulting in inhibition of cell migration (corresponding to the bell-shaped dose dependence described above), this is the only group that is protected from the development of obesity. Furthermore, this hypothesis reconciles the contradicting data that both overexpression of PAI-1 and absence of PAI-1 can protect against the development of obesity (12, 13). This confirms the earlier observations that the ratio stromal cells/adipocytes is lower in both PAI-1 KO and PAI-1 transgenic mice when compared with their WT counterparts after feeding mice a HFD (13, 14). Furthermore, the current study shows that even though bone marrow-derived PAI-1 only contributes minimally to total PAI-1 levels in both plasma and epididymal fat, it is enough even for the PAI-$1^{+/-}$→KO combination to contribute to the development of obesity. The importance of PAI-1 production in the microenvironment rather than the total plasma PAI-1 levels can also be seen by the protection PAI-$1^{-/-}$ marrow affords WT mice against the expansion of the visceral fat pad. Because PAI-1 is produced in the marrow-derived cells (*e.g.* macrophages), this may facilitate the migration of those same cells.

PAI-1 also has important effects on the metabolic consequences of obesity. In agreement with the visceral fat data and previous observations (12), insulin levels after 24 weeks on a HFD suggest protection against insulin resistance in mice receiving PAI-$1^{-/-}$ marrow. Glucose levels for PAI-$1^{-/-}$→KO animals are also lower than in the other groups. Although both of these measures are consistent with a benefit of PAI-1 deficiency, definitive conclusions cannot be drawn because of the relatively small sample size in the present study. Whether other cells originating in the bone marrow (*e.g.* preadipocytes) are responsible for these observations cannot be excluded. However, the correlations between the macrophage marker CD68 with typical markers of obesity and more specific visceral fat pad maturation suggest an influence of macrophages. As such, this study is the first to demonstrate that bone marrow constit-

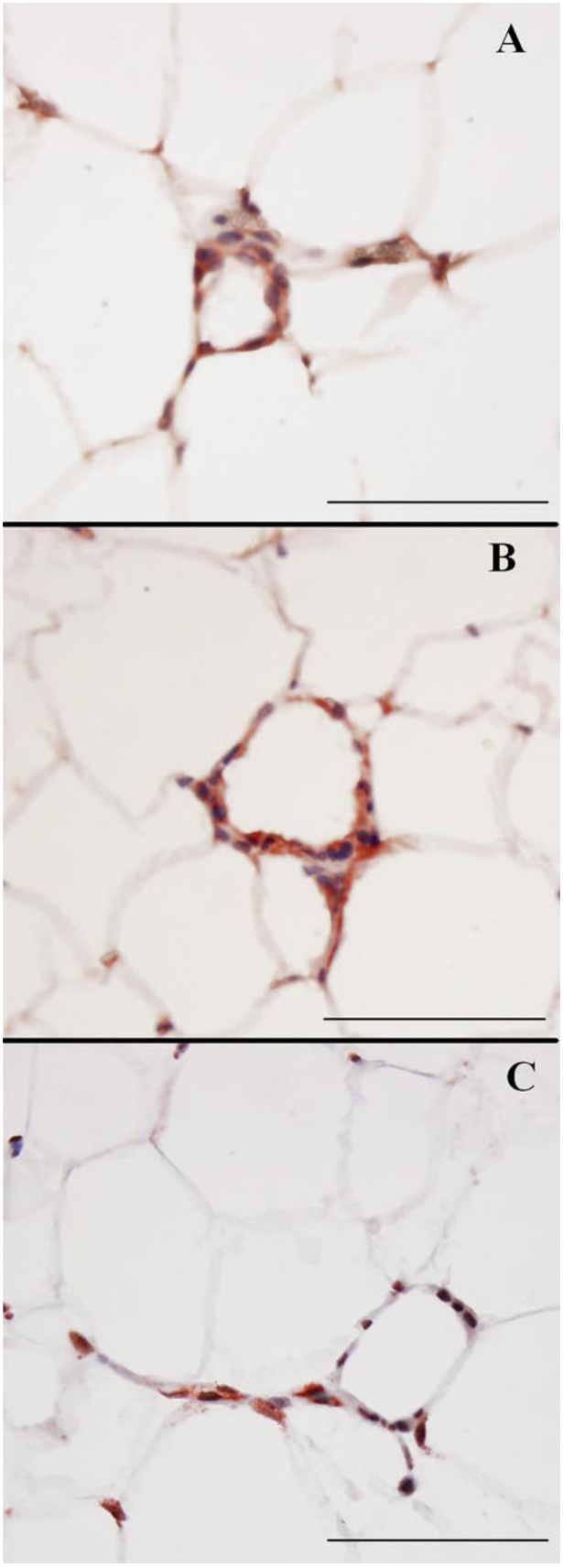

FIGURE 6. **Macrophages are an important source of PAI-1 in adipose tissue.** *A*, immunohistochemical analysis of sections of the epididymal fat pad for F4/80, a macrophage marker, shows that even though macrophages are detected throughout the entire fat pad, the majority of them are localized surrounding fat cells. *B*, staining for PAI-1 indicates that these macrophages are an important source of PAI-1. *C*, The presence of blood vessels, as indicated by immunohistochemical analysis for von Willebrand factor, in the proximity of the macrophages suggests that they entered the fat pad through these vessels. *Bar*, 100 μm.

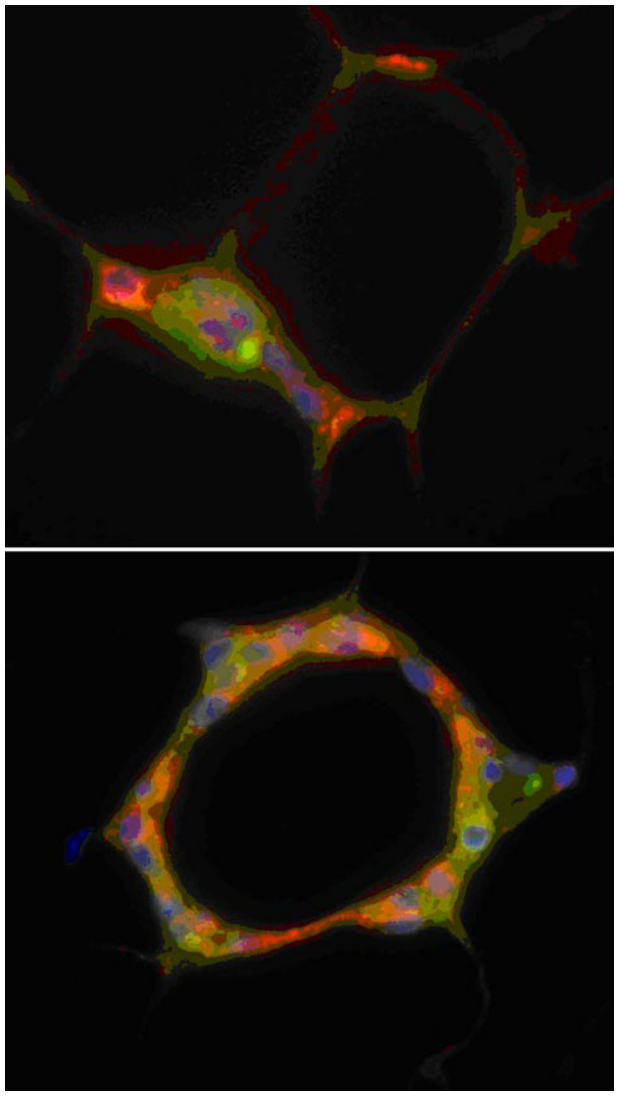

FIGURE 7. **Bone marrow-derived macrophages are an important source of PAI-1 in adipose tissue.** Analysis of the epididymal fat pad isolated from B6D2F1/J WT mice transplanted with bone marrow from a transgenic mouse expressing EGFP driven by the proximal −2.9 kilobases of the human PAI-1 promoter shows that Mac3 (*red-orange*)-positive cells express EGFP (*green*). These cells are bone marrow-derived macrophages and express PAI-1 (*i.e.* EGFP expression). The figure shows that even though macrophages are present throughout the entire fat section (*upper panel*), a vast number of them surround adipocytes (*lower panel*). Nuclei are shown in *blue*.

uents play a role in coordinating the accumulation of visceral fat and suggests that cellular and humoral factors originating in the bone marrow influence the development of obesity. Because previous studies have identified visceral fat as the most important source of adipose-derived PAI-1 (8, 21, 22), our study focused on the epididymal fat pad. Although it is possible that PAI-1 influences the accumulation and histology of other fat depots, the design and focus of the present study precludes any meaningful analysis of this question. However, the impact on the visceral fat mass is of special interest since most detrimental

consequences of obesity, including insulin resistance, type 2 diabetes, and ischemic cardiovascular disease are associated with visceral obesity. From this perspective we speculate that the development and application of specific PAI-1 antagonists leads to reduction in visceral fat mass with secondary improvement in metabolic profile and may reduce the risk of co-morbidities.

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