Adipose Tissue-Derived Soluble Fms-Like Tyrosine Kinase 1 Is an Obesity- Relevant Endogenous Paracrine Adipokine

Florian Herse, John N. Fain, Juergen Janke, Stefan Engeli, Christian Kuhn, Norbert Frey, Herbert A. Weich, Astrid Bergmann, Kai Kappert, S. Ananth Karumanchi, Friedrich C. Luft, Dominik N. Muller, Anne C. Staff, Ralf Dechend

Abstract—Adipose tissue growth depends on angiogenesis. We tested the hypothesis that adipose tissue produces factors relevant to angiogenesis. We obtained fat biopsies in 2 different patient cohorts, cultured adipose-derived stem cells and studied mature adipocytes. We performed microarray, RT-PCR, and Western blotting; studied a rat obesity/metabolic syndrome model; and conducted viral gene transfer experiments in leptin-deficient mice. The microarray identified the splice variant of the vascular endothelial growth factor receptor, the soluble fms-like tyrosine kinase 1 (sFlt-1), as an antiangiogenesis candidate. We verified the expression findings and found that sFlt-1 was secreted by isolated mature human adipocytes. Tumor necrosis factor-α decreased sFlt-1 expression in mature adipocytes, whereas hypoxia had no effect. Separating cells from adipose tissue showed that the highest sFlt-1 expression was present in adipose-tissue nonfat cells rather than in the adipocytes themselves. We also found that sFlt-1 expression and sFlt-1 release by adipose-tissue explants were inversely correlated with body mass index of the corresponding patients but was directly correlated with adiponectin expression. In the obesity/metabolic syndrome rat model, we observed that circulating sFlt-1 levels and sFlt-1 expression in adipose tissue were also inversely correlated with body weight. To model our putative antiangiogenic factor further, we next overexpressed sFlt-1 by viral transfer in a mouse genetic model of leptin deficiency and observed that the transfected mice gained less weight than controls. We suggest that sFlt-1 could act as a paracrine factor inhibiting adipose tissue growth. Local sFlt-1 may regulate angiogenic potential and thereby influence adipose tissue mass.

Key Words: adipogenesis • soluble fms-like tyrosine kinase receptor 1 • sFlt1 • obesity • adipocytes

Angiogenesis is essential to many processes, including development, reproduction, and repair. 1,2 Pathological angiogenesis is not only important for tumor growth but also for several nonneoplastic, “angiogenesis-dependent” diseases, such as peptic ulcers, ocular neovascularization, rheumatoid arthritis, atherosclerosis, and obesity. 3–5 In contrast to most adult tissues, adipose tissue exhibits an enormous plasticity and can grow, regress, and regrow depending on needs and behavior. 6–8 Recent work showed that obesity involves the coupling of angiogenesis and angiogenesis. 3,9 Angiogenesis regulates adipose tissue by affecting the number, growth, and remodeling of the adipose tissue vasculature. 4,10–12 Adipose tissue is highly vascular, because several capillaries surround each adipocyte. 5,13,14 Accordingly, adipose tissue has been used clinically to promote wound healing and revascularization. 3,15 Furthermore, adipose tissue is an expandable endocrine gland that produces and secretes growth factors, free fatty acids, hormones, and cytokines, including leptin, adiponectin, vascular endothelial growth factor (VEGF), interleukin (IL) 6, and tumor necrosis factor (TNF)-α. VEGF is responsible for most of the angiogenic capacity and is upregulated during angiogenesis. 6,7,15 In growing adipose tissue, vessels contribute to angiogenesis by switching on angiogenic pathways, thereby altering the balance between angiogenic factors and their inhibitors. 1,14 Local endothelial cells produce several growth factors and cytokines and communicate with adipocytes in a paracrine fashion to promote their growth and expansion. 3,13,16 Adipocytes may also signal endothelial cells by producing proangiogenic factors, inhibitors, and cytokines.
sFlt-1 release stems from adipose tissue nonfat cells. Values as mean±SEM; *P<0.01 for SV cells and mature adipocytes vs adipose tissue nonfat cells (n=12).

Figure 1. Soluble fms-like tyrosine kinase 1 (sFlt-1) expression in adipose tissue and its compartments. A, mRNA expression of sFlt-1 in isolated adipose-derived stem cells (ADSCs) and isolated mature adipocytes from human adipose tissue. The 10-fold differences was significant with *P<0.01. B, sFlt-1 release from ADSCs incubated for 5 days and mature adipocytes incubated for 3 or 5 days. *P<0.05 for 5 days vs 3 days. (n.d. indicates not detectable; n=4). C, sFlt-1 release from adipose tissue nonfat cells, stromal vascular cells (SV cells), and mature adipocytes is shown. sFlt-1 production stems from adipose tissue nonfat cells. Values as mean±SEM; *P<0.01 for SV cells and mature adipocytes vs adipose tissue nonfat cells (n=12).

Materials and Methods
We obtained adipose tissue from women after internal review board approval with informed written consent. A detailed method section for collecting patients specimens and cell isolation is described in the online Data Supplement (please see http://hyper.ahajournals.org).

RNA Isolation, Microarray, and RT-PCR
Our microarray experiments of adipose tissue that adhere to Minimum Information About a Microarray Experiment guidelines, are described elsewhere. Total mRNA was isolated with the Qiagen RNAeasy mini kit (including the RNase-Free DNase set) (Qiagen). RNA was reverse transcribed into cDNA by using the Transcriptor First Strand cDNA synthesis kit from Roche Diagnostics and were analyzed by real-time quantitative PCR on an ABI 7500 sequence detection system (PE Biosystems) or Roche LightCycler 480 Real-Time RT-PCR system (Roche Diagnostics). Primer and probes were designed with PrimerExpress 2.0 (Applied Biosystems). Sequences are indicated in the online Data Supplement.

Western Blot and ELISA
Mature adipocytes were separated from medium after indicated period and lysed by vortexing in radioimmunoprecipitation assay buffer (Cell Signaling). After centrifugation, protein was used for sodium dodecyl sulfate-gel electrophoresis, followed by blotting on membrane. The membrane was stained specific for Flt-1 (antihuman VEGF receptor-1/Flt-1, ReliaTech) and β-actin (13E5; Cell Signaling). Densitometric analyses were done by Image-J 1.41 software (National Institutes of Health). The soluble splice variants of the VEGF fms-like tyrosine kinase receptor 1 (sFlt-1) levels in plasma or medium were determined by ELISA (human soluble VEGF receptor 1/Flt-1 Quantikine ELISA Kit, R&D Systems). Food consumption was determined. Local authorities (LAGeSo, Berlin, Germany) approved the animal protocols that complied with criteria outlined by the American Physiological Society.

Statistics
All of the parameters were tested for normal distribution, and group differences were analyzed by t test, ANOVA, Mann-Whitney U test, or χ² test as appropriate. Relations between parameters were examined by means of Pearson correlation coefficients. P<0.05 was considered statistically significant.

Results
sFlt-1 Expression in Human Adipose Tissue
The microarray showed that sFlt-1 was expressed in human adipose tissue. We confirmed the finding with real-time RT-PCR. sFlt-1 expression was 10-fold higher (P<0.01) in mature adipocytes freshly isolated from human subcutaneous adipose than in adipose-derived stem cells (Figure 1A). Isolated human mature adipocytes stably secreted sFlt-1 protein into the medium (Figure 1B). Adipose-derived stem cell did not secret sFlt-1 during culturing. We then focused on sFlt-1 release from adipose-tissue nonfat cells, the undigested tissue matrix, the stromal vascular (SV) cells, and mature adipocytes (Figure 1C). We found that nonfat cells in adipose tissue released most of the sFlt-1, whereas the contribution of mature adipocytes and SV cells was similar.

sFlt-1 Regulation in Isolated Mature Human Adipocytes
We reasoned that tissue hypoxia could influence angiogenesis and sFlt-1 production. We incubated isolated mature adipocytes in normal (20% O₂) or 3% O₂ for 48 hours, which increased VEGF-A expression (3.8-fold change; P<0.01) but did not alter sFlt-1 expression (Figure 2A). Insulin and angiotensin II also had no effect, whereas TNF-α increased VEGF-A expression (fold change: 2.3; P<0.05) while decreasing sFlt-1 expression (3.8-fold; P<0.05) in the cells (Figure 2B). We confirmed these findings with Western blotting (Figure 2C). Quantitative analysis substantiated the reduced sFlt-1 protein expression on TNF-α stimulation (Figure 2D).

Animal Studies
Rats transgenic for the human renin gene (hREN) are an established obesity/metabolic syndrome model. Serum and adipose tissue of these obese rats were described earlier and investigated for sFlt-1 expression. Male B6.V-Lep/H11006/J (ob/ob mice) were purchased from Charles River and treated by IV injection with 1×10⁶ plaque-forming unit adenovirus encoding LacZ or sFlt-1 sequence as indicated. The adenovirus has been described elsewhere. We determined PLIN2 expression in epididymal fat and plasma sFlt-1 levels in the circulation with ELISA (mouse soluble VEGF receptor 1/Flt-1 Quantikine ELISA Kit, R&D Systems). Food consumption was determined. Local authorities (LAGeSo, Berlin, Germany) approved the animal protocols that complied with criteria outlined by the American Physiological Society.

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Correlation of sFlt-1 and Body Mass Index

We compared sFlt-1 expression in subcutaneous fat from women across a broad range of body mass index (BMI) values. The sFlt-1 expression was inversely correlated with BMI ($r = -0.36; P = 0.002$; Figure 3A) and directly correlated with adiponectin expression ($r = 0.33; P = 0.01$; Figure 3B). In the patients with high circulating TNF-α levels (first quartile), the sFlt-1 expression in fat tissue was significantly lower than in those with lower TNF-α levels (fourth quartile; Figure 3C). In a different and independent cohort, we confirmed the same inverse relationship ($r = -0.55; P = 0.008$) between BMI and Flt-1 release by subcutaneous fat, across a range of BMI values (Figure 3D). These findings were not confined to subcutaneous fat, because Flt-1 release by omental fat was
also inversely correlated with BMI ($r = -0.48; P = 0.02$; Figure 3E).

The inverse correlation (Figure 4A) between sFlt-1 expression in epididymal fat tissue and body weight ($r = -0.74; P = 0.001$) was also present in an obese rat model, transgenic for human renin.19 The male obese rats had a lower sFlt-1 mRNA expression in their adipose tissue compared with age-matched controls ($P<0.01$; Figure 4A). Reduced circulating sFlt-1 levels were seen in the obese rats, and an inverse correlation between sFlt-1 level and body weight ($r = -0.89; P = 0.001$) was also observed in the serum (Figure 4B).

**sFlt-1 Overexpression Reduces Obesity**

Because induction of insulin resistance by TNF-α reduced adipocyte-specific sFlt-1 expression in cell culture and because we found inverse correlations with BMI and direct correlations with adiponectin, we hypothesized that sFlt-1 overexpression could reduce adipose tissue growth. We, therefore, overexpressed sFlt-1 with adeno-viral-gene transfer in a mouse model of obesity (ob/ob mice). Control male mice received adenovirus harboring the β-galactosidase coding gene LacZ. Over 123 days, AdLacZ mice gained more weight than AdsFlt-1 mice (Figure 5A). We could not attribute the difference to food intake (Figure 5B). Circulating sFlt-1 levels increased in ob/ob mice receiving the sFlt-1 gene by a factor of 29-fold but not in control ob/ob mice (Figure 5C). The adipophillin (PLIN2) expression, a marker for lipid accumulation, was also lower in the epididymal fat of the mice receiving sFlt-1 (Figure 5D). We tested whether the ob/ob mice receiving sFlt-1 would have a reduced weight gain as compared with mice receiving LacZ (Figure 5A). Over 68 days, mice receiving sFlt-1 gained significantly ($P<0.05$) less weight than control mice.

**Discussion**

The novel findings in our study are the identification of sFlt-1 production by adipose tissue and the potential regulatory role of this molecule, as a regulator of adipose tissue burden, as suggested by correlations in humans and in a rat model for obesity. We observed that TNF-α downregulates sFlt-1 in isolated mature adipocytes. We underscored this inference by overexpressing sFlt-1 in an established murine obesity model and showed that sFlt-1 influences weight gain and lipid accumulation in this model.

The concept that leaner patients with no signs of insulin resistance have higher sFlt-1 levels in adipose tissue that may prevent angiogenesis and thus minimize obesity represents a novel potential molecular mechanism. In growing adipose tissue, the switch to an angiogenic phenotype represents an imbalanced production of angiogenic factors and inhibitors, resulting in an increased angiogenic net balance.4,9,14 We argue that a reduced secretion of sFlt-1 by adipose tissue is part of this mechanism. Ambati et al21 showed that sFlt-1 secretion locally prevents endothelial cell stimulation and subsequent angiogenesis in the cornea. This important goal is achieved by local secretion of sFlt-1 from retinal cells. The stimulus can be interrupted by hyperoxia.21

Several groups have shown that hypoxia and angiotensin II upregulate sFlt-1 in endothelial cells, trophoblasts, and other cell types.22,23 We showed that sFlt-1 regulation in isolated mature adipocytes differs from that described in placenta and endothelial cells, because hypoxia and angiotensin II had no effect on sFlt-1 expression in mature adipocytes. Nonetheless, TNF-α downregulates sFlt-1 expression. TNF-α is an important overexpressed inflammatory cytokine in adipose tissue in obesity.24 Genetic TNF-α inhibition restored insulin sensitivity in vitro and in vivo in an earlier study.25 Chronic treatment with anti–TNF-α antibodies improves insulin sensitivity in both lean and obese patients, indicating that TNF-α may be a major contributor in the pathogenesis of obesity-induced insulin resistance.25 The molecular mechanisms of TNF-α–induced sFlt-1 downregulation are unknown. Recently, other investigators showed that peroxisome proliferator-activated receptor-γ, concomitant activation of the p53 tumor suppressor, and estrogen receptors induced Fli-1 transcription.26,27 Both factors, peroxisome proliferator-activated receptor and estrogen, are downregulated by TNF-α.28 Our data show that lean patients have higher sFlt-1 levels.
with adipocyte hypertrophy and hyperplasia. These processes are accompanied by angiogenesis, which is essential for adipogenesis and correct tissue function. The vasculature has a causal role in determining adipocyte growth, regression, and physiological functions by controlling the number of microvessels and by remodeling existing vessels. High levels of sFlt-1 might, therefore, restrain adipose tissue growth via inhibition of local angiogenesis. Adipogenesis and angiogenesis uncoupling in adipose tissue occurred in several intervention studies. All showed that disrupting adipose tissue vasculature by angiogenesis inhibitors leads to fat tissue reduction. Rupnick et al documented the phenomenon in ob/ob mice. Bräkenhielm et al showed that the selective angiogenesis inhibitor, TNP-470, prevented obesity in high-caloric, diet-fed mice and in genetically leptin-deficient ob/ob mice. Rupnick et al underscored the variability among the angiogenesis inhibitors regarding effects on body weight. Our sFlt-1 results correspond with those reported by Rupnick et al in their studies of endostatin and thalidomide. Tam et al observed that VEGF inhibition by blockade of the VEGF receptor 2, rather than VEGF receptor 1, prevented diet-induced obesity. They also showed that formation of new vessels in fat tissue during obesity is attributed to VEGF-dependent angiogenesis rather than de novo vasculogenesis.

Finally, we observed that sFlt-1 concentrations were higher in the adipose tissue nonfat cells than in the mature adipocyte cell fraction. The exact nature of the adipose tissue nonfat cells and the SV cells is not known; however, the nonfat cell fraction that we studied probably contains all of the cells resisting collagen digestion, including vascular smooth muscle cells, endothelial cells, and connective tissue fibroblasts. Recently the preparation of the SV subfractions from adipose tissue was described. This technique will be very useful for further investigations. We believe that this finding is particularly important. The implication is that SV cells or other cells are the primary sources for sFlt-1 rather than the mature adipocytes themselves. A similar state of affairs has been described for several other adipokines. For instance, Fain investigated 37 adipokines and found that, for 30, the release was higher in the adipose tissue nonfat cells including SV cells compared with the adipocytes themselves. We suggest a novel regulatory role for sFlt-1 in terms of supporting a link between angiogenesis and fat mass production. However, angiogenesis is complex, and future studies will have to confirm that sFlt-1 expression in adipocytes is indeed inducing local antiangiogenesis. The definitive impact of sFlt-1 on adipose tissue development and the role in the cross-talk to endothelial cell warrants further experimental proof. One drawback of our study is that we have tested our constructs only in a single, artificial model of obesity. To gain more insight in the molecular mechanism, adipose-specific overexpression of sFlt-1 and different obesity models should be tested. Our hypothesis has mechanistic and therapeutic implications, which underscore the role of the vasculature in the regulation of fat mass and obesity.

**Perspectives**

We identified sFlt-1 as a potential angiogenesis regulator in adipose tissue. Although we showed that adipocytes produce...
sFlt-1, other nonfat cells in adipose tissue may be more important. In contrast to the cornea, hypoxia is not a major regulatory mechanism. The robust inverse correlations between sFlt-1 expression and BMI in humans or body weight in rats underscore a potential role of sFlt-1 in regulating obesity. Our preliminary therapeutic trial in a mouse model suggests that the pathway could be therapeutically relevant. Our findings may be relevant to the interrelationship between obesity and hypertension.

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**Disclosures**

None.

**References**

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ONLINE SUPPLEMENT

Adipose tissue-derived sFlt1 is an obesity-relevant endogenous paracrine adipokine

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Supplemental methods

Patient specimen and cell isolation

For gene expression studies, we used subcutaneous abdominal adipose tissue from 69 postmenopausal healthy women with body mass index (BMI) 20-44 obtained by needle biopsies from the periumbilical region. For the sFlt-1 release studies, we used omental and subcutaneous adipose tissue from 10 obese women (mean BMI=33) who were undergoing open abdominal surgery and from 12 morbidly obese women (mean BMI=46) who were undergoing laparoscopic gastric bypass. Mature adipocytes, their precursor cells called, adipose-derived stem cells (ADSC), and adipose tissue nonfat cells were isolated and cultured as described previously. 1-3 Briefly, fat was digesting by collagenase for 2h. The collagenase digest was then separated from the undigested tissue, the adipose tissue nonfat cells, by filtration. The stromal-vascular (SV) cells were separated from mature adipocytes by centrifugation of the filtered collagenase digest while the tissue nonfat cells were those remaining in the tissue after collagenase digestion. The SV cells were defined as those cells isolated by collagenase digestion that were deposited at the bottom of the tube after centrifugation, whereas the mature adipocytes were those cells that floated on the surface.

Primer and probes

Following primer and probes were used in realtim RT-PCR. Soluble Flt1 (GenBank accession number: U01134) 5´-AATCAGAGGTGAGCCTGCAAC-3´ (forward-primer), 5´-TGGTACAATCATCCTTTGTGCTTT-3´ (reverse-primer), 5´-FAM-AAAAGGCTGTATTCTCTGGATCTCCAAATTT-TAMRA-3´ (probe); VEGFa (GenBank accession number: NM_003376) 5´-TACCTCCACCAGCGTCAAGT-3´ (forward-primer), 5´-GATGATTCTGCCCCTCCTCTT-3´(reverse primer), 5´-TAMRA-TCCAGGCTGACCCATGG-FAM-3´(probe), adiponectin (GenBank accession number: NM_004797) 5´-GCTCTGCTTCTCGATCATCTC-3´ (forward primer), 5´-ACGCTCTCTCCCTCCCATACA-3´ (reverse primer), 5´-FAM-AGGTGGGGCAAGTGCTGGT-TAMRA-3´ (probe), adipophilin (PLIN2) (GenBank accession number: NM_007408) 5´-GACCTCTGTGCCCATGAC-3´ (forward primer), 5´-GTATTGGCAACCGAATTGT-3´ (reverse primer) and the endogenous-control Eukaryotic 18S rRNA (GenBank accession number: X03205) (PE Biosystems).

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