Antiangiogenic Actions of Vascular Endothelial Growth Factor-A$_{165}^{b}$, an Inhibitory Isoform of Vascular Endothelial Growth Factor-A, in Human Obesity

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Background—Experimental studies suggest that visceral adiposity and adipose tissue dysfunction play a central role in obesity-related cardiometabolic complications. Impaired angiogenesis in fat has been implicated in the development of adipose tissue hypoxia, capillary rarefaction, inflammation, and metabolic dysregulation, but pathophysiological mechanisms remain unknown. In this study, we examined the role of a novel antiangiogenic isoform of vascular endothelial growth factor-A (VEGF-A), VEGF-A$_{165}^{b}$, in human obesity.

Methods and Results—We biopsied paired subcutaneous and visceral adipose tissue in 40 obese subjects (body mass index, 45±8 kg/m$^2$; age, 45±11 years) during bariatric surgery and characterized depot-specific adipose tissue angiogenic capacity using an established ex vivo assay. Visceral adipose tissue exhibited significantly blunted angiogenic growth compared with subcutaneous fat (P<0.001) that was associated with marked tissue upregulation of VEGF-A$_{165}^{b}$ (P=0.004). The extent of VEGF-A$_{165}^{b}$ expression correlated negatively with angiogenic growth (r=-0.6, P=0.006). Although recombinant VEGF-A$_{165}^{b}$ significantly impaired angiogenesis, targeted inhibition of VEGF-A$_{165}^{b}$ with neutralizing antibody stimulated fat pad neovascularization and restored VEGF receptor activation. Blood levels of VEGF-A$_{165}^{b}$ were significantly higher in obese subjects compared with lean control subjects (P=0.02), and surgical weight loss induced a marked decline in serumVEGF-A$_{165}^{b}$ (P=0.003).

Conclusions—We demonstrate that impaired adipose tissue angiogenesis is associated with overexpression of a novel antiangiogenic factor, VEGF-A$_{165}^{b}$, that may play a pathogenic role in human adiposopathy. Moreover, systemic upregulation of VEGF-A$_{165}^{b}$ in circulating blood may have wider-ranging implications beyond the adipose milieu. VEGF-A$_{165}^{b}$ may represent a novel area of investigation to gain further understanding of mechanisms that modulate the cardiometabolic consequences of obesity. (Circulation. 2014;130:1072-1080.)

Key Words: angiogenesis modulating agents | intra-abdominal fat | metabolism | obesity | vascular endothelial growth factor A | vascular endothelial growth factors

Obesity with its associated metabolic complications has emerged as one of the most critical healthcare problems in the United States and worldwide, with nearly 70% of the US population currently overweight or obese.1,2 Angiogenesis, the generation of new blood vessels, is critical for adequate fat expansion and adipose tissue remodeling.1 Clinical studies show that adipose tissue angiogenic responses are blunted in human obesity, particularly in visceral depots, and are associated with inflammation and metabolic dysfunction.4–7 Strategies aimed at therapeutic modulation of adipose vascularization to improve metabolism have focused on stimulating the primary regulator of tissue angiogenesis, vascular endothelial growth factor-A (VEGF-A).8–10 In experimental models, adipose tissue overexpression of VEGF-A promotes neovascularization and improves insulin sensitivity and glucose metabolism.9,10 Conversely, adipose-specific VEGF-A knockouts display capillary rarefaction, inflammation, and metabolic collapse.10 These data collectively provide strong evidence that qualitative features of fat and altered tissue homeostasis as a function of impaired vascular support play a central role in shaping systemic phenotypes.

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Clinical studies demonstrate that subcutaneous adipose tissue exhibits higher capillary density and angiogenic capacity compared with the visceral depot. Despite consistent published data demonstrating higher VEGF-A expression in visceral fat, this paradoxical finding remains unexplained in human studies of obesity. Although initially described in the oncological literature, it is now recognized that alternative VEGF-A gene splicing may generate a number of VEGF-A isoforms that differ in their biological action. Therefore, proximal splicing that includes an exon 8a sequence results in the proangiogenic VEGF-A165a, whereas distal splicing inclusive of exon 8b yields the antiangiogenic isoform, VEGF-A165b. VEGF-A165a exhibits a binding affinity similar to that of VEGF-A165b to VEGF receptor-2 (VEGFR-2), but it fails to activate receptor phosphorylation as a result of a lack of binding to the neuropilin-1 coreceptor, consequently impairing angiogenesis. To date, essentially nothing is known about the biological relevance of VEGF-A165b in obesity-related cardiometabolic disease.

In this study, we aimed to characterize the role of 2 major VEGF-A splice variants with opposing actions—proangiogenic, VEGF-A165a, and antiangiogenic, VEGF-A165b—in mediating angiogenic responses in subcutaneous and visceral human adipose tissue. Additionally, we sought to gain evidence that antiangiogenic VEGF-A165b is overexpressed systemically and favorably modified after bariatric surgical weight loss in obese subjects.

Methods

Study Subjects
Consecutive obese men and women (body mass index \( \geq 30 \) kg/m\(^2\); age \( \geq 18 \) years) with severe long-standing obesity enrolled in the Boston Medical Center Bariatric Surgery Program were recruited into the study. Samples of subcutaneous and visceral adipose tissue were collected intraoperatively from both the lower abdominal wall and greater omentum, respectively, during planned bariatric surgery, as previously described. Each subject provided 1 biopsy specimen from the subcutaneous depot and 1 specimen from the visceral depot (paired samples). No subject provided \( >1 \) fat sample per depot. A subset of obese individuals were followed up prospectively, and serum analyses were performed before and after a 2 months after bariatric surgery and compared with analyses from lean control subjects (body mass index, 18–25 kg/m\(^2\)) recruited through general public advertisement. Subjects with unstable medical conditions or pregnancy were not eligible for gastric bypass surgery and thus were excluded. The study was approved by the Boston University Medical Center Institutional Review Board, and written consent was obtained from all participants.

Anthropometric and Biochemical Measures

During a single outpatient visit before planned bariatric surgery, clinical characteristics, including blood pressure, height, weight, and waist circumference, were measured, and cardiovascular risk factors were recorded. Fasting blood was taken via an antecubital vein for biochemical parameters, including lipids, glucose, insulin, glycosylated hemoglobin, and high-sensitivity C-reactive protein, and serum analyses of VEGF-A165a, and VEGF-A165b isoforms. Homeostasis model assessment was used as the index of insulin sensitivity. All biochemical analyses were performed by the Boston Medical Center clinical chemistry laboratory.

Angiogenesis Assay of Adipose Tissue

Fat biopsy samples freshly collected during bariatric surgery were immediately placed in sterile endothelial cell basal media-2 (Lonza), and angiogenesis studies were performed by previously published methods. Briefly, fat pads were finely minced with scissors and enzymatically digested with 1 mg/mL collagenase type 1 (Worthington Biochemical) for 30 minutes at 37°C. Digested pieces of adipose tissue was passed through 100-µm nylon filter (BD Falcon) and washed in endothelial cell basal media-2. Pieces \( \approx 1 \) mm\(^3\) were embedded on ice in 250 µL per well of growth factor-depleted Matrigel (BD Discovery) on a 24-well plate and incubated for 30 minutes at 37°C. After polymerization of Matrigel, wells were covered with 500 µL endothelial cell basal media-2 supplemented with growth factors (Lonza) and cultured at 37°C for 7 days. Half of the media was removed and replaced with fresh media every other day. Sprouts growing from the adipose tissue explant along the perimeter of each sample under \( \times 100 \) magnification were counted by a blinded investigator, as previously described.

Immunofluorescence Staining

Adipose tissue embedded in Matrigel was fixed in 4% paraformaldehyde (Sigma Aldrich) and stained with endothelium-specific markers: anti-mouse CD31 (1:100; Pierce Thermo Scientific), anti-rabbit VEGFR-2 (1:50; Cell Signaling), and anti-rabbit von Willebrand factor (1:50; Dako), as previously published. DAPI stain (Molecular Probes, Invitrogen) was used for cell nucleus identification. Fat tissue was also stained for CD90 (BD Pharmingen) and CD73 (Invitrogen) as markers for mesenchymal stem cells. Immuno-positive depots were imaged with a fluorescence microscope (Nikon Eclipse Ti) at \( \times 10 \) magnification for CD31 and VEGFR-2 and at \( \times 20 \) magnification for von Willebrand factor. Digital images of the cells were captured with an Andor Clara E Camera.

Neutralizing Antibody Experiments

To examine the effects of neutralizing VEGF-A165b antibody (R&D Systems) on the angiogenic capacity of visceral adipose tissue, fat pads were processed as described above, and endothelial cell basal media-2 supplemented with growth factors but not VEGF was used. Nonspecific mouse IgG or VEGF-A165b-neutralizing antibody (R&D Systems clone 56-1, catalog No. MAB3045) was used at concentration of 10 µg/mL. Recombinant VEGF-A165b protein (R&D Systems, catalog No. 3045-VE) was used at concentration of 500 ng/mL. Half of the media and treatment was removed and replaced every other day for sprout analyses. Angiogenesis was quantified from the tissue explant by a blinded investigator.

For whole adipose tissue incubation with neutralizing antibody, fat tissue was minced and placed in 1 mL endothelial cell basal media-2 supplemented with growth factors (but not VEGF) containing either mouse IgG control or VEGF-A165b antibody at 10 µg/mL for 24 hours. Tissue was collected and snap-frozen in liquid nitrogen and stored at \(-80°C\) for analysis of protein expression levels of VEGF-A165b, VEGF-A165a, VEGFR-2 (Y951), VEGFR-2, phosphorylated extracellular signal-regulated kinase (ERK) 1/2 (Y204,197), and total ERK1/2 using Western blot.

Flow Cytometry

Adipose tissue macrophages were isolated as previously described. Briefly, whole adipose tissue samples were cut into small pieces, gently homogenized in RPMI using disposable tissue grinders (Fisher Brand), and filtered through 70-µm cell strainers (BD Falcon, Bedford, MA) to obtain single-cell suspension. Macrophages were isolated by dual density gradient (Histopaque-1077 and Histopaque-1119, Sigma Aldrich). Isolated cells were incubated with fluorescence-activated cell sorter analysis permeabilization solution 2 (BD Biosciences) for 30 minutes at 37°C and then measured by flow cytometry for cells positive for both CD14 (BD Pharmingen) and human (h) VEGF-A165a (which does not distinguish isoforms; R&D Systems).

Western Immunoblot Analyses

VEGF-A165b antibody for Western blot was purchased from Abcam (catalog No. 14994). The polyclonal antibody against human VEGF-A165a, C-terminal (Exon 8a)–specific antibody (VEGF-A165a), was generated by immunization of a rabbit with a KLH-conjugated peptide TRCRDKPRR, corresponding to the last 9 amino acids of
VEGF-A$_{165a}$, using standard immunization techniques. Proteins were extracted from adipose tissue by homogenization in liquid nitrogen, followed by the addition of ice-cold lysis buffer (50 mmol/L Tris- HCl [pH 7.4], 150 mmol/L NaCl, 1 mmol/L EDTA, 1% Triton X-100, 0.25% SDS) supplemented with 1 mmol/L phenylmethylsulfonyl fluoride, 1 mmol/L sodium orthovanadate, protease inhibitor cocktail, and phosphatase inhibitors I and II (Sigma Aldrich). Samples were assayed for protein content by use of the Bradford method. Protein 20 to 30 μg was subjected to electrophoresis in 12% SDS–polyacrylamide gel under reducing conditions and then blotted to a polyvinylidene difluoride membrane using the Bio-Rad Transblot Turbo Transfer system, blocked with Odyssey Blocking Buffer (Lior Biosciences), and incubated overnight with the respective antibodies at 4°C with primary mouse or rabbit anti-human antibodies (1:500–1:1000). The amount of protein loaded was the same for both depots for each specific subject. Immunostained membranes for VEGF-A$_{165b}$ (Abcam), phosphorylated ERK1/2 (Y204/197; Abcam, MitoSciences), and total ERK1/2 (BD Biosciences) were incubated with infrared dye–conjugated secondary antibodies and visualized and quantified with the LI-COR Odyssey system. Membranes probed for VEGF-A$_{165a}$ (from Dr David Bates), VEGFR-2 phosphorylated (Y951; Cell Signaling), and VEGFR-2 (Cell Signaling) were visualized and quantified with the use of horseshadish peroxidase–conjugated anti-rabbit or anti-mouse IgG (R&D Systems) for 1 hour at room temperature. Immune complexes were detected with the enhanced chemiluminescence detection system (Bio-Rad). Densitometric analysis of the bands was performed with the ImageQuant LAS 4000 biomolecular imaging system (GE Healthcare). ImmunobLOTS were normalized to β-actin (Cell Signaling).

**Immunoprecipitation**

Immunoprecipitation was performed with G-Sepharose–coupled beads. Beads were incubated with mouse anti-hVEGFR-2 (Cell Signaling) antibody (1:100) for 1 hour, followed by overnight incubation at 4°C with 300 μg protein in the presence of protease and phosphatase inhibitor cocktail (Sigma Aldrich). The eluate was subjected to SDS-PAGE. Mouse anti-human VEGF-A$_{165b}$ at 1:500 dilution was blotted with the use of standard Western blot protocols.

**Serum VEGF-A$_{165a}$ and VEGF-A$_{165b}$ Protein**

Serum protein samples were resolved on SDS-PAGE for Western blot analysis, and VEGF-A$_{165a}$ and VEGF-A$_{165b}$ protein expression was measured in both lean and obese subjects. Additionally, serum VEGF-A$_{165a}$ and VEGF-A$_{165b}$ were measured in obese patients before and after bariatric surgical weight loss. Immunopositive blots were normalized to total protein stained with MemCode Reversible Protein Stain (Thermo Scientific).

**Statistics**

Group differences in clinical characteristics of subjects were analyzed with t tests or χ$^2$ tests with SPSS 20.0. Other analyses were performed with GraphPad Prism 6.0 software. Area under the curve of the plot for cumulative angiogenic growth (quantified as capillary sprout count) over the time period of 7 days was examined with GraphPad Prism 6.0. Differences between subcutaneous and visceral tissue in angiogenic studies, VEGF-A protein isoform expression and receptor signaling, and neutralizing antibody experiments were analyzed with paired t tests. Serum measures of VEGF-A isoforms before and after weight loss were examined by paired t tests. All other analyses were performed with unpaired t tests with correction for unequal variances with the Welch approximation, as indicated in the figure legends. A value of P<0.05 was accepted as statistically significant. Data are presented as mean±SD unless otherwise indicated.

**Results**

**Quantification of Angiogenesis in Subcutaneous and Visceral Adipose Tissue**

A total of 40 subjects were recruited who provided subcutaneous and visceral adipose tissue samples. Clinical characteristics of the subjects (Table) are consistent with those of the bariatric population at our medical center. In agreement with prior data,7 angiogenic capacity was significantly impaired in visceral compared with subcutaneous fat pads quantified by either area under the curve plot of capillary growth (area under the curve, 63±11 for visceral versus 127±17 for subcutaneous; P<0.001) over 7 days (Figure 1A) or total capillary branch counts at 7 days of culture (34±25 for visceral versus 63±30 for subcutaneous; P<0.001; Figure 1A in the online-only Data Supplement). Illustrations of preserved (subcutaneous) and blunted (visceral) angiogenic capacity are shown in Figure 1B. Subjects with diabetes mellitus exhibited markedly reduced capillary growth in the visceral depot compared with nondiabetics (Figure 1C), whereas no difference was noted in the subcutaneous depot. In line with prior published data, we confirmed that sprout extensions from central fat pad represent burgeoning capillary networks that immunostained positively for endothelial-specific markers, including CD31, VEGFR-2, and von Willebrand factor (Figure 2A, 2B, and 2C, respectively).7 Lastly, stains for CD73 and CD90 as mesenchymal stem cell markers were negative (Figure II in the online-only Data Supplement).

**Depot-Specific VEGFR-2 Signaling in Adipose Tissue.**

We and others have demonstrated a “paradoxical” increase of VEGF-A$_{total}$ mRNA and protein expression in whole visceral fat despite impaired angiogenic potential.6,11 Using flow cytometry, we similarly observed upregulation of VEGF-A$_{total}$ expression...
in adipose macrophages that correlated negatively with capillary growth (Figure IIIA and IIIB in the online-only Data Supplement, respectively). Furthermore, we demonstrated that proangiogenic activation of VEGFR-2 through phosphorylation of its tyrosine kinase receptor VEGFR-2 at Y951 (kinase insert domain; VEGFR-2 p(Y951))22 was significantly impaired in the visceral compared with the subcutaneous depot by Western blot analysis (P=0.007; n=20 per group; Figure 3A). However, total VEGFR-2 protein expression was not different between depots (Figure IV in the online-only Data Supplement). As shown in Figure 3B, the extent of VEGFR-2 phosphorylation correlated positively with the angiogenic capacity (r=0.7, P=0.001).

Depot-Specific Characterization of VEGF-A165a and VEGF-A165b in Adipose Tissue

Altered VEGFR-2 signaling in visceral adipose tissue led us to characterize protein expression patterns of the major proangiogenic (VEGF-A165a) and antiangiogenic (VEGF-A165b) isoforms using isoform-specific antibodies. VEGF-A165a protein was not different between subcutaneous versus visceral depots (P=0.8; Figure V in the online-only Data Supplement), whereas VEGF-A165b expression was 1.3±0.2-fold higher in visceral fat (P=0.004; n=23 per group; Figure 4A). Furthermore, visceral adipose tissue VEGF-A165b expression was significantly higher in subjects with diabetes mellitus compared with nondiabetics.
There was no correlation between VEGF-A<sub>165a</sub> expression and capillary growth; however, adipose VEGF-A<sub>165b</sub> correlated significantly with lower angiogenic capacity ($r=-0.6$, $P=0.006$; Figure 4B), providing support that it negatively regulates vascular proliferation. Moreover, coimmunoprecipitation experiments demonstrated greater VEGF-A<sub>165b</sub> coupling with its receptor VEGFR-2 in visceral fat (Figure 4C), indicating higher VEGF-A<sub>165b</sub> occupancy of VEGFR-2 ($P=0.03$). These data led us to hypothesize that the antiangiogenic action of VEGF-A<sub>165b</sub> predominated despite a seemingly “VEGF-$\text{A}_{\text{total}}$–abundant” adipose milieu.

**Effect of Neutralizing VEGF-A<sub>165b</sub> Antibody on Angiogenic Responses**

To confirm that VEGF-A<sub>165b</sub> is involved in angiogenic impairment, the addition of recombinant hVEGF-A<sub>165b</sub> protein (rhVEGF-A<sub>165b</sub>) to culture media significantly reduced angiogenic growth of visceral adipose tissue ($P=0.01$; $n=6$; Figure 5A). In contrast, treatment with a neutralizing VEGF-A<sub>165b</sub> antibody significantly bolstered angiogenesis in fat by 56±29% compared with IgG control ($P=0.008$; $n=10$; Figure 5B). Combining rhVEGF-A<sub>165b</sub> with VEGF-A<sub>165b</sub> antibody abolished the positive angiogenic effects of the neutralizing antibody (Figure 5C). These data provide strong support for the functional significance of VEGF-A<sub>165b</sub> as a negative modulator of angiogenesis in human fat.

Figure 4. Differential protein expression of vascular endothelial growth factor (VEGF)-A<sub>165b</sub> in subcutaneous (S) vs visceral (V) adipose tissue. A, Visceral adipose tissue ($n=23$) exhibited significant upregulation of VEGF-A<sub>165b</sub> protein expression vs subcutaneous depot ($n=23$; $P=0.004$). B, Increased adipose tissue protein expression of VEGF-A<sub>165b</sub> correlated with reduced angiogenic capacity ($r=-0.6$, $P=0.006$). C, Coimmunoprecipitation of VEGF-A<sub>165b</sub> and its receptor, VEGFR-2, was significantly higher in visceral vs subcutaneous adipose tissue ($n=3$ in each group, $P=0.03$). Data are presented as mean±SEM. AUC indicates area under the curve; and IP, immunoprecipitation.

**Effect of VEGF-A<sub>165b</sub>–Neutralizing Antibody on VEGFR-2 Phosphorylation and ERK Activation**

Incubation of whole visceral adipose tissue with VEGF-A<sub>165b</sub>–neutralizing antibody significantly suppressed VEGF-A<sub>165b</sub> protein expression ($P=0.025$; $n=10$; Figure 6A) without affecting VEGF-A<sub>165a</sub> protein (Figure VII in the online-only Data Supplement). Importantly, VEGF-A<sub>165b</sub> suppression via incubation with the neutralizing antibody increased the ratio of VEGFR-2 p(Y951) to VEGFR-2 ($P=0.02$; Figure 6B). Furthermore, reduced VEGF-A<sub>165b</sub> expression correlated negatively with upregulation of VEGFR-2 p(Y951; $r=-0.7$, $P=0.03$; Figure 6C). Restoration of phospho-VEGFR-2 and suppression of VEGF-A<sub>165b</sub> led to significant upregulation of the ratio of phospho-ERK1/2 to total ERK1/2 ($P=0.007$; Figure 7A). Additionally, VEGF-A<sub>165b</sub> inhibition correlated significantly with activation of ERK1/2 phosphorylation ($r=-0.7$, $P=0.03$; Figure 7B). These combined findings suggest a key role of VEGF-A<sub>165b</sub> in antiangiogenic signaling in adipose tissue.

**Serum Levels of VEGF Isoforms in Obesity and the Effect of Weight Loss**

We compared serum levels of VEGF-A<sub>165b</sub> protein by Western immunoblotting and demonstrated significantly higher antiangiogenic VEGF-A<sub>165b</sub> protein levels in obese ($n=19$) compared with lean ($n=15$) subjects ($P=0.02$; Figure 8A). In contrast, proangiogenic VEGF-A<sub>165a</sub> was significantly lower
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in obese (n=14) compared with lean (n=11) subjects (P=0.03; Figure 8B). In a cohort of obese individuals who were followed up prospectively for 10±2 months after bariatric surgery, serum VEGF-A165b declined significantly (P=0.003; n=14) after a 30±9% weight reduction (Figure 8C) without a significant change in VEGF-A165a (Figure 8D). In obese subjects, there was no significant difference in VEGF-A165b protein levels in diabetics (1.5±0.2 arbitrary units; n=10) versus nondiabetics (1.4±0.2 arbitrary units; n=9). Clinical characteristics of lean subjects and obese individuals before and after weight loss are given in Tables I and II in the online-only Data Supplement.

Discussion

We describe for the first time an endogenous antiangiogenic isoform of VEGF-A, VEGF-A165b, that is overexpressed in human visceral fat and is associated with impaired angiogenesis in adipose tissue. Targeted VEGF-A165b inhibition restored proangiogenic VEGFR-2 phosphorylation and ERK activation and stimulated angiogenesis in visceral fat pads, suggesting that this approach may promote vasculogenesis in fat and potentially influence metabolism. Importantly, we observed markedly elevated blood levels of VEGF-A165b in obese subjects that decreased significantly after bariatric surgical weight loss. This latter finding has potentially important clinical implications in that systemic upregulation of a circulating antiangiogenic factor in obesity may have wider-ranging implications beyond the adipose milieu that could support pathological changes in other organ systems, including coronary and peripheral circulations.23

Adequate vascularization and expansion of adipose tissue are critically important for whole-body metabolic homeostasis.3 Strategies to limit adipose tissue growth and to attempt to combat obesity with antiangiogenic interventions have failed to confer any metabolic benefit.3,24 Rather, angiogenic insufficiency has been implicated in the development of adipose tissue dysfunction, hypoxia, capillary rarefaction, and inflammation, which trigger metabolic dysregulation and insulin resistance.4–6 Experimental studies show that stimulating adipose vascularization improves insulin sensitivity, whereas VEGF-A deficiency promotes capillary dropout and metabolic dysfunction.10 Therefore, enhanced adipose tissue vascularization may serve as a therapeutic approach for the generation of healthy adipocytes25 and preserved metabolism in the face of obesogenic stress, strengthening the growing paradigm that “quality” in addition to quantity of fat plays a significant role in shaping metabolic and cardiovascular phenotypes in human obesity.13,20,26

A major novel finding in our study is the identification of significant upregulation of antiangiogenic VEGF-A165b in the visceral fat of obese subjects. We performed a number of experiments confirming the pathological actions of VEGF-A165b in human tissue. We demonstrated that its upregulation correlates

Figure 5. Effects of recombinant human vascular endothelial growth factor (VEGF)-A165b (rhVEGF-A165b) and VEGF-A165b-neutralizing antibody (VEGF-A165b Ab) on angiogenesis in adipose tissue. A, Addition of rhVEGF-A165b protein (500 ng/mL) significantly reduced the sprouting capacity of visceral adipose tissue explants vs vehicle control (area under the curve [AUC], 79±18 vs 53±14; n=6; P=0.01). B, VEGF-A165b-neutralizing antibody (10 µg/mL; VEGF-A165b antibody) increased angiogenic capacity vs IgG control (10 µg/mL; AUC, 67±20 vs 81±20; n=10; P=0.008). C, Combination of VEGF-A165b antibody (10 µg/mL) and rhVEGF-A165b (500 ng/mL) reversed angiogenic potentiation of VEGF-A165b antibody vs IgG control (AUC, 83±19 vs 78±19; n=9; P=0.14). Data are presented as mean±SEM.

Figure 6. Effects of vascular endothelial growth factor (VEGF)-A165b-neutralizing antibody on phosphorylation of VEGF receptor (VEGFR-2). A, Addition of VEGF-A165b-neutralizing antibody significantly suppressed VEGF-A165b protein expression (n=10; P=0.025). B, Suppression of VEGF-A165b protein expression with VEGF-A165b-neutralizing antibody increased the ratio of VEGFR-2 p(Y951) to VEGFR-2 (n=10; P=0.02). C, Degree of VEGF-A165b suppression correlated significantly with higher protein expressions of VEGFR-2 p(Y951) (r=-0.7, P=0.03; n=10). Data are presented as mean±SEM.
negatively with capillary growth and that recombinant VEGF-A_{165b} significantly impairs angiogenesis. Conversely, targeted inhibition of VEGF-A_{165b} with neutralizing antibody stimulated fat pad angiogenesis by activating VEGFR-2 phosphorylation without altering VEGF-A_{165a}.

Further, we showed that suppression of VEGF-A_{165b} correlated significantly with increased ERK phosphorylation, which has been shown to confer cellular proliferation and survival in endothelial cells. Taken together, these findings suggest that reduced VEGF-A_{165b} may promote a VEGF-A_{165a}–VEGFR-2 interaction and activate proangiogenic signaling in fat, as previously demonstrated in nonadipose tissue.

Our results therefore provide a potential mechanistic explanation for the seemingly paradoxical clinical observation of increased VEGF-A expression in the face of angiogenic impairment by demonstrating that the antiangiogenic isoform VEGF-A_{165b} may predominate in disease states.

Compared with VEGF-A_{165a}, very little is known about VEGF-A_{165b} or its potential pathophysiological role in human disease, but it is expressed in a range of tissues, including human microvascular endothelial cells, kidney, skeletal muscle, pituitary, and eyes. Its pathogenic role has been described in systemic sclerosis in which VEGF-A_{165b}–neutralizing antibody significantly improved angiogenesis in human skin microvascular endothelial cells via activation of VEGFR-2 phosphorylation.

Conversely, a potential therapeutic use has been explored in the treatment of proliferative diabetic retinopathy in which VEGF-A_{165b} significantly inhibited retinal neovascularization. Because alternative VEGF splicing is increasingly being linked to the origin of specific cancers, clinical applications have also been considered for the prevention of tumor growth.

Relatively little is known about the regulation of VEGF-A alternative splicing. Insulin growth factor-1 and tumor necrosis factor-α promote formation of VEGF-A_{165a}, whereas transforming growth factor-β favors VEGF-A_{165b} expression. Insulin growth factor-1 modulates splicing of VEGF isoforms by preferential use of the proximal splice site to upregulate proangiogenic expression that may be modulated by protein kinase C and serine-arginine rich protein kinases 1 and 2.

In this study, we observed that the diabetic state was associated with upregulated VEGF-A_{165b} expression and blunted angiogenic capacity in visceral fat. Although the “chicken or egg” dilemma remains, pharmacological treatment with rosiglitazone has been shown to simultaneously improve adipose tissue angiogenesis and insulin sensitivity. Furthermore, clinical imaging studies with computed tomography have linked qualitative measures of adipose tissue lipid and vascular density to adverse cardiometabolic profiles such as insulin resistance, hypertension, and dyslipidemia. It is thus becoming increasingly clear that qualitative features of adipose tissue, including its vascularity, could play an important role in the pathogenesis of obesity-induced cardiometabolic complications.

Another major finding in our study was the demonstration of markedly increased serum levels of antiangiogenic VEGF-A_{165b} in obese compared with lean subjects. Detection of VEGF-A_{165b} in the systemic circulation raises the possibility that elevated VEGF-A_{165b} may contribute to vascular disease and ischemia beyond the adipose environment. Impaired angiogenic regulation in key vascular territories, including coronary, renal, cerebral, and peripheral beds, has the potential to contribute to the clinical expression of coronary and peripheral arterial disease, renal dysfunction, and stroke. Importantly, we showed that marked weight loss induced by bariatric surgery, the only weight reduction intervention shown to reduce cardiovascular risk, significantly lowers VEGF-A_{165b} levels, demonstrating the feasibility of modulating antiangiogenic isoforms with clinical intervention.

It remains unclear whether upregulation of VEGF-A_{165b} in visceral adipose tissue is pathogenic or a compensatory mechanism that regulates adipose remodeling. It is possible that analogous to a neoplastic process, growing adipose tissue is highly dependent on its vascular support and that VEGF-A_{165b} overexpression may represent an endogenous mechanism to attempt to limit ectopic adipose expansion by depriving it of oxygen and nutrients to “the point of death.” Therefore, it is also possible that VEGF-A_{165b} levels decrease with weight loss because the stimulus for angiogenesis is reduced with weight reduction because there is no longer a physiological demand for adipose expansion. Lastly, VEGF-A_{165b} may play a stabilizing role in the innate vasculature of other organs, including coronary and peripheral circulation, by curbing uncontrolled or immature angiogenesis, although essentially nothing is known about these processes in the context of human obesity.

Figure 7. Effects of vascular endothelial growth factor (VEGF)-A_{165b}–neutralizing antibody (Ab) on phosphorylation of extracellular signal-regulated kinase (ERK) 1/2. A, Suppression of VEGF-A_{165b} protein expression with VEGF-A_{165b}–neutralizing antibody increased the ratio of phospho-ERK1/2 (Y204/197) to total ERK1/2 (n=10; P<0.007) B, Degree of VEGF-A_{165b} suppression correlated significantly with higher ratio of phospho-ERK1/2 to total ERK1/2 (r=−0.7, P=0.03; n=10). Data are presented as mean±SEM.
Our study has several limitations. We did not have access to adipose tissue, particularly the visceral depot, from lean subjects; thus, we did not study angiogenesis or VEGF-A isoform expression in lean subjects, which will be the topic of future investigation. Additionally, we were not able to rebiopsy visceral fat tissue in obese subjects after weight loss because this would require a repeat invasive abdominal operation that cannot be justified without clinical indication. Although we have shown that VEGF-A165b exhibits antiangiogenic actions in human adipose tissue, compensatory changes or isoform regulation of other angiogenic mediators in fat such as angiotensin-49 and thrombospondin-139 was not examined. Lastly, we did not comprehensively investigate all VEGF-A isoforms, and we acknowledge that other antiangiogenic isoforms that modulate adipose tissue angiogenesis may exist.

Conclusions
We demonstrate overexpression of the novel antiangiogenic factor VEGF-A165b, which may play a key pathogenic role in obesity-related cardiovascular and metabolic disease. VEGF-A165b may represent a novel area of investigation and potential intervention to modulate angiogenesis in human adipose tissue and possibly cardiometabolic consequences of obesity.

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Disclosures
None.

References


**CLINICAL PERSPECTIVE**

Adequate vascularization and expansion of adipose tissue are critically important for whole-body metabolic homeostasis. Experimental studies suggest that impaired angiogenesis in adipose tissue is associated with the development of adipose tissue hypoxia, inflammation, and metabolic dysfunction in obesity. In this study, we describe for the first time a novel antiangiogenic isoform of vascular endothelial growth factor-A (VEGF-A), VEGF-A(165b), that is overexpressed in human visceral fat and is associated with impaired angiogenesis in adipose tissue. Targeted VEGF-A(165b) inhibition restored proangiogenic VEGF receptor-2 phosphorylation and extracellular signal-regulated kinase activation and stimulated angiogenesis in human fat. Importantly, serum levels of antiangiogenic VEGF-A(165b) were elevated in obese compared with lean subjects and decreased significantly after bariatric surgical weight loss. This latter finding has potentially important clinical implications because systemic upregulation of a circulating antiangiogenic factor in obesity could support additional pathological vascular changes in systemic targets beyond the adipose milieu. VEGF-A(165b) may play a key pathogenic role in obesity-related adiposopathy and may represent a novel area of investigation and potential intervention to modulate cardiometabolic consequences of obesity.
Antiangiogenic Actions of Vascular Endothelial Growth Factor-A<sub>165b</sub>, an Inhibitory Isoform of Vascular Endothelial Growth Factor-A, in Human Obesity

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Supplemental Material
Supplemental figure 1: Angiogenesis comparison between subcutaneous and visceral adipose tissue. Angiogenic capacity was blunted in visceral vs. subcutaneous fat quantified by capillary branch numbers at day 7 (Visceral: 34 ± 25 vs. Subcutaneous: 63 ± 31, p<0.001) in 25 subjects who provided paired samples demonstrating reduced angiogenesis in visceral vs. subcutaneous fat.
Supplemental figure 2: Immunofluorescence of adipose tissue sprouts. Adipose tissue sprouts stained positively for endothelial cell specific marker von Willebrand factor (vWF), but were negative (absent) for mesenchymal stem cell markers (A) CD90 and (B) CD73. (DAPI: nuclear stain)
Supplemental Figure 3: Depot-specific VEGF-A$_{total}$ positive macrophages (double VEGF-A$_{total}$ and CD14 positive cells) in adipose tissue assessed by flow cytometry. A) Visceral adipose tissue (n=9) had significantly higher VEGF-A$_{total}$ positive macrophages vs. subcutaneous depot (n=10), $p=0.01$. B) There was a significant inverse correlation between percent of VEGF-A$_{total}$ macrophages and angiogenesis in adipose tissue ($r=0.5$, $p=0.04$).
Supplemental figure 4: Comparison of VEGFR-2 protein expression between subcutaneous and visceral adipose tissue. There was no difference in total VEGFR-2 protein expression between the two depots (n=11 in each group, p=NS), using western blot analysis.
Supplemental figure 5: VEGF-A_{165a} protein expression in subcutaneous vs. visceral adipose tissue. There was no significant difference in VEGF-A_{165a} protein expression between subcutaneous (S) vs. visceral (V) depot.
Supplemental figure 6: VEGF-A$_{165}$b protein expression in visceral adipose tissue in subjects with or without diabetes. Mean VEGF-A$_{165}$b protein expression was significantly lower in subjects without diabetes (n=15) vs. with diabetes (n=12), p<0.05, unpaired t-test.
Supplemental figure 7: Effect of VEGF-A165b neutralizing antibody on VEGF-A165a protein expression. There was no significant difference in VEGF-A165a protein expression after treatment of visceral adipose tissue with VEGF-A165b neutralizing antibody vs. IgG control, n=12, p=0.2
**Supplementary Table 1: Clinical characteristics of subjects with serum VEGF-A isoform analyses.**

<table>
<thead>
<tr>
<th>Clinical parameter</th>
<th>Lean (n=15)</th>
<th>Obese (n=19)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight (kg)</td>
<td>63 ± 8</td>
<td>122 ± 22</td>
<td>0.006</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>21 ± 2</td>
<td>44 ± 8</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Waist circumference (cm)</td>
<td>80 ± 8</td>
<td>128 ± 16</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Insulin (mIU/mL)</td>
<td>3.1 ± 2.1</td>
<td>15.8 ± 17</td>
<td>0.006</td>
</tr>
<tr>
<td>Glucose (mg/dL)</td>
<td>89 ± 8</td>
<td>143 ± 73</td>
<td>0.008</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>0.7 ± 0.5</td>
<td>3.1 ± 1.3</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>5.3 ± 0.5</td>
<td>7.0 ± 1.9</td>
<td>0.002</td>
</tr>
<tr>
<td>hsCRP (mg/dL)</td>
<td>0.8 ± 1.5</td>
<td>4.8 ± 6.3</td>
<td>0.001</td>
</tr>
<tr>
<td>Systolic BP (mmHg)</td>
<td>117 ± 10</td>
<td>130 ± 14</td>
<td>0.006</td>
</tr>
<tr>
<td>Diastolic BP (mmHg)</td>
<td>71 ± 6</td>
<td>75 ± 10</td>
<td>0.1</td>
</tr>
<tr>
<td>Total cholesterol (mg/dL)</td>
<td>180 ± 26</td>
<td>189 ± 32</td>
<td>0.4</td>
</tr>
<tr>
<td>HDL-C (mg/dL)</td>
<td>63 ± 17</td>
<td>49 ± 8</td>
<td>0.003</td>
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<tr>
<td>LDL-C (mg/dL)</td>
<td>99 ± 17</td>
<td>109 ± 33</td>
<td>0.3</td>
</tr>
<tr>
<td>Triglycerides (mg/dL)</td>
<td>91 ± 14</td>
<td>154 ± 42</td>
<td>0.2</td>
</tr>
</tbody>
</table>
**Supplementary Table 2: Clinical characteristics of obese subjects before and after weight loss.**

<table>
<thead>
<tr>
<th>Clinical parameter</th>
<th>Baseline</th>
<th>Following weight loss</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight (kg)</td>
<td>140 ± 25</td>
<td>99 ± 22</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>49 ± 7</td>
<td>34 ± 7</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Waist circumference (cm)</td>
<td>134 ± 13</td>
<td>107 ± 16</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Insulin (mIU/mL)</td>
<td>12.9 ± 5.2</td>
<td>4.2 ± 2.1</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Glucose (mg/dL)</td>
<td>109 ± 30</td>
<td>94 ± 11</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>3.2 ± 1.4</td>
<td>1.0 ± 0.5</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>6.4 ± 0.9</td>
<td>5.4 ± 0.6</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>hsCRP (mg/dL)</td>
<td>4.5 ± 4.4</td>
<td>2.2 ± 2.8</td>
<td>0.09</td>
</tr>
<tr>
<td>Systolic BP (mmHg)</td>
<td>131 ± 10</td>
<td>132 ± 17</td>
<td>0.8</td>
</tr>
<tr>
<td>Diastolic BP (mmHg)</td>
<td>75 ± 8</td>
<td>75 ± 9</td>
<td>0.8</td>
</tr>
<tr>
<td>Total cholesterol (mg/dL)</td>
<td>182 ± 36</td>
<td>172 ± 20</td>
<td>0.3</td>
</tr>
<tr>
<td>HDL-C (mg/dL)</td>
<td>44 ± 7</td>
<td>52 ± 7</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>LDL-C (mg/dL)</td>
<td>117 ± 37</td>
<td>104 ± 19</td>
<td>0.2</td>
</tr>
<tr>
<td>Triglycerides (mg/dL)</td>
<td>108 ± 43</td>
<td>82 ± 28</td>
<td>0.03</td>
</tr>
</tbody>
</table>