Roles of degree of fat deposition and its localization on VEGF expression in adipocytes

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Miyazawa-Hoshimoto, Saori, Kazuo Takahashi, Hideaki Bujo, Naotake Hashimoto, Kazuo Yagui, and Yasushi Saito. Roles of degree of fat deposition and its localization on VEGF expression in adipocytes. Am J Physiol Endocrinol Metab 288: E1128–E1136, 2005. First published December 21, 2004; doi:10.1152/ajpendo.00003.2004.—Vascular endothelial growth factor (VEGF) is an important angiogenic factor and is expressed in wide variety of cell types. In this study, we investigated the mechanism of VEGF production in adipocytes in three sets of experiments. First, to clarify the relation between plasma VEGF concentrations and their expressions in adipose tissues, we investigated the genetically obese db/db and KK-Ay mice. Plasma VEGF concentrations in obese mice were significantly higher than in controls and were related to adiposity. VEGF expressions in visceral fat were enhanced during growth and were related to fat deposition. Next, to demonstrate the relation between VEGF production and lipid accumulation in adipocytes, we analyzed VEGF mRNA expression and its protein secretion in 3T3-L1 cells. VEGF production was enhanced during lipid accumulation in 3T3-L1 cells after adipocyte conversion. Next, to clarify the role of anatomic localization on VEGF expression in adipocytes, we implanted 3T3-L1 cells into visceral or subcutaneous fat in athymic mice. 3T3-L1 cells implanted into the mesenteric area expressed more VEGF mRNA than that into the subcutaneous area. Plasma VEGF concentration in the mice implanted in visceral fat was higher than in controls. These results suggest that both the anatomic localization and the lipid accumulation are important for the VEGF production in adipocytes.

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rodent chow (352 kcal/100 g, CE-2; CLEA Japan). Male mice were used in the studies reported here. Animal care and procedures were approved by the Animal Care Committee of Chiba University School of Medicine.

Body weight and adiposity. Body weights of db/db and db/+ mice were measured at every 2 wk from the time they were 6 wk old throughout the study. Blood samples were also obtained from the retroorbital venous plexus of the mice fasted more than 16 h. The db/db and db/+ mice were killed at 6, 10, 14, and 18 wk of age by cervical dislocation before adipose tissues were collected. Mesenteric adipose tissues were used as visceral fat, and inguinal subcutaneous adipose tissues were used as subcutaneous fat in the studies reported here. The white adipose tissues were weighted on an analytic balance and processed for cell counts as described previously (13). Briefly, minced adipose tissues were incubated with PBS containing collagenase S-1. The tissue fragments were removed by passage through a 250-μm nylon screen. The isolated cells were then stained with methylene blue, and aliquots were placed on a Neubauer hemocytometer. Total cell counts were measured using a light microscope.

Total RNA and protein extraction from adipose tissues in obese mice. Mesenteric and subcutaneous adipose tissues of db/db and db/+ mice were processed for total RNA isolation using ISOGEN reagents according to the manufacturer’s instructions. In another set of experiments, the adipose tissues were homogenized in an ice-cold buffer containing 50 mM Tris-HCl (pH 7.4), 1 mM EDTA, 1 mM dithiothreitol, 5 mM MgCl2, 130 mM NaCl, 1% NP-40, 10 μM 4-amidino-phenylmethanesulfonyl fluoride, and 5 μM leupeptin. Insoluble materials in the tissue were removed by centrifugation at 12,000 g at 4°C for 20 min. After centrifugation, tissue extracts were collected. Moreover, total RNA in the isolated adipocytes of mesenteric fat was also prepared. The mesenteric adipose tissue was digested with collagenase S-1 and passed through a 250-μm nylon screen to remove tissue debris. Then, the isolated cells, containing adipocytes and vascular-stromal cells, were separated by centrifugation. After the adipocytes were allowed to float, the vascular-stromal cells were removed from the bottom layer. The floating layer, as adipocyte fraction, was washed three times with PBS. Finally, the isolated adipocytes were collected and processed for cell counts, using a Neubauer chamber as described above. To compare directly the cellular expression of VEGF in adipocyte, 2 × 10⁴ cells were processed for total RNA isolation using ISOGEN reagent. The KK-A¹ and C57BL/6 mice were killed at 16 wk of age by cervical dislocation before mesenteric and inguinal subcutaneous fat was collected for total RNA isolation.

3T3-L1 cells culture and differentiation. 3T3-L1 preadipocytes were cultured with DMEM containing 10% FBS at 37°C in a 5% CO2 incubator. Adipocyte differentiation was carried out by changing to a differentiation medium containing 10 μg/ml insulin, 0.25 μM dexamethasone, and 0.5 mM 3-isobutyl-1-methylxanthine. After 48 h, the medium was replaced with a maturation medium containing 5 μg/ml insulin, and cells were maintained in this medium until use. Every week after differentiation, the cells were washed with PBS and then cultured in fresh DMEM medium alone. After incubation for 24 h, the conditioned media were collected. In another set of experiments, the cells were processed for total RNA isolation using an RNeasy Mini Kit.

In vitro endothelial tube formation assay. HUVECs were grown in EBM-2 medium containing 10% FBS. Formation of capillary tube-like structures by HUVECs was assessed in a Matrigel-based assay as previously described (8). Briefly, HUVECs were incubated with MCDB131 containing 2% FBS for 48 h prior to tube formation assay. Cells (7 × 10⁴) were plated onto 300 μl of the conditioned medium derived from pre- or postdifferentiated 3T3-L1 cells in the presence of anti-mouse VEGF-neutralizing antibody. Three different phase-contrast microscopic low-power fields (×100) per well were photographed. The total length of capillary tubes in each photograph was measured using a scale ruler.

Preadipocyte transplantation. 3T3-L1 cells were implanted into athymic mice as described previously (19). Briefly, 3T3-L1 preadipocytes were grown to near confluence, trypsinized, and suspended in DMEM with 10% FBS. After centrifugation, cell pellets were resuspended in PBS and injected 1 × 10⁷ cells (500 μl) through 22-gauge needles into the mesenteric area near the small intestine or the subcutaneous fat area of athymic mice of the BALB/C strain under...
anesthetization by intraperitoneal injection with pentobarbital sodium. Mice were housed in microisolator cages under specific pathogen-free conditions during whole experiments. Four weeks after implantation, the mice were killed by cervical dislocation under anesthetization before mesenteric or subcutaneous fat area was collected. Total RNA of mesenteric and subcutaneous fat was isolated using ISOGEN reagent. Blood samples were also obtained from the retroorbital venous plexus of the mice fasted more than 16 h.

**Measurement of immunoreactive VEGF.** Plasma samples were prepared by centrifugation at 1,500 g for 15 min at 4°C. After centrifugation, the plasma fraction was collected and stored at −70°C until use. The extracts of adipose tissues and the conditioned media from pre- and postdifferentiated 3T3-L1 cells were also stored at −70°C until use. VEGF concentrations of plasma, extracts from adipose tissues, and conditioned media were measured with an enzyme-linked immunosorbent assay system (R&D Systems, Minneapolis, MN).

**RT-PCR.** To evaluate the contents of VEGF expression in adipose tissues and 3T3-L1 cells, 0.4 μg of total RNA was amplified by OneStep RT-PCR kit using the indicated specific primers. To compare directly the VEGF expressions in adipocytes of mesenteric fat during growth, total RNA prepared from 2 × 10⁴ cells was also amplified using the specific primers. The contents of GLUT4, peroxisome proliferator-activated receptor-γ (PPARγ), and β-actin were also amplified by RT-PCR. The RT-PCR products were run on 1.5% agarose and stained with ethidium bromide. The relative signal intensities of the PCR products were determined with luminescent image analyzer LAS-1000 (Fuji Photo Film, Tokyo, Japan). mRNA amounts were normalized to levels of β-actin mRNA, which served as endogenous standard.

**Primers.** The following primers were designed for RT-PCR analysis using in this study: VEGF, 5'-GCGGGCTGCTGATCAGTCA-3' (forward) and 5'-TCACGCCTTTGGCTTTGCAC-3' (reverse); β-actin, 5'-TGGATCCTGCTGATCAGTCA-3' (forward) and 5'-TAAAACGCGCTGATCAGTCA-3' (reverse); GLUT4, 5'-GGCATTTGTGGCTTGATCAGTC-3' (forward) and 5'-GGGTTTCACCTCCTGCTCTAA-3' (reverse); PPARγ, 5'-GACATCCAA-
GACAACCTGCTG-3' (forward) and 5'-GCAATCAATAGAAG-GAACACG-3' (reverse). RT-PCR products for VEGF were 716 bp (VEGF188), 644 bp (VEGF164), and 512 bp (VEGF120), respectively. The signal intensity of the 644-bp product was analyzed in this study. Products of 349, 413, and 258 bp were predicted for β-actin, GLUT4, and PPARγ, respectively.

Statistical analysis. Statistical analyses were performed using Statview J-4.5. Statistical analysis was performed with a t-test. All of the results reported herein were confirmed by repeating the experiments with different occasions. A value of \( P < 0.05 \) indicated statistical significance.

RESULTS

Growth-dependent changes of plasma VEGF concentration in \( db/db \) mice. We measured circulating VEGF concentrations in \( db/db \) mice, a strain of the mouse models for obesity, to demonstrate the role of fat accumulation and its effect on VEGF levels in vivo. Plasma VEGF concentrations were increased during growth in both \( db/+ \) and \( db/db \) mice (Fig. 1A). At 10 wk old, plasma VEGF concentrations in \( db/db \) mice were significantly higher than in \( db/+ \) mice. Moreover, plasma VEGF concentrations were significantly correlated with body weight (Fig. 1B).

Growth-dependent changes of VEGF mRNA expressions and protein contents in visceral and subcutaneous fat of \( db/db \) mice. VEGF mRNA was detected in both visceral and subcutaneous fat in \( db/db \) mice. Expression levels of VEGF mRNA in visceral fat were increased 3.0-fold in 14-wk-old mice compared with those in 6-wk-old mice (Fig. 2A). VEGF expressions in subcutaneous fat were also increased during growth, but its enhancement was smaller than in visceral fat. Moreover, tissue contents of VEGF in visceral fat were significantly increased in 14-wk-old mice compared with those in 6-wk-old mice (Fig. 2B). However, the VEGF contents in subcutaneous fat were almost the same in 6- and 14-wk-old mice. These data suggest that an enhanced expression of the VEGF gene in visceral fat mainly contributes to the elevated plasma concentrations.

Effect of fat accumulation on VEGF expression in white adipose tissues of \( db/db \) mice. Whole tissue weights of mesenteric and subcutaneous fat were increased gradually during growth (Fig. 3A). Total cell counts were significantly decreased during growth only in mesenteric adipose tissues (Fig. 3B). A significant correlation between fat weight and VEGF expression levels was observed in mesenteric adipose tissue but not in subcutaneous adipose tissue (Fig. 4A). Moreover, cellular levels of VEGF expression were calculated from the results of mRNA expression levels and total cell counts in adipose tissues and positively correlated for adiposity in mesenteric adipose tissue but not in subcutaneous adipose tissue (Fig. 4B).

Growth-dependent change of VEGF expression in adipocytes of visceral area of \( db/db \) mice. VEGF expressions in adipocyte fraction were increased during growth (Fig. 5). Cellular expression levels of VEGF mRNA in visceral adipocytes were increased sevenfold in 18-wk-old mice compared with those in 6-wk-old mice. The correlation between fat weight and VEGF expression levels was observed in mesenteric adipose tissue but not in subcutaneous adipose tissue (Fig. 4A). Moreover, cellular levels of VEGF expression were calculated from the results of mRNA expression levels and total cell counts in adipose tissues and positively correlated for adiposity in mesenteric adipose tissue but not in subcutaneous adipose tissue (Fig. 4B).
with those in 6-wk-old mice. These results suggest that circulating VEGF concentrations in db/db mice were increased by the enhancement of VEGF mRNA expression in visceral adipocytes.

Plasma concentration and tissue expression of VEGF in KK-A^y mice. To demonstrate the correlation between VEGF expression and adiposity in another model of obesity, we analyzed KK-A^y mice. Plasma VEGF concentrations were significantly increased in both 8- and 16-wk-old KK-A^y mice compared with those in age-matched control mice (Fig. 6A). Moreover, expression levels of VEGF mRNA in visceral fat were significantly increased in KK-A^y mice compared with those in control mice (Fig. 6B). These results suggest that circulating VEGF concentrations in KK-A^y mice as well as in db/db mice were increased by the enhancement of VEGF mRNA expression in visceral fat.

Change of VEGF expressions during differentiation and maturation process in 3T3-L1 cells. We performed RT-PCR analysis for the gene expression of VEGF, PPAR-γ, and GLUT4 in cultured 3T3-L1 cells. VEGF mRNA was expressed even in the preadipocyte condition (Fig. 7A), and its expression was enhanced during adipocyte conversion. Especially, the expression levels of VEGF mRNA were significantly increased 14 days after differentiation (Fig. 7B). Both PPAR-γ and GLUT4 expressions were gradually enhanced during differentiation (Fig. 7A). These results suggest that expression levels of VEGF mRNA in 3T3-L1 cells were enhanced during lipid accumulation.

VEGF concentrations of conditioned media cultured with pre- and postdifferentiated 3T3-L1 cells. 3T3-L1 cells secreted VEGF proteins into culture medium even in the preadipocyte condition (Table 1), and the VEGF protein secretion was enhanced during adipocyte conversion. Especially, the VEGF concentrations in conditioned medium were increased fourfold 14 days after differentiation compared with those of predifferentiation. These results suggest that protein secretion as well as mRNA expression of VEGF in 3T3-L1 cells were enhanced during lipid accumulation. The biological activity of VEGF should be examined to know the role of VEGF in physiological and pathological conditions. Therefore, we demonstrated the angiogenic activity of conditioned medium from cultured adipocytes.

Enhancement of tube formation activity in HUVECs by addition of conditioned medium cultured with 3T3-L1 cells. VEGF secreted from both pre- and postdifferentiated 3T3-L1 cells had stimulatory activity toward HUVECs in tube formation (Fig. 8, A and B). The stimulatory activity in the condi-
tioned medium derived from postdifferentiated 3T3-L1 cells was three times higher than in predifferentiated cells. Moreover, anti-VEGF-neutralizing antibody apparently inhibited the stimulatory tube formation activity in both pre- and postdifferentiated 3T3-L1 cells. These findings suggest that 3T3-L1 cells secrete the bioactive form of VEGF protein.

Effect of implantation of 3T3-L1 preadipocytes into mesenteric or subcutaneous fat area of nude mice on VEGF expression. We performed RT-PCR analysis for gene expressions of VEGF, PPARγ, and GLUT4 in the mesenteric or subcutaneous fat area implanted with 3T3-L1 cells. As shown in Fig. 9A, the content of VEGF expression was increased fourfold in the mesenteric fat implanted with 3T3-L1 cells compared with those in sham-operated control mice. In contrast, VEGF expression of subcutaneous fat was almost the same in the mice implanted with 3T3-L1 cells into the subcutaneous area and controls. Moreover, PPARγ expression was enhanced only in mesenteric fat implanted with 3T3-L1 cells but not in subcutaneous fat. The expression levels of GLUT4 in both mesenteric and subcutaneous fat implanted with 3T3-L1 cells were higher than in controls.

The plasma VEGF concentration increased after implantation of 3T3-L1 cells into the mesenteric area, and these reached 381 ± 63 pg/ml at 4 wk. However, the mice injected in the subcutaneous area did not show any difference from control mice (Fig. 9B).

DISCUSSION

In the first set of experiments, we showed that the plasma VEGF concentrations gradually increased during growth in both db/db and db/+ mice. After 10 wk of age, however, plasma VEGF concentrations were higher in db/db mice than in db/+ mice. The db/db mice are considered to be an obesity model because fat deposition is the primary change. Then we analyzed the correlation between plasma VEGF concentration and body weight. The plasma VEGF concentration in db/db

![Figure 5](image_url) Time course change of VEGF expressions in adipocyte fraction prepared from mesenteric fat of db/db mice. Visceral fat was cut into small pieces and digested using collagenase S-1. Then, the tissues were suspended with PBS and separated by centrifugation. The floating layer was collected as adipocyte fraction. Images show RT-PCR products of VEGF amplified from total RNA prepared from 2 × 10⁶ cells of adipocytes at 6, 10, 14, and 18 wk after birth. The PCR products of VEGF were densitometrically analyzed, and the relative amounts at 6 wk old were set to 1.0. Results are represented as means and SD. Arrowhead, VEGF₁₆₄; *, VEGF₁₈₈.

![Figure 6](image_url) A: comparison of plasma VEGF concentrations between KK-A'y and C57BL/6 mice. Plasma VEGF concentrations were measured in 8- and 16-wk-old KK-A'y and C57BL/6 mice. Results are represented as means + SD. B: comparison of VEGF gene expressions in visceral and subcutaneous adipose tissues between KK-A'y and C57BL/6 mice. Images show RT-PCR products of VEGF amplified from total RNA in mesenteric and subcutaneous adipose tissues at 16 wk after birth. The PCR products of VEGF were densitometrically analyzed, and the relative amounts in visceral adipose tissues of control mice were set to 1.0. Results are represented as means + SD. Arrowhead, VEGF₁₆₄; *, VEGF₁₈₈.
mice was significantly related to their body weight, the same as our previous results in human subjects (15). These results suggest that plasma VEGF may be determined by body fat deposition in mice.

In our previous report (15), plasma VEGF concentrations were revealed to be dependent on visceral fat accumulation. Therefore, to clarify the VEGF expressions with regard to whether subcutaneous or visceral adipose tissue affects plasma VEGF concentration in \( db/db \) mice, we examined mRNA expressions and protein contents of VEGF in visceral and subcutaneous adipose tissues during growth. The mRNA ex-

Table 1. VEGF concentrations of conditioned media cultured with pre- and postdifferentiated 3T3-L1 cells

<table>
<thead>
<tr>
<th>Differentiation periods</th>
<th>Days</th>
<th>0</th>
<th>7</th>
<th>14</th>
<th>21</th>
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<td>VEGF concentration, ng/ml</td>
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<td>0.9±0.2</td>
<td>2.4±0.5</td>
<td>2.6±0.2</td>
</tr>
<tr>
<td>Total protein concentration, mg/ml</td>
<td></td>
<td>0.4±0.1</td>
<td>0.4±0.1</td>
<td>0.4±0.2</td>
<td>0.4±0.2</td>
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Values are means ± SD. VEGF, vascular endothelial growth factor.
Expression levels of VEGF in visceral fat were more enhanced than in subcutaneous fat. Furthermore, the protein contents were enhanced in visceral fat but not in subcutaneous fat. These results suggest that plasma VEGF concentrations are revealed to be dependent on visceral fat accumulation even in mice.

The expression levels of TNF-α and PAI-1 in adipocytes are reported to be directly related to the degree of differentiation from preadipocytes and to be dependent on their anatomic location (12, 14, 20, 21). Therefore, we demonstrated the degree of fat accumulation in adipocytes and the correlation between VEGF mRNA expressions and adiposity in subcutaneous and mesenteric adipose tissues. Whole tissue weights of mesenteric and subcutaneous fat were increased gradually during growth. However, total cell counts were significantly decreased during growth in mesenteric fat but not in subcutaneous fat. A significant correlation between VEGF mRNA expressions and weight and adiposity in mesenteric adipose tissue was observed, but not in subcutaneous adipose tissue. These results suggest that the increase of weight in mesenteric adipose tissue is dependent on fat accumulation in adipocytes but not in subcutaneous adipose tissue. Moreover, VEGF expression is dependent on the levels of fat deposition in adipocytes. Then, we isolated the adipocyte fraction from mesenteric fat and examined VEGF mRNA expression in adipocytes. VEGF expression levels in the adipocyte fraction were also increased during growth.

Next, we examined the relation between VEGF expression and degree of differentiation using 3T3-L1 cells, an established adipocyte cell line. VEGF mRNA was expressed even in the preadipocyte condition, and its expression was enhanced after adipocyte conversion. Especially, the expression levels of VEGF mRNA were significantly increased 14 days after differentiation. Furthermore, the levels of VEGF protein secretion were almost the same level as gene expression. These results suggest that VEGF production may be dependent on the lipid accumulation (maturation) as well as the time lapse after adipocyte conversion rather than adipocyte differentiation from preadipocytes in 3T3-L1 cells.

We next determined whether VEGF protein secreted from 3T3-L1 cells has some biological activities. To know the role of VEGF in physiological or pathological conditions, we examined the effect of conditioned medium derived from 3T3-L1 cells on in vitro tube formation activity in HUVECs. The conditioned media from pre- and postdifferentiated 3T3-L1 cells enhanced angiogenesis in vascular endothelial cells. These results suggest that VEGF protein secreted from adipocytes may play some roles in the pathological neovascularization observed in diabetic retinopathy or atherosclerosis.

In the third set of experiments, the effects of anatomic localization on fat accumulation and VEGF production in adipocytes were analyzed using a cell implantation technique. 3T3-L1 cells implanted into the mesenteric area of athymic mice expressed more VEGF mRNA than that implanted into the subcutaneous area. The expression of PPARγ was also higher in 3T3-L1 cells implanted into the mesenteric area than into the subcutaneous area, and plasma VEGF concentrations in the mice implanted with 3T3-L1 cells into the mesenteric area were higher than in the subcutaneous area. These results suggest that a certain mechanism may exist in the visceral fat area to make implanted 3T3-L1 cells for enhanced VEGF production.

In summary, these results from in vitro and in vivo experiments indicate that VEGF expression in adipocytes is possibly differentiation as well as time (age) dependent after adipocyte conversion and may be determined by the site of body distribution. Further experiments are required to clarify the mechanism of enhanced expression of VEGF in visceral fat.

GRANTS

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REFERENCES


