0013-7227/05/$15.00/0
Printed in U.S.A.

Endocrinology 146(10):4545–4554

doi: 10.1210/en.2005-0532

Modulation of Angiogenesis during Adipose Tissue Development in Murine Models of Obesity

Gabor Voros, Erik Maquoi, Diego Demeulemeester, Natalie Clerx, Désiré Collen, and H. Roger Lijnen

Center for Molecular and Vascular Biology, Katholieke Universiteit Leuven, B-3000 Leuven, Belgium

Development of vasculature and mRNA expression of 17 pro- or antiangiogenic factors were studied during adipose tissue development in nutritionally induced or genetically determined murine obesity models. Subcutaneous (SC) and gonadal (GON) fat pads were harvested from male C57Bl/6 mice kept on standard chow [standard fat diet (SFD)] or on high-fat diet for 0–15 wk and from male ob/ob mice kept on SFD. Ob/ob mice and C57Bl/6 mice on high-fat diet had significantly larger SC and GON fat pads, accompanied by significantly higher blood content, increased total blood vessel volume, and higher number of proliferating cells. mRNA and protein levels of angiopoietin (Ang)-1 were down-regulated, whereas those of thrombospondin-1 were up-regulated in developing adipose tissue in both obesity models. Ang-1 mRNA levels correlated negatively with adipose tissue weight in the early phase of nutritionally induced obesity as well as in genetically determined obesity. Placental growth factor and Ang-2 expression were increased in SC adipose tissue of ob/ob mice, and thrombospondin-2 was increased in both their SC and GON fat pads. mRNA levels of vascular endothelial growth factor (VEGF)-A isoforms VEGF-B, VEGF-C, VEGF receptor-1, -2, and -3, and neuropilin-1 were not markedly modulated by obesity. This modulation of angiogenic factors during development of adipose tissue supports their important functional role in obesity. (*Endocrinology* 146: 4545–4554, 2005)

IN EARLY STAGE development of adipose tissue, adipogenesis is tightly associated with angiogenesis (1). Adipose tissue, unlike other organs, grows and develops continuously throughout life. *In vitro* studies revealed that differentiating adipocytes and adipose tissue explants trigger blood vessel formation (2, 3) and that, in turn, adipose tissue endothelial cells (ECs) promote preadipocyte differentiation (4). Furthermore, adipose tissue growth in mice can be impaired with angiogenesis inhibitors (5).

Many pro- and antiangiogenic components have been identified. Vascular endothelial growth factor (VEGF)-A is a major angiogenic factor that stimulates proliferation and migration of ECs (6). Three forms of VEGF-A are produced in the mouse as a result of alternative splicing (VEGF-A121, VEGF-A165, and VEGF-A189). VEGF-B also promotes angiogenesis and is implicated in extracellular matrix degradation via activation of plasminogen (7, 8). VEGF-C plays an important role both in angiogenesis and lymphangiogenesis (9). Placental growth factor (PlGF), a VEGF homolog, stimulates angiogenesis in a variety of conditions *in vivo* and is a key molecule in regulating the angiogenic switch in pathological conditions (10). The members of the VEGF family bind to transmembrane tyrosine kinase receptors (VEGF-R1, VEGF-R2, and VEGF-R3) (11). VEGF-A165, PlGF, and VEGF-B also bind to another transmembrane receptor, neuropilin (Np)-1. Np-1 functions as an enhancer of VEGF-R2 activation (12), and inactivation of the *Np-1* gene causes disturbances in development of the vascular and nervous system (13). Fibroblast growth factor (FGF)-2 is also a potent stimulator of EC proliferation and angiogenesis (14) and enhances adipocyte differentiation *in vivo* (15). Another signaling system contributing to vessel maintenance, growth, and stabilization involves the tyrosine kinase with Ig and epidermal growth factor homology domains (TIE)-2 receptor, which binds the angiopoietins (Ang)-1 and -2. Unlike Ang-2, which activates TIE-2 on some cells but blocks it on others, Ang-1 consistently activates TIE-2. The role of Ang-1 in vascularization is pleiotropic and context-dependent (16). Ang-1 tightens vessels by affecting junctional molecules (17), by promoting the interaction between ECs and mural cells and by recruiting pericytes (18). Ang-2 may stimulate vessel growth by loosening endothelial-peri-EC interactions and degrading the extracellular matrix (19). Ang-2 synergizes with VEGF to stimulate angiogenesis (20); but when insufficient angiogenic signals are provided, Ang-2 causes EC death and blood vessel regression (21). Thrombospondins (TSP)-1 and -2 are matricellular proteins that inhibit angiogenesis *in vivo* and impair migration and proliferation of cultured microvascular ECs (22). Although it is generally accepted, yet unproven *in vivo*, that angiogenesis is ongoing during development of obesity, little is known about the expression and the role of these different pro- and antiangiogenic components in adipose tissue.

The aims of this study, therefore, were: 1) to monitor development of the vascular network in adipose tissue during nutritionally induced obesity in mice, 2) to measure

First Published Online July 14, 2005

Abbreviations: Ang, Angiopoietin(s); DAPI, 4′-6-diamidino-2-phenylindole; EC, endothelial cell; FGF, fibroblast growth factor; GON, gonadal; HFD, high-fat diet; HRP, horseradish peroxidase; Np, neuropilin; PAI, plasminogen activator inhibitor; PlGF, placental growth factor; PPAR, peroxisome proliferator-activated receptor; rRNA, ribosomal RNA; RU, relative units; SC, subcutaneous; S-V, stromal-vascular; TBS, Tris-buffered saline; TIE, tyrosine kinase with Ig and epidermal growth factor homology domains; TSP, thrombospondin; VEGF, vascular endothelial growth factor; vWF, vonWillebrand factor; WT, wild type.

***Endocrinology* is published monthly by The Endocrine Society (http://www.endo-society.org), the foremost professional society serving the endocrine community.**

mRNA levels of pro- and antiangiogenic factors in murine adipose tissue and to investigate their modulation by diet-induced or genetically determined obesity, 3) to measure protein levels of modulated factors, 4) to examine the cellular localization of their expression, and 5) to study expression patterns during differentiation of murine 3T3-F442A preadipocytes, as in an *in vitro* model of adipogenesis.

Materials and Methods

Obesity model

Five-week-old male wild-type (WT) mice of about 20 g (100% C57Bl/6 genetic background) were kept on a standard chow [4% (wt/wt) fat, standard fed diet (SFD); KM-04-k12; Muracon, Carfil, Oud-Turnhout, Belgium] or were given a high-fat diet (HFD) [21% (wt/wt) fat from milk and 49% (wt/wt) carbohydrate from sucrose and corn starch, HFD; Harlan TD 88137, Zeist, The Netherlands] for 0, 2, 5, or 15 wk (n = 12 in each group). The SFD contained 13% kcal as fat with a caloric value of 10.9 kJ/g, with corresponding values of 42% and 20.1 kJ/g for the HFD. Nine-week-old male ob/ob mice (n = 5) and corresponding WT mice (n = 5) were purchased from Charles River Laboratories, Inc. (Wilmington, MA). The mice were euthanized with an overdose (60 mg/kg ip) of Nembutal (Abbott Laboratories, North Chicago, IL). Intraabdominal (gonadal, GON) and inguinal subcutaneous (SC) fat pads were removed and the wet weight determined. All animal experiments were approved by the local ethical committee (Katholieke Universiteit Leuven, P03112) and were performed in accordance with the guiding principles of the American Physiological Society and The International Society on Thrombosis and Hemostasis (23).

Measurement of blood content

Blood content of the adipose tissues was quantified using a radiolabeled tracer (^{131}I-BSA; Draximage, Quebec, Canada). C57Bl/6 mice after 2, 5, or 15 wk on SFD or HFD (n = 7 in each group) were anesthetized with a combination of ketamin and xylazine, and a heparinized catheter was inserted into the jugular vein. After the administration of 2 mg NaI (Sigma, St. Louis, MO), 100 kBq ^{131}I-BSA was injected as a bolus. Two minutes later, the mouse was killed, and a blood sample was taken from the tail vein (24). SC and GON fat pads and the blood sample were weighed, and ^{131}I was counted using a Minaxi 5000 γ-counter (Packard Instrument Company, Meriden, CT). The amount of blood in the adipose tissue was calculated as: (^{131}I-BSA/g tissue)/(^{131}I-BSA/g blood).

Morphometric and histological analysis

The number of adipocytes and their mean size were determined as described elsewhere (25). Blood vessels were visualized by staining 10-μm paraffin sections with the biotinylated Bandeiraea (Griffonia) Simplicifolia BSI lectin (Sigma) followed by signal amplification with the Tyramide Signal Amplification Cyanine System (PerkinElmer, Boston, MA). For each mouse (n = 5), at least 12 randomly selected fields in nine to twelve sections were analyzed by computer-assisted image analysis, and data were then averaged per animal. The ratio of blood vessel area to total section area was then normalized to the total adipose tissue mass to estimate total blood vessel area in the fat pad. Cell proliferation was evaluated by staining the paraffin sections with anti-Ki-67 antibody (DakoCytomation, Glostrup, Denmark), amplified with the Tyramide Signal Amplification Cyanine System. Cell nuclei were visualized by staining with 4′-6-diamidino-2-phenylindole (DAPI, Vector Laboratories, Inc., Burlingame, CA). Colocalization of proliferating cells with ECs was performed by staining the sections with both lectin and anti-Ki-67 antibody. To avoid aspecific reactions, lectin was visualized with Alexa 568-labeled straptavidin (Molecular Probes, Inc., Eugene, OR), and Ki-67 was detected with horseradish peroxidase (HRP)-labeled secondary and tertiary antibody followed by signal amplification with the Tyramide Signal Amplification Cyanine System. Macrophages were visualized by staining paraffin sections with anti-Mac-3 antibody (PharMingen, San Diego, CA), amplified with the Tyramide Signal Amplification Cyanine System (n = 5). Mac-3 positive cells were counted in a blinded way (staining and counting were performed by two independent observers) and were expressed as a percentage of the total number of cell nuclei, determined by DAPI staining, as described above. For each immunostaining, a negative control without the primary antibody was also prepared, which did not show detectable staining (not shown).

Adipose tissue dissociation

SC or GON fat pads dissected from a male ob/ob mouse were used to separate mature adipocytes from stromal-vascular (S-V) cells, as described elsewhere (26). Briefly, minced fat pads were digested with 0.15 mg/ml Liberase Blendzyme 3 (Roche Molecular Biochemicals, Indianapolis, IN) at 37 C for 90 min. Mature adipocytes were separated from S-V cells by their ability to float upon low-speed (600 $\times$ *g*) centrifugation. The two cell populations were used immediately for RNA extraction. To monitor EC contamination of the adipocyte fraction, vonWillebrand factor (vWF) mRNA levels were measured by quantitative real-time PCR.

Culture and differentiation of 3T3-F442A cells

3T3-F442A preadipocytes were cultured in basal medium: DMEM/nutrient mix F12 (1:1; Life Technologies, Inc., Merelbeke, Belgium) containing 100 mM pantothenate, 1 mM biotin, 2.5 mM glutamine, 15 mM HEPES, and supplemented with 10% (vol/vol) fetal bovine serum (Life Technologies). To induce differentiation, cells were seeded at 3.6×10^4 cells/cm^2 and grown to confluency in basal medium with 10% fetal bovine serum. Confluent cultures (d 0) were washed in serum-free basal medium and treated for 5 d with an induction medium: basal medium supplemented with BSA (100 mg/liter), ITS (10 mg/liter insulin, 5.5 mg/liter transferin, 5 mg/liter selenium; Sigma), 10 nM dexamethasone, 250 mM methylisobutylxanthine, and 1 nM T_3. Cultures were then switched to a differentiation medium (basal medium supplemented with ITS and T_3) for 2 wk (induction and differentiation media were renewed every 2–3 d). Cellular viability was assessed by cell counting, Trypan Blue dye exclusion assay, and WST-1 (Roche Molecular Biochemicals) assay as described previously (27). To assess the extent of preadipocyte differentiation, cytosolic triglyceride content was quantified by determining Nile Red uptake, as monitored by flow cytometric analysis (FACSCalibur; Becton Dickinson, San Jose, CA), and the expression profile of several preadipocyte and adipocyte markers [preadipocyte factor-1, peroxisome proliferator-activated receptor (PPAR)-γ, and glycerophosphate dehydrogenase] was determined (28). 3T3-F442A cells were differentiated in two independent experiments.

RNA isolation

SC and GON fat pads were homogenized using lysing matrix tubes (Qbiogene, Carlsbad, CA) in a Hybaid ribolyzer (Thermo, Waltham, MA). Total RNA was extracted from homogenized adipose tissues, isolated adipocytes, S-V cells, and 3T3-F442A cells using the RNA Easy Qiagen Kit (QIAGEN, Valencia, CA) and treated with ribonuclease free deoxyribonuclease (QIAGEN) according to the manufacturer's instructions. Integrity of extracted RNA was validated from the intensity of 18S and 28S ribosomal RNA bands on agarose gels. RNA concentrations were measured using the RiboGreen RNA quantification kit (Molecular Probes). RNA samples were diluted in water and stored at −80 C. To investigate the expression of pro- and antiangiogenic factors *in vivo*, an initial screening was performed. Therefore, adipose tissue samples were divided into 16 groups (SC or GON adipose tissue from mice on 2, 5, or 15 wk on SFD or HFD and SC or GON adipose tissue from WT or ob/ob mice). After extracting total RNA, the same amount of RNA was pooled from adipose tissues in the same group; thus 16 RNA pools were created. The expression profile of pro- and antiangiogenic factors was first measured in these pools, and the mRNA levels of factors that were modulated at least by 2-fold in both obesity models (*i.e.* Ang-1 and TSP-1) were subsequently determined in individual samples. For prescreening, the samples were analyzed in triplicate, and the whole analysis was repeated two times.

Oligonucleotide primers and probes

The design of oligonucleotide primers and probes specific for the different targets (Table 1) was described elsewhere (25). Briefly, sense

TABLE 1. Sequence of primers and probe (5′–3′) used for the RT-PCR assays

mRNA species	Sense	Probe	Antisense
Ang-1	CAACAACAACAGCATCCTGCA	AAGCAACAACTGGAGCTCATGGACACAGT	TGCAAAGGCTGACAAGGTTATG
Ang-2	CTGTGCGGAAATCTTCAAGTCA	CACCAGTGGCATCTACACACTGACCTTCC	CCTTGATCTCCTCTGTGGAGTTG
TIE-1	ACCTGTGCTGAGCTCTACGAGAA	CTATCGCATGGAGCAGCCTCGAAACTG	CATCAGCTCGTACACTTCGTCAT
TIE-2	AACCAACAGTGATGTCTGGTCCTAT	TGCCTCCTAAGCTAACAATCTCCCAGAGCAATA	GCACGTCATGCCGCAGTA
VEGF-A 121	TGCAGGCTGCTGTAACGATG	TGTCTTTCTTTGGTCTGCATTCACATCGG	CCTCGGCTTGTCACATTTTTCT
VEGF-A 165	TGCAGGCTGCTGTAACGATG	TGTCTTTCTTTGGTCTGCATTCACATCGG	GAACAAGGCTCACAGTGATTTTCT
VEGF-A 189	TGCAGGCTGCTGTAACGATG	TGTCTTTCTTTGGTCTGCATTCACATCGG	CTCCAGGATTTAAACCGGGATT
VEGF-B	TGCCATGGATAGACGTTTATGC	CGTGCCACATGCCAGCCCA	TGCTCAGAGGCACCACCAC
VEGF-C	AAGACCGTGTGCGAATCGA	AAAGGACAGTCCTGGATCACAATGCTTCA	ACACAGCGGCATACTTCTTCAC
VEGF-R1	TGGCCAGAGGCATGGAGT	TCTGTCCTCCAGAAAGTGCATTCATCGG	TCGCAAATCTTCACCACATTG
VEGF-R2	GGAGAAGAATGTGGTTAAGATCTGTGA	CATCTCCTTTTCTGACATAATCCGGGTCTTTATAAATGTC	ACACATCGCTCTGAATTGTGTATACT
VEGF-R3	GGTTCCTGATGGGCAAAGG	CGGCGAGCCCCACTTGTCCA	TCAGTGGGCTCAGCCATAGG
PIGF	TTCAGTCCGTCCTGTGTCCTT	ACCACAGCAGCCACTACAGCGACTCA	GCACACAGTGCAGACCTTCA
FGF-2	GCGACCCACACGTCAAACTA	TGTGCCAACCGGTACCTTGCTATGAAG	AGCCAGCAGCCGTCCAT
Np-1	ACACCTGAGCTTCGGACGTT	TCACCTCTCTCCACAAGGTTCATCAGGATC	CCACTGTGTGTGGCTCTCTCA
TSP-1	AACAAAGGACCTCCAAGCTATCTG	TGCGCACCATCGTGACCACTCTG	GGGAGGCCGCTTCAGC
TSP-2	GAGCTCTCTGGACTGCACGTC	GACACTCTCTCCAGGTTCTTGCTCAGCTG	TTTCATTTTCTGCAAAGATTCGG
vWF	GCATCTACGGCGCTATCATGTAT	TTGGCCACATCCTCACATACACGCC	TGTGGTGACCGTGCCATCT
PPAR-γ	CTGTCGGTTTCAGAAGTGCCT	CCCAAACCTGATGGCATTGTGAGACA	ATCTCCGCCAACAGCTTCTC

and antisense primers (Eurogentec, Seraing, Belgium) annealing to distinct exons were selected, and different concentrations of the primer pairs were first tested on positive control RNA samples. Primers were always used in concentrations to yield only one band of the correct size as demonstrated on polyacrylamide gels. The amplified DNA was sequenced and was found to be correct for all targets.

Quantitative real-time PCR (RT-PCR)

mRNA expression levels were determined by quantitative RT-PCR. Reverse transcription reactions were performed from 200 ng total RNA at 48 C during 60 min with the Taqman Reverse Transcription Kit and 5 μM random hexamers (Applied Biosystems). Quantitative RT-PCR was performed in the ABI-prism 7700 sequence detector using the Taqman PCR core reagents kit (Applied Biosystems). Samples containing the highest amount of each target were used to create standard curves (10-, 20-, 40-, 80-, 320-, and 1280-fold dilution of the transcribed cDNA). All of these standard curves were linear, with r^2 values more than 0.98. In the same run, CT values of the samples (20-fold diluted cDNA) were measured, and relative expression levels were determined. As a housekeeping gene, the expression of 18S ribosomal RNA was measured in the same way, using the Taqman Ribosomal RNA Control Kit (Applied Biosystems) according to the manufacturer's protocol [18S ribosomal RNA (rRNA) levels were relatively stable across different samples; differences were always less than 20%]. The mRNA levels of the targets were normalized to those of 18S for each sample. PCR amplifications were performed in duplicate wells, using the following conditions: 2 min at 50 C and 10 min at 94 C followed by 40 two-temperature cycles (15 sec at 95 C and 1 min at 60 C).

Immunoprecipitation and immunoblotting

Because Ang-1 antigen levels were undetectable with standard Western blotting, we concentrated the antigen by immunoprecipitation. SC and GON adipose tissues were homogenized in a lysis buffer (10 mM Na phosphate, 150 mM NaCl; pH 7.2) containing 0.2% Na azide and a complete protease inhibitor cocktail (Roche Molecular Biochemicals), followed by incubation with detergents (1% Triton X-100, 0.1% sodium dodecyl sulfate, 0.5% Na deoxycholate) for 2 h at 4 C. Protein concentrations were measured with the BCA protein assay (Pierce Biotechnology, Rockford, IL) according to the manufacturer's protocol.

Protein extracts (1 mg protein) were incubated with 5 μg polyclonal goat anti-Ang-1 antibody (N-18; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) overnight, followed by an additional overnight incubation with Protein G Sepharose beads (Amersham Pharmacia Biotech, Uppsala, Sweden). After washing the beads three times with PBS containing a complete protease inhibitor cocktail, Ang-1 was eluted with sample buffer containing 20 mM 2-mercaptoethanol and boiled for 8 min. Samples were loaded on 10% sodium dodecyl sulfate-polyacrylamide gels. After electroblotting, nitrocellulose membranes were blocked with 5% milk powder in Tris-buffered saline (TBS) (TBS-Tween containing 150 mM NaCl, 10 mM Tris, 0.05% Tween 20; pH 7.5) for at least 1 h. After an overnight incubation with anti-Ang-1 antibody, membranes were washed with TBS-Tween and incubated with HRP-labeled rabbit anti-goat secondary antibody (1:2000; DakoCytomation) for 2 h. Protein bands were detected using the enhanced chemiluminescence plus detection kit (Amersham Biosciences, Little Chalfont, UK) and expressed as relative units (RU) after background correction.

ELISA

A home-made ELISA was used to measure TSP-1 protein levels in SC and GON adipose tissue extracts. Ninety-six-well plates (Costar, Cambridge, MA) were coated with a monoclonal mouse anti-TSP-1 antibody, followed by incubation of samples and standards. Bound TSP-1 was labeled with a biotinylated monoclonal mouse anti-TSP-1 antibody and HRP-labeled streptavidine, followed by a color reaction with o-phenylenediamine. Mouse TSP-1 was obtained by lysing platelet-rich plasma with 1% triton in PBS and was used to make a standard dilution curve. The results are expressed as percentage of the undiluted platelet lysate (linear range of the standard curve between 0.33 and 0.015% of the undiluted platelet lysate). The interassay coefficient of variation was 8.9%, and the intraassay coefficient of variation was 3.4%. The dilution curves of the platelet lysate and the samples were parallel (not shown).

Statistical analysis

Data are reported as mean ± SEM. Statistical significance between groups is evaluated using nonparametric *t* testing. Correlations are examined using the nonparametric Spearman's rank correlation coefficient. The threshold for significance is set at $P < 0.05$.

Results

Increased vascularization in fat pads of obese mice

C57Bl/6 mice kept on HFD for 2, 5, or 15 wk had a significantly higher total body weight than age-matched mice on SFD (26.4 ± 0.6 *vs.* 21.1 ± 0.7 g after 2 wk, 32.9 ± 0.9 *vs.* 25.4 ± 0.4 g after 5 wk, and 46.3 ± 1.77 *vs.* 30.0 ± 0.62 g after 15 wk, all $P < 0.001$), and the weight of their SC and GON adipose tissues was significantly higher (Table 2). Analysis of SC and GON adipose tissue sections showed that the average adipocyte area was significantly increased in mice fed a HFD. The blood mass/tissue mass ratios, evaluated from *in vivo* distribution of ^{131}I-BSA, were lower in obese,

TABLE 2. Composition of SC and GON adipose tissues from C57Bl/6 mice kept on SFD or HFD for 2–15 wk

Weeks	SFD						HFD					
	SC			GON			SC			GON		
	2	5	15	2	5	15	2	5	15	2	5	15
Fat pad weight (mg)	180 ± 8.8	210 ± 14	350 ± 56	180 ± 18	280 ± 19	590 ± 94	490 ± 35[a]	800 ± 66[a]	1300 ± 110[a]	670 ± 44[a]	1200 ± 93[a]	1600 ± 62[a]
Adipocyte size (μm^2)	280 ± 14	340 ± 31	370 ± 40	360 ± 28	570 ± 20	650 ± 48	650 ± 50[a]	830 ± 110[a]	1300 ± 46[a]	940 ± 39[a]	1300 ± 130[a]	2200 ± 38[a]
Blood vessel size (μm^2)	92 ± 7.6	74 ± 5.5	49 ± 2.8	52 ± 1.9	50 ± 2.6	49 ± 3.4	62 ± 4.4[a]	48 ± 2.8[a]	46 ± 2.5	48 ± 2.2	41 ± 1.8[a]	54 ± 3.3
Blood vessel density ($1/mm^2$)	1600 ± 57	1400 ± 100	1400 ± 100	1200 ± 55	850 ± 40	790 ± 41	1000 ± 44[a]	770 ± 51[a]	740 ± 30[a]	790 ± 30[a]	410 ± 20[a]	490 ± 19[a]
Blood mass (mg)	6.7 ± 0.36	7.6 ± 0.50	7.3 ± 0.73	8.6 ± 1.2	9.4 ± 0.90	9.2 ± 1.0	8.4 ± 0.35[a]	13 ± 0.95[a]	19 ± 1.8[a]	14 ± 1.7[a]	27 ± 2.5[a]	28 ± 2.0[a]
Proliferating cells (%)	2.2 ± 0.63	1.9 ± 0.38	2.2 ± 0.29	3.7 ± 0.47	2.8 ± 0.61	2.2 ± 0.67	7.0 ± 1.3[a]	4.9 ± 1.8	2.6 ± 0.49	9.0 ± 1.5[a]	4.5 ± 0.86	3.8 ± 0.69
Macrophages (%)	7.1 ± 0.79	7.0 ± 0.54	7.2 ± 0.56	8.1 ± 1.2	10 ± 0.91	11.2 ± 1.0	11 ± 1.2[a]	14 ± 1.5[a]	13 ± 3.0[a]	13 ± 0.64[a]	15 ± 1.9[a]	18 ± 3.3[a]

Data are mean ± SEM.
[a] $P < 0.05$ *vs.* SFD.

compared with lean, adipose tissues (not shown), but the total amount of blood was significantly higher (Table 2). The total blood content of SC or GON fat pads from mice on SFD or HFD correlated well with the adipose tissue mass [both rho (ρ) = 0.9 with $P < 0.000001$]. Staining of blood vessels in the adipose tissue sections showed a smaller vessel size and lower vessel density in tissues of obese mice, but a positive correlation was observed between the total blood vessel area and SC or GON fat pad mass when data on SFD and HFD were pooled ($\rho = 0.34$ with $P = 0.074$ for SC adipose tissue, and $\rho = 0.79$ with $P < 0.000001$ for GON adipose tissue). Staining with an anti-Ki-67 antibody revealed that the ratio of proliferating cells was significantly increased in SC and GON adipose tissue sections from mice on HFD for 2 wk, but not for 5 or 15 wk, compared with mice on SFD (Table 2). Double staining revealed that Ki-67-positive nuclei colocalize with lectin-positive ECs (Fig. 1). Staining of macrophages with an anti-Mac-3 antibody showed that the ratio of macrophages to total number of cell nuclei was significantly higher in all adipose tissue sections from obese, compared with lean, mice (Table 2); it correlated positively with adipose tissue mass ($\rho = 0.87$ with $P < 0.0001$ for SC adipose tissue, and $\rho = 0.91$ with $P < 0.0001$ for GON adipose tissue).

Genetically obese ob/ob mice kept on SFD also had a significantly higher total body weight (41 ± 1.2 *vs.* 27 ± 0.62 g), SC (1600 ± 47 *vs.* 150 ± 16 mg), and GON adipose tissue mass (2800 ± 260 *vs.* 270 ± 30 mg, all $P < 0.001$), compared with the WT mice. Blood vessel density ($1/mm^2$) in their adipose tissues was significantly decreased (430 ± 20 *vs.* 1200 ± 100, $P < 0.01$ for SC adipose tissue, and 390 ± 21 *vs.* 830 ± 87, $P < 0.05$ for GON adipose tissue), but the total blood vessel area in the whole fat pad was significantly

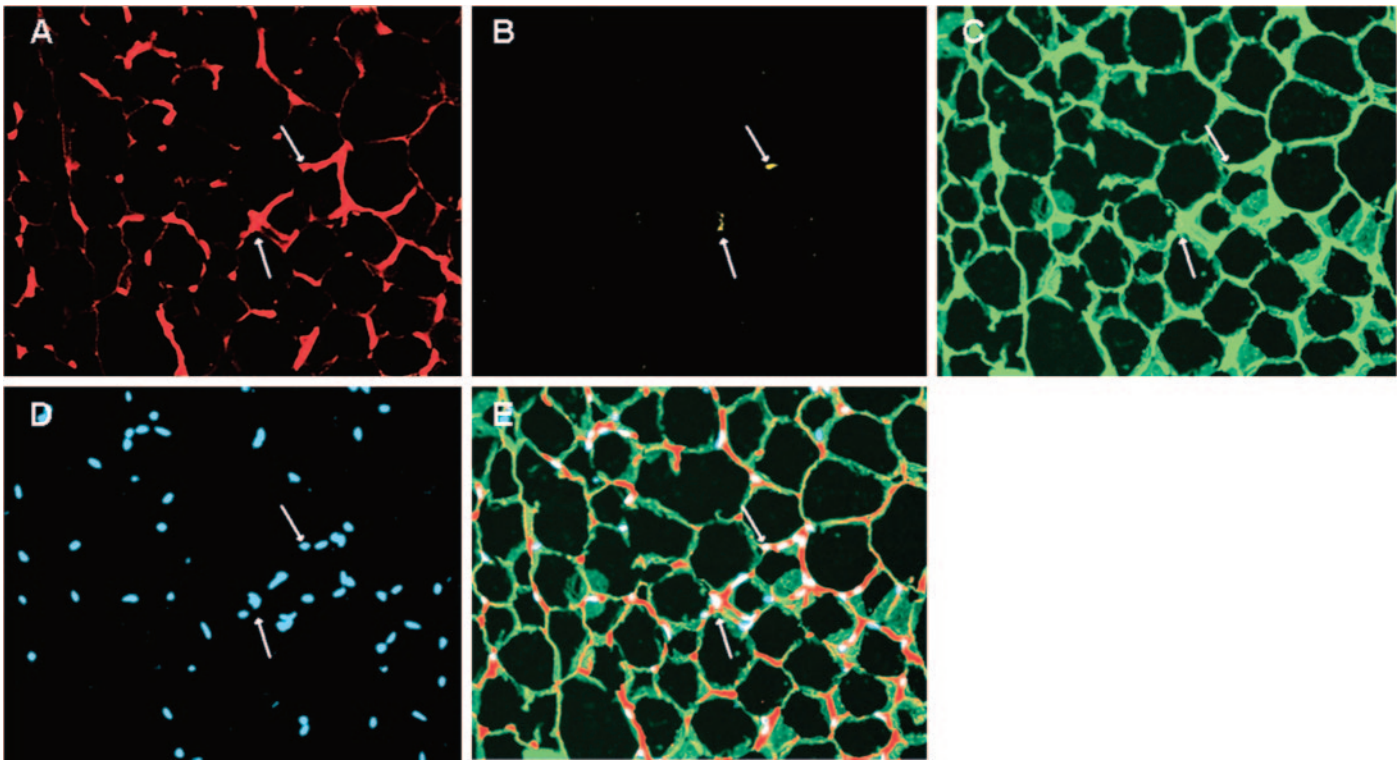

FIG. 1. Double staining of SC or GON adipose tissue with Bandeiraea Simplicifolia lectin and anti-Ki-67. A, Lectin staining; B, anti-Ki-67 staining; C, autofluorescence of the tissue; D, DAPI staining; E, superposition.

increased, compared with WT mice. A positive correlation was observed between the total blood vessel area and SC or GON fat pad mass ($\rho = 0.77$ with $P < 0.01$ for SC adipose tissue, and $\rho = 0.93$ with $P < 0.001$ for GON adipose tissue). The macrophage content was significantly higher in adipose tissues of ob/ob, compared with WT, mice (17 ± 0.99 *vs.* $5.1 \pm 0.43\%$ for SC and 27 ± 2.1 *vs.* $6.7 \pm 0.87\%$ for GON adipose tissue; both $P < 0.001$).

Adipose tissue expression of pro- and antiangiogenic molecules

The expression pattern of 17 transcripts of interest involved in angiogenesis was first investigated in the RNA pools of SC and GON adipose tissue from C57Bl/6 mice kept on SFD or HFD for 2, 5, or 15 wk (Table 3). The expression of pro- and antiangiogenic factors was relatively constant in adipose tissue samples from mice on SFD (not shown), with the exception of Ang-1 and TSP-1 (see below). Ang-1 was down-regulated in SC adipose tissue after 5 wk of HFD and in GON adipose tissue after 2 and 5 wk, compared with mice kept on SFD for the same duration. The expression patterns of Ang-2 and TIE-2 did not show marked modulation. TIE-1 mRNA levels were 1.8-fold higher in the SC adipose tissue after 2 and 15 wk on HFD. The expression of TSP-1 was more than 2-fold higher in both fat pads after 5 wk on HFD but showed a down-regulation after 2 wk in the GON adipose tissue and after 15 wk in both adipose tissues. TSP-2 mRNA levels in SC tissues were stable during the diet but increased in GON tissues. The three isoforms of VEGF-A, VEGF-B, VEGF-C, the three VEGF receptors, Np-1, and PlGF did not show marked modulations. The expression of FGF-2 was overall lower in HFD than in SFD samples, most obviously in SC adipose tissue after 5 wk. The mRNA levels of these proteins were also measured in the adipose tissue of 9-wk-old ob/ob and corresponding WT mice (Table 3). Ang-1 was also strongly down-regulated in both SC and GON adipose tissues of ob/ob, compared with WT, mice. Ang-2 mRNA levels were increased more than 2-fold in SC, but not in GON, adipose tissue, and the expression of TIE-1 and TIE-2 remained unaltered. The VEGF family members, VEGF receptors, FGF-2, and Np-1 mRNA levels were not markedly modulated, albeit sometimes decreased. PlGF expression was 2-fold up-regulated in SC adipose tissue. TSP-1 mRNA levels were 5-fold increased in SC adipose tissue, whereas TSP-2 expression was 2-fold increased in both adipose tissues of ob/ob mice. PPAR-γ mRNA levels were not markedly modulated in SC or GON adipose tissue by either nutritionally induced or genetically determined obesity (Table 3).

The mRNA levels of Ang-1 and TSP-1, the two targets modulated at least 2-fold in both obesity models, were subsequently measured in individual samples from both diet studies. This confirmed the expression pattern seen in the initial screening (Fig. 2). Compared with mRNA levels in 5-wk-old C57Bl/6 mice on SFD (wk 0), Ang-1 expression was down-regulated in SC adipose tissue after 15 wk and in GON adipose tissue after 2, 5, and 15 wk in mice on SFD (Fig. 2A). In mice fed a HFD, Ang-1 mRNA levels were down-regulated in both adipose tissues after 2, 5, and 15 wk, compared with wk 0. Furthermore, Ang-1 mRNA levels were significantly lower in SC adipose tissue after 5 wk and in GON adipose tissue after 2 and 5 wk on HFD, compared with SFD. TSP-1 expression was up-regulated in SC adipose tissue after 15 wk and in GON adipose tissue after 2, 5, and 15 wk in mice on SFD, compared with 5-wk-old mice (Fig. 2B). In mice fed a HFD, TSP-1 mRNA levels were up-regulated in both adipose tissues after 5 and 15 wk, compared with wk 0. TSP-1 mRNA levels were significantly lower in SC adipose tissue after 15 wk, in GON adipose tissue after 2 and 15 wk, but significantly higher in both adipose tissues after 5 wk on HFD, compared with SFD. Expression of Ang-1 was down-regulated in SC and GON adipose tissue and that of TSP-1 up-regulated in SC adipose tissue of ob/ob, compared with

TABLE 3. Expression of pro- and antiangiogenic molecules and PPAR-γ in SC and GON adipose tissue of mice

Weeks	C57Bl/6[a]						ob/ob[b]	
	SC			GON			SC	GON
	2	5	15	2	5	15	9	9
Ang-1	73	43	96	22	48	71	35	16
Ang-2	134	76	111	118	86	120	225	75
TIE-1	182	110	183	101	94	140	130	110
TIE-2	86	74	129	94	111	135	89	61
VEGF-A 121	103	99	85	94	108	95	79	52
VEGF-A 165	92	74	75	79	76	84	79	55
VEGF-A 189	97	80	90	108	106	116	92	74
VEGF-B	89	61	97	125	101	81	75	54
VEGF-C	99	68	173	105	99	120	143	97
VEGF-R1	95	52	95	95	96	107	49	79
VEGF-R2	78	66	89	104	87	74	105	43
VEGF-R3	109	76	106	77	62	99	179	87
PIGF	154	81	73	99	82	122	218	96
FGF-2	79	36	64	82	70	55	87	59
Np-1	111	104	95	84	79	92	116	72
TSP-1	86	272	30	35	215	37	518	99
TSP-2	127	111	114	61	103	179	180	229
PPAR-γ	137	79	143	89	108	87	130	109

[a] mRNA expression level relative to the SFD (=100%).
[b] mRNA expression level relative to WT mice (=100%).

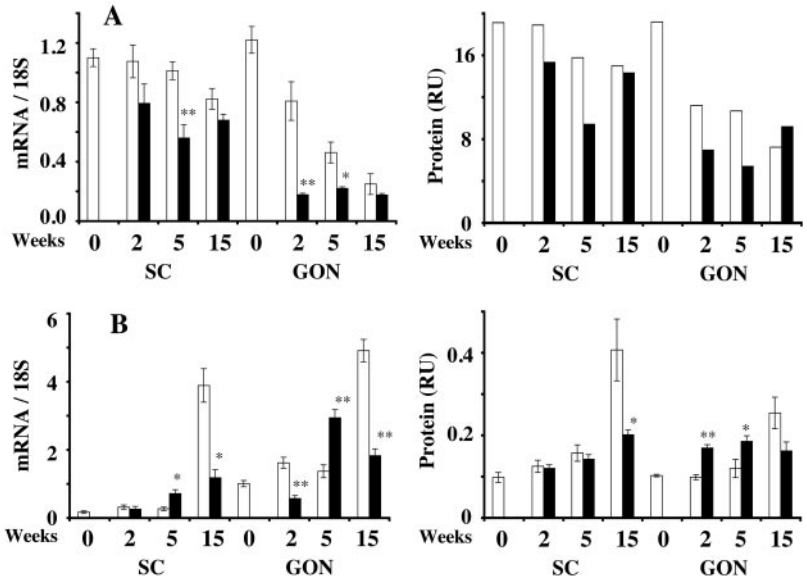

FIG. 2. mRNA expression and protein level of Ang-1 (A) and TSP-1 (B) in nutritionally induced obesity. Mice (n = 12) were kept on SFD (*white bars*) or HFD (*black bars*) for 0–15 wk. mRNA and protein levels were measured in SC and GON adipose tissue; mRNA levels were normalized to 18S rRNA, and protein levels were expressed in RU. *, $P < 0.01$ and **, $P < 0.001$ *vs.* SFD. Data are mean ± SEM, with the exception of Ang-1 protein levels that were measured by immunoprecipitation and Western blotting on pooled samples.

WT, mice (Fig. 3). Ang-1 mRNA levels correlated negatively with adipose tissue weights in the early phase of nutritionally induced obesity. In SC adipose tissue, the correlation was significant after 2 and 5 wk ($\rho = -0.39$ with $P < 0.01$) and in GON adipose tissue after 2 wk ($\rho = -0.88$ with $P < 0.0001$). Ang-1 expression did not change between 5 and 15 wk in SC adipose tissue and between 2 and 15 wk in GON adipose tissue from mice on HFD, and no correlation was found between tissue weights and Ang-1 expression in this later phase. Adipose tissue weights showed significant negative correlations with Ang-1 mRNA levels in genetically determined obesity ($\rho = -0.72$ with $P < 0.02$ for SC adipose tissue, and $\rho = -0.75$ with $P < 0.02$ for GON adipose tissue).

Protein levels of Ang-1 and TSP-1 in obesity

Ang-1 protein levels were generally compatible with the mRNA expression pattern (Fig. 2A). In nutritionally induced obesity, protein levels were lower in both adipose tissues of mice after 2, 5, and 15 wk on SFD or HFD, compared with wk 0. In accordance with significant differences in mRNA levels, lower Ang-1 protein levels were found in SC adipose tissue after 5 wk and in GON adipose tissue after 2 and 5 wk on HFD, compared with SFD. TSP-1 protein expression in mice on SFD (Fig. 2B) was significantly increased in SC and GON adipose tissue after 15 wk, compared with wk 0. In mice fed a HFD, TSP-1 protein levels were significantly higher in SC adipose tissue after 5 and 15 wk and in GON adipose tissue after 2, 5 and 15 wk, compared with wk 0. TSP-1 protein levels in mice on HFD were lower in the SC and GON adipose tissue after 15 wk but higher in GON adipose tissue after 2 and 5 wk, compared with SFD. Also in genetically determined obesity, Ang-1 protein levels were lower and TSP-1 protein levels significantly higher in both SC and GON adipose tissue of ob/ob mice, compared with WT mice (Fig. 3).

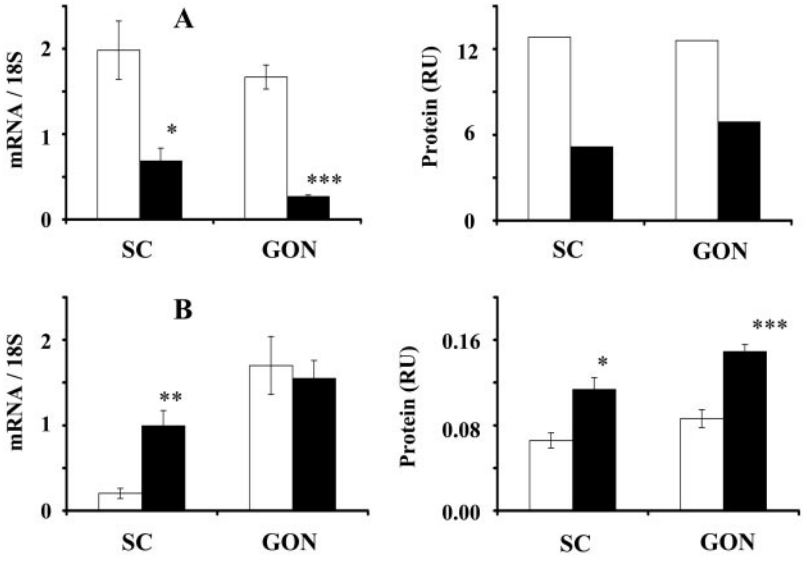

FIG. 3. mRNA and protein levels of Ang-1 (A) and TSP-1 (B) in genetic obesity. mRNA and protein levels were measured in SC and GON adipose tissue of 5 WT (*white bars*) or ob/ob (*black bars*) mice; mRNA levels were normalized to 18S rRNA, and protein levels were expressed in RU. *, $P < 0.05$; **, $P < 0.01$ and ***, $P < 0.001$ *vs.* WT. Data are mean ± SEM, with the exception of Ang-1 protein levels that were measured by immunoprecipitation and Western blotting on pooled samples.

Cellular localization

The expression patterns of the targets that were modulated in the two obesity models and of those expected to be selectively expressed were investigated in the separated cell populations. Ang-1, Ang-2, PlGF, TSP-1, and -2 mRNAs were detected in both cell fractions. TIE-1 was mainly expressed by S-V cells and by some mature adipocytes, especially in SC adipose tissue. TIE-2 and the three VEGF receptors were nearly exclusively expressed by the S-V cells in both SC and GON adipose tissues (Fig. 4). Low expression levels of vWF (ranging from 2–7% of those observed in the corresponding S-V fractions) were detected in the mature adipocyte fractions, indicating the presence of a low number of contaminating ECs (29).

Expression during in vitro adipogenesis

Differentiation of 3T3-F442A preadipocytes resulted in the appearance of cells characterized by higher intensities of Nile Red fluorescence (indicating intracellular lipid accumulation) detected at 530 nm (Fig. 5). mRNA levels of preadipocyte factor-1 decreased, whereas the expression of PPAR-γ, a marker of early differentiation, and that of glycerophosphate dehydrogenase, a marker of late differentiation, increased (data not shown). mRNAs for Ang-1, the VEGF-A isoforms, and PlGF showed an up-regulation during adipocyte differentiation. FGF-2 and Np-1 expression were transiently up-regulated, whereas the expression of Ang-2 was high in confluent cultures but decreased as adipocytes matured. TSP-1 and TSP-2 expression was down-regulated 5 d after induction but increased again during further differentiation. VEGF-B, VEGF-C, and VEGF-R1 mRNA levels were less modulated. TIE-1, TIE-2, VEGF-R2, and VEGF-R3 were not expressed at detectable levels by the 3T3-F442A cells.

Discussion

Modulation of development of the vascular network in adipose tissue may constitute a strategy to affect obesity (2). Therefore, it is important to obtain information on expression and functional role of pro- and antiangiogenic components. In this study, we characterized some aspects of blood vessel development in nutritionally induced or genetically determined murine models of obesity. We found that the total blood content of the SC and GON fat pads correlated well with the adipose tissue mass. Different morphological or functional changes of the vasculature can account for this observation. One possible explanation is that the perfusion of the tissue increases due to vasodilatation and opening of preexisting, but nonperfused, capillaries. In a previous study, increased perfusion and decreased vascular resistance were shown in SC and GON fat pads of obese, compared with lean, rats (30). Blood vessel staining confirmed a positive correlation between fat pad mass and the total blood vessel area in both obesity models, supporting the hypothesis that the increased blood content of obese adipose tissue is not only the consequence of functional modulations of the vasculature but is also caused by growth of the vascular network. To identify factors involved in adipose tissue-related angiogenesis, the expression pattern of 17 pro- or antiangiogenic molecules was monitored. Adipose tissue development is a partly physiological and a partly pathological process. On the basis of previously published data, the genes that we studied are likely to play crucial roles in both physiological and pathological angiogenesis (6, 7, 10).

Ang-1 expression was significantly down-regulated in SC fat pads after 5 wk and in the GON adipose tissue after 2 and 5 wk on HFD *vs.* SFD. A significant negative correlation was found between Ang-1 mRNA levels and adipose tissue mass in the early phase of nutritionally induced and in genetically determined obesity. Ang-1 protein levels, measured in both obesity models, were overall compatible with mRNA expression. In a recent study, reduced Ang-1 expression was reported in mice losing weight, and an inverse correlation was found between Ang-1 mRNA levels and the rate of weight change, independent of the direction (31). In our study Ang-1 mRNA and protein expression were decreased (compared with wk 0) in the early phase of nutritionally induced obesity, when mice gained weight faster, and they further decreased in the fat pads of mice on SFD between 5 and 15 wk. Ang-1 mRNA and protein levels were considerably lower, compared with wk 0 (especially in GON adipose tissue) in mice after 15 wk on both SFD and HFD, when the body weight was stable. These data support the concept that Ang-1 mRNA levels are not merely regulated by weight change in developing or regressing adipose tissue. Lower Ang-1 levels may result in increased plasticity of vessels,

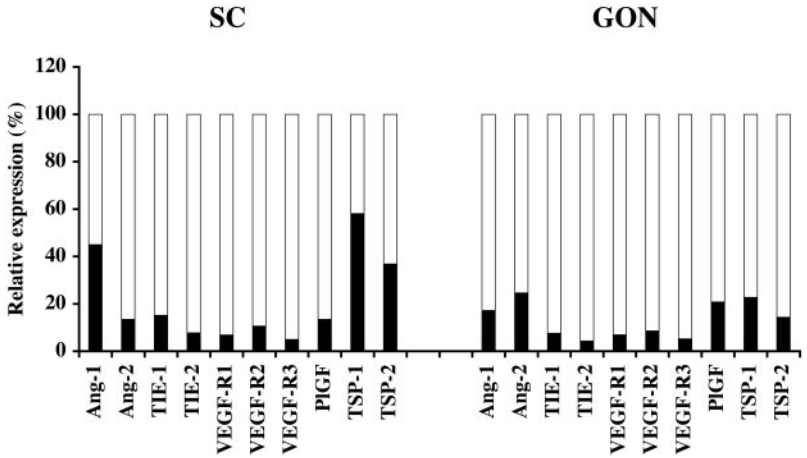

FIG. 4. Cellular localization of mRNA in isolated adipocytes (*black bars*) expressed relative to the S-V cells (*white bars*) derived from SC and GON adipose tissue (ob/ob genetic background).

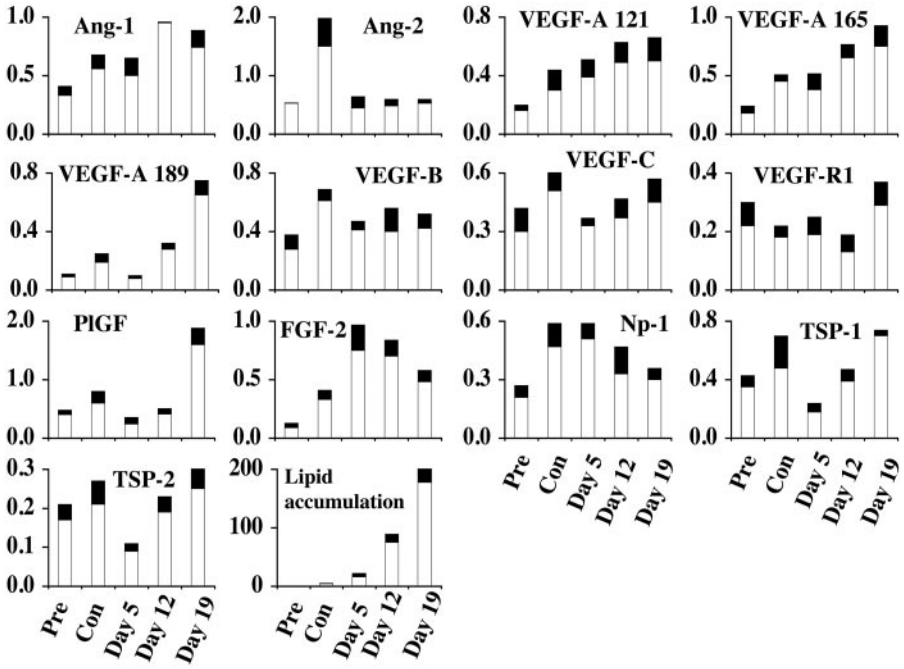

FIG. 5. Expression of angiogenic factors during *in vitro* adipogenesis. Total RNA was extracted from 3T3-F442A cells at different stages of differentiation: Pre, preconfluent; Con, confluent (d 0); d5, d12, and d19, postconfluent cultures after 5, 12, and 19 d, respectively. mRNA levels were normalized to 18S rRNA. Intracellular lipid accumulation during preadipocyte differentiation, as monitored by the intensity of Nile Red fluorescence, is also shown. *White bars* represent mRNA levels obtained in one experiment, and *total bars* (*white plus black*) represent mRNA levels from a second experiment.

increased vascular permeability, and smaller lumen diameters, which all characterize the vasculature of obese adipose tissue. There are similarities, but also clear differences, between TSP-1 mRNA and protein expression patterns both in nutritionally induced and genetically determined obesity (Figs. 2B and 3B). This different regulation may be due to translational regulation of TSP-1 expression or differences in its degradation (32).

The opposite modulation of Ang-1 (down-regulated) and TSP-1 (up-regulated) expression during adipose tissue development may have a common mechanism. Indeed, inhibition of TIE-2 signaling *in vitro* has been shown to up-regulate TSP-1 in ECs (33).

TSP-1 is mainly expressed by intraabdominal adipose tissue of rats and humans (34, 35). In this study, we found that TSP-1 expression was strongly modulated by the degree of obesity and was mainly observed in GON adipose tissue after 2 and 5 wk on HFD and in the WT littermates of ob/ob mice. Gene expression is differently regulated in SC and GON adipose tissues. This may be explained by the fact that SC and GON fat pads have substantially different characteristics. In humans, it is well established that accumulation of intraabdominal adipose tissue is a higher risk factor for vascular complications than the SC fat pad (36). They produce different amounts of plasminogen activator inhibitor (PAI)-1, PAI-2, leptin, ILs, and TSP-1 (37). In mice, after 5 wk, the growth rate of GON adipose tissue is generally slower, compared with SC adipose tissue. These differences might all contribute to the different regulations of angiogenic factors in different fat pads. However, there are factors (Ang-1) that are similarly regulated in the distinct fat pads of the different models.

TSPs are known antiangiogenic factors; and, when the growth rate of adipose tissue stabilizes, their higher expression may limit further blood vessel formation. TSPs are expressed by 3T3-F442A cells and are down-regulated after induction of differentiation, followed by a later up-regulation. A similar expression pattern was found in NIH-3T3 (38) and in 3T3-L1 (39) cells during differentiation into adipocytes; in other studies, however, TSP-1 was shown to be up-regulated 1–3 h after induction of differentiation in 3T3-F442A (40) and 3T3-L1 (41) preadipocytes. Whether a higher amount of TSPs produced by differentiated adipocytes only has an impact on vascular elements or, in an autocrine manner, on the adipocytes themselves needs further investigation.

The expression of Ang-2 and TIE-2 remained relatively constant in the two *in vivo* obesity models, except for a 2-fold up-regulation of Ang-2 in the SC adipose tissue of ob/ob mice (Table 3). This may be important for the intensive modulation of vasculature in the developing adipose tissue of ob/ob mice, because Ang-2 is often expressed together with VEGF-A at sites of neoangiogenesis (42). In our study, the expression of VEGF-A isoforms, VEGF-B, VEGF-C, and the three VEGF receptors was not markedly modulated in either obesity model. Still, adipose tissue and mature adipocytes are known to produce high amounts of VEGF-A (43). A recent study in overweight and obese male subjects did not show significant modulation of the serum concentration of VEGF or Ang-2 (44). Both VEGF-A mRNA and protein levels were found to be higher in 14-wk-old, compared with 6-wk-old, db/db mice and in obese KK-A^Y mice, compared with WT mice (45). The difference with our results may be explained by differences in the experimental conditions. First, in our experiments, we used the nutritionally induced murine obesity model and studied ob/ob and WT mice, in which regulation of VEGF-A expression might be different. Second, we normalized VEGF-A expression to 18S rRNA levels and not to β-actin mRNA. β-actin is significantly down-regulated in obese, compared with lean, SC adipose tissue from human

subjects (46). If this is also the case in murine models of obesity, normalization of mRNA or protein levels to β-actin might result in a wrong interpretation. In our samples the expression of 18S rRNA was very similar in all samples investigated. Further *in vivo* studies with inhibition of VEGF receptors will be required to elucidate the exact role of VEGF in obesity. FGF-2 was generally down-regulated in nutritionally induced and genetic obesity but up-regulated in differentiating 3T3-F442A cells. These data differ from a study showing higher FGF-2 mRNA levels in massively obese humans, compared with lean ones (47), possibly due to the fact that, in our *in vivo* models, obesity mainly results from adipocyte hypertrophy, whereas in massively obese persons, adipocyte hyperplasia may also occur. In the latter case, increased levels of FGF-2, an enhancer of adipocyte differentiation, may be more relevant.

Regulation of mRNA expression of some pro- and antiangiogenic factors was considerably different between the two diet models. For example, expression of Ang-2 and PlGF was up-regulated in the SC adipose tissue, and that of VEGF-R2 was down-regulated in the GON adipose tissue of ob/ob, compared with WT, mice, but it was unchanged in nutritionally induced obesity. Leptin-deficiency apparently results in faster adipose tissue formation and a higher degree of obesity than the HFD used in this study. The observed differences may be explained by the enhanced adipose tissue formation or by the lack of leptin itself, which has been found to affect the expression of several angiogenic components (48).

It is increasingly recognized that macrophages are playing a role in adipose tissue development. As reported in other studies (49), we found a strong correlation between the macrophage content and the adipose tissue mass in our *in vivo* models. Because these immune cells produce angiogenic factors and are abundant in adipose tissue, they may significantly contribute to the enlargement of the tissue vasculature.

Hypoxia may be increased during adipose tissue development, which, in turn, would trigger blood vessel formation. Several mRNAs induced by hypoxia (PAI-1, TSP-1) are increased in obesity. On the other hand, the expression of VEGF isoforms, which are also strongly induced by hypoxia and leptin, is unchanged in our *in vivo* samples. Thus, if there is hypoxia during adipose tissue development, it only stimulates the expression of a limited number of targets; VEGF expression is clearly also regulated by additional factors.

In summary, in nutritionally induced or genetic obesity in mice, fat pad growth is accompanied by increased vascularization. During adipose tissue development, Ang-1 is markedly down-regulated, whereas TSP-1 is up-regulated in SC and GON adipose tissue. Less extensive modulations were found in PlGF, TSP-2, and FGF-2 mRNA levels. Expression of other VEGF family members and Np-1 was not markedly changed in these obesity models. These findings do not exclude the possibility that modifying VEGF signaling may have an effect on adipose tissue development, but suggest a potential role for other pro- and antiangiogenic factors in obesity-related angiogenesis. Further studies are required to elucidate their functional role in obesity.

Acknowledgments

Skillful technical assistance by A. De Wolf, L. Frederix, K. Umans, and B. Van Hoef is gratefully acknowledged.

Received May 3, 2005. Accepted July 1, 2005.

Address all correspondence and requests for reprints to: H. R. Lijnen, Center for Molecular and Vascular Biology, Katholieke Universiteit Leuven, Campus Gasthuisberg, Onderwijs en Navorsing, Herestraat 49, B-3000 Leuven, Belgium. E-mail: roger.lijnen@med.kuleuven.ac.be.

This work was supported by the "Fonds voor Wetenschappelijk Onderzoek-Vlaanderen" (G.0281.04) and by the Interuniversity Attraction Poles (IUAP, P5/02).

References

1. **Hausman GJ, Richardson RL** 1983 Cellular and vascular development in immature rat adipose tissue. J Lipid Res 24:522–532
2. **Castellot Jr JJ, Karnovsky MJ, Spiegelman BM** 1982 Differentiation-dependent stimulation of neovascularization and endothelial cell chemotaxis by 3T3 adipocytes. Proc Natl Acad Sci USA 79:5597–5601
3. **Montesano R, Mouron P, Orci L** 1985 Vascular outgrowths from tissue explants embedded in fibrin or collagen gels: a simple *in vitro* model of angiogenesis. Cell Biol Int Rep 9:869–875
4. **Varzaneh FE, Shillabeer G, Wong KL, Lau DC** 1994 Extracellular matrix components secreted by microvascular endothelial cells stimulate preadipocyte differentiation *in vitro*. Metabolism 43:906–912
5. **Rupnick MA, Panigrahy D, Zhang CY, Dallabrida SM, Lowell BB, Langer R, Folkman MJ** 2002 Adipose tissue mass can be regulated through the vasculature. Proc Natl Acad Sci USA 99:10730–10735
6. **Carmeliet P, Ferreira V, Breier G, Pollefeyt S, Kieckens L, Gertsenstein M, Fahrig M, Vandenhoeck A, Harpal K, Eberhardt C, Declercq C, Pawling J, Moons L, Collen D, Risau W, Nagy A** 1996 Abnormal blood vessel development and lethality in embryos lacking a single VEGF allele. Nature 380: 435–439
7. **Silvestre JS, Tamarat R, Ebrahimian TG, Le-Roux A, Clergue M, Emmanuel F, Duriez M, Schwartz B, Branellec D, Levy BI** 2003 Vascular endothelial growth factor-B promotes *in vivo* angiogenesis. Circ Res 93:114–123
8. **Olofsson B, Korpelainen E, Pepper MS, Mandriota SJ, Aase K, Kumar V, Gunji Y, Jeltsch MM, Shibuya M, Alitalo K, Eriksson U** 1998 Vascular endothelial growth factor B (VEGF-B) binds to VEGF receptor-1 and regulates plasminogen activator activity in endothelial cells. Proc Natl Acad Sci USA 95:11709–11714
9. **Karkkainen MJ, Haiko P, Sainio K, Partanen J, Taipale J, Petrova TV, Jeltsch M, Jackson DG, Talikka M, Rauvala H, Betsholtz C, Alitalo K** 2004 Vascular endothelial growth factor C is required for sprouting of the first lymphatic vessels from embryonic veins. Nat Immunol 5:74–80
10. **Luttun A, Tjwa M, Moons L, Wu Y, Angelillo-Scherrer A, Liao F, Nagy JA, Hooper A, Priller J, De Klerck B, Compernolle V, Daci E, Bohlen P, Dewerchin M, Herbert JM, Fava R, Matthys P, Carmeliet G, Collen D, Dvorak HF, Hicklin DJ, Carmeliet P** 2002 Revascularization of ischemic tissues by PlGF treatment, and inhibition of tumor angiogenesis, arthritis and atherosclerosis by anti-Flt1. Nat Med 8:831–840
11. **Carmeliet P, Collen D** 1997 Molecular analysis of blood vessel formation and disease. Am J Physiol 273:H2091–H2104
12. **Soker S, Takashima S, Miao HQ, Neufeld G, Klagsbrun M** 1998 Neuropilin-1 is expressed by endothelial and tumor cells as an isoform specific receptor for vascular endothelial growth factor. Cell 92:735–745
13. **Kitsukawa T, Shimono A, Kawakami A, Kondoh H, Fujisawa H** 1995 Overexpression of a membrane protein, neuropilin, in chimeric mice causes anomalies in the cardiovascular system, nervous system and limbs. Development 121:4309–4318
14. **Bikfalvi A, Klein S, Pintucci G, Rifkin DB** 1997 Biological roles of fibroblast growth factor-2. Endocr Rev 18:26–45
15. **Kawaguchi N, Toriyama K, Nicodemou-Lena E, Inou K, Torii S, Kitagawa Y** 1998 *De novo* adipogenesis in mice at the site of injection of basement membrane and basic fibroblast growth factor. Proc Natl Acad Sci USA 95: 1062–1066
16. **Takagi H, Koyama S, Seike H, Oh H, Otani A, Matsumura M, Honda Y** 2003 Potential role of the angiopoietin/tie2 system in ischemia-induced retinal neovascularization. Invest Ophthalmol Vis Sci 44:393–402
17. **Thurston G, Rudge JS, Ioffe E, Zhou H, Ross L, Croll SD, Glazer N, Holash J, McDonald DM, Yancopoulos GD** 2000 Angiopoietin-1 protects the adult vasculature against plasma leakage. Nat Med 6:460–463
18. **Carlson TR, Feng Y, Maisonpierre PC, Mrksich M, Morla AO** 2001 Direct cell adhesion to the angiopoietins mediated by integrins. J Biol Chem 276:26516–26525
19. **Gale NW, Thurston G, Hackett SF, Renard R, Wang Q, McClain J, Martin C, Witte C, Witte MH, Jackson D, Suri C, Campochiaro PA, Wiegand SJ, Yancopoulos GD** 2002 Angiopoietin-2 is required for postnatal angiogenesis

and lymphatic patterning, and only the latter role is rescued by angiopoietin-1. Dev Cell 3:411–423
20. **Visconti RP, Richardson CD, Sato TN** 2002 Orchestration of angiogenesis and arteriovenous contribution by angiopoietins and vascular endothelial growth factor (VEGF). Proc Natl Acad Sci USA 99:8219–8224
21. **Hackett SF, Wiegand S, Yancopoulos G, Campochiaro PA** 2002 Angiopoietin-2 plays an important role in retinal angiogenesis. J Cell Physiol 192:182–187
22. **Armstrong LC, Bornstein P** 2003 Thrombospondins 1 and 2 function as inhibitors of angiogenesis. Matrix Biol 22:63–71
23. **Giles AR** 1987 Guidelines for the use of animals in biomedical research. Thromb Haemost 58:1078–1084
24. **Luttun A, Brusselmans K, Fukao H, Tjwa M, Ueshima S, Herbert JM, Matsuo O, Collen D, Carmeliet P, Moons L** 2002 Loss of placental growth factor protects mice against vascular permeability in pathological conditions. Biochem Biophys Res Commun 295:428–434
25. **Voros G, Maquoi E, Collen D, Lijnen HR** 2004 Influence of membrane-bound tumor necrosis factor (TNF)-α on obesity and glucose metabolism. J Thromb Haemost 2:507–513
26. **Rodbell M** 1964 Metabolism of isolated fat cells. I. Effects of hormones on glucose metabolism and lipolysis. J Biol Chem 239:375–380
27. **Maquoi E, Noel A, Frankenne F, Angliker H, Murphy G, Foidart JM** 1998 Inhibition of matrix metalloproteinase 2 maturation and HT1080 invasiveness by a synthetic furin inhibitor. FEBS Lett 424:262–266
28. **Maquoi E, Munaut C, Colige A, Collen D, Lijnen HR** 2002 Modulation of adipose tissue expression of murine matrix metalloproteinases and their tissue inhibitors with obesity. Diabetes 51:1093–1101
29. **Samad F, Yamamoto K, Loskutoff DJ** 1996 Distribution and regulation of plasminogen activator inhibitor-1 in murine adipose tissue *in vivo*: induction by tumor necrosis factor-α and lipopolysaccharide. J Clin Invest 97:37–46
30. **Crandall DL, Goldstein BM, Lizzo FH, Gabel RA, Cervoni P** 1986 Hemodynamics of obesity: influence of pattern of adipose tissue cellularity. Am J Physiol 251:R314–R319
31. **Dallabrida SM, Zurakowski D, Shih SC, Smith LE, Folkman J, Moulton KS, Rupnick MA** 2003 Adipose tissue growth and regression are regulated by angiopoietin-1. Biochem Biophys Res Commun 311:563–571
32. **Adams JC, Lawler J** 2004 The thrombospondins. Int J Biochem Cell Biol 36:961–968
33. **Niu Q, Perruzzi C, Voskas D, Lawler J, Dumont DJ, Benjamin LE** 2004 Inhibition of Tie-2 signaling induces endothelial cell apoptosis, decreases akt signaling, and induces endothelial cell expression of the endogenous anti-angiogenic molecule, thrombospondin-1. Cancer Biol Ther 3:402–405
34. **Ramis JM, Franssen-van Hal NL, Kramer E, Llado I, Bouillaud F, Palou A, Keijer J** 2002 Carboxypeptidase E and thrombospondin-1 are differently expressed in subcutaneous and visceral fat of obese subjects. Cell Mol Life Sci 59:1960–1971
35. **Hida K, Wada J, Zhang H, Hiragushi K, Tsuchiyama Y, Shikata K, Makino H** 2000 Identification of genes specifically expressed in the accumulated visceral adipose tissue of OLETF rats. J Lipid Res 41:1615–1622
36. **Sharma AM** 2002 Adipose tissue: a mediator of cardiovascular risk. Int J Obes Relat Metab Disord 26:S5–S7
37. **Fain JN, Madan AK, Hiler ML, Cheema P, Bahouth SW** 2004 Comparison of the release of adipokines by adipose tissue, adipose tissue matrix, and adipocytes from visceral and subcutaneous abdominal adipose tissues of obese humans. Endocrinology 145:2273–2282
38. **Okuno M, Arimoto E, Nishizuka M, Nishihara T, Imagawa M** 2002 Isolation of up- or down-regulated genes in PPARγ-expressing NIH-3T3 cells during differentiation into adipocytes. FEBS Lett 519:108–112
39. **Burton GR, Nagarajan R, Peterson CA, McGehee Jr RE** 2004 Microarray analysis of differentiation-specific gene expression during 3T3-L1 adipogenesis. Gene 329:167–185
40. **Shang CA, Thompson BJ, Teasdale R, Brown RJ, Waters MJ** 2002 Genes induced by growth hormone in a model of adipogenic differentiation. Mol Cell Endocrinol 189:213–219
41. **Inuzuka H, Nanbu-Wakao R, Masuho Y, Muramatsu M, Tojo H, Wakao H** 1999 Differential regulation of immediate early gene expression in preadipocyte cells through multiple signaling pathways. Biochem Biophys Res Commun 265:664–668
42. **Goede V, Schmidt T, Kimmina S, Kozian D, Augustin HG** 1998 Analysis of blood vessel maturation processes during cyclic ovarian angiogenesis. Lab Invest 78:1385–1394
43. **Zhang QX, Magovern CJ, Mack CA, Budenbender KT, Ko W, Rosengart TK** 1997 Vascular endothelial growth factor is the major angiogenic factor in omentum: mechanism of the omentum-mediated angiogenesis. J Surg Res 67:147–154
44. **Silha JV, Krsek M, Sucharda P, Murphy LJ** 7 June 2005 Angiogenic factors are elevated in overweight and obese individuals. Int J Obes Relat Metab Disord, advance online publication
45. **Miyazawa-Hoshimoto S, Takahashi K, Bujo H, Hashimoto N, Yagui K, Saito Y** 2004 The roles of degree of fat deposition and its localization on VEGF expression in adipocytes. Am J Physiol Endocrinol Metab 288:E1128–E1136
46. **Fisher RM, Hoffstedt J, Hotamisligil GS, Thorne A, Ryden M** 2002 Effects of obesity and weight loss on the expression of proteins involved in fatty acid metabolism in human adipose tissue. Int J Obes Relat Metab Disord 26:1379–1385
47. **Teichert-Kuliszewska K, Hamilton BS, Deitel M, Roncari DA** 1992 Augmented production of heparin-binding mitogenic proteins by preadipocytes from massively obese persons. J Clin Invest 90:1226–1231
48. **Suganami E, Takagi H, Ohashi H, Suzuma K, Suzuma I, Oh H, Watanabe D, Ojima T, Suganami T, Fujio Y, Nakao K, Ogawa Y, Yoshimura N** 2004 Leptin stimulates ischemia-induced retinal neovascularization: possible role of vascular endothelial growth factor expressed in retinal endothelial cells. Diabetes 53:2443–2448
49. **Weisberg SP, McCann D, Desai M, Rosenbaum M, Leibel RL, Ferrante Jr AW** 2003 Obesity is associated with macrophage accumulation in adipose tissue. J Clin Invest 112:1796–1808

***Endocrinology* is published monthly by The Endocrine Society (http://www.endo-society.org), the foremost professional society serving the endocrine community.**