Adipose Tissue Endothelial Cells from Obese Human Subjects: Differences Among Depots in Angiogenic, Metabolic, and Inflammatory Gene Expression and Cellular Senescence

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Running title: Human adipose endothelial cells

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Submitted 23 March 2010 and accepted 28 July 2010.

Additional information for this article can be found in an online appendix at http://diabetes.diabetesjournals.org

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**Objective:** Regional differences among adipose depots in capacities for fatty acid storage, susceptibility to hypoxia, and inflammation likely contribute to complications of obesity. We defined the properties of endothelial cells (EC) isolated from subcutaneous and visceral adipose tissue (SAT and VAT) biopsied in parallel from obese subjects.

**Research Design and Methods:** The architecture and properties of the fat tissue capillary network were analyzed using immunohistochemistry and flow cytometry. CD34+/CD31+ EC were isolated by immunoselection/depletion. Expression of chemokines, adhesion molecules, angiogenic factor receptors, as well as lipogenic and senescence-related genes were assayed by real-time PCR. Fat cell size and expression of hypoxia-dependent genes were determined in adipocytes from both fat depots.

**Results:** Hypoxia-related genes were more highly expressed in VAT than SAT adipocytes. VAT adipocytes were smaller than SAT adipocytes. Vascular density and EC abundance were higher in VAT. VAT-EC exhibited a marked angiogenic and inflammatory state with decreased expression of metabolism-related genes, including endothelial lipase, GPIHBP1, and PPAR gamma. VAT-EC had enhanced expression of the cellular senescence markers, IGFBP3 and γ-H2AX, and decreased expression of SIRT1. Exposure to VAT adipocytes caused more EC senescence-associated β-galactosidase activity than SAT adipocytes, an effect reduced in the presence of VEGFA neutralizing antibodies.

**Conclusions:** VAT-EC exhibit a more marked angiogenic and pro-inflammatory state than SAT-EC. This phenotype may be related to premature EC senescence. Potentially, VAT-EC contribute to hypoxia and inflammation in VAT.

The endothelium plays a major role in regulating exchange of leukocytes, nutrients, and oxygen between blood and tissues. The extent of the capillary network and endothelial cell (EC) characteristics are major determinants of growth and function of adipose tissue (AT) (1). Indeed, angiogenesis and adipogenesis have been shown, through distinct approaches, to be tightly linked (2-4). Moreover, lipogenesis is dependent on lipoprotein lipase (LPL) and the newly discovered endothelial cell-surface glycoprotein, glycosylphosphatidylinositol-anchored high density lipoprotein binding protein 1 (GPIHBP1) which are anchored to the EC that line the luminal surface of capillaries (5; 6). Additionally, a recent study demonstrated that endothelial targeting of peroxisome proliferator-activated receptor gamma (PPARγ) regulates the metabolic response to high fat diet in mice (7). Little is known about regional variation in the properties of fat tissue EC. Given their central role in lipid metabolism and inflammation, we tested the hypothesis that EC and their microenvironments differ among human fat depots in obesity. We tested our hypothesis by comparing abdominal subcutaneous to omental EC isolated in parallel from the same obese human subjects for the following reasons. 1) These two depots are highly clinically relevant, with omental fat being more heavily implicated in metabolic syndrome and adverse clinical consequences of obesity than subcutaneous fat (8). 2) Omental fat becomes more extensively
Human adipose endothelial cells infiltrated with immuno-inflammatory cells, including macrophages and T-lymphocytes, than subcutaneous fat in obesity (9-12). 3) More is known about differences in metabolism, fatty acid handling, gene expression, and mechanisms of growth in these two depots in humans with obesity than most other depots (13; 14). 4) These two depots differ in fat cell size in response to obesity as well as lipogenesis and lipolysis (15; 16).

We found that EC isolated from omental fat had reduced expression of genes related to metabolism and increased expression of genes related to angiogenesis and inflammation compared to subcutaneous cells. Conditioned medium prepared from fat cells of these two depots from obese subjects have distinct effects on EC from lean subjects. We found that cellular senescence, a pro-inflammatory state, is more evident in omental than subcutaneous fat-derived EC, potentially contributing to regional variation in fat tissue function.

RESEARCH DESIGN AND METHODS

Materials. Collagenase type 1 was purchased from Worthington Biochemical Corporation (Lakewood, USA). Kits for CD34+ and CD31+ cells were, respectively, from StemCell Technologies (Grenoble, France) and Dynal-Biotech (Invitrogen, Cergy-Pontoise, France). Culture media, including endothelial cell basal medium (ECBM), were from Promocell (Heidelberg, Germany). Antibodies for flow cytometry were from BD-Biosciences (Le-Pont-de-Clai, France) or Caltag (Invitrogen, Cergy-Pontoise, France). Antibodies for immunofluorescence were from DakoCytomation (Denmark, CD31), Epitomics (USA, CD34), Zymed (Invitrogen, France, ICAM1), Santa-cruz (Cliniscience, France, GPIHPB1), Upstate (Millipore, France, phospho-histone-γ-H2AX), and Molecular Probes (Invitrogen, USA, secondary antibodies coupled to AlexaFluor-488 or -546). Chemicals were from Sigma (Saint-Quentin-Fallavier, France).

Subjects and isolation of mature adipocytes, stromal-vascular fraction (SVF) cells, and EC from human subcutaneous and visceral AT (SAT and VAT). Paired biopsies from abdominal SAT (periumbilical) or VAT (greater omental) were obtained from subjects undergoing bariatric surgery for obesity. Subjects had been weight stable for at least three months before surgery and included 29 women and 1 man (mean body mass index [BMI] ± standard error of the mean [SEM]=43.35±1.05 kg/m², range 34.5-59.1; mean age ± SEM=40.4±1.93 years, range 26-61). All subjects gave informed consent. Five subjects were hypertensive, 6 had hypercholesterolemia, and 3 had type 2 diabetes. The mean time since obesity ± SEM had been diagnosed was 18.2±1.4 years. Abdominal SAT were obtained from 51 non-obese healthy women undergoing plastic surgery for cosmetic purposes (mean BMI ± SEM =23.11±0.221, range 19.5-27.2; mean age ± SEM =43.57±1.753, range 24-74). Fat collection protocols were approved by the Institutional Research Board of Inserm and the Toulouse University Hospital Ethics Committee. AT (2 to 10 g) was processed immediately after removal. Adipocytes and SVF were obtained by collagenase digestion as previously described (10). After digestion and filtration of the adipocyte suspension (250 µm mesh nylon sieves), adipocytes were washed three times with Krebs-Ringer bicarbonate buffer supplemented with 10 mM Hepes (KRBH) and 0.1% fatty acid free bovine serum albumin (BSA; pH=7.4). Mature adipocytes were suspended in ECBM supplemented with 0.1% BSA (1/10, vol/vol). Five µL of cell suspension were transferred onto plastic slides. Three different calibrated fields were examined to measure adipocyte diameters using NIS software (Nikon, Champigny-sur-Marne, France). SVF cells
were counted and analysed by flow cytometry for EC number determination. At least 100,000 cells (in 100 µL phosphate buffer saline [PBS]/0.5% BSA, 2 mmol/L EDTA) were incubated (20 min, 4°C) with FITC-CD31 and PerCP-CD34 antibodies or the appropriate isotype controls. After washing, the labeled cells were analysed by flow cytometry using a FACSCalibur flow cytometer and Diva software (BD-Biosciences). When the quantity of SVF cells was sufficient (11 subjects, paired SAT and VAT), native CD34+/CD31+ cells (EC) were isolated by immunoselection/depletion as previously described (10). Mature adipocytes and EC were either lysed and stored at -20°C for mRNA extraction or further processed.

**Mature adipocyte culture.** SAT and VAT mature adipocytes were placed in fibrin gels (1.5 mg fibrinogen/ml ECBM supplemented with 25 units/ml thrombin; 1/3, vol/vol) and cultured in ECBM/0.1% BSA supplemented with 100 units/ml penicillin and 100 µg/ml streptomycin. After 24 h, the adipocyte-conditioned media were collected and frozen at -80°C.

SAT mature adipocytes were cultured in ECBM supplemented with 0.1% BSA, 100 units/ml penicillin, and 100 g/ml streptomycin in CLINICell® 25 culture cassettes (1/3, vol/vol) in normoxia or hypoxia chambers (1% O2; Sanyo, Avon, France). After 24 h, mature adipocytes were lysed and stored at -20°C for RNA extraction.

**Immunohistochemistry analyses.** Tissue immunofluorescent staining of human SAT and VAT cut into small pieces (0.5-1 mm³) was performed. After fixation in paraformaldehyde (PFA) 4% (1 h) and subsequent washing in PBS, AT pieces were incubated for 30 min in PBS/2% BSA in the presence of 0.1% Triton. For γ-H2AX staining, AT pieces were permeabilized for 5 min in 95% ethanol and 5% acetic acid and then incubated for 30 min in a Tris saline buffer (TBS)/3% BSA. AT pieces were incubated for 1 h with primary antibody (CD31 [1/50], CD34 [1/200], ICAM1 [1/50], GPIHBP1 [1/40], γ-H2AX [1/200]). After several washing steps (PBS/0.2% Tween or TBS), AT pieces were incubated 30 min in PBS/2% BSA/0.1% Triton or TBS/3% BSA and then incubated with the corresponding fluorescently-labeled secondary antibodies (AlexaFluor-488 or -546, 1/200). After washing, AT pieces were incubated 10 min with 10 µg/mL Hoechst 33342 to stain nuclei. They were placed between two mounting slides and examined with a fluorescence (Nikon) or confocal (Zeiss 510) microscope.

Quantification of phospho-γ-H2AX positive nuclei in human SAT and VAT was performed on three calibrated fields using NIS software (Nikon, Champigny-sur-Marne, France) and normalized to the total number of nuclei (Hoescht 33342 staining).

**RNA isolation and real-time PCR.** Total RNA was extracted from EC and mature adipocytes and real-time PCR analyses were performed as previously described (10). Primers (Taqman® gene expression assays from Applied Biosystems, Lifetechnologies, France) are listed in the Supplemental Table in the online appendix available at http://diabetes.diabetesjournals.org.

**Culture and treatment of SAT-EC.** Senescence-assoaciated-β-galactosidase (SA-β-gal) activity was determined in human SAT-EC isolated from non-obese healthy women (mean BMI ± SEM=25.34±1.033, range 22.0-28.5; mean age ± SEM =48.5±3.074, range 35-57) and treated or not with adipocyte-conditioned media from SAT and VAT (pretreated or not for 1 h with 1 µg/mL VEGFA neutralizing antibody [R&D systems, Lille, France] or with VEGFA [10 ng/mL; Peprotech, Levallois-Perret, France]). Cells were fixed (5 min, room temperature) in 0.5% glutaraldehyde, washed with PBS, and incubated at 37°C for 16 h with fresh SA-β-gal staining solution (1 mg/ml of 5-bromo-4-chloro-3-indolyl galactopyranoside in
dimension formamide) supplemented with 0.12 mM potassium ferrocyanide, 0.12 mM potassium ferricyanide, and 1 mM MgCl₂ (pH 6). The enzymatic reaction was stopped with water. Cells positive for SA-β-gal activity were counted by phase contrast microscopy through scanning the whole well and normalized to total number of cells determined in four different fields with Hoescht 33342 nuclear staining using NIS software.

**Statistical analyses.** Statistical analyses were performed with GraphPad Software (San Diego, USA). Values are expressed as means ± SEM of (n) independent experiments. Correlations were determined with the Spearman test. Comparisons between the two groups (paired biopsies from SAT and VAT) were analysed by paired t-tests. Comparisons among groups were made by one way analysis of variance (ANOVA) followed by a Dunnett post-hoc test. Differences were considered significant when P<0.05.

**RESULTS**

**Adipocyte size and hypoxia-related gene expression in human subcutaneous and visceral AT (SAT and VAT).** Human mature adipocytes were isolated from paired biopsies of SAT and VAT from obese subjects. Adipocytes were classified according to their diameter (i.e., small, with a diameter less than 60 µm, and large, with a diameter more than 100 µm) and the expression of genes was analyzed by real-time PCR. The proportion of large adipocytes was higher in SAT than VAT (Table 1). Expression of hypoxia-related genes, such as hypoxia-inducible factor (HIF)-1α, and certain HIF1-responsive genes (vascular endothelial growth factor A [VEGFA] and glucose transporter 1 [GLUT1]), was higher in VAT than SAT adipocytes (Figure 1A). In contrast, other genes regulated by hypoxic conditions and induced by HIF-1α, including leptin and the fasting-induced adipose factor (FIAF), exhibited the inverse profile (Figure 1A). To define further the impact of hypoxia and adipocyte size on adipocyte gene expression, correlations among transcript levels and percentages of large adipocytes were calculated. Adipocyte transcript levels of VEGFA and GLUT1 were positively correlated with HIF1-α mRNA, irrespective of fat depot origin (* P=0.03; Spearman r=0.2425, n=60 [SAT and VAT] and ** P<0.0001; Spearman r=0.4744, n=60 [SAT and VAT], respectively). However, leptin and FIAF mRNAs were positively correlated with the percentage of large adipocytes (* P=0.012; Spearman r=0.3302, n=60 [SAT and VAT] and * P=0.014; Spearman r=0.3231, n=60 [SAT and VAT], respectively). No correlation was found between the percentage of large adipocytes and transcript levels of HIF-1α, GLUT1, or VEGFA. Finally, the specific impact of low oxygen tension on adipocyte VEGFA and GLUT1 expression was confirmed by real-time PCR analysis of mature SAT adipocytes maintained in culture for 24 h under normoxic or hypoxic (1% O₂) conditions. VEGFA and GLUT1 transcript levels were increased under hypoxic culture conditions whereas leptin and FIAF were not altered substantially (Figure 1B). Thus, human VAT adipocytes exhibit hypoxia-related characteristics, with increased HIF-1α, VEGFA, and GLUT1 expression, independently of fat cell size.

**Vascular network and EC abundance in human SAT and VAT.** To test if a less extensive vascular network in VAT contributes to higher hypoxia-related gene expression in VAT than SAT in obese subjects, SAT and VAT were analyzed using three dimensional confocal immunofluorescence microscopy for the EC markers, CD34 and CD31. The apparent density of the vascular network, determined from colocalized CD34 and CD31 positive signals, was greater in VAT than SAT (Figures 2B and 2A, respectively). This was
confirmed by flow cytometry of SVF from both depots. The number of CD34+/CD31+ cells, normalized for tissue weight, was significantly higher in VAT than SAT (Figure 2C). Thus, capillary density is higher in human VAT than SAT in obese subjects. To note, the EC number in SAT of lean (n=42) subjects was similar to that of obese subjects (20378±2554 vs 25476±3211 cells/g AT, respectively; p=0.1), indicating that growth of SAT is associated with concomitant expansion of its capillary network.

The EC phenotype of human SAT and VAT. To determine if hypoxia-related gene expression in VAT adipocytes is related to endothelial dysfunction, native human CD34+/CD31+ cells were isolated by immunoselection/depletion from SAT and VAT from obese subjects in those biopsies that were of sufficient size. Genes involved in inflammation (chemokines and adhesion molecules), angiogenesis, metabolism, and cellular senescence were analyzed by real-time PCR. Many of the genes encoding pro-inflammatory and angiogenic factor receptors were up-regulated in VAT compared to SAT-EC, including CC motif ligand 20 (CCL20), chemokine CXC motif ligand 8 (CXCL8), intercellular adhesion molecule-1 (ICAM1), vascular endothelial growth factor receptor 2 (VEGFR2), leptin receptor (Leptin R), and neuropilin 1 and 2 (NRP1 and 2; Figures 3A and 3B, respectively). Expression of genes involved in metabolism, such as endothelial lipase (EL), LPL, PPARγ, and GPIHBP1, tended to be higher in SAT than VAT-EC, although differences for EL and LPL did not reach statistical significance (Figure 3C). Immunohistochemical analyses confirmed the increased expression of ICAM1 and decreased expression of GPIHBP1 in EC from VAT compared to SAT in obese subjects (Figure 4B-4C and 4E-4F, respectively). To note, both proteins were expressed at similar levels in SAT from lean and obese subjects (Figure 4A, 4D).

Moreover, real-time PCR analyses performed in isolated SAT-EC from lean subjects (n=6) did not differ substantially in CCL20, CXCL8, ICAM1, Leptin R, NRP2, and EL compared to obese SAT-EC (data not shown). Finally, expression of the deacetylase, SIRT1, was decreased while insulin-like growth factor binding protein 3 (IGFBP3), a gene up-regulated in senescent EC, was increased in VAT-EC from obese subjects compared to SAT-EC (Figures 5A and 5B, respectively). No differences in SIRT1 and IGFBP3 expression were detected in SAT-EC from lean and obese subjects (data not shown). To test if cellular senescence is more extensive in VAT than SAT-EC, immunofluorescence analyses were performed using an antibody directed against γ-H2AX, a marker of senescent nuclei. γ-H2AX positive nuclei were observed in both SAT and VAT (Figures 5C and 5D, respectively), but at a higher density in VAT-EC (3.3-fold increase in VAT vs SAT, p=0.0004 **, n=10).

Effects of the SAT and VAT microenvironment on senescence of human EC. To define effects of the SAT and VAT microenvironments on EC, native SAT-EC from non-obese women were treated with SAT and VAT adipocyte-conditioned media. Adipocyte-conditioned media clearly increased the number of SA-β-gal positive EC compared to control basal medium (Figures 6B and 6A, respectively). Moreover, native SAT-EC treated with VAT conditioned medium developed a significantly higher percentage of senescent cells than the same native SAT-EC cells treated with SAT-derived conditioned medium (Figure 6C). EC proliferation was significantly increased by both SAT and VAT adipocyte-conditioned media, but to a greater extent in VAT than SAT-EC (Figure 6D). Finally, to analyse the potential factor(s) involved in such a senescence-promoting effect of adipocyte-conditioned media, SAT-EC were treated with VEGFA alone or with SAT- and VAT-
derived conditioned media in the presence of neutralizing VEGFA antibody. Whereas VEGFA increased the number of SA-β-gal positive EC, the presence of a neutralizing VEGFA antibody reduced significantly the effects of SAT- and VAT-conditioned media (Figure 6C).

DISCUSSION

We found greater expression of hypoxia-related genes and smaller sizes of VAT than SAT adipocytes in obese subjects. The increased hypoxia in VAT is not likely to be a consequence of capillary rarefaction since vascular density, as well as EC number, were higher in VAT than SAT. However, the VAT-EC phenotype in obese subjects was markedly pro-angiogenic and inflammatory, with decreased expression of metabolism-related genes, including EL, GPIHBP1, and PPARγ. This phenotype of VAT-EC in obese subjects could be related to premature EC senescence, as suggested by expression of the senescence markers, IGFBP3 and γ-H2AX, as well as decreased expression of SIRT1.

AT is regionally distinct in terms of function, adipokine production, and inflammation. Adipocytes from VAT appear to have reduced capacity for lipogenesis (17) and greater capacity for lipolysis than SAT cells (15), with VAT containing more pro-inflammatory immune cells than SAT (12). In the present study and consistent with other reports (18; 19), marked hypertrophy of SAT compared to VAT adipocytes was observed in obese subjects. Increased expression of the hypoxia-related genes, HIF-1α, VEGFA, and GLUT1 was found in VAT compared to SAT adipocytes. Despite previous studies reported that leptin and FIAF are induced under hypoxic conditions (20; 21), we found they were lower in VAT than SAT. Irrespective of AT location, both leptin and FIAF transcript levels correlated with adipocyte size, as noted by others for leptin (22). Expression of VEGFA and GLUT1 was tightly linked with that of HIF-1α. Moreover, SAT adipocytes maintained under low oxygen conditions had higher VEGFA and GLUT1 expresssion than VAT, while the expression of both leptin and FIAF was not affected substantially. Together, the present results indicate that hypoxia-related processes are more highly activated in VAT than SAT and are not related to extent of adipocyte hypertrophy in obese subjects. Moreover, qualitative analyses by confocal microscopy and flow cytometry, using both the EC markers CD34 and CD31 simultaneously, reveal that capillary network density was higher in VAT than in SAT. Therefore, hypoxia in VAT is unlikely to be a consequence of capillary rarefaction.

Treatment of native SAT-EC originating from normal non-obese women with conditioned media originating from VAT adipocytes from obese subjects led to marked proliferation compared to conditioned media from SAT adipocytes from the same subjects. This suggests the microenvironment of VAT is more pro-angiogenic than SAT and is consistent with the greater capillary density in VAT than SAT. Whether increased pro-angiogenic induction by VAT adipocytes is related to hypoxia- and inflammation-related events remains to be determined. Our results indicate that VAT-EC from obese subjects exhibited a markedly pro-inflammatory and angiogenic activated state, with increased expression of chemokines, adhesion molecules, and angiogenic factor receptors. This phenotype could contribute to the greater abundance of pro-inflammatory immune cells in VAT than SAT (12). EL and GPIHBP1 are EC-specific metabolic genes, at least in murine models (23). Together with PPARγ (the main regulator of GPIHBP1), these genes were down-regulated in VAT compared to SAT. A recent study in murine models of diet-induced obesity highlighted the key role of PPARγ in the modulation of EC function and metabolic alterations associated with obesity (7). Together our results show,
although the capillary density of VAT is higher than that of SAT, that VAT-EC exhibit a phenotype characterized by marked activation of inflammatory and angiogenic pathways associated with altered metabolic function in obese subjects.

This pro-angiogenic, pro-inflammatory phenotype might be related to premature endothelial cellular senescence. Indeed, senescent EC exhibit an activated state that may induced either by extensive cell replication, leading to premature irreversible cell growth arrest, or by various stresses, including oxidative stress (24). Decreased expression of SIRT1, together with increased expression of IGFBP3 and γ-H2AX, have been noted in senescent, replicatively exhausted human EC (25-28), indications of cellular senescence we found in VAT-EC from obese subjects. Moreover, since conditioned media originating from VAT adipocytes increased the number of SA-β-gal positive EC, it is tempting to speculate that the chronic pro-angiogenic microenvironment of VAT promotes premature EC senescence, leading to endothelial dysfunction. Interestingly, VEGFA alone enhanced the number of SA-β-gal positive EC, although to a lesser extent than adipocyte-conditioned media. Moreover, neutralization of VEGFA reduced the senescence-promoting effect of the SAT and VAT adipocyte-conditioned media, suggesting that VEGFA might be an adipocyte-derived factor involved in premature adipose tissue EC senescence, consistent with its effects on EC senescence in other systems (29; 30).

Cellular senescence in other cell types is associated with a senescent secretory phenotype, with increased production of pro-inflammatory cytokines, chemokines, and extracellular matrix-modifying proteins, as well as angiogenic factors (31-35). It will be important to define the secreted protein profile of senescent VAT-EC and SAT-EC and to determine whether such a phenotype is involved in accumulation of immuno-inflammatory cells. Consistent with this possibility, we previously showed that treatment of SAT-EC with SAT adipocyte-conditioned media led to increased diapedesis of blood-derived monocytes (10).

The subjects in our study were obese. Whether regional variation in fat tissue EC properties are already present in lean subjects or arise as a consequence of obesity needs to be determined. Since cellular senescence is increased in obesity (36) and since obesity is associated with aortic endothelial cell senescence (37), it is tempting to speculate that obesity could induce senescence in VAT-EC to a greater extent than in SAT-EC. Consistent with this speculation, our study shows that SAT-EC from lean and obese subjects did not exhibit marked phenotypic differences or numbers relative to AT weight. If comparative studies of VAT-EC from lean and obese subjects validate our speculation, interventions that limit accumulation of senescent EC or their pro-inflammatory state could improve the approaches to limit inflammation due to obesity and its complications.

**Author Contributions.** A.V. research data, wrote manuscript, contributed discussion, J.G. research data, reviewed/edited manuscript, contributed discussion, P.D. research data, D.E. research data, M.A.M research data, C.S. research data, M.L. reviewed/edited manuscript, contributed discussion, A.B. research data, wrote manuscript, reviewed/edited manuscript, contributed discussion, P.C. research data, T.T. reviewed/edited manuscript, contributed discussion, J.L.K. reviewed/edited manuscript, contributed discussion.

**ACKNOWLEDGMENTS**
We thank Dr. Bénarous, MD., plastic surgeon, Clinique du Parc, Toulouse, France, for his technical support. We are grateful for the
excellent technical support and advice of B. Payre and Romina D’Angelo, Cellular Imaging Facility IFR150-Rangueil/TRI Plateform, Toulouse, France, for confocal microscopy.

Funding sources. This study was supported by grants from INSERM (AVENIR), the Agence Nationale de la Recherche (ANR “RIOMA”), the European Union (FP7 ADAPT HEALTH-F2-2008-2010), the Laboratoires Sérobiologiques Division of COGNIS, NIH grants AG13925 and AG031736 (J.L.K.), the Noaber Foundation (J.L.K.), and the Ted Nash Long Life Foundation (J.L.K.). This work was sponsored by the University Hospital of Toulouse (regulatory and ethics submission No. 06 029 03).

Disclosures. Nothing to disclose

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Table 1. Human SAT and VAT adipocyte sizes

|                | SAT (% ± SEM) | VAT (% ± SEM) | P value |
|----------------|--------------|---------------|---------|
| Adipocytes <60 µm | 29.1±3.6     | 33.8±3.0      | 0.12 ns |
| Adipocytes >100 µm | 33.2±3.0     | 19.1±2.2      | 0.0003**|

Data are percentages±SEM of small (< 60 µm) and large adipocytes (100 µm) in paired samples of SAT and VAT from n=30 subjects. * P<0.05, ** P<0.01; paired t tests between SAT and VAT.

Figure legends

Figure 1: Hypoxia-related genes in human SAT and VAT adipocytes
(A) Comparison of mature adipocyte gene expression in SAT and VAT (n=30). Hypoxia inducible factor 1 or 2, alpha subunit (HIF-1α, HIF-2α), glucose transporter 1 (GLUT1), vascular endothelial growth factor A (VEGFA), and fasting-induced adipose factor (FIAF). Open bars: genes upregulated and solid bars: genes downregulated in VAT compared to SAT. Results are expressed as fold differences between VAT and SAT as means±SEM. * P<0.05, ** P<0.01; paired t tests between SAT and VAT.
(B) SAT adipocyte gene expression under normoxic (Nx) and hypoxic conditions (1% O2) (n=6). Open bars: gene expression under normoxic conditions and solid bars: gene expression under hypoxic conditions. Results are expressed as means±SEM ** P<0.01; paired t tests between normoxic and hypoxic conditions.

Figure 2: Vascular network and EC number in human SAT and VAT
(A-B) Representative photomicrographs of three dimensional confocal immunofluorescence analyses of human (A) SAT and (B) VAT using antibodies directed against CD34 (green) and CD31 (red) (n=5). Original magnification ×10.
(C) Flow cytometry analyses were performed on freshly harvested SVF using fluorescently-labeled antibodies directed against CD34 and CD31 (n=30). Open bar: SAT and solid bar: VAT. Results are means±SEM of the number of CD34+/CD31+ cells per gram of AT * P<0.05; paired t tests between the two AT depots.

Figure 3: Expression of inflammatory, angiogenic, and metabolic genes in human SAT- and VAT-EC
(A-C) Comparison of SAT and VAT-EC gene expression. CC motif ligand 20 (CCL20), CC motif ligand 2 (CCL2), chemokine CXC motif ligand 8 (CXCL8), platelet endothelial cell adhesion molecule-1 (PECAM1), intercellular adhesion molecule-1 (ICAM1), vascular endothelial growth factor receptor 1 and 2 (VEGFR1 and 2), leptin receptor (Leptin R), neuropilin 1 and 2 (NRPI and 2), endothelial lipase (EL), lipoprotein lipase (LPL), peroxisome proliferator-activated receptor gamma (PPARγ), and GPI-anchored HDL-binding protein 1 (GPIHBP1). Open bars: genes upregulated and solid bars: genes downregulated in VAT compared to SAT. Results are expressed as fold differences between VAT and SAT-EC as means±SEM (n=7 to 11) * P<0.05, ** P<0.01; paired t tests between SAT and VAT-EC.

Figure 4: Expression of ICAM1 and GPIHBP1 proteins in SAT and VAT
Representative photomicrographs of immunofluorescence analyses of human SAT from lean subjects (n=4, 5) (A, D), obese subjects (n=6, 9) (B, E) and VAT from obese subjects (n=6, 9) (C, F) using antibodies directed against ICAM1 (green) and CD34 (red) (A, B, C) GPIHBP1 (green) (D, E, F) with nuclear staining with Hoescht 33342 (blue). Original magnification ×40.
**Figure 5: Markers of senescence in native EC in SAT and VAT**

(A-B) Expression of (A) sirtuin 1 (SIRT1, n=9) and (B) insulin-like growth factor binding protein 3 (IGFBP3, n=11) in SAT and VAT-EC. Open bars: SAT-EC and solid bars: VAT-EC. Results are expressed as means±SEM * P<0.05, ** P<0.01; paired t tests between SAT- and VAT-EC.

(C-D) Representative photomicrographs of immunohistofluorescence analyses of human (C) SAT and (D) VAT using antibody directed against phospho-γ-H2AX (red) and nuclear staining (blue) with Hoescht 33342 (n=10). Original magnification ×40.

**Figure 6: Effect of the SAT and VAT microenvironment on EC senescence and proliferation**

(A-B) Representative photomicrographs of native SAT-EC cultured in the presence (B) or absence (A) of adipocyte-conditioned media (n=6). Original magnification ×20.

(C) Percentage of SA-β-gal positive EC. Results are expressed as means±SEM as percent of control in the presence of VEGFA (n=3) (dotted) or adipocyte-conditioned media from SAT (open) or VAT (solid) treated (hatched) or not with VEGFA neutralizing antibody (n=3) * P<0.05, **, P<0.01 vs. control; $ P<0.05, $$ P<0.01, adipocyte-conditioned media alone vs. containing VEGFA neutralizing Ab.

(D) Number of EC expressed as percentage of control. Hatched bar: control, open bar: SAT and solid bar: VAT. Results are expressed as means±SEM as percent of control * P<0.05, ** P<0.01, n=3.
Figure 1

A

[Fold difference in VAT gene expression compared to SAT]

B

[Gene expression (% control)]

Human adipose endothelial cells
Figure 2

A

B

C

CD34+CD31+ cell number (lg of AT)

SAT

VAT

*
Figure 3

A

- CCL20
- CCL2
- CXCL8
- PECAM1
- ICAM1

Fold difference in VAT gene expression compared to SAT

B

- VEGFR1
- VEGFR2
- Leptin R
- NRP1
- NRP2

Fold difference in VAT gene expression compared to SAT

C

- EL
- LPL
- PPARγ
- GPIHBP1

Fold difference in VAT gene expression compared to SAT

Human adipose endothelial cells
Figure 5

A  

**SIRT1**

Gene expression (AU)

|       | SAT | VAT |
|-------|-----|-----|
|       | 0.3 | 0.4 |

B  

**IGFBP3**

Gene expression (AU)

|       | SAT | VAT |
|-------|-----|-----|
|       | 8   | 10  |

C  

Image of SAT and VAT cells

D  

Image of SAT and VAT cells with different staining
Figure 6

A
Control

B
+ Adipocyte-conditioned medium

C

D

SA-βgal positive EC (% control)

EC cell number (% control)

VegfA  SAT  +Ab  VAT  +Ab

Ctrl  SAT  VAT

**  *  *