Remodeling Phenotype of Human Subcutaneous Adipose Tissue Macrophages

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Background—Adipose tissue macrophages (ATMs) have become a focus of attention recently because they have been shown to accumulate with an increase in fat mass and to be involved in the genesis of insulin resistance in obese mice. However, the phenotype and functions of human ATMs are still to be defined.

Methods and Results—The present study, performed on human subcutaneous AT, showed that ATMs from lean to overweight individuals are composed of distinct macrophage subsets based on the expression of several cell surface markers: CD45, CD14, CD31, CD44, HLA-DR, CD206, and CD16, as assessed by flow cytometry. ATMs isolated by an immunoselection protocol showed a mixed expression of proinflammatory (tumor necrosis factor-α, interleukin-6 [IL-6], IL-23, monocyte chemoattractant protein-1, IL-8, cyclooxygenase-2) and antiinflammatory (IL-10, transforming growth factor-β, alternative macrophage activation–associated cc chemokine-1, cyclooxygenase-1) factors. Fat mass enlargement is associated with accumulation of the CD206+/CD16− macrophage subset that exhibits an M2 remodeling phenotype characterized by decreased expression of proinflammatory IL-8 and cyclooxygenase-2 and increased expression of lymphatic vessel endothelial hyaluronan receptor-1. ATMs specifically produced and released matrix metalloproteinase-9 compared with adipocytes and capillary endothelial cells, and secretion of matrix metalloproteinase-9 from human AT in vivo, assessed by arteriovenous difference measurement, was correlated with body mass index. Finally, ATMs exerted a marked proangiogenic effect on AT-derived endothelial and progenitor cells.

Conclusions—The present results showed that the ATMs that accumulate with fat mass development exhibit a particular M2 remodeling phenotype. ATMs may be active players in the process of AT development through the extension of the capillary network and in the genesis of obesity-associated cardiovascular pathologies.

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Key Words: angiogenesis ■ inflammation ■ leukocytes ■ metalloproteinases ■ obesity

Tissue macrophages exhibit a broad range of physiological functions such as the clearance of apoptotic cells and the remodeling and repair of tissues. Thought to originate from blood-circulating monocytes, they show a high degree of heterogeneity, depending mostly on the microenvironment of their anatomic locations.1 In addition, at the site of inflammation, monocytes are recruited and differentiate into macrophages. In vitro experiments performed with mouse and human monocyte-derived macrophages have defined several activation states. Among them, the classic M1 polarization state defines the proinflammatory macrophages and initiates the inflammatory reaction, whereas the reparative antiinflammatory macrophages, or M2 state, terminate the inflammatory process.2

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Several markers, including cell surface receptors, chemokines, cytokines, and free radical–producing and matrix-degrading enzymes, have been described as hallmarks of the macrophage phenotype such as tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), inducible nitric oxide synthase (iNOS), and cyclooxygenase-2 (COX-2) expressed in M1 macrophages and IL-10 and transforming growth factor-β (TGF-β) in M2 macrophages.3,4 Conversely to the activated macrophages, the resident macrophage populations of normal or pathological tissues in humans are not fully defined. However, increasing evidence has shown that macrophages associated with pathological tissues exhibit a peculiar profile.
For example, tumor-associated macrophages, characterized by the production of proangiogenic factors such as TGF-β, TNF-α, matrix metalloproteinase-9 (MMP-9), and vascular endothelial growth factor-A (VEGF-A), have been shown to be associated with tumor progression and metastasis.5 Macrophages present in atherosclerotic plaques have been involved in plaque progression and recently have been shown to release gelatinase activity in vivo.6 Recently, the population of resident macrophages present in adipose tissue (ie, adipose tissue macrophages [ATMs]) has been the focus of particular attention. Overweight and obese patients are known to exhibit a higher risk of developing type 2 diabetes and cardiovascular pathologies and are characterized by a moderate plasma increase in several inflammatory markers such as acute-phase proteins, IL-6, and monocyte chemoattractant protein-1 (MCP-1).7 We and others have shown that the number of macrophages in human subcutaneous and visceral ATs increases with the degree of adiposity.8–10 Similar data have been reported in mouse models of obesity.11,12 In mice, macrophage accumulation within the fat mass induced by a high-fat diet appeared to be more related to the insulin-resistant state associated with obesity than to the increase in the fat mass.12 Indeed, distinct approaches have shown that both the recruitment and proinflammatory activation of ATMs are required for the development of insulin resistance in obese mice.11,13,14 Given the large heterogeneity in macrophage subsets and phenotypes and the described discrepancies between human and murine macrophages,3,5 the present study was undertaken to characterize the phenotype of the human ATMs, to define their contribution to the production of adipokines, and to analyze their potential changes related to the degree of adiposity, as well as their function within the fat mass.

**Methods**

**Materials**

Chemicals were from Sigma (St Quentin Fallavier, France). Collagenase NB4 was from Serva (Coger, Paris, France). Selection kits for CD34+ and CD14+ cells were from StemCell Technologies (Grenoble, France), and magnetic microbeads coupled with anti-CD31 antibodies were from Dynal Biotech (Invitrogen, Cergy Pontoise, France). Culture media were from Promocell (Heidelberg, Germany). Antibodies for flow cytometry analysis were from BD Biosciences (Le Pont de Claix, France) or Caltag (Invitrogen).

**Isolation of the Stroma-Vascular Fraction Cells and Mature Adipocytes From Human AT**

Human AT was obtained from healthy women undergoing elective procedures for fat removal for esthetic purposes. Their body mass index (BMI) ranged from 20 to 30 kg/m² (ie, lean to overweight). The protocol of fat collection was approved by the Institutional Research Board of INSERM and Toulouse University Hospital. Liposuction aspirates from subcutaneous abdominal AT were processed immediately after removal. The stroma-vascular fraction (SVF) cells and mature adipocytes were isolated as previously described.8,16 SVF cells were further analyzed by flow cytometry (age, 42±1 years; BMI, 24.4±0.3 kg/m²; n=78). The distinct cell fractions of the SVF (age, 49±3 years; BMI, 25.5±0.7 kg/m²; n=17) were isolated using an immunoselection/depletion protocol as previously described.8,16 Freshly isolated CD34+/CD31− cells defined as progenitor cells, CD34+/CD31+ cells defined as capillary endothelial cells, and CD34−/CD14− cells defined as macrophages were either lysed in RLT lysis buffer and stored at –20°C for mRNA extraction or cultured for further analysis. Conditioned media from capillary endothelial cells and macrophages were obtained from cells cultured for 24 hours in endothelial cell basal medium (ECBM)/0.1% bovine serum albumin (BSA) (200 000 cells/cm²) and from mature adipocytes as previously described.8

**Flow Cytometry Analysis**

In the flow cytometry analysis, 100 000 cells of the SVF or whole-blood samples (age, 36±1 years; BMI, 22.9±0.7 kg/m²; n=4) were incubated with fluorescein isothiocyanate-conjugated antibody (CD31, HLA-DR, CD16, CD62L), PerCP-conjugated antibody (CD45), phycoerythrin (PE)-conjugated antibody (CD14), and allophycocyanin (APC)-conjugated antibody (CD206, CD44, CCR2). An equal number of cells was labeled with the respective isotype control. Analyses were performed with a FACSCalibur flow cytometer and the CellQuest Pro software (BD Biosciences).

For cell sorting, mononuclear cells from the SVF were isolated by labeling total SVF on Histopaque-1077 (Sigma). After centrifugation (800g for 30 minutes), ATMs were directly isolated by the CD14-coupled beads immunoselection approach and maintained overnight in ECBM/0.1% BSA. ATMs were labeled with PE-conjugated anti-CD16 or respective isotype control (BD Biosciences), and CD14+ /CD16- and CD14+ /CD16+ cells were separated with an Epics ALTRA cell sorter (Beckman Coulter, Fullerton, Calif). Purity of the sorted cells was analyzed by flow cytometry (96% to 98% purity). For K67 experiments, isolated ATMs were permeabilized in 70% ethanol (overnight at 4°C) before labeling and flow cytometry analysis.

**RNA Extraction and Real-Time Polymerase Chain Reaction**

Total RNA was extracted from ATMs and blood monocytes with the RNasy kit (Qiagen). The RNA concentration was determined with a fluorometric assay (Ribogreen, Invitrogen). RNA was reverse transcribed with the Superscript II kit (Invitrogen). Reverse transcription also was performed without the superscript enzyme on RNA samples to ensure the absence of contaminating genomic DNA. Primers for TNF-α, IL-6, IL-8, IL-10, IL-23, TGF-β, MCP-1, alternative macrophage activation-associated cc chemokine 1 (AMAC-1), iNOS, arginase 1, COX-1, COX-2, MMP-2, MMP-9, VEGF-A, lymphatic vessel endothelial hyaluronan receptor 1 (LYVE-1), and lipoprotein lipase were from Applied Biosystems (Courtaboeuf, France) (Hs00174128_m1, Hs00174131_m1, Hs_00174103_m1, Hs00174086_m1, Hs00372324_m1, Hs00171257_m1, Hs00234140_m1, Hs00268113_m1, Hs00167257_m1, Hs00163660_m1, Hs00168776_m1, Hs00153133_m1, Hs00234422_m1, Hs00234579_m1, Hs00173626_m1, Hs00272659_m1, and Hs00173425_m1, respectively). The amplification reaction was performed in duplicate on 15-ng cDNA samples in a final volume of 20 μL in 96-well reaction plates (Applied Biosystems) in a GeneAmp 7500 detection system. All reactions were performed under the same conditions: 50°C for 2 minutes, 95°C for 10 minutes, 40 cycles of 95°C for 15 seconds, and 60°C for 1 minute. Results were analyzed with the GeneAmp 7500 software, and all values were normalized to the levels of 18S rRNA.

**Gelatin Zymography**

Proteins with gelatinolytic activity (ie, MMP-2 and MMP-9) were identified as previously described.17

**In Situ Arterial and Venous MMP-9 Plasma Determination**

In situ arteriovenous differences were measured in healthy subjects (age, 36.07±3.29 years; BMI, 24.82±0.67 kg/m²; n=15). They ate a low-fat evening meal and were studied the next morning after an overnight fast. The studies were approved by the Central Oxford Research Ethics Committee, and all subjects gave
Informed consent. A catheter (22 gauge×10 cm; Secalon Hydrocat, Ohmeda, Swindon, UK) was introduced into a small vein on the abdominal wall and advanced into the superficial epigastric vein until its tip lay near, but superior to, the inguinal ligament. As described previously,18,19 blood obtained in this way has all the characteristics of the venous drainage from AT. A cannula was inserted retrogradely into a vein draining a hand that was warmed in a box (65°C) to provide arterialized blood.20 After 30 minutes of rest, blood samples were taken simultaneously from the arterialized vein and subcutaneous abdominal catheter. Plasma MMP-9 was quantified with an ELISA kit (Chemicon/Millipore, St Quentin en Yvelines, France).

**In Vitro Angiogenesis Assay**

Tube-like formation was assessed with capillary endothelial cells isolated from AT. Then, 200 μL per well growth factor-reduced matrigel was poured into 48-well plates and polymerized (30 minutes at 37°C). Next, 15,000 cells per well were plated on the polymerized matrigel and treated with ECBM/0.1% BSA (control) or macrophage-conditioned medium for 24 hours. The branching formation was observed by phase microscopy (Nikon, Germany), and the length of cytoplasmic extensions was measured with LUCIA image software on 3 distinct fields (Nikon, Germany).

**Progenitor Cell Culture**

Isolated progenitor cells (120,000 cells/cm²) were plated on fibronectin-coated 48-well plates in ECBM/10% FCS. After 24 hours, adipogenic and angiogenic differentiation was induced by ECBM supplemented with 0.5 ng/mL VEGF, 20 ng/mL insulin growth factor-I, 66 nmol/L insulin, 10 μg/mL transferrin, 1 nmol/L triiodothyronine, and 1 μg/mL rosiglitazone. Cells were cultured either in a mixture (vol/vol) of 2% defined medium and macrophage-conditioned medium or in a mixture (vol/vol) of 2% defined medium and ECBM/0.1% BSA (control). Media were changed every 2 days until day 8. Cells were then either scraped in PBS/2% BSA, followed by a 2-hour incubation with CD31 mouse monoclonal antibody (1/10) (Dako, Trappes, France). After washing (PBS/0.2% Tween), cells were incubated for 1 hour with the corresponding fluorescence-labeled second antibody (goat anti-mouse coupled to AlexaFluor 488, Invitrogen) (1/100). Cells were washed and incubated for 10 minutes with 10 μg/mL Hoescht 33258 (Invitrogen). The fluorescently labeled cells were washed with distilled water, incubated for 15 minutes with Oil Red O solution (0.3% in 60% isopropanol), and washed again before direct observation with a fluorescence microscope (Nikon).

**Tissue Immunochemistry**

Tissue immunochemical analyses were performed on freshly harvested human subcutaneous AT cut into small pieces. After fixation in acetone (30 minutes at 4°C), the tissue pieces were incubated for 15 minutes in PBS/0.1% Triton for permeabilization and for 30 minutes in PBS/2% BSA, followed by an overnight incubation with mouse monoclonal antibody against CD45 (Chemicon) and rabbit polyclonal antibody against Ki67 (Dako) (1/50). After washing (PBS/0.2% Tween), the tissue pieces were incubated for 1 hour with the corresponding fluorescent-labeled second antibodies (goat anti-mouse or goat anti-rabbit coupled to AlexaFluor 546 or 488; Invitrogen) (1/200). The pieces were washed again, incubated for 10 minutes with 10 μg/mL Hoescht 33258, and washed once in PBS. They were then placed between 2 mounting slides and examined with a fluorescence microscope.

**Statistical Analysis**

Values are given as mean±SE for separate experiments. Correlations were performed with Spearman’s rank correlation for nonnormally distributed data, and comparisons between groups were analyzed by either a paired t test or 1-way ANOVA, followed by a Newman-Keuls post hoc test (Prism 4, GraphPad Software). Differences were considered significant when \( P<0.05 \).

The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.
Results

Characterization of the CD14+ Cells as AT Resident Macrophages

Multiparameter fluorescent-activated cell sorter analyses were performed on freshly isolated SVF of human subcutaneous AT from lean to overweight patients. As depicted in Figure 1A and 1B, ATMs, characterized by the expression of the common leukocyte antigen CD45, the monocyte differentiation antigen CD14, the leukocyte surface glycoprotein CD44, the class II histocompatibility antigens HLA-DR, and the platelet and endothelial cell adhesion molecule CD31, were constituted mainly by cells positive for the mannose receptor CD206. Within the CD14+/CD206+ population, 2 distinct cell subsets were identified on the basis of the expression of the immunoglobulin G Fc receptor III (CD16), with the major population characterized as CD14+/CD206+/CD16-. Total ATMs were isolated from the SVF by a CD14 immunoselection approach, and the expression of known markers of the macrophage differentiation, ie, CD206, lipoprotein lipase, and MMP-9, was studied in freshly isolated ATMs and blood monocytes by flow cytometry and real-time polymerase chain reaction (PCR) analyses. ATMs specifically expressed all 3 macrophage markers, together with LYVE-1, recently described in a murine macrophage subset involved in tissue remodeling21 (Figure 2A). In addition, flow cytometry analyses showed that ATMs lack the expression of CCR2 and CD62L (Figure 2B), both of which are involved in the recruitment of inflammatory monocytes. Moreover, immunohistochemical and flow cytometry analyses using Ki67 antibodies identified proliferative ATMs within the AT (Figure 2C and 2D).

ATM Phenotype and Secretory Activity

The ATM expression of M1 and M2 activation state markers (ie, cytokines and chemokines, iNOS, arginase,
pared with the arterial plasma (Figure 3D).

Arteriovenous measurements revealed that the epigastric gelatinolytic activity released by adipocytes, macrophages, and endothelial cells (Figure 3C). Finally, the production of MMP-9 by human AT was shown in situ. Indeed, MMP-9 was found to be expressed mainly in ATMs, whereas VEGF-A and arginase-1 and COX) was assessed by real-time PCR. As depicted in Table 1, total ATMs expressed both M1 and M2 markers. Markers related to arginine metabolism, arginase-1 and iNOS, were poorly or not expressed, respectively. No marked differences in gene expression between the subtypes of ATM sorted on the basis of CD16 expression were observed, although the CD14+/CD16- ATMs tended to exhibit higher transcript levels both for the M1 markers IL-6 and MCP-1 and for LYVE-1 (Table 2). To assess the specific contribution of ATMs in the secretory activity of human AT, the expression of the inflammatory cytokines MCP-1 and IL-6, as well as the transcripts for adipokines involved in remodeling processes such as the proangiogenic VEGF-A, MMP-2, and MMP-9, was determined by real-time PCR in mature adipocytes, freshly isolated ATMs, and capillary endothelial cells from AT. Comparative analyses clearly showed that MCP-1 and IL-6 are expressed mainly in human AT capillary endothelial cells, as shown in Figure 3A. Interestingly, MMP-9 was found to be expressed mainly in ATMs, whereas VEGF-A and MMP-2 (data not shown) were found to be expressed in the 3 cell types (Figure 3B). The ATM-specific expression of MMP-9 was confirmed by comparative analysis of the gelatinolytic activity released by adipocytes, macrophages, and endothelial cells (Figure 3C). Finally, the production of MMP-9 by human AT was shown in situ. Indeed, arteriovenous measurements revealed that the epigastric venous plasma that drains the subcutaneous abdominal AT was statistically significantly enriched in MMP-9 compared with the arterial plasma (Figure 3D).

Table 1. M1 and M2 Macrophage Activation Markers Expressed in Human ATMs

<table>
<thead>
<tr>
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<th>M1 Markers</th>
<th>M2 Markers</th>
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<tr>
<td>Proinflammatory or antiinflammatory cytokines</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TNF-α</td>
<td>++</td>
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<tr>
<td>IL-6</td>
<td>+</td>
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<td>IL-23</td>
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<tr>
<td>IL-10</td>
<td>++</td>
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<tr>
<td>TGF-β</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>Chemokines</td>
<td></td>
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<tr>
<td>MCP-1 (CCL-2)</td>
<td>+</td>
<td></td>
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<tr>
<td>IL-8 (CXCL-8)</td>
<td>+++</td>
<td></td>
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<tr>
<td>AMAC-1 (CCL-18)</td>
<td>++</td>
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<tr>
<td>Arginine metabolism enzymes</td>
<td>-</td>
<td>+/-</td>
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<tr>
<td>iNOS</td>
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<tr>
<td>ARG-1</td>
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<tr>
<td>Cyclooxygenases</td>
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<tr>
<td>COX-2</td>
<td>++</td>
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<tr>
<td>COX-1</td>
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Table 2. Expression of Macrophage Markers in Human Total ATMs and CD14+/CD16+ and CD14+/CD16- ATM Subtypes

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<thead>
<tr>
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<th>Total ATMs</th>
<th>CD14+/CD16+</th>
<th>CD14+/CD16-</th>
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<tbody>
<tr>
<td>IL-6</td>
<td>++</td>
<td>+</td>
<td>+</td>
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<tr>
<td>TGF-β</td>
<td>++</td>
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<tr>
<td>MCP-1 (CCL-2)</td>
<td>++</td>
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<tr>
<td>IL-8 (CXCL-8)</td>
<td>++</td>
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<tr>
<td>COX-2</td>
<td>++</td>
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<tr>
<td>COX-1</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>MMP-9</td>
<td>++</td>
<td>+</td>
<td>+</td>
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<tr>
<td>LYVE-1</td>
<td>+</td>
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Total ATMs and CD14+/CD16+ and CD14+/CD16- ATM subtypes were isolated from the SVF (n=4 to 7), and real-time PCR analysis of relevant macrophage markers was assessed by mean 2^ΔΔCt×1000 and categorized as follows: 0.001<+/−<0.01; 0.01<+/−<0.1; 0.1<+/−<1; and 1<+/++.

Influence of the Degree of Adiposity on the ATMs

The percentage of CD14+ cells within the SVF increased with the BMI of the patients (data not shown). However, the positive statistically significant correlation between AT percentage and the degree of adiposity was found to be restricted to the CD45+/CD14+/CD206- population (P=0.019; Spearman r=0.2664; n=61) (Figure 4A). Indeed, BMI did not influence the percentage of the CD45+/CD14+/CD206- cell subset within the SVF (P=0.2; Spearman r=−0.1094; n=61, data not shown). Furthermore, the number of CD45+/CD14+/CD206-/CD16- cells normalized to the weight of AT correlated positively with BMI (P=0.010; Spearman r=0.2986; n=60; Figure 4B), whereas the number of CD45+/CD14+/CD206-/CD16- cells remained constant (P=0.189; Spearman r=−0.1159; n=60; data not shown). In parallel with the increased number of CD45+/CD14+/CD206-/CD16- cells, the difference measurement in MMP-9 concentration between arterial and epigastric venous plasma was statistically positively correlated with the BMI of the subjects, and the ATM expression of LYVE-1 was increased (Figure 4B and 4C, respectively). Finally, to assess a potential change in the activation state of the ATMs with the development of AT, the expression of the M1 and M2 markers was studied in ATMs. Among the studied genes, only IL-8 and COX-2 transcript levels were found to be downregulated (Figure 5A). Treatment of isolated ATMs for 24 hours with leptin led to a reduction in IL-8 and COX-2 expression, whereas adiponectin did not modify their expression (Figure 5B), suggesting that increased leptin production and secretion with obesity might contribute to the changes in IL-8 and COX-2 expression in ATMs.

Human ATMs Control Adipogenesis and Angiogenesis

The potential effects of ATMs on the differentiation of AT progenitor cells were investigated on primary cultures of human AT-derived CD34+/CD31- cells under culture conditions that allowed the expression of specific adipocyte and endothelial cell markers, as previously described. The treatment of AT progenitor cells with AT-conditioned
media for 8 days resulted in reduced adipogenesis, as shown by the decreased Oil Red O staining associated with the reduction in triglyceride content of the cells (Figure 6A). Conversely, the number of cells expressing endothelium-specific genes such as CD31 and the organization in capillary-like structures increased in the presence of secretory products derived from human ATMs. Indeed, the length of the network of cells positive for CD31 showed a statistically significant increase (2-fold increase compared with control). Note that similar effects were observed when cells were treated with CD14+/H11001/CD16+/H11002-conditioned media (1.8-fold increase in CD31-positive network length compared with control; P<0.01; n=4; data not shown).

The proangiogenic effect of ATMs was confirmed with an in vitro angiogenesis assay using AT-derived capillary endothelial cells cultured on growth factor–reduced matrigel. Indeed, as depicted in Figure 6B, ATM-conditioned media promoted the migration and organization of human AT capillary endothelial cells (2.3-fold increase in tube length; P<0.05; n=5).

**Discussion**

Human subcutaneous AT-derived CD14+ cells that were positive for a combination of common monocyte/macrophage markers, ie, CD45, CD14, CD44, CD31, and HLA-DR, also expressed markers distinct from the circulating blood monocytes. Indeed, they are composed mainly of cells expressing CD206, described as being expressed in M2-activated macrophages,23 as well as in tissue-resident macrophages such as tumor-associated macrophages24 and ATMs, as recently described by a study that appeared during the preparation of this article.25 Moreover, freshly isolated ATMs expressed specific macrophage differentiation markers, ie, lipoprotein lipase and MMP-9,21,26 as well as the newly described macrophage marker LYVE-1.21 On the basis of the combined expression of CD206 and CD16,2 ATMs subsets were identified. Comparison of the M1 and M2 gene pattern expressed by ATMs sorted by CD16 revealed discrete discrepancies restricted to higher expression of IL-6, MCP-1, and LYVE-1 in the CD16+ subset, demonstrating that the CD16 expression in human ATMs is not clearly associated with a specific M1 or M2 activation state. However, a marked difference between both subsets was revealed with the growth of the fat mass because only the CD206+/CD16− cells accumulated with increased adiposity, suggesting that both subsets may have distinct origin. Replenishment of tissue macrophages relies on a blood CD16− monocyte subset.27,28 In addition, self-renewal of tissue macrophages through local proliferation has been described.29 Macrophage recruitment to inflammatory sites involves the blood CD16− monocyte subset that coexpresses CCR2 and CD62L.27,28 It is thus tempting to speculate that the CD206+/CD16− ATMs might represent resident macrophages deriving from CD16− monocytes.
whereas the CD206+/CD16− ATMs may originate from recruitment of inflammatory CD16+ monocytes. However, neither CCR2 nor CD62L was detected in the AT CD14+ cells, and the AT minor CD14+/CD206−/CD16+ monocyte subset remained constant with AT growth, suggesting that this population corresponds to contaminating blood cells more than to inflammatory infiltrating monocytes recruited by the growing tissue. Although a rapid local differentiation of inflammatory infiltrating monocytes into macrophages, as recently described in a mouse model of atherosclerosis, cannot be excluded, enhanced local proliferation might be involved in the accumulation of CD206+/CD16− cells. Indeed ATMs exhibited proliferative abilities as shown by the positive Ki67 labeling. However, additional experiments are needed to clearly state the relative contribution of blood-derived cells and local proliferation in the ATM accumulation. Nevertheless, the phenotype exhibited by ATMs in the growing fat mass did not correspond to a classic inflammatory phenotype because both M1 markers, IL-8 and COX-2, were found to be downregulated, whereas LYVE-1 expression was enhanced.

Interestingly, a recent study performed on mice after skeletal injury demonstrated that the phagocytic activity may switch inflammatory recruited monocytes into antiinflammatory macrophages. Moreover, it is now well accepted that the tissue microenvironment can influence the macrophage phenotype.

Leptin and adiponectin are specifically produced and released by adipocytes, and the levels of leptin concentration are directly linked to the degree of adiposity. Leptin treatment of ATMs led to decreased expression of IL-8 and COX-2, whereas adiponectin had no effect. Taken together, these results strongly suggest that the changes in the microenvironment of the growing human AT and, more precisely, in leptin concentration may be involved in the modulation of ATM phenotype.

Extension of the capillary network has been shown to accompany the fat mass enlargement in mice and in humans. It has been suggested that adipocytes exert control over their own vascularization through their production of a wide range of proangiogenic factors. However, an increasing number of reports show that tissue macrophages, particularly tumor-associated macrophages, and M2-activated macrophage subsets also might promote the formation of new blood vessels and particularly in angiogenesis and atherosclerosis. MMP-9, a key enzyme involved in remodeling processes and particularly in angiogenesis and atherosclerosis. Moreover, because the plasma concentration of MMP-9 increased after passage through the subcutaneous AT and was found to be positively correlated with the BMI of the patients, the present study demonstrates for the first time that MMP-9 is a true adipokine; ie, it is produced and released in the systemic circulation by the human AT. Moreover, it strongly suggests that the reported increase in MMP-9 plasma concentration with obesity might be related to the accumulated ATMs. The remodeling function of ATMs was further confirmed by the finding that ATMs expressed the receptor for hyaluronan, LYVE-1, and that the ATM expression of LYVE-1 was closely positively correlated with fat mass enlargement. LYVE-1+ macrophage subsets, recently described in mice, have been suggested to be specifically involved in tissue remodeling during tumor growth and wound healing and in the angiogenesis of the epididymal fat pad. In agreement with such observations, the present data showed that ATM-conditioned media stimulated the AT-derived endo-

![Figure 4. ATM populations with respect to AT growth. A and B, Multicolor fluorescent-activated cell sorter analyses were performed on the SVF of patients with increasing BMI using fluorescence-labeled antibodies CD14-PE, CD45-PerCP, CD206-APC, and CD16-FITC. A, Percentage of CD45+/CD14+/CD206+ cells within the SVF (P<0.05; Spearman r=0.2664; n=61) and (B) cell number of CD45+/CD14+/CD206+/CD16+ per 1 mg AT (P<0.05; Spearman r=0.2986; n=60). C, Correlation between arteriovenous MMP-9 plasma concentration, determined by ELISA, and BMI (P<0.05; Spearman r=0.53; n=15). D, Correlation between LYVE-1 expression assessed by real-time PCR in isolated ATMs and BMI (P<0.001; Spearman r=0.73; n=17).]
Moreover, secretory products originating from ATMs decreased adipogenesis, an observation in agreement with a recent study using different approaches, but conversely increased angiogenesis by the human AT-derived progenitor cells. Although additional experiments are needed to clearly delineate the macrophage-derived factors responsible for such effects, the present results demonstrate that ATMs exhibit proangiogenic properties associated with an antiadipogenic effect.

The present study showed that human subcutaneous ATMs are composed of distinct macrophage subsets. Fat mass enlargement is associated with the accumulation of CD206+/CD16− ATMs that exhibit a remodeling phenotype characterized by decreased proinflammatory factors IL-8 and COX-2 and increased LYVE-1 expression. Recent studies performed in mice have clearly shown that obesity induced by either a high-fat diet or leptin deficiency is associated with the accumulation of M1 inflammatory monocyte/macrophages expressing iNOS, TNF-α, and CCR2. \cite{11-13} However, the accumulation of ATM in mice occurs after a long period of time under high-fat feeding and appears more related to the development of insulin resistance than directly linked to the extension of the fat mass. In the present study, increased ATM number was already observed in the subcutaneous AT of overweight healthy women and thus was independent of the potential direct effects of a high-fat diet, recently shown to be associated with increased endotoxemia and established obesity-associated pathologies. Moreover, discrepancies between mice and human macrophages are well documented, especially concerning iNOS and arginase-1 expression. \cite{15,40} The present results suggest that ATMs may be active players in the process of AT development through the extension of the capillary network and in the genesis of obesity-associated pathologies through their production of the proatherogenic MMP-9. Furthermore, the ATM-mediated antiadipogenic effect may contribute to lipotoxicity by promoting the ectopic deposition of free fatty acids in non-ATs.

**Figure 5.** ATM phenotype with respect to AT growth. A, Real-time PCR analyses using specific Taqman probes for IL-8 and COX-2 were performed on immunoselected ATMs from patients with distinct BMIs. Correlations between ATM IL-8 and COX-2 transcript levels and BMI (P < 0.01, Spearman r = −0.58, n = 17; P < 0.05, Spearman r = −0.47, n = 17, respectively). B, Expression of IL-8 and COX-2 in ATMs cultured for 24 hours in ECM/0.1% BSA (control, white bar) in the presence of leptin (2 ng/mL, black bar) or adiponectin (20 ng/mL, hashed bar). Values are mean ± SEM (arbitrary units) of 6 independent experiments. *P < 0.05 vs control.

**Figure 6.** Roles of ATM-conditioned media in AT remodeling. A, Immunoselected AT progenitors cells were cultured in a medium allowing both adipogenic and angiogenic differentiation supplemented or not with macrophage (ATM)-conditioned media. After 8 days, cells were fixed and stained with antibody against CD31 and Oil Red O. In parallel, DNA and triglyceride contents were determined. Representative photomicrographs from 5 independent experiments are shown. Hoescht 33258 (blue) was used to stain nuclei. Values are mean ± SEM of 5 independent experiments normalized to DNA and expressed as percentage of the control. B, Capillary endothelial cells were isolated from AT, plated on matrigel, and cultured for 24 hours with control or macrophage (ATM)-conditioned media. Representative photomicrographs from 5 independent experiments are shown. *P < 0.05, **P < 0.01 vs control medium.
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Disclosures
None.

References
Obesity is characterized by a systemic low-grade inflammatory state that is thought to contribute to the genesis of obesity-associated cardiovascular diseases and type 2 diabetes mellitus. However, the cellular and molecular mechanisms underlying the link between fat mass enlargement, inflammation, and obesity-associated pathologies remain to be clearly defined. Adipose tissue (AT) itself produces a wide range of adipokines with inflammatory properties, and its excessive development has been associated in humans and mice with accumulation of AT macrophages (ATMs). The present study, performed in subcutaneous ATs from lean to overweight individuals, showed that ATMs coexpressed proinflammatory and antiinflammatory factors. Furthermore, ATMs specifically produced and released the key matrix remodeling enzyme matrix metalloproteinase-9 compared with mature adipocytes and AT capillary endothelial cells. Interestingly, the secretion of matrix metalloproteinase-9 from human AT in vivo, assessed by arteriovenous difference measurement, was correlated with the body mass index of the patients. An increase in body mass index also was found to be associated in ATMs with lower expression levels of 2 proinflammatory factors (ie, interleukin-8 and cyclooxygenase-2) and a higher expression level of the remodeling marker lymphatic vessel endothelial hyaluronan receptor-1. Finally, ATMs exerted marked in vitro proangiogenic effects on AT-derived capillary endothelial and progenitor cells. These overall results indicate that the human ATMs that accumulate within the fat mass during its growth exhibit a particular remodeling phenotype. ATMs may thus be active players in AT development via their proangiogenic effects but also in the genesis of obesity-associated cardiovascular pathologies through their release of matrix metalloproteinase-9.
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