From Blood Monocytes to Adipose Tissue–Resident Macrophages

Induction of Diapedesis by Human Mature Adipocytes

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Obesity has been suggested to be a low-grade systemic inflammatory state, therefore we studied the interaction between human adipocytes and monocytes via adipose tissue (AT)-derived capillary endothelium. Cells composing the stroma-vascular fraction (SVF) of human ATs were characterized by fluorescence-activated cell sorter (FACS) analysis and two cell subsets (resident macrophages and endothelial cells [ECs]) were isolated using antibody-coupled microbeads. Media conditioned by mature adipocytes maintained in fibrin gels were applied to AT-derived ECs. Thereafter, the expression of endothelial adhesion molecules was analyzed as well as the adhesion and transmigration of human monocytes. FACS analysis showed that 11% of the SVF is composed of $CD14^+/CD31^+$ cells, characterized as resident macrophages. A positive correlation was found between the BMI and the percentage of resident macrophages, suggesting that fat tissue growth is associated with a recruitment of blood monocytes. Incubation of AT-derived ECs with adipocyte-conditioned medium resulted in the upregulation of EC adhesion molecules and the increased chemotaxis of blood monocytes, an effect mimicked by recombinant human leptin. These results indicate that adipokines, such as leptin, activate ECs, leading to an enhanced diapedesis of blood monocytes, and suggesting that fat mass growth might be linked to inflammatory processes. *Diabetes* 53:1285–1292, 2004

The concept that adipose tissue (AT) is a simple fat storage facility has evolved over the last few years, and current thinking leans toward its recharacterization as a multifunctional secretory organ (1). Indeed, mature adipocytes are involved in endocrine, paracrine, and autocrine regulatory processes (2), through the secretion of a large number of multifunctional molecules now termed adipocytokines or adipokines (3). In addition to playing key roles in the regulation of the lipid and glucose homeostasis, adipokines modify physiological processes, such as hematopoiesis, reproduction, and feeding behavior, and may mediate the genesis of the multiple pathologies associated with an increased fat mass (4,5). Inflammatory processes contribute to the endothelial dysfunction and vascular remodeling that are associated with hypertension and atherosclerosis (6,7), disorders that are clearly linked to obesity. Moreover, in humans, the development of AT has been associated with an increased production of inflammatory markers, including adhesion molecules (P-selectin, intercellular adhesion molecule-1, and plasma E-selectin) and inflammatory cytokines (tumor necrosis factor-α, interleukin-6, interleukin-8, and the monocyte chemoattractant protein 1) (8,9). It is therefore tempting to speculate that adipocytes, via the production of adipokines, are directly involved in the genesis of systemic and vascular inflammation. In the present study, we report the presence of resident macrophages in the stroma-vascular fraction (SVF) of human AT, the number of which is positively correlated with the BMI of AT donors. Furthermore, mature human adipocytes release soluble factors that increase the diapedesis of human blood monocytes across a layer of AT-derived capillary endothelial cells (ECs), an effect that can be mimicked by human recombinant leptin.

RESEARCH DESIGN AND METHODS

RPMI-1640 medium was purchased from Gibco (Invitrogen, Carlsbad, CA) and the EC growth medium and EC basic medium from Promocell (Heidelberg, Germany). The Easysep CD34- and CD14-coupled magnetic beads were from StemCell Technologies (Meylan, France), and the CD31-coupled magnetic Dynabeads were from Dynal (Hamburg, Germany). The human α-thrombin was provided by Hemochrom Diagnostica (Essen, Germany). Ficoll gradient (Biocoll) and collagenase were from Biochrom (Berlin, Germany), and recombinant human leptin and interferon (IFN)-γ were from PeproTech (Cell Concept, Umkirch, Germany). The vibrant phagocytosis system was from Molecular Probes (Leiden, the Netherlands). The mouse monoclonal intracellular adhesion molecule (ICAM)-1 antibody and the goat polyclonal platelet/EC adhesion molecule (PECAM)-1 antibodies used for the Western blot analysis were from Santa Cruz Biotechnology (Santa Cruz, CA). The peroxidase-coupled secondary mouse and goat antibodies were from Calbiochem (Darmstadt, Germany). The mouse monoclonal antibodies directed against von Willebrand factor and CD45 were from Dako (Glostrup, Denmark) and Cymbius biotechnology (Hofheim, Germany), respectively. The anti-rabbit or anti-mouse antibodies conjugated with Alexa Fluor 488 were from Molecular Probes. The labeled mouse IgG coupled with phycoerythrin (PE), fluorescein

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AT, adipose tissue; EC, endothelial cell; FACS, fluorescence-activated cell sorter; FITC, fluorescein isothiocyanate; GM-CSF, granulocyte macrophage–colony stimulating factor; ICAM, intracellular adhesion molecule; IFN, interferon; LPS, lipopolysaccharide; MCP, monocyte chemoattractant protein; MIP, macrophage inflammatory protein; PECAM, platelet/EC adhesion molecule; PerCP, peridinin chlorophyll-α protein; SVF, stroma-vascular fraction.

TABLE 1
Primer sequences

	Forward	Reverse
hGM-CSF	GCAGCCTCACCAAGCTCAAG	GGAGGGCAGTGCTGTTTGTAG
hMIP-1α	TTTGCTCTGAGAGTTCCCCTG	AGCCACTCGGTTGTCACCA
hMCP-1	CGCCTCCAGCATGAAAGTCT	ATGAAGGTGGCTGCTATG
hIL-8	ACCGGAAGGAACCATCTCACT	GGAAGGCTGCCAAGAGAGC
hLeptin	CCTTCCAGAAACGTGATCCAA	GGCCAGCACGTGAAGAAGAT
18S	GGAGGTCATTGGTGATTACTTTGGA	AGAGCCCCAAGGATATTTGACA

h, human; IL, interleukin.

isothiocyanate (FITC), peridinin chlorophyll-α protein (PerCP), and the labeled mouse monoclonal antibodies against CD14 and CD45 were from BD Biosciences (Bedford, MA), and the labeled mouse monoclonal antibody against CD31 was from Cymbius biotechnology (Hofheim, Germany). VectaShield mounting medium was provided by Vector Laboratories (Burlingame, CA). All other products were from Sigma (Munich, Germany).

Isolation of the SVF of human AT. Human subcutaneous AT was obtained from patients undergoing plastic surgery by lipoaspiration of the gluteal regions or lipectomy of the abdominal region (mean BMI 25 ± 1 and 25.3 ± 0.5 kg/m^2, respectively). Human visceral AT was obtained from patients undergoing abdominal tumor surgery (colorectal carcinoma, pancreas, and stomach) that was not associated with obvious inflammatory disease (mean BMI 26.3 ± 0.8 kg/m^2). The study was approved by the ethics committee of the University Hospital of Frankfurt am Main. After digestion of the AT in a collagenase solution (300 units/ml in PBS and 2% BSA), followed by a 200*g* centrifugation, the pellet containing the SVF was incubated for 10 min in an erythrocyte-lysing buffer (155 mmol/l NH_4Cl, 5.7 mmol/l K_2HPO_4, and 0.1 mmol/l EDTA) and finally resuspended in PBS/2% FCS and sequentially filtered through 100-, 70-, and 40-μm sieves. The isolation of the human preadipocytes was performed as described (10).

Human mature adipocyte isolation and preparation of the adipocyte-derived secretions. Adipocytes were isolated as described (11). Briefly, after digestion of the AT in the collagenase solution for 20 min, the resulting suspension was filtered (210-μm polyamid filter) and washed with PBS. Mature adipocytes (~400,000) were included in fibrin gels (1.5 mg fibrinogen/ml EC basic medium supplemented with 25 units/ml α-thrombin) and cultured in RPMI medium containing 100 units/ml penicillin, 100 μg/ml streptomycin, 4 mmol/l L-glutamin, 1 mmol/l sodium pyruvate, 1% modified Eagle's medium vitamins, and 1% modified Eagle's medium nonessential amino acids (RPMI-S). Control gels were prepared without adipocytes. After 24 h, the adipocyte-conditioned media were collected and frozen at −80°C.

Isolation of the ECs and macrophages from the SVF. The SVF, homogenized at the maximal concentration of 40 million cells per ml of PBS/2% FCS, was incubated at room temperature for 15 min with 100 μl of Easysep positive-selection cocktail per milliliter of cell suspension, followed by a 10-min incubation period with 50 μl of Easysep magnetic nanoparticles per milliliter of cell suspension. After successive magnetic sorting steps and washes with PBS/2%FCS, the CD34$^+$ cell fraction was finally suspended in 1 ml PBS/0.1% BSA, whereas the CD34$^-$ subset, collected after each sorting step, was centrifuged at 200*g* and homogenized in 1 ml PBS/2%FCS.

The CD34$^+$ cell fraction was used to isolate the capillary ECs with 50 μl of CD31-coupled magnetic microbeads (Dynal Biotech, Hamburg, Germany) per milliliter of CD34$^+$ cell fraction. After 20 min of incubation at 4°C under constant shaking, the cell suspension containing the beads was briefly homogenized in 10 ml PBS/0.1%BSA and placed in front of the magnet for 1 min. After two washes in PBS/0.1% BSA, the magnetic bead–coupled fraction, which corresponds to the CD34$^+$/CD31$^+$ cell population, was collected, centrifuged for 10 min at 200*g*, and cultured on fibronectin-coated dishes in EC growth medium.

The CD34$^-$ cell fraction was used to isolate the resident macrophages with 50 μl of CD14-coupled magnetic microbeads (Dynal Biotech) per milliliter of CD34$^-$ cell fraction. After 20 min of incubation at 4°C under constant shaking, the cell suspension containing the beads was briefly homogenized in 10 ml PBS/2%FCS and placed in front of the magnet for 1 min. After two washes in PBS/0.1% BSA, the magnetic bead–coupled fraction, which corresponds to the CD34$^-$/CD14$^+$ cell population, was collected and centrifuged for 10 min at 200*g*.

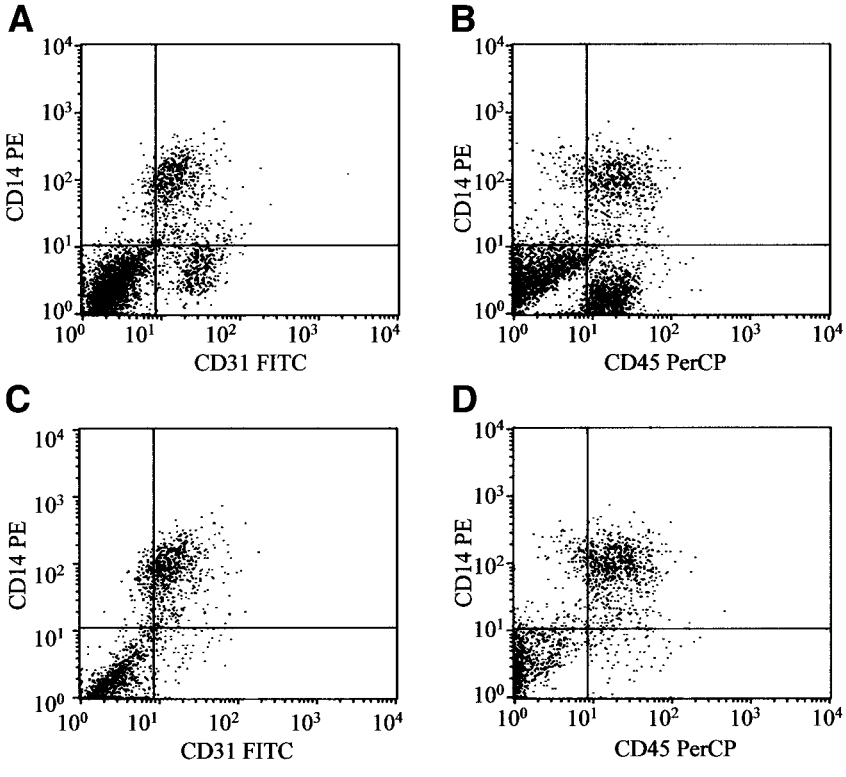

FIG. 1. Flow cytometry analysis of the SVF. Representative triple-color FACS analysis performed on total SVF using CD14(PE) and CD31(FITC) antibodies (*A*) or CD14(PE) and CD45(PerCP) antibodies (*B*). Representative FACS analysis of the gated CD45$^+$ population (*C*) and of the gated CD31$^+$ population (*D*).

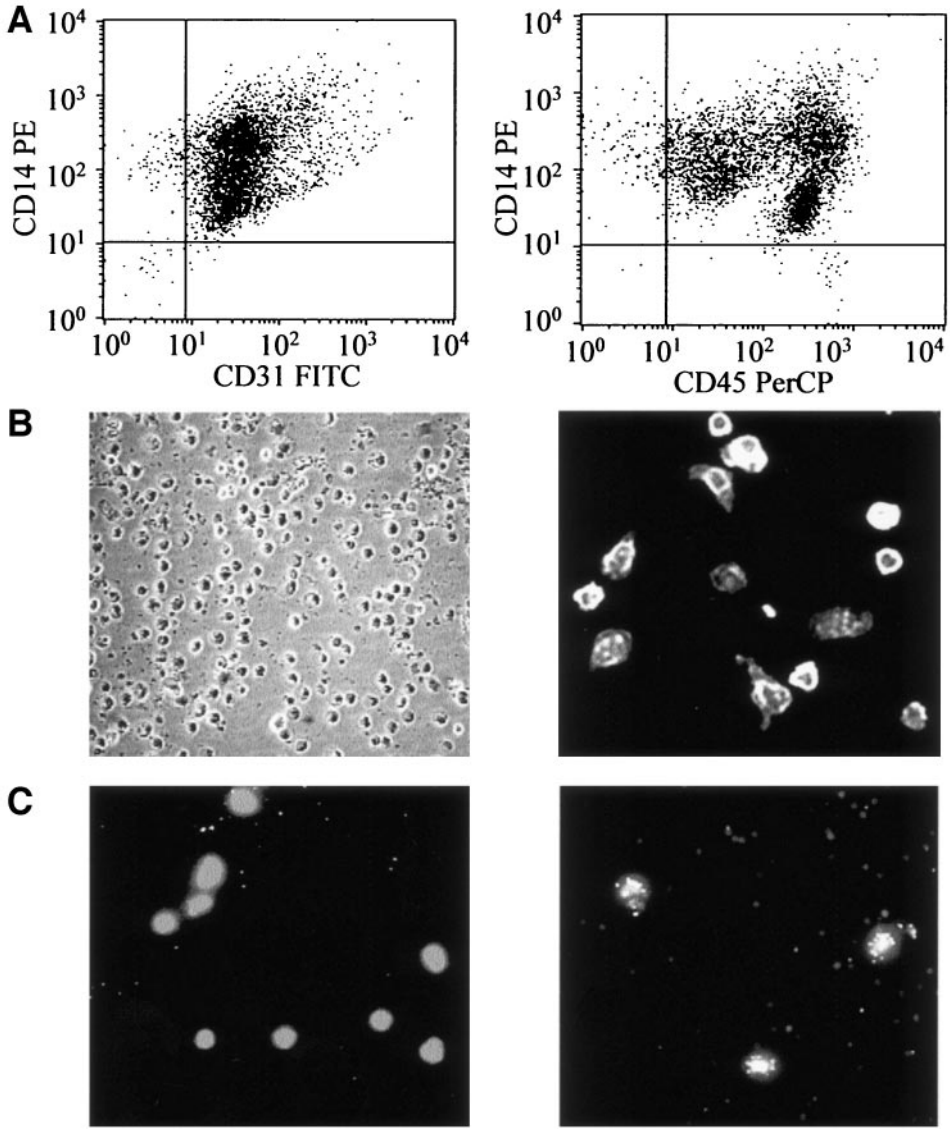

FIG. 2. Characterization of the isolated CD14$^+$ cells. *A*: Representative FACS analysis of CD14$^+$ cells using antibodies against CD14(PE), CD45(PerCP), and CD31(FITC). *B*: Phase-contrast microscopy (×100 magnification) and immunocytochemical analysis using an antibody against CD45 of the cultured CD14$^+$ cells. *C*: Phagocytosis assays using FITC-labeled *E. coli* performed on the CD14$^+$ cells stimulated (*right panel*) or not (*left panel*) with 5 μg/ml LPS and 240 units/ml IFN-γ (n = 5).

Fluorescence-activated cell sorter analysis. Cell sorter (FACS) analyses were performed using the freshly harvested SVF as well as the SVF-derived CD14$^+$/CD34$^-$ cell population. Cells were suspended in 100 μl PBS containing 0.5% BSA and 2 mmol/l EDTA and incubated (30 min at 4°C) with labeled monoclonal antibodies (0.5 mg/100 ml CD14-PE, 0.25 mg/100 ml CD45-PerCP, or 1.0 mg/100 ml CD31-FITC) or the appropriate isotype control. After extensive washing with PBS, the labeled cells were analyzed by flow cytometry using a FACSCalibur flow cytometer and CellQuest Pro software (Becton Dickinson, Erembodegem-Aalst, Belgium).

Phagocytosis assay. Freshly isolated CD14$^+$/CD34$^-$ cells were cultured in RPMI supplemented with 10% FCS for 4 h and thereafter treated or not with bacterial lipopolysaccharide (LPS) (serotype 0111:B4, 5 μg/ml) and IFN-γ (240 units/ml) overnight. After media removal, 200 μl of sonicated FITC-labeled *Escherichia coli* bioparticle mix (Molecular Probes) was added to the cells and incubated for 2 h at 37°C. After three washes with PBS, the cells were fixed with 4% paraformaldehyde and the presence of green fluorescent *E. coli* particles in the cell cytoplasma, an index of phagocytotic activity, was assessed by fluorescence microscopy.

Immunohistochemistry. Immunohistochemical analyses were performed using cultured CD14$^+$/CD34$^-$ and CD31$^+$/CD34$^+$ cells. After fixation, cells were incubated for 1 h at room temperature with a dilution of the relevant primary antibodies and, after washing in PBS containing 0.2% Tween, for 1 h with the corresponding fluorescence-labeled second antibodies.

Transmigration assays. Human blood monocytes were isolated from buffy coats obtained from healthy donors (Transfusion Center of Frankfurt Hospital) using a Biocoll gradient solution (density 1.077) (12) and labeled for 40 min with 10 μmol/l calcein acetoxymethyl ester (13). Confluent capillary ECs were incubated overnight with conditioned medium from either mature adipocytes or from preadipocytes, with recombinant human leptin or with control medium. Following incubation, labeled monocytes (30,000 cells/0.5 ml RPMI-S) were added to the capillary ECs for 1 h. The adhesion of the monocytes was assessed using a fluorescence microscope (14), and the number of adherent monocytes was expressed as the percentage of the endothelial nuclei.

Monocyte transendothelial migration was assessed on 3-μm pore, fibronectin-coated, microchemotaxis chambers (Costar, Cambridge, MA) seeded at the density of 100,000 capillary ECs/cm^2. After 5–6 days in culture, the cells were incubated overnight with adipocyte-conditioned media, recombinant human leptin, or control medium before exposure to labeled monocytes (130,000 cells/100 μl RPMI-S, 4 h). Monocyte transmigration was evaluated by counting the number of labeled monocytes attached to the lower surface of the wells (15).

RNA extraction and real-time PCR. Total RNA was extracted from human mature adipocytes, CD14$^+$ cells, and blood monocytes using a RNeasy kit (Qiagen, Hilden, Germany) and its concentration determined using a Ribogreen fluorometric assay (Molecular Probes). RNA (1 μg) was reverse transcribed using the thermoScript RT system from Life Technologies (Invitrogen, Carlsbad, CA). Reactions were also performed without reverse transcriptase to provide a control for contamination of samples with genomic DNA (Table 1).

The PCR mixtures were prepared with SYBR Green Master Mix (Applied Biosystems, Foster City, CA). For the ribosomal RNA quantification (18S rRNA) and the CD36 probe (Assays-on-Demand; Applied Biosystems), the PCR contained the primers, the fluorogenic probe mix, and the TaqMan Universal PCR Master mix (Applied Biosystems). All amplification reactions were performed in duplicate from 20 ng cDNA using the Mx4000 Multiplex

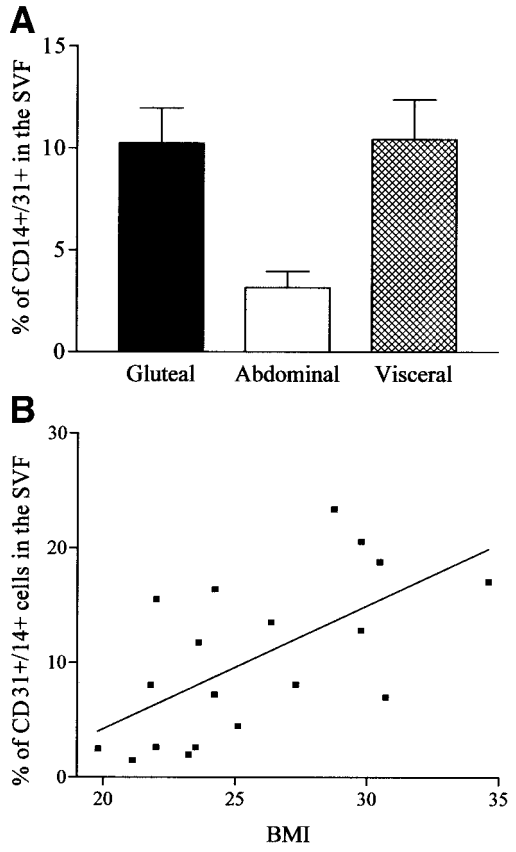

FIG. 3. Influence of the source of AT and BMI. *A*: Percentage of the CD14$^+$/CD31$^+$ population determined by FACS analysis of SVF of gluteal (n = 19), abdominal (n = 7), and visceral (n = 17) AT samples. *B*: Correlation between the percentage of CD14$^+$/CD31$^+$ cells in the gluteal SVF and BMI (n = 19, P = 0.0045, r^2 = 0.3863).

Quantitative PCR System (Stratagene, La Jolla, CA) using the following conditions: 50°C for 2 min and 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. Results were analyzed with Strategene MX4000 software, and all values were normalized to the levels of the ribosomal RNAs.

Western blot analysis. Capillary ECs, stimulated overnight with either adipocyte-conditioned or control media, were lysed on ice for 20 min in Nonidet lysis buffer (20 mmol/l Tris/Cl, pH 7.5, 150 mmol/l NaCl, 10 mmol/l NaPPi, 20 mmol/l NaF, 1% Nonidet P40, 25 mmol/l β-glycerophosphate, 2 mmol/l orthovanadate, 10 μmol/l ocadeic acid, 4 μl/ml phenylmethylsulfonyl fluoride, and 12 μl/ml of protease inhibitor mix). Proteins (20 μg) were separated by SDS-PAGE under denaturing conditions and transferred to nitrocellulose membranes. Membranes were blocked overnight at 4°C, incubated with primary antibody for 90 min, rinsed, and incubated with peroxidase-coupled secondary antibodies for 60 min. The immunocomplexes were visualized using a chemiluminescence reagent (Amersham, Freiburg, Germany), and the autoradiographs were scanned by an imaging densitometer.

Data analysis. Values are given as means ± SE of n independent experiments. Statistical analyses were performed using Student's t test or one-way ANOVA, followed by Bonferroni t test where appropriate. Differences were considered significant where P was <0.05.

RESULTS

Identification and characterization of a population of resident macrophages in human ATs. Three-color FACS analysis of the freshly harvested SVF from human subcutaneous ATs was performed using antibodies directed against the leukocyte common antigen precursor (CD45), the PECAM-1 (CD31), and the LPS receptor (CD14). FACS analysis of the CD14 versus CD31 labeling (Fig. 1*A*) revealed two distinct cell subsets: a CD31$^+$ and CD14$^-$ cell population (14 ± 1.5% of the total SVF) and a double CD31$^-$ and CD14$^+$ cell population (10.5 ± 1.5% of the total SVF). FACS analysis of the CD14 and CD45 labeling (Fig. 1*B*) clearly identified a CD45$^+$ and CD14$^-$ cell population (19 ± 5% of the total SVF) as well as a double CD45$^-$ and CD14$^+$ cell population (11.5 ± 2% of the total SVF). The double-positive populations (CD31$^+$/CD14$^+$ and CD45$^+$/CD14$^+$) were found to be triple positive when gated for the third marker (CD45 and CD31, respectively) (Fig. 1*C* and *D*).

To further characterize the CD14$^+$/CD31$^+$/CD45$^+$ cell population, three-color FACS analysis was performed on the freshly extracted CD14$^+$ cells. All cells expressed both CD31 and CD45 (Fig. 2*A*). Moreover, in short-term culture the cells exhibited a typical rotund morphology with unilobular nuclei (Fig. 2*B*). Real-time PCR analysis using specific primers for the thrombospondin receptor (CD36) showed the presence of the CD36 gene in the transcripts of the CD14$^+$ cells in a level similar to that found in the blood monocytes (ratio of 1.09 ± 0.05, n = 10, using blood monocytes as reference). Treatment with LPS and IFN-γ led to an uptake of FITC-labeled *E. coli* into the cytoplasma of the cells (Fig. 2*C*, *right panel*), whereas no specific labeling could be detected in untreated cells (Fig. 2*C*, *left panel*), indicating that the CD14$^+$ cell population exhibits phagocytotic activity. It is important to note that the CD14$^+$/CD31$^+$ cell population could be detected by FACS analysis independent of the anatomical source (i.e., subcutaneous gluteal and abdominal as well as visceral) of the AT (Fig. 3*A*). Moreover, because a significant positive correlation was found between the percentage of the CD14$^+$/CD31$^+$ cells in the SVF and the BMI, it appears that the extent of AT growth is linked to the number of macrophages resident therein (Fig. 3*B*).

Effect of adipocyte-conditioned medium on the transmigration of blood monocytes through a capillary EC layer. To determine whether adipocytes are able to recruit blood monocyte/macrophages into AT, we studied the effects of the adipocyte-derived factors on the adhesion and transmigration of monocytes to/through AT-derived capillary ECs. A 2.5-fold increase in the adhesion of labeled monocytes was observed on the capillary ECs preincubated with the adipocyte-derived factors compared with ECs pretreated with control media ($P < 0.01$, n = 5) (Fig. 4*A*). Similarly, the migration of labeled-monocytes through a layer of ECs, cultured on transwell filters, was markedly enhanced when the endothelium was pretreated with adipocyte-conditioned medium (threefold increase compared with control medium, $P < 0.001$, n = 3) (Fig. 4*B*). A chemotactic effect was only observed using medium from mature adipocytes. Conditioned medium from undifferentiated preadipocytes had no effect on the monocyte adhesion/transmigration (Fig. 5).

Activation of the AT-derived capillary ECs by adipocyte-conditioned medium. To assess whether the chemotactic effect of the adipocyte-conditioned medium was associated with an EC activation, we determined the effect of the adipocyte-conditioned media on the endothelial expression of the ICAM-1 and PECAM-1. Higher levels of ICAM-1 and PECAM-1 were expressed in ECs pretreated with conditioned medium from mature adipocytes as assessed by immunocytochemical (Fig. 6*A* and *B*) and Western blot analyses (1.9- and 1.2-fold increase for

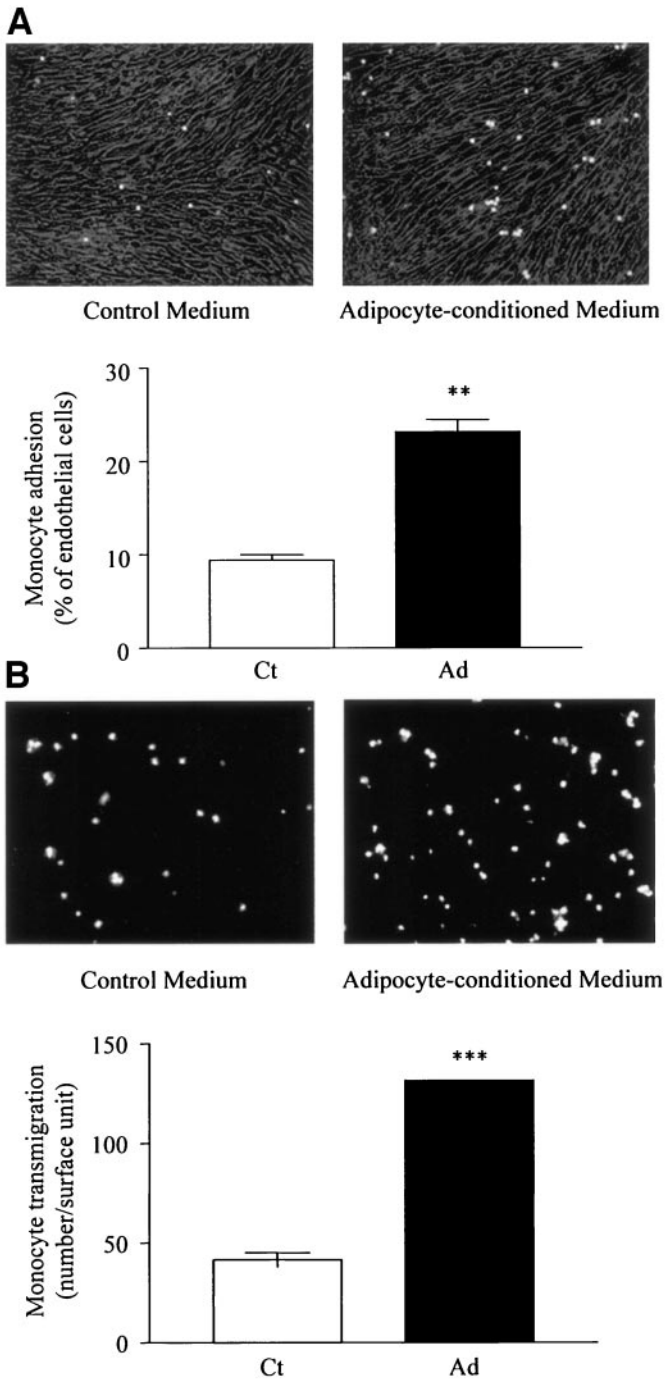

FIG. 4. Effect of adipocyte-derived factors on the diapedesis of blood monocytes. *A*: Adhesion of calcein-labeled monocytes to AT-derived ECs pretreated or not with adipocyte-conditioned media. Representative fluorescence microscopy analyses are shown, and values are means ± SE of the number of adherent monocytes expressed as the percentage of the endothelial nuclei ($n = 5$). **$P < 0.01$ versus control. *B*: Transmigration of calcein-labeled monocytes through AT-derived ECs pretreated or not with adipocyte-conditioned media. Representative fluorescence microscopy analyses are shown, and values are means ± SE of the number of transmigrated monocytes per surface unit of the lower part of the well ($n = 3$). ***$P < 0.001$ versus control.

ICAM-1 and PECAM-1, respectively, $n = 4$, $P < 0.05$) (Fig. 6*C*).

Nature of the adipocyte-derived product involved in the enhanced adhesion of blood monocytes. To determine the nature of the adipocyte-derived factors involved in the enhanced adhesion of the blood monocytes, mature adipocyte-derived medium was either heated to denaturate proteins or treated with ether to selectively deplete the adipocyte secretions from lipid components. Lipid depletion did not affect the chemotactic effect of the adipocyte-conditioned medium (Fig. 7*A*), whereas protein denaturation reduced it markedly (twofold decrease compared with native medium, $n = 4$, $P < 0.01$).

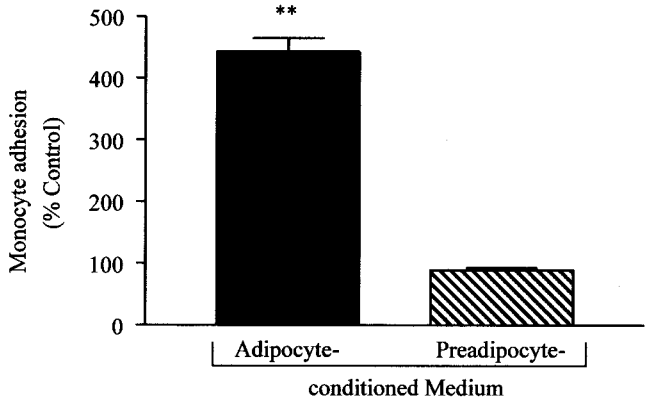

FIG. 5. Specificity of adipocyte-derived factors. The values are mean ± SE of the percentage of adherent monocytes to AT-derived ECs pretreated or not with adipocyte- or preadipocyte-conditioned media, $n = 3$, $P < 0.01$ versus control.**

To further characterize the adipocyte-derived proteins potentially involved in the chemotactic effect of the adipocyte-conditioned medium, real-time PCR analysis was performed on mature adipocyte mRNA. Monocyte chemoattractant protein (MCP)-1, interleukin-8, and leptin were expressed in human mature adipocytes (Fig. 7*B*), whereas no specific signal could be detected for granulocyte macrophage–colony stimulating factor (GM-CSF) or macrophage inflammatory protein (MIP)-1α. Moreover, increasing concentrations of recombinant human leptin (from 10 to 1,000 ng/ml) resulted in an increased adhesion and transmigration of blood monocytes in a concentration-dependent manner (Fig. 7*C* and *D*). The maximal effect was observed for 1,000 ng/ml leptin (3.5-fold increase in monocyte adhesion, $P < 0.001$, $n = 4$, and 7-fold increase in monocyte transmigration, $P < 0.01$, $n = 4$).

DISCUSSION

Obesity is rapidly becoming a major public health concern because it is clearly associated with increased risks of cardiovascular pathologies, diabetes, and cancer. However, the link between an excess of fat and the development of such pathological states remains to be elucidated, as do the mechanisms involved in the expansion and development of the AT. While many studies have focused on the role of the adipocytes and their precursors cells, the preadipocytes, other cell types within AT, especially cells composing the so-called SVF, might play a determinant role in disease progression. Recently, a key role of the microcirculation within AT in the increase of the fat mass has been demonstrated in a genetic model of animal obesity (16) and in severe combined immunodeficient (SCID) mice after implantation of preadipocytes (17). In humans, little data are available regarding the nature of the cell types composing the SVF. On the basis of immunohistochemical analysis, one report described the pres-

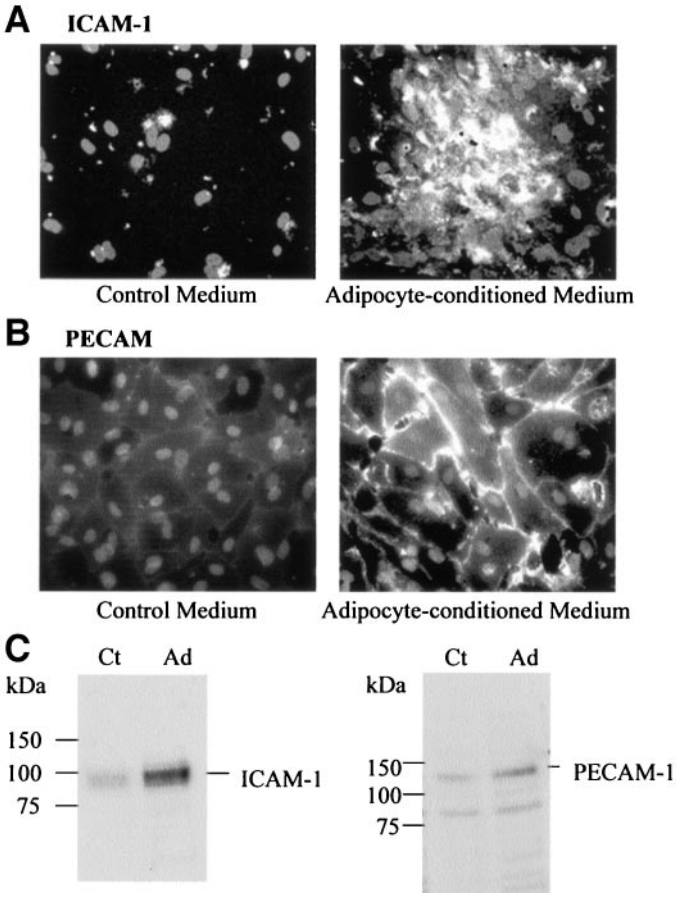

FIG. 6. Activation of AT-derived ECs by adipocyte-derived factors. AT-derived ECs were incubated with either adipocyte-conditioned medium or control medium. *A* and *B*: Immunocytochemical analyses performed using ICAM-1 and PECAM-1 antibodies, respectively (n = 3). *C*: Western blot analyses using ICAM-1 and PECAM-1 antibodies (n = 4).

ence of ECs as well as stromal cells, blood cells, and tissue macrophages in the human mammary and visceral ATs (18). In the present study, the FACS analysis revealed a cell population that expressed the cell surface markers characteristic of the monocyte/macrophage lineage, i.e., CD14 (LPS receptor), CD31 (PECAM-1), and CD45 (leukocyte common antigen). Moreover, the isolation of the $CD14^+$ cells using CD14-coupled magnetic microbeads allowed us to demonstrate that the $CD14^+$ cells expressed the CD36 gene, thus excluding the granulocyte lineage, and exhibited in vitro macrophage phenotype and activity. The population of resident macrophages appears to constitute an important component of the SVF since macrophages could be detected in subcutaneous and in visceral AT samples. Tissue macrophages have been shown to perform diverse functions, such as protecting against microorganisms and exerting cytotoxic activity against tumor cells, in addition to regulating local homeostasis via the production of growth factors and cytokines. Macrophages are also present in human tumors, where they are thought to contribute to the promotion of tumor angiogenesis, and in atherosclerotic plaques, where they are important for intracellular lipid accumulation and foam cell formation as well as the modulation of vascular cell function and growth (19–21). Since the number of resident macrophages present in AT was found to correlate positively with BMI, it is tempting to speculate that macrophages might contribute to the growth of the fat mass in a manner similar to that described in tumors. Notably, such a correlation between the amount of AT resident macrophages and obesity has also been observed in various mouse models of obesity and in human subcutaneous AT, as has been reported by two works (22,23) that appeared during the preparation of the study.

AT resident macrophages could originate from either blood monocytes or from preadipocytes. Indeed, preadipocytes have been reported (24,25) to differentiate into macrophages, but the reported differentiation of rodent preadipocytes took place in an environment favored by macrophages. It therefore seems more likely that the macrophage population found in AT originates as a consequence of the recruitment of blood monocytes. Tissue infiltration of blood monocytes is a complex phenomenon that involves several steps, including the activation of the capillary endothelium and the increased expression of adhesion molecules, such as ICAM-1, and the adhesion of blood monocytes, followed by their transmigration across the endothelium and differentiation into macrophages. Ourresults indicate that mature human adipocytes, via the production of soluble factors, stimulated the diapedesis of blood monocytes. The latter response was related to the activation of the AT-derived capillary ECs and an increase in the expression of ICAM-1 and PECAM-1. It should be mentioned that increased plasma concentrations of soluble cell adhesion molecules (E-selectin, VCAM-1, ICAM-1, and von Willebrand factor) have been reported (26,27) in overweight and obese individuals, suggesting that increased fat mass is associated with an early systemic endothelial activation. Thus, adipocyte-derived factors that are released in the systemic circulation might play an important role in the activation of ECs. Among the cytokines or "adipokines" reported to be produced by adipocytes are inflammatory factors, such as interleukin-8 and tumor necrosis factor-α, and chemotactic factors, such as MCP-1, MIP-1, and GM-CSF. Since preadipocyte-conditioned medium exerted no effect on monocyte adhesion, the candidate mediators of monocyte diapedesis can be restricted to those produced and released by differentiated adipocytes. This means, for example, that tumor necrosis factor-α, which is produced by human preadipocytes but not adipocytes (28), could be ruled out. The data obtained by real-time PCR on RNA extracted from mature adipocytes confirmed the low expression of interleukin-8 and MCP-1 (28,29) and the absence of the MIP-1 and GM-CSF in mature human fat cells (28). Leptin, as expected, was strongly expressed in mature adipocytes, and this adipokine was able to mimic the effects of adipocyte-conditioned medium on monocyte adhesion and transmigration. Since the leptin receptor is known to be expressed in ECs derived from human AT (18) and leptin induces an oxidative stress in ECs (30,31), the results of the present study strongly suggest that leptin could elicit EC activation and the infiltration of circulating blood monocytes into AT. However, it has to be noticed that in the models of leptin-deficient and leptin receptor–deficient mice, the amount of resident macrophages still correlates with the degree of obesity, as has been recently reported (22,23),

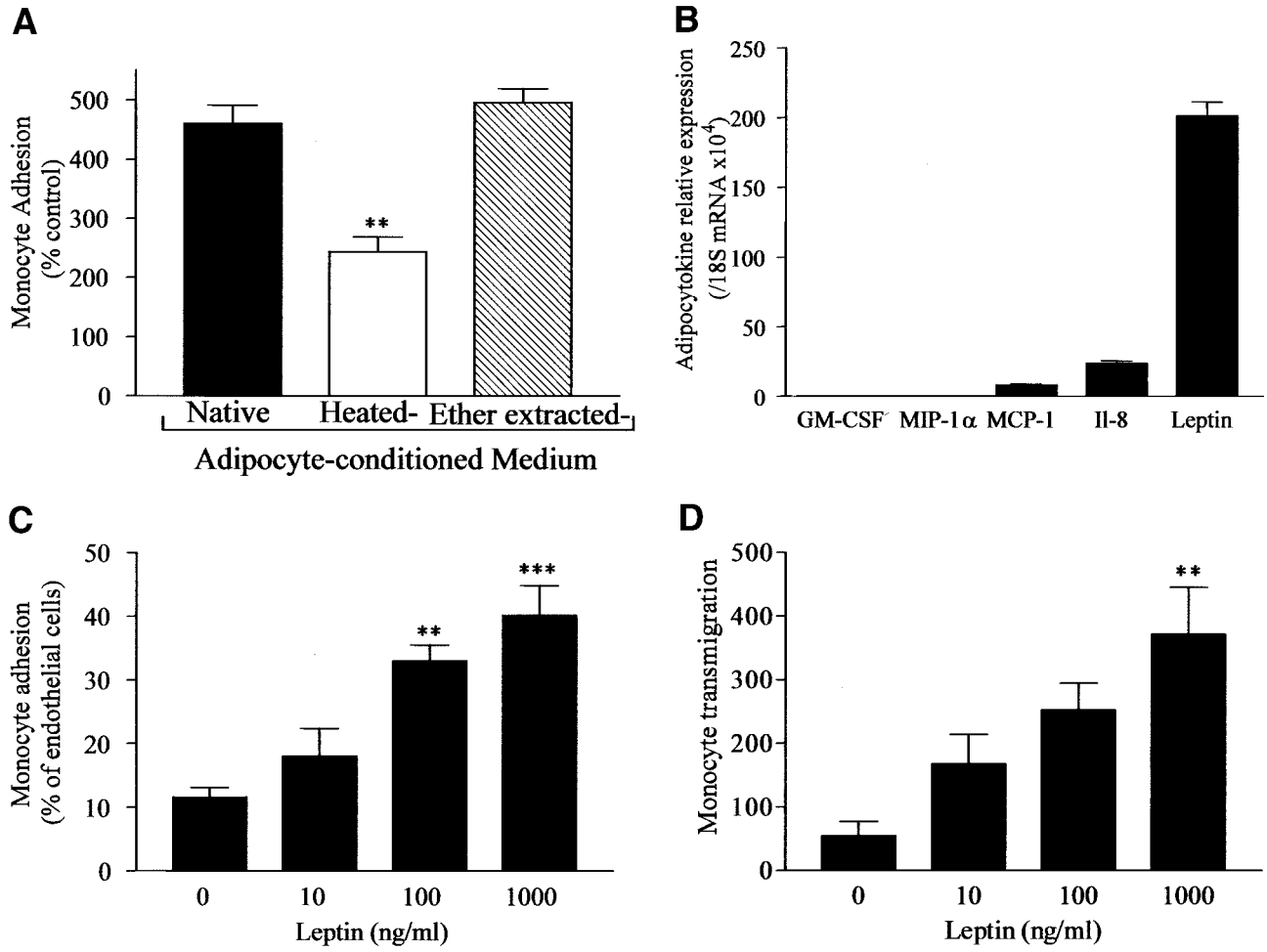

FIG. 7. Nature and characterization of the adipocyte-derived factors. *A*: Adhesion of monocytes to AT-derived ECs pretreated or not with native, heated, or ether-extracted adipocyte-conditioned medium. The number of adherent monocytes was expressed as the percentage of the endothelial nuclei, and the values are means ± SE of the percentage of control (n = 4). **P < 0.01. *B*: mRNA expression of GM-CSF, MIP-1α, MCP-1, interleukin-8, and leptin in mature adipocytes. Values are means ± SE of the normalized values to 18S (n = 4). *C*: Adhesion of monocytes to AT-derived ECs pretreated with increasing concentrations of human recombinant leptin. The number of adherent monocytes was expressed as the percentage of the endothelial nuclei, and the values are means ± SE (n = 4). **P < 0.01, ***P < 0.001 versus control. *D*: Transmigration of monocytes to AT-derived ECs pretreated with increasing concentrations of human recombinant leptin. The number of transmigrated monocytes was counted per surface unit of the lower part of the well, and values are means ± SE (n = 4). **P < 0.01 versus control.

suggesting that adipokines other than leptin may participate in the adipocyte-mediated diapedesis.

In conclusion, adipokines, most likely leptin, derived from mature human adipocytes, activate microvascular ECs, and thus promote monocyte diapedesis and the accumulation of macrophages within AT. It is tempting to speculate that the increase in fat mass that is associated with the enhanced release of adipocyte-derived factors, such as leptin into the circulation, contributes to the development of a systemic inflammatory state and may therefore be involved in the pathogenesis of obesity-associated vascular pathologies.

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