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Abstract

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Reference

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Production of Chemokines by Perivascular Adipose Tissue
A Role in the Pathogenesis of Atherosclerosis?

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Objective—Obesity is associated with an increased risk for cardiovascular disease. Although it is known that white adipose tissue (WAT) produces numerous proinflammatory and proatherogenic cytokines and chemokines, it is unclear whether adipose-derived chemotactic signals affect the chronic inflammation in atherosclerosis.

Methods and Results—Histological examination showed that perivascular WAT (pWAT) is in close proximity to vascular walls, particularly at sites that have a tendency to develop atherosclerosis. In rodents, the amount of pWAT is markedly increased by a high-fat diet. At a functional level, supernatant from subcutaneous and pWAT strongly induced the chemotaxis of peripheral blood leukocytes. The migration of granulocytes and monocytes was mostly mediated by interleukin-8 and monocyte chemoattractant protein-1, respectively, whereas both chemokines contributed to the migration of activated T cells. Moreover, pWAT produces these chemokines, as shown by immunohistochemistry and by explant culture. The accumulation of macrophages and T cells at the interface between pWAT and the adventitia of human atherosclerotic aortas may reflect this prochemotactic activity of pWAT.

Conclusions—Human pWAT has chemotactic properties through the secretion of different chemokines, and we propose that pWAT might contribute to the progression of obesity-associated atherosclerosis. (***Arterioscler Thromb Vasc Biol.* 2005;25:2594-2599.)**

Key Words: chemokines ■ perivascular adipose tissue ■ chemotaxis ■ obesity ■ inflammation

Obesity is characterized by an excess of body fat and is associated with an increased risk for cardiovascular disease.[1] Obesity is also linked to a state of chronic inflammation[2] associated with the production of pro- and antiinflammatory cytokines secreted by white adipose tissue (WAT), such as interleukin (IL)-6, tumor necrosis factor (TNF)-α, IL-1β, and the IL-1 receptor antagonist.[3] WAT is also known to produce chemokines such as IL-8 (also called CXCL8)[4,5] and monocyte chemoattractant protein-1 (MCP-1, also called CCL2).[5,6]

Atherosclerosis is characterized by the accumulation of macrophages and T lymphocytes in the wall of large arteries, a process in which chemokines play an important role.[7,8] Many of the chemoattractant factors involved in the development of atherosclerosis are expressed in the atherosclerotic plaque and produced by endothelial and smooth muscle cells.[8] The importance of chemokines in the development of atherosclerosis has been demonstrated in a low-density lipoprotein (LDL) receptor-deficient mouse model where the invalidation of the MCP-1 gene prevented lipid deposition and macrophage infiltration in the aortic wall.[8] Similarly, irradiated LDL receptor-deficient mice received a transplant of bone marrow cells lacking the CXCR2 (murine IL-8 homologue receptor), resulting in the reduced accumulation of monocytes/macrophages in the vascular intima.[9]

Because obesity is a metabolic and chronic inflammatory condition associated with cardiovascular complications, it is tempting to speculate that proinflammatory cytokines produced by WAT contribute to the progression of obesity-associated atherosclerosis. Given that WAT has been shown to secrete numerous cytokines, sometimes with opposing actions (eg, IL-1 and IL-1 receptor antagonist), we first examined leukocyte chemotaxis by assessing the net effect of the secretory products from human WAT. We then examined the production of chemotactic cytokines by perivascular adipose tissue (pWAT), an adipose depot found in close proximity to the vascular wall, as well as the presence of inflammatory cells in its vicinity. The data from this study are consistent with the theory that pWAT may play an active role in the progression of atherosclerosis.

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Methods

Human Adipose Tissue and Aorta

After authorization from the Ethical Committee of the University Hospital of Geneva and informed consent from the patients were obtained, samples of subcutaneous adipose tissue (scWAT) were collected from 5 patients undergoing abdominal plastic surgery. Samples of human aortas and scWAT were obtained from the same patient undergoing cardiovascular surgery. The results from these experiments represent the means of 4 different patients.

Adipose Tissue Culture

Supernatants of WAT explants were prepared from human scWAT and pWAT by culturing 0.3 g tissue/mL of medium for 48 hours. Adipocytes and stromal cells of human WAT were isolated by collagenase digestion as previously described.[3,10] For detailed Methods please see the online-only Data Supplement at http://www.atvbaha.org.

Chemotaxis

Peripheral blood leukocytes (PBLs) were prepared from fresh blood of healthy donors by Ficoll-Plaque density gradient according to the manufacturer's instructions (Amersham Bioscience). Peripheral blood mononuclear cells (PBMCs) and granulocytes were isolated and pooled to form the PBL fraction.[11] Monocytes were further purified from the PBMC fraction by cold-induced aggregation,[12] and T cells were further depleted of contaminant cells by nylon-wool column chromatography.[13] The purity of leukocytes was assessed by fluorescence cell sorting (fluorescence-activated-cell sorter; for details, please see the Data Supplement).

Migration tests were performed for 15 to 60 minutes in 24-well transwell plates with a pore size of 5 μm from Costar (Omnilab) in a cell culture incubator (37°C, 5% CO_2). Blocking antibodies against IL-8 or MCP-1 (R&D) were added to the supernatant for 30 minutes before the migration assay. Negative controls were performed using normal goat immunoglobulin G (IgG; Santa Cruz Biotechnology).

Measurement of Cytokines and DNA

Quantitative measurements of MCP-1 and IL-8 secretion in conditioned media were performed using duoset enzyme-linked immunosorbant assay (ELISA) development systems according the manufacturer's instructions (R&D Systems). The secretion profiles of scWAT and pWAT from each patient were compared using human cytokine antibody arrays covering 120 different cytokines (C series 1000; Raybiotech) according to the manufacturer's recommendations. Total DNA was isolated from adipose explants, adipocytes, or stromal cells using the GenomicPrep Cells and Tissue DNA Isolation kit (Amersham).

Morphological Analysis of pWAT

All animal experiments were conducted in accordance with the protocol approved by the veterinary offices of the canton of Geneva. Wistar rats obtained from Charles River (St-Germain sur l'Arbresle, France) were fed for 7 weeks with either a normal diet (n=5) or chow rich in pork fat (n=5) (19%, Provimi Kliba) and then killed by decapitation. The aorta and the associated WAT were dissected from the iliac arteries to the aortic arch. The aorta was then fixed overnight in phosphate-buffered saline with 4% formaldehyde and processed for paraffin embedding. The hematoxylin-eosin staining was performed on 5-μm sections. For morphometric analysis methods, please see the Data Supplement.

Immunohistological Analysis

For hematoxylin-eosin staining and immunohistological analysis, aortic sections were processed as described above. IL-8 was detected by immunofluorescence staining with a polyclonal goat antibody (Santa Cruz Biotechnology) and a secondary fluorescein isothiocyanate-coupled donkey anti-goat IgG antibody (Santa Cruz Biotechnology). Negative controls were performed using normal goat IgG (Santa Cruz Biotechnology).

Immunohistological staining of macrophages, T lymphocytes, and MCP-1 was performed using monoclonal antibodies against human CD68, CD3 (DakoCytomation AG), and MCP-1 (Alexis), respectively. Binding was revealed with a biotin-labeled secondary antibody that reacted with a streptavidin-biotin complex-horseradish peroxydase (DakoCytomation AG) and using 3,3′-diaminobenzidin and hydrogen peroxide as substrate. Sections were counterstained with hematoxylin. The negative controls were performed using normal mouse IgG (DakoCytomation AG).

For cell counting, 4 different fields of view on each slide were analyzed at 20× magnification. The total number of cells expressing CD68 or CD3 per field was calculated using the Leica Qwin software (Leica).

Statistical Methods

Results are expressed as mean±1 standard error of the mean (SEM). The nonparametric Mann–Whitney U test was applied where appropriate, and ANOVA was used for morphometric analysis. All tests were performed using SYSTAT 10 software (SPSS).

Results

Human WAT Secretes Chemotactic Factors

Although it is known that human WAT is a source of a variety of cytokines and chemokines, it was unclear whether their net effect would suffice to induce chemotaxis. We addressed this question by using an in vitro test of leukocyte migration. The conditioned medium from cultures of human scWAT was tested for its capacity to induce the migration of PBLs in transwells (Figure 1A). As shown in Figure 1B, the addition of supernatant of human WAT cultures induced the migration of PBLs 8-fold compared with control medium. To determine which type of leukocytes was recruited, PBLs were fractionated into granulocytes, monocytes, and T cells. As shown in Figure 1C, chemotaxis of granulocytes, monocytes, and IL-2–activated T cells was effectively stimulated by the medium conditioned by human scWAT (7.1-, 3.4-, and 2.5-fold respectively, compared with control), whereas no chemotaxis of resting T cells was observed. Supernatant from human WAT induced the migration of T cells after stimulation by IL-2, however, which is known to induce the expression of chemokine receptors.[14]

Because IL-8 and MCP-1 were previously shown to be produced by human WAT,[4–6] we tested the possibility of these chemokines being responsible for the chemotaxis induced by adipose tissue. Preincubation of the supernatant from WAT with blocking antibodies against IL-8 resulted in the complete inhibition of the migration of granulocytes and monocytes, whereas an anti–MCP-1 antibody blocked the chemotaxis of monocytes and activated T cells (Figure 1C). In contrast, the preincubation of WAT explants with a normal goat IgG (Figure 1C) or an anti–interferon γ inducible protein 10 kD (IP-10) antibody did not show any effect on the chemotaxis of granulocytes, monocytes, or activated T cells (data not shown).

To examine which cells in WAT produce MCP-1 and IL-8, we fractionated human WAT in adipocytes and stromal cells before measuring chemokine secretion. WAT explants, primary adipocytes and the stromal vascular fraction, were prepared from the same patient. Because adipocytes constitute 85% to 95% of all cells in WAT based on measurements

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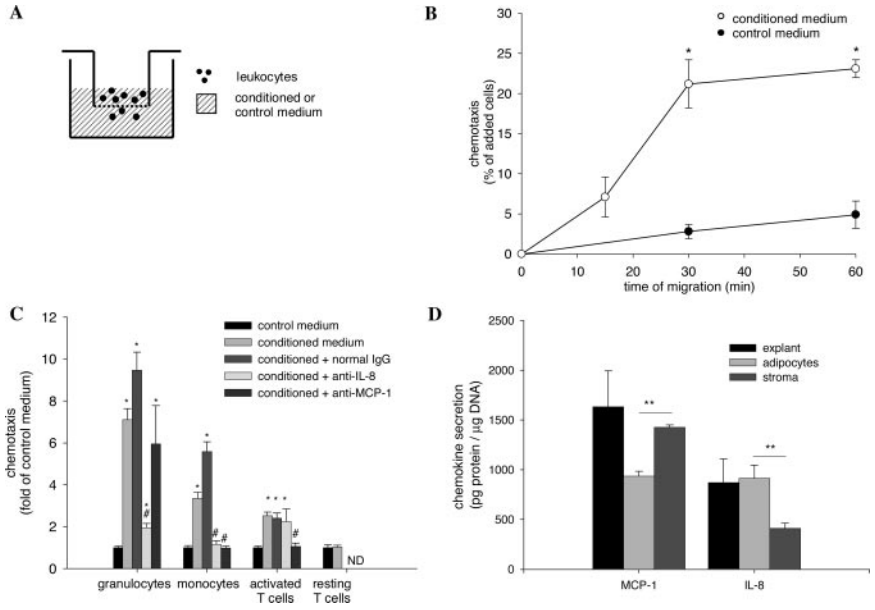

Figure 1. Chemotactic activity of human scWAT. A, Control medium or supernatant of human adipose tissue cultured for 48 hours (conditioned) was placed in the lower chamber of a transwell and 200 000 PBLs, isolated granulocytes, monocytes, or activated T lymphocytes were added to the upper chamber. B, Migration of PBLs was performed for up to 60 minutes, and cells were counted after 15, 30, and 60 minutes in the presence of control (black circles) or conditioned medium (open circles). *$P<0.05$ as compared with control medium. C, The migration of isolated granulocytes, monocytes, or resting or activated T cells was assessed after 30 minutes in the presence of control medium, conditioned medium, or conditioned medium preincubated with normal goat IgG (conditioned + normal IgG) as a negative control, or blocking antibody for IL-8 (conditioned + anti-IL-8) or MCP-1 (conditioned + anti MCP-1) at 37°C for 30 minutes before the migration assay. *$P<0.005$ as compared with control medium; #$P<0.05$ as compared with conditioned medium. D, Human WAT explants (black bars) were fractionated in isolated adipocytes (light gray bars) and the stromal fraction (dark gray bars) and cultured for 48 hours. The secretion of IL-8 and MCP-1 in the supernatant was measured by ELISA and normalized for total DNA. **$P<0.05$ between adipocytes and stromal fraction.

of the content in genomic DNA, this cell type is quantitatively the major source of MCP-1 and IL-8. Stromal cells produced more MCP-1 and less IL-8 than adipocytes when normalized for DNA content of each fraction, however (Figure 1D).

pWAT Is Increased by a High-Fat Diet in Rats

Considering that WAT is a source of proatherogenic chemokines known to act in a paracrine manner by forming local concentration gradients, we explored the proximity of WAT to the vasculature. Histology showed that pWAT is in close proximity to the vascular wall of the rat aorta, with a distance of only 50 to 100 μm between adipocytes and the smooth muscle cells of the media (Figure 2A). Occasionally, adipocytes even infiltrated the adventitia (data not shown). Given the significant quantity of perivascular fat and its proximity to the vascular wall, we then examined the effects of a high-fat diet for 7 weeks on the mass of pWAT in rats. The amount of periaortic fat was increased significantly in animals exposed to a high-fat diet, predominantly at the level of the abdominal aorta (1.9-fold, segment C) and the iliac arteries (1.7-fold, segment D) (Figure 2B). Because this localization of pWAT coincides with the predilection sites for atherosclerosis, we quantified pWAT in 4 distinct segments of the aorta (A, aortic arch; B, thoracic aorta; C, abdominal aorta; and D, iliac arteries; Figure IA, inset, available online at http://atvb.ahajournals.org) of rats fed either a normal or a high-fat diet. Regional differences between the upper and lower segments of the aorta were found: The abdominal aorta and iliac arteries were surrounded by 4- and 10-fold larger amounts of pWAT, respectively, compared with the aortic arch in rats fed a normal diet. In rats fed a high-fat diet, the pWAT in the abdominal aorta and iliac arteries was increased 5- and 12-fold, respectively (Figure IA). Compared with the aortic arch, the adipocytes in the pWAT surrounding the abdominal aorta and the iliac arteries were larger and more than 2-fold and more than 5-fold more numerous after the high-fat diet (Figure IB and IC). In contrast, the feeding of a high-fat diet had no effect on the pWAT area and adipocyte number in the aortic arch and thoracic aorta (Figure IB and IC).

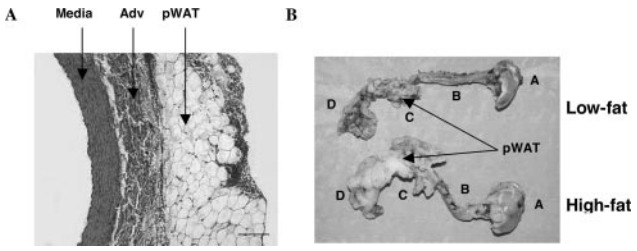

Figure 2. Effect of a high-fat diet on pWAT mass in rats. A, The sections of abdominal rat aorta were stained with hematoxylin-eosin. Adv indicates adventitia. B, Macroscopic difference in the quantity of periaortic fat between rats fed a normal or high-fat diet for 7 weeks. A indicates aortic arch; B, thoracic aorta; C, abdominal aorta, and D, iliac arteries.

Production of Chemokines by Human pWAT

Similarly to what we have observed in rodents, pWAT is also present adjacent to the vascular wall in the human aorta (Figure 3A). We used immunohistochemistry and antibody arrays to examine whether human pWAT also produced chemokines. As shown in Figure 3B, pWAT of human atherosclerotic aorta stained strongly positive for IL-8 and MCP-1, the latter being produced in a nonuniform manner by small groups of adipocytes. Although these results appear to show the presence of these chemokines inside the small cytoplasmic rim of adipocytes, the possibility that some of the immunostaining arises from the intercellular space can not be formally excluded by light microscopy.

To characterize the cytokine profile of human pWAT with regard to subcutaneous depots, we used a cytokine antibody array allowing the qualitative assessment of 120 cytokines. A

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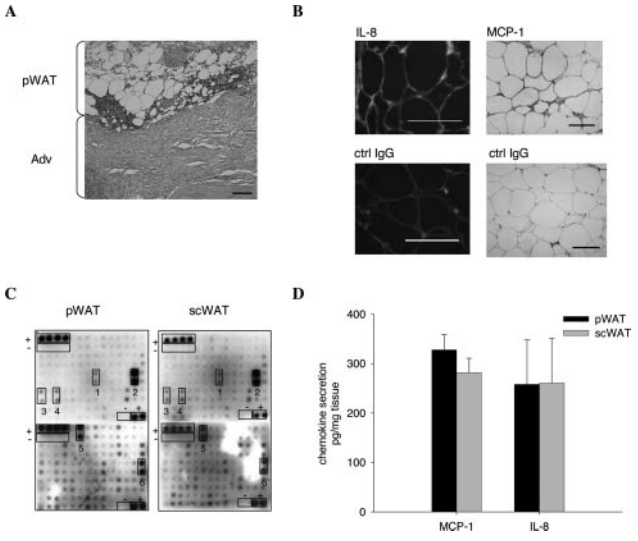

Figure 3. Chemokine production by human pWAT. A, Section of a human atherosclerotic aorta stained with hematoxylin-eosin. Adv indicates adventitia. Lines represent the internal scale of 100 μm. B, The pWAT of a human atherosclerotic aorta was immunostained for IL-8 (upper left panel), and normal goat IgG (lower left panel) was used as a negative control. The pWAT of a human atherosclerotic aorta was also immunostained for MCP-1 (upper right panel), and the negative control was performed with a normal mouse IgG (lower right panel). C, Cytokine antibody arrays were used for the simultaneous detection of 120 cytokines. Two antibody-coated membranes were incubated with conditioned medium from human pWAT (left panel) and scWAT (right panel). + indicates positive control; -, negative control; 1, IL-1Ra; 2, IL-6; 3, leptin; 4, MCP-1; 5, adiponectin; and 6, IL-8. D, Comparison of the secretion of MCP-1 and IL-8 by human pWAT and scWAT from the same patients (n=4). Explants from 4 individuals were cultured for 48 hours before chemokines were measured in the supernatant.

similar cytokine pattern was found in pWAT and scWAT of the same patient (Figure 3C). As MCP-1 and IL-8 are well known proatherogenic chemokines produced by human adipose tissue and their production is increased in obesity, we compared their secretion by human pWAT and scWAT explants from the same patients (n=4). Human pWAT was found to produce amounts of MCP-1 and IL-8 similar to the amount of scWAT (Figure 3D).

Human pWAT Is Associated With a Monocytic and Lymphocytic Infiltrate

Given the secretion of chemoattractants by human pWAT, we used immunostaining to examine the presence of infiltrating leukocytes. We found that macrophages (CD68+ cells) and T lymphocytes (CD3+ cells) accumulated preferentially at the interface between the pWAT and adventitia of atherosclerotic aortas (Figure 4A and 4B). In contrast, dendritic CD1a+ and natural killer CD56+ cells were not detected in this zone and were preferentially present within the vascular wall (data not shown). For comparison, we also stained macrophages and T cells in scWAT and pWAT surrounding peripheral arteries, which are rarely affected by atherosclerosis. In both adipose depots, very few cells stained positive for CD68 (0.68±0.3 and 0.5±0.2 cells/20× field in scWAT and pWAT of peripheral arteries, respectively) and CD3 (1.9±0.5 and 1.3±0.4 cells/20× field in scWAT and pWAT of peripheral arteries, respectively), whereas macrophages and T lymphocytes were markedly increased in pWAT of atherosclerotic aorta (9.3±1.63 macrophages and 5.2±1.6 T cells/20× field), particularly at the interface between pWAT and the adventitia (53.2±3.6 macrophages and 142.2±23.4 T cells/20× field) (Figure 4C and 4D).

To confirm the chemotactic properties of pWAT in vitro, we performed the chemotaxis assay of PBLs in response to conditioned medium. Indeed, migration of leukocytes induced by pWAT supernatant was 6-fold higher compared with the unconditioned medium. No difference was observed, however, between pWAT and scWAT (Figure 4E).

We also tested the effect of the supernatant of pWAT on purified granulocytes, monocytes, and activated T cells in the presence or absence of blocking anti-chemokine antibodies. PWAT supernatant induced the migration of all 3 cell types, albeit with different potencies (granulocytes: 14×; monocytes: 10×; and activated T cells: 2.6×; Figure 4F). The preincubation of the supernatant from pWAT with blocking antibodies against IL-8 resulted in the complete inhibition of the migration of granulocytes and monocytes, whereas an anti–MCP-1 antibody blocked the chemotaxis of monocytes and activated T cells (Figure 4F). In contrast, the preincubation of supernatant from pWAT with normal goat IgG did not show any effect on the chemotaxis of granulocytes, monocytes, or activated T cells.

Discussion

WAT is an active endocrine and paracrine organ secreting various pro- and antiinflammatory cytokines and chemokines. We report that human adipose tissue exerts a strong chemotactic effect on monocytes, granulocytes, and T lymphocytes that is mainly mediated by MCP-1 and IL-8. Both chemokines are produced by monocytes, granulocytes, and fibroblasts,[15,16] and earlier studies have reported their production by adipocytes as well as their increased secretion in human obesity.[4,5,17,18] The functional relevance remained unexplored, however, although both chemokines are potent proatherogenic factors[8] and IL-8 was also shown to induce the adhesion of monocytes and the proliferation of endothelial cells.[15]

Chemokines are known to act locally through the formation of concentration gradients rather than systemically. We therefore examined the spatial relationship between WAT and large arteries. Histological studies of the human abdominal aorta showed a close proximity of adipocytes producing IL-8 and MCP-1 to the smooth muscle cells of the media and the endothelial cells of the vasa vasorum. This is of particular interest because both chemokines can induce smooth muscle cell proliferation[19–21] and IL-8 is angiogenic for endothelial cells.[22,23] Moreover, it has been shown that smooth muscle cells and endothelial cells possess functional chemokine receptors, including the receptors for MCP-1 and IL-8.[24] Given the preferential accumulation of pWAT at sites predisposed to atherosclerosis, such as the abdominal aorta and iliac bifurcation, and the observation that this depot markedly increases after a high-fat diet in rodents, it is tempting to speculate that pWAT might contribute to chronic vascular inflammation.

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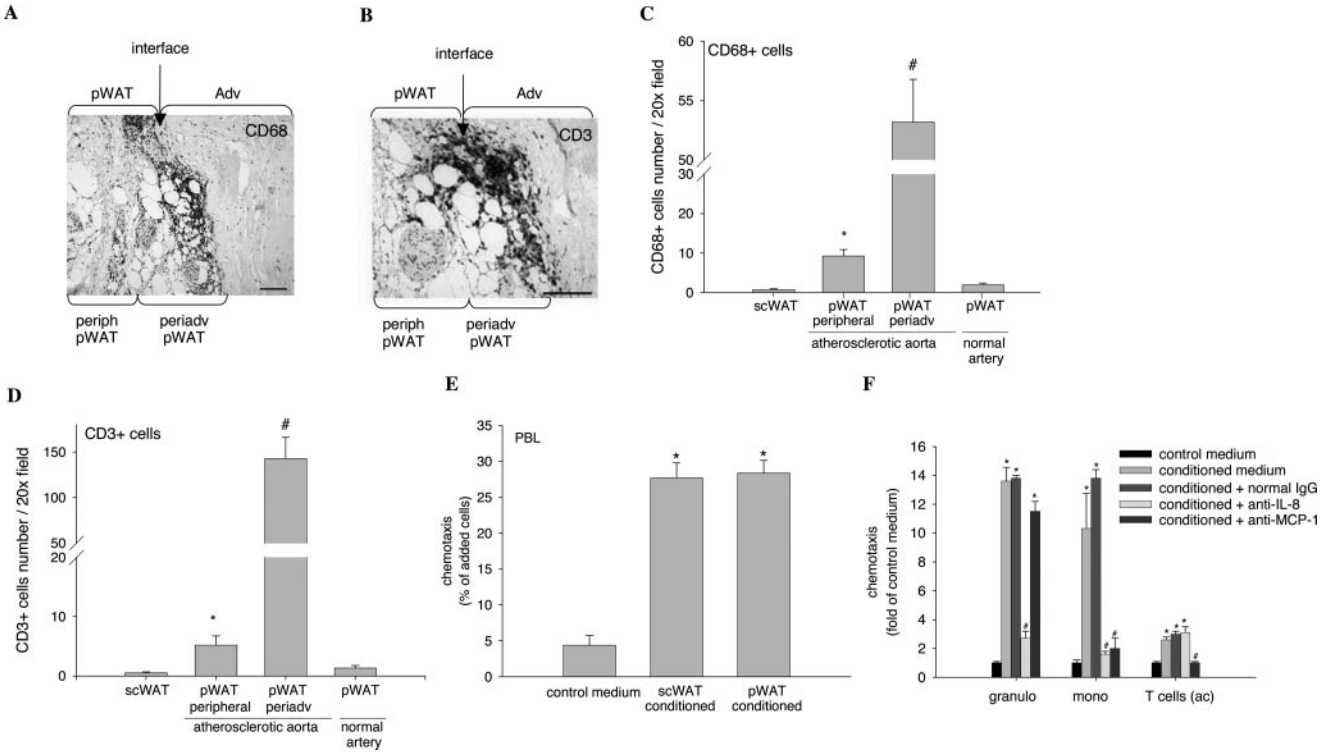

Figure 4. Leukocyte infiltration in pWAT of human atherosclerotic aortas. A, A human atherosclerotic aorta was immunostained for the macrophages specific antigen CD68. Adv indicates adventitia; periph, peripheral; and periadv, periadventitial. Lines represent the internal scale of 100 μm. B, The same aorta was also immunostained for the T lymphocyte specific antigen CD3. C, Quantification of CD68 positive cells (CD68+) in human scWAT (n=4), in pWAT of peripheral arteries (n=4), and at the interface between pWAT and adventitia (n=3), as well as in more peripheral pWAT of atherosclerotic aortas (n=4). *$P<0.001$ for peripheral pWAT of an atherosclerotic aorta compared with scWAT and pWAT of peripheral artery; #$P<0.001$ compared with scWAT, pWAT of normal artery, and peripheral pWAT of aorta. D, Quantification of CD3 positive T lymphocytes (CD3+) as described above. *$P=0.002$ for peripheral pWAT of an atherosclerotic aorta compared with scWAT and $P=0.018$ for peripheral pWAT of an atherosclerotic aorta compared with pWAT of peripheral artery; #$P<0.001$ compared with scWAT, pWAT of normal artery, and peripheral pWAT of aorta. E, Chemotactic activity of human pWAT. Control medium or supernatant of either human subcutaneous or perivascular adipose tissue cultured for 48 hours was placed in the lower chamber of a transwell and 200 000 PBLs were added to the upper chamber. Migration of PBLs was performed for up to 30 minutes before the cells were counted. F, The chemotactic activity of human pWAT on the migration of isolated granulocytes (granulo), monocytes (mono), or activated T lymphocytes [T cells (ac)] was assessed after 30 minutes in the presence of control, conditioned medium, or conditioned medium preincubated with normal goat IgG (conditioned + normal IgG) as a negative control and a blocking antibody for IL-8 (conditioned + anti-IL-8) or MCP-1 (conditioned + anti-MCP-1) at 37°C for 30 minutes before the migration assay.

Indeed, human pWAT and scWAT secrete similar amounts of chemokines, and pWAT has comparable chemoattractant properties. Hence, the 2 WAT depots are not fundamentally different in this respect. Given its strategic location in proximity to the vascular wall, however, pWAT might represent a more relevant risk factor for the progression of atherosclerosis.

Paracrine interactions between various tissues and anatomically associated adipocytes have been previously proposed, eg, in lymph nodes where perinodal WAT suppresses lymphocyte proliferation and lymphoid cells increase WAT lipolysis.[25] pWAT also releases a vascular anti-contractile factor, the perturbations of which could contribute to obesity-related hypertension.[26] pWAT surrounding arterioles in skeletal muscles has been proposed to be implicated in insulin resistance through the local production of TNF-α by the periarteriolar adipocytes.[27] The putative paracrine role of adipose depots on local atherogenesis was recently illustrated in epicardial WAT, which has been shown to produce significantly higher levels of inflammatory factors than scWAT, including IL-1β, IL-6, MCP-1, and TNF-α.[28] Those authors have also shown that the degree of local inflammation in epicardial fat did not correlate with the plasma concentration of these factors. The local infiltration by leukocytes, however, was associated with the production of these cytokines by the epicardial adipose depots.[28] Similarly, others have speculated that the presence of WAT surrounding epicardial arteries leads to an amplification of vascular inflammation, even more so as the mass of epicardial fat is increased in obesity.[29]

Recently, obesity has been shown to be associated with the infiltration of scWAT by macrophages representing 50% of total cells,[30] a finding compatible with a chemotactic effect of WAT. Only 5% of cells found within our samples of scWAT were macrophages, however. In addition, we show that macrophages, as well as T lymphocytes, preferentially accumulate at the interface between pWAT and the adventitia of atherosclerotic aorta. Consistent with these results, it has been

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reported that few inflammatory cells are present in normal pWAT, but leukocytes invade this tissue 24 hours after angioplasty.[31] Because few leukocytes are present in normal pWAT and scWAT, it is unlikely that infiltrated leukocytes are the main source of chemokines secreted by these depots, especially as isolated primary adipocytes secrete an amount of cytokines similar to that seen with adipose explants. Moreover, our data obtained from immunohistochemistry show that the adipocytes themselves are the major source of chemokines, rather than the relatively sparse infiltrating inflammatory cells.

In summary, we have demonstrated that scWAT and pWAT have strong chemotactic activity that is mainly mediated by the secretion of IL-8 and MCP-1. These factors are likely to contribute to the infiltration of leukocytes at the interface between human pWAT and the adventitia of atherosclerotic aortas. Hence, our findings show the novel concept that pWAT is a diet-regulated adipose depot that may contribute to the progression of obesity-associated vascular complications. The effect of other obesity-related risk factors, such as dyslipidemia, hypertension, and insulin resistance on pWAT is currently unexplored, but it is conceivable that these factors modulate the adipogenesis and functionality of pWAT depots, eg, through of local shear stress.

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Additional or detailed methods

Adipose tissue culture

Supernatants of WAT explants were prepared from human scWAT and pWAT as follows: 0.3 g of minced adipose tissue explants was placed in 1 ml of M199 medium supplemented with 1% streptomycin and penicillin and 5% fetal calf serum (FCS) for 18 h. The medium was then changed and explants were cultured for an additional 48 h before the supernatants were collected. Adipocytes were isolated by collagenase digestion (6 mg / g WAT; Worthington Biochemical Corp., Lakewood, NJ) in low-glucose (1 g / L) DMEM with 1% penicillin, streptomycin and BSA fraction V (20 mg / ml; Sigma, Buchs, Switzerland) at 37 C in a rocking bath for 30 minutes. Adipocytes and the stromal fraction were then separated from undigested tissue by filtration through a 350-μm nylon mesh before the separating centrifugation (10 minutes at 200 x g).[10] Adipocytes from 0.6 g of WAT were resuspended in 2 ml of M199 containing 5% FCS, 1% penicillin and streptomycin. After 24 h, culture medium was changed and the adipocytes were cultured for additional 48 h in the same medium. The pellet of the stromal fraction from 5 g of WAT was resuspended in 1 ml of low-glucose DMEM containing 10% FCS, 1% glutamine, 1% penicillin and streptomycin. The cells were grown for 4 days before the medium was changed with FCS reduced to 5% and the cells were cultured for 48 h.

T cells activation and purity of leukocyte fractions

T cells were activated in the presence of phytohemaglutinin (PHA 1 μg / ml) for 48 h and then grown in the presence of interleukin-2 (20 U / ml; Biogen, Cambridge, MA) and 5% human serum AB (Blood Bank of the University Hospital, Geneva, Switzerland) for 7-10 days.

The purity of blood monocytes and T cells was assessed by fluorescence activated cell sorting (FACS) using fluorescein isothiocyanate-coupled CD3 and CD16 and phycoerythrin-coupled

CD14, and CD19 antibodies (FACScan cell sorter; BD Biosciences Pharmingen, Basel, Switzerland). The monocyte fraction was 96% pure with 3 % of T cells, 0.5 % of B lymphocytes and 0.5 % of natural killer (NK) cells, while activated T cells were 98% pure, with 0.5% of monocytes, 0.5% of B cells and 1% of NK cells. The granulocyte population was analyzed by May-Grünwald-Giemsa staining (95% purity with 3.5% of monocytes and 1.5% of lymphocytes).

Measurement of cytokines and DNA

Secretion profile of 120 cytokines by scWAT and pWAT from the same patient were compared using human cytokine antibody arrays (C series 1000; Raybiotech, Norcross, GA) according to the manufacturer's recommendations. Briefly, antibody-coated membranes were first incubated with conditioned medium from WAT explants. The membranes were then washed and biotin-labeled antibodies were added. After a second washing step, the membranes were incubated with horseradish peroxydase-conjugated streptavidin. Finally a detection reagent was added and membranes were exposed on X-ray film.

Morphometrical analysis of pWAT (Figure I online)

For morphometrical analysis the aortas were separated into four segments: the aortic arch (segment A, from the heart to the same level on the retro-cardiac side), the thoracic aorta (segment B, up to the diaphragm), the abdominal aorta (segment C, up to the iliac bifurcation), and the iliac arteries (segment D, from the iliac bifurcation up to 1 cm in maximal length). The morphometrical measurements were performed on 10 paraffin embedded sections of 5 μm thickness taken every 100 μm in each aortic segment of 5 controls and 5 rats fed a high-fat diet. First, the area of pWAT was measured in each section. Only WAT lobules with direct contact with the vascular wall or a distance of maximally one 5x

field of view from the vascular wall were measured. Secondly, the number of adipocytes in pWAT was determined. Finally, 10 adipocytes were randomly selected in each section of pWAT and their cross-sectional surface was measured. In order to correct for the potential minor tilting in the preparation of the histological slides, the number of adipocytes in pWAT was determined per section and the area of pWAT was normalized to the area of the aortic media. All measurements were made using Leica Qwin software (Leica, Glattbrugg, Switzerland) at a 5x magnification.

For cell counting, four different fields of view on each slide were analyzed at 20x magnification. The total number of cells expressing CD68 or CD3 per field was calculated using the Leica Qwin software (Leica, Glattbrugg, Switzerland).

Legend: Figure I

Morphometric analysis of pWAT in rats fed a normal or a high-fat diet

Morphometric comparison of rats fed either a normal (low fat, black bars, n=5) or a high-fat diet for 7 weeks (high fat, grey bars, n=5) depending on the aortic segment.

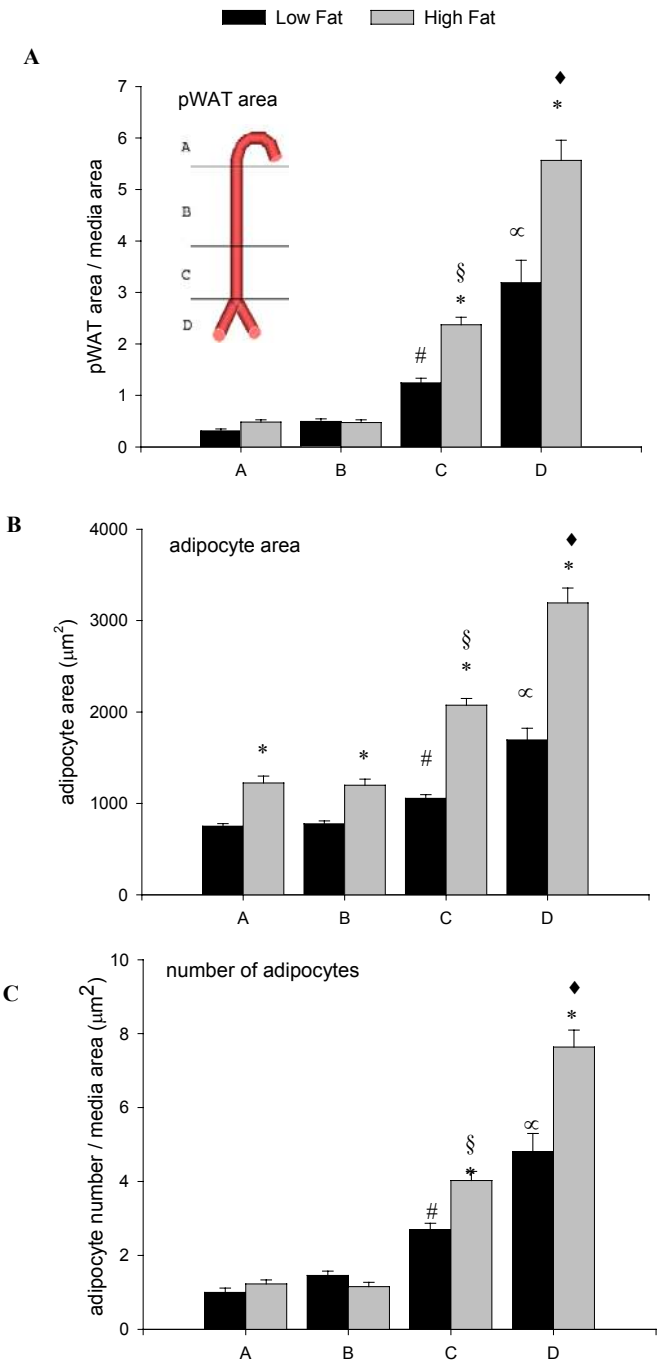
A. The area of pWAT was measured by quantifying the total adipose surface in direct contact with the vascular wall and normalized to the area of the media. Insert: schematic representation of the four aortic segments analyzed (A, arch; B, thoracic; C, abdominal; D, iliac arteries).

B. The size of adipocytes was measured by quantifying their cross-sectional surface.

C. The number of adipocytes was assessed by counting the adipocytes per pWAT area and normalized to the area of the media. The results are expressed relative to segment A in the control animals.

All results are expressed as the mean ± 1 SEM. * p<0.001 in comparison with rats fed normal diet, # p<0.001 comparison with low fat A and B segments, § p<0.001 in comparison with high fat A and B segments, ∝ p<0.001 in comparison with low fat A, B and C segments, ♦ p<0.001 comparison with high fat A, B and C segments.

Figure I



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Production of Chemokines by Perivascular Adipose Tissue: A Role in the Pathogenesis of Atherosclerosis?

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