Stromal Cells Are the Main Plasminogen Activator Inhibitor-1–Producing Cells in Human Fat
Evidence of Differences Between Visceral and Subcutaneous Deposits

Delphine Bastelica, Pierre Morange, Bruno Berthet, Hélène Borghi, Odile Lacroix, Michel Grino, Irène Juhan-Vague, Marie-Christine Alessi

Abstract—Elevated plasma plasminogen activator inhibitor (PAI)-1 observed during insulin resistance has been connected with an excessive PAI-1 adipose tissue secretion mainly by visceral fat. Our aim was to compare the localization of PAI-1 in human visceral and subcutaneous fats. PAI-1 secretion was also investigated in vitro during human adipocyte differentiation. PAI-1 antigen and mRNA were localized in the stromal area of the tissue and were also present in a few CD14-positive monocytes, in direct contact with adipocytes. In addition, in subcutaneous tissue, PAI-1 mRNA contents, determined by using real-time polymerase chain reaction, were higher in the stromal fraction than in the adipocyte fraction. PAI-1 mRNA-positive cells were 5-fold more frequent in the visceral area than in the subcutaneous stromal area ($P<0.004$). Such a difference was also observed for PAI-1 mRNA content between both whole adipose tissues. In contrast to leptin, during adipocyte differentiation, PAI-1 secretion did not follow adipocyte maturation. In situ hybridization in culture did not reveal PAI-1 mRNA in lipid-filled cells. Our results demonstrate that PAI-1 production is mainly due to stromal cells, which were more numerous in the visceral than in the subcutaneous depot. These results could explain the strong relationship observed between circulating PAI-1 levels and the accumulation of visceral fat. (Arterioscler Thromb Vasc Biol. 2002;22:173-178.)

Key Words: fibrinolysis ■ obesity ■ differentiation ■ visceral fat ■ real-time polymerase chain reaction Taqman

Plasminogen activator inhibitor (PAI)-1 is a fibrinolytic inhibitor whose elevated plasma concentration is thought to contribute to the increased susceptibility to atherogenesis described in insulin-resistant patients with obesity.

Interestingly, some reports have underlined the independent association of body adiposity and plasma PAI-1 levels, mainly visceral fat, in mice and in humans. PAI-1 expression has been reported to be upregulated in adipose tissue from obese mice and humans concomitantly with increased plasma PAI-1 levels, and it has been proposed that the increase in adipose tissue PAI-1 observed in obesity could be the result of tumor necrosis factor-α disturbance, which specifically accompanies insulin resistance.

The precise origin of PAI-1 expression in adipose tissue is not definitively established, because PAI-1 synthesis has been attributed or not to adipocytes, depending on which model was used. Several groups have documented PAI-1 synthesis by murine adipocyte cell lines. Recently, Crandall et al observed that human stromal cells issued from adipose tissue produced PAI-1 after 24 hours of incubation in medium containing serum, whereas Birgel et al identified PAI-1 antigen in stromal preadipocytes differentiated into adipocytes. In untreated CB6 mice, Samad et al localized PAI-1 mRNA by in situ hybridization in the smooth muscle cells of the tunica media of the vessels, but a positive signal was also observed in >50% of the cells that morphologically resembled adipocytes. In humans, we have previously shown, by use of RNase protection assay, that PAI-1 mRNA was mainly present in the stromal fraction of freshly collected adipose tissue from obese patients, whereas, conversely, immunolocalization performed with isolated adipocytes also evidenced the protein at the adipocyte level. Until now, the precise localization of PAI-1 in human adipose tissue sections has not been evaluated. Several studies have emphasized the relationship between visceral fat and plasma PAI-1. In mice, Shimomura described a strong PAI-1 expression in visceral fat. Using human adipose tissue explants maintained...
in culture or differentiating adipocytes, we and others have observed a higher PAI-1 secretion by the visceral fat.\(^\text{11,20,21}\)

The purpose of the present work was to identify the localization of PAI-1 synthesis from cryosections of freshly collected human adipose tissue from obese patients or from human adipose cells in primary culture by immunolocalization and in situ hybridization and to compare the levels of PAI-1 expression between visceral and subcutaneous fat territories.

Methods

Population and Sample Collection

Visceral and subcutaneous adipose tissues were obtained during gastropasty from 26 patients (22 women, 4 men) aged 41±12 (mean±SD) years, with mean±SD body mass index (BMI) 41±6.3 kg/m\(^2\), which is weight (kilograms) divided by height squared (meters). Mean±SD (range) values for plasma PAI-1 antigen and activity levels were 52±36 (12 to 165) ng/mL and 26±29 (1 to 140) IU/mL, respectively. Fasting insulinemia was 7.3±6.7 (mean±SD) \(\mu\)U/mL. The fasting triglyceride level was 1.56±0.65 (mean±SD) g/L. All subjects were white and did not suffer from any ongoing disease (infection, cancer). Investigations were conducted according to the principles expressed in the declaration of Helsinki.

After resection, adipose tissue was immediately put into dry ice and rapidly transported to the laboratory. The separation of mature adipocytes from stromal cells was performed with subcutaneous adipose tissue samples obtained from 8 women during abdominal liposcopy; mean±SD (range) values for BMI and age were 23±3.7 (20 to 31) kg/m\(^2\) and 33±10 (18 to 54) years, respectively.

Venous blood samples were obtained just before anesthesia. For PAI-1 antigen and activity, samples were drawn into chilled trisodium citrate tubes, centrifuged as previously described\(^\text{11}\) to obtain platelet free plasma, and stored at −80°C until use.

PAI-1 Antigen Determination

Supernatant PAI-1 antigen was assayed as previously described.\(^\text{22}\) Plasma PAI-1 antigen and activity were assayed by using Asserachrom PAI-1 (Diagnostica Stago) and Chromolize (Biopool) kits, respectively.

RNA Extraction

Total RNA was extracted by using the method of Chomczynski and Sacchi.\(^\text{23}\) The integrity of the RNA was confirmed by electrophoresis in ethidium bromide–containing agarose gels, and the RNA concentration was determined spectrophotometrically.

Quantitative RT-PCR TaqMan

Reverse transcription (RT) was performed as previously described.\(^\text{11}\) Amplification was performed in a final volume of 25 \(\mu\)L with an Abiprism 7700 (Perkin Elmer). Amplification was performed in 25 \(\mu\)L on an ABI Prism 7700 (Perkin Elmer). The polymerase chain reaction (PCR) conditions were as follows: 2 minutes at 50°C, 10 minutes at 95°C followed by 40 cycles of 2-step PCR reaction denaturation at 95°C for 15 seconds, and annealing extension at 60°C for 60 seconds. Each sample contained 2 \(\mu\)L cDNA in 1:10 TaqMan Universal PCR Master Mix (Applied Biosystems) and 400 mmol/L of primers (Life Technologies) and probe (FAM labeled, Applied Biosystems). Primer express software (Applied Biosystems) was used to design primers and probes for PAI-1 mRNA and 18S rRNA (housekeeping gene). For PAI-1 mRNA quantification, primers were 5’-CAG AAA GTG AAT GTC GAT AAC-3’ and 5’-GGA AGG GTC TCT GCA TGA TGA T-3’, and the probe was 5’-AAG GTG GCC TCC TCA TCC ACA GC-3’ (GenBank accession No. M16006). The primers for 18S rRNA were 5’-CCA CCA CTA CCT GGA AGG AAG-3’ and 5’-TTT TTT TTC GTG TTC ACC TCC CC-3’, and the probe for 18S rRNA was 5’-CGC GCA AAT TAC CCA CTC CGC AC-3’ (GenBank accession No. X03205).

The number of cycles required to generate a threshold of 0.03 fluorescence units was determined in triplicate for each sample. Results were accepted if the coefficient of variation was <0.5 for the number of cycles required. Each experiment included a standard curve for the individual amplicon. Results were expressed as femtograms of PAI-1 mRNA per nanograms of 18S rRNA.

Separation of Mature Adipocytes From Stromal Cells and Cell Culture

Stromal and adipocyte cell fractions were isolated by collagenase digestion as previously described.\(^\text{20}\) Semiquantitative RT-PCRs for von Willebrand factor (vWF) and leptin mRNA were performed on each fraction to determine their degree of purity. For cell cultures, the stromal cell fraction was incubated with erythrocyte-lysing buffer that contained 154 mmol/L NaCl, 10 mmol/L KHCO\(_3\), and 0.1 mmol/L EDTA for 10 minutes. Cells were repeatedly washed and resuspended in DME/F-12 Ham medium (vomol) supplemented with 15 mmol/L Na\(\text{HCO}_3\), 33 mmol/L L-glutamine, and 17 mmol/L pantethenate. After cell attachment (SonicSeal Slides, Nunc), the medium was switched to a differentiation mixture that consisted of the same medium enriched with 252 \(\mu\)mol/L isobutylmethylxanthine, insulin transferrin selenium (ITS, Sigma Chemical Co; 1:100), 10 mmol/L dexamethasone, and 1 mmol/L triiodothyronine. After 4 days of incubation, isobutylmethylxanthine was retrieved. Medium was then changed every 72 hours. Differentiated adipocytes were defined as cells, the cytoplasm of which was completely filled with lipid droplets. Leptin was quantified in the culture medium (Human Leptin Quantikine, R&D Systems) as a marker of adipocyte maturation. After 21 days of culture, cells were fixed in 4% paraformaldehyde.

Immunohistochemistry

Sections (20 to 25 \(\mu\)m) were cut in a cryostat microtome (Leica) at −20°C and put onto slides (Superfrost Plus, CML). Dried slides were fixed for 5 minutes in acetone and then washed for 5 minutes in PBS. Labeling was performed with the DAKO LSAB kit according to the manufacturer’s instructions. A mix of 4 monoclonal antibodies kindly provided by Prof P. Declerck (Faculty of Pharmaceutical Sciences, Leuven, Belgium) was used at a final concentration of 5 \(\mu\)g/mL to detect PAI-1. Two monoclonal antibodies directed against CD68 and CD14 (Dako) allowed the identification of cells from the monocyte/macrophage lineage. They were diluted at 1:100 and 1:20, respectively. Two antibodies directed against vWF (rabbit polyclonal antibody from Dako, dilution 1:300) and CD34 (Dako) were used to evidence endothelial cells. Anti–\(\alpha\)-actin (rabbit antibody, diluted 1:50, Sigma) and anti–cytokertatin M18 (mouse monoclonal antibody, diluted 1:100, Sigma) antibodies were used to identify smooth muscle cells and mesothelial cells, respectively. Antibodies against leptin were kindly provided by Prof R. Negrel (Center of Biochemistry, Université de Nice Sophia Antipolis, Nice, France). Slides were then counterstained with Harris’ hematoxylin.

Double labeling was performed by combining a first incubation step with anti-CD14 antibodies (1:20). Then, slides were subjected to goat anti-mouse antibodies linked to alkaline phosphatase. Staining was performed by using the fast blue substrate (Vectastain) from Vector in the presence of levamisole during 20 minutes at obscurity. Slides were then rinsed with PBS and incubated for 15 minutes in normal mouse serum (1:10). Slides were then incubated for 30 minutes with the mix of 4 anti–PAI-1 monoclonal antibodies coupled to peroxidase. After a further wash, peroxidase was revealed by using amino-9-ethylcarbazole (AEC) in the presence of \(\text{H}_2\text{O}_2\) for 20 minutes. Controls without antibodies were assessed under the same conditions.

In Situ Hybridization

PAI-1 sense and antisense riboprobes were produced as previously described.\(^\text{24}\) Leptin riboprobes were used to obtain a labeling of adipocytes. The leptin probe was a 301-bp fragment (from base 57 to 357) subcloned in pPCRscript (Strategene) linearized with BsmBI (antisense) and with NotI (sense). Tissues were processed and hybridized with \(^{35}\)S-labeled PAI-1 or leptin riboprobes as previously described.\(^\text{23}\) Slides were subsequently dipped into nuclear emulsion (Ilford K5, Ilford) diluted 1:2 and exposed for 30 days. After development, slides were counterstained with neutral nuclear red.
Results

Immunolocalization of PAI-1 Antigen in Human Adipose Tissue

Sixteen paired adipose samples of visceral and subcutaneous origin were immunostained for PAI-1 antigen. The population was composed of 12 women and 3 men (aged 42±13 [range 23 to 66] years, BMI 42±6.8 [range 33 to 57] kg/m², respectively). Visceral adipose tissue contained 5-fold more positive cells than the stroma of the subcutaneous tissue (67.6±14.7 [range 12 to 165] cell/mm², respectively). The pattern of PAI-1 expression was compared with that of leptin, which is produced by adipocytes. PAI-1 expression differed from that of leptin because it was mainly expressed in the stromal area from visceral tissue (Figure 2A) as well as from subcutaneous tissue (Figure 2B) and in small cells in close contact with adipocytes but not within adipocytes (Figure 2C), whereas leptin was observed mainly in the small cytoplasmic rim of adipocytes (Figure 2D). Slides hybridized with PAI-1 mRNA sense probe were negative (data not shown).

The counterstaining performed allowed quantification of the number of PAI-1 mRNA–positive cells per square millimeter of the stromal surface from both territories. The percentage of the quantified surface did not differ between the 2 fat territories. Interestingly, the stroma of the visceral tissue contained 5-fold more positive cells than the stroma of the subcutaneous tissue (67.6±61.2 [range 9.5 to 166.8] versus 13.6±24.2 [range 0 to 66.2] cell/mm², respectively; P=0.004; n=15). This difference was also observed when the amounts of PAI-1 mRNA were measured by quantitative RT-PCR in both tissues. Indeed, the visceral tissue contained 4.7-fold more PAI-1 mRNA than did the subcutaneous tissue (141±155 [range 7.6 to 637] versus 29.8±29.4 [range 0.7 to 102] fg PAI-1 mRNA/ng 18S rRNA, respectively; P=0.001; n=21).

Immunolocalization and In Situ Hybridization of PAI-1 mRNA in Cultured Human Adipocytes

Adipocyte differentiation was followed for 21 days. A 4-fold increase in PAI-1 antigen accumulation was observed between days 4 and 10. Then, after a plateau of 6 days, PAI-1 antigen accumulation progressively decreased. This pattern of production is in contrast to that of leptin, which continued to increase (Figure 3). The decrease of PAI-1 antigen accumu-
Six cases of meningitis were unreported, presumably due to unnoticed signs of meningitis.

Cell types in culture proved to be heterogeneous. We could distinguish some round cells labeled with antibodies against CD68 (Figure 4C) and identified as macrophages. Leptin was found in cells full of lipids (Figure 4D) as well as in more largely spread cells free of lipids (data not shown). PAI-1 antigen was detected in several types of cells: fully differentiated adipocytes, cells free of lipids (Figure 4A), and cells that morphologically resembled those positive for CD68 (Figure 4B). The intensity of the PAI-1 signal obtained at day 14 was higher than that observed at day 21 (data not shown).

In situ hybridization for PAI-1 was performed on cultures at different periods of time. PAI-1 mRNA labeling was mainly observed in small cells but not in lipid-filled cells. As for PAI-1 antigen, we observed a decrease in PAI-1 mRNA signal intensity preceding that of PAI-1 antigen (Figure 2E through 2F).

PAI-1 Expression in Mature Adipocytes and Stromal Cells From Human Subcutaneous Adipose Tissue

Subcutaneous adipose tissues were subjected to collagenase digestion, as described in Methods, to separate the adipocytes from the stromal cells. A complete depletion of the stromal cells from the adipocyte fraction was not obtained, inasmuch as vWF mRNA was detected in both fractions, whereas leptin mRNA was only detected in the adipocyte fraction (data not shown). Total RNA was then obtained to quantify PAI-1 mRNA by quantitative RT-PCR. The stromal fraction contained much more PAI-1 mRNA than did the adipocyte fraction. Stromal cells versus adipocyte fractions were, respectively, as follows: 6.05 versus 1.43, 116.82 versus 11.57, and 16.47 versus 0.35 fg PAI-1 mRNA/ng 18S rRNA.

Discussion

The present results show that in human adipose tissue, stromal cells are, in vivo, the main source of PAI-1, even in obese patients with high plasma PAI-1 levels. In contrast to Samad and Loskutoff,19 we did not find PAI-1 mRNA in mature adipocytes. Indeed, PAI-1 mRNA was never detected in the cytoplasmic part of adipocytes but rather in small cells in close contact with the latter. Moreover, by use of quantitative RT-PCR assay on isolated cell fractions from adipose

Figure 4. Immunolocalization of PAI-1 antigen and specific cell markers in human cultured adipocytes from 2 lean women (aged 42 and 55 years, BMI 23.4 and 24.4 kg/m², respectively). PAI-1 protein is detected in several cell types, including fully differentiated adipocytes, cells free of lipids (A), and cells that morphologically look like CD68-positive cells (B). CD68-positive cells are shown (C). Antibodies against leptin show a strong staining of cells full of lipids (D). No signal was observed when primary antibodies were omitted. Peroxidase is revealed by using AEC substrate (red), and nuclei are stained with Harris’ hematoxylin (blue). Bar=10 μm for all panels.

Figure 2. In situ hybridization of PAI-1 and leptin mRNA in fresh-frozen, subcutaneous, visceral adipose tissue and during adipocyte differentiation. Tissue sections from obese subjects are subjected to PAI-1 antisense probe (A through C). The number of PAI-1–expressing cells was higher in the visceral area (A) than in the subcutaneous stromal area (B). In the adipocyte area from both tissues, positive cells are in close contact to adipocytes (C). As an adipocyte marker, leptin antisense probe labeling shows a more diffuse signal in the cytoplasmic rim of adipocytes (D). In situ hybridization of the PAI-1 antisense probe on human adipocytes, obtained from a lean woman (aged 55 years, BMI 24.4 kg/m²), is shown during differentiation (E and F). At day 8, a strong signal is observed in small cells (E) but not in lipid-filled cells (F, arrow). From day 8 (E) to day 14 (F), this signal intensity drastically decreases. Nuclei are stained with neutral red. Bar=25 μm (A through D), and bar=35 μm (E and F).

Figure 3. Leptin (open circles, broken line) and PAI-1 (solid circles, solid line) secretion during adipocyte differentiation. Adipocytes were isolated from the adipose tissue of lean subjects. Results of a representative experiment from a lean woman (31 years, BMI 20.7 kg/m²) are shown. Data represent the mean±SD of 5-well values for PAI-1 and of adipocytes cultured in a 25-cm² flask for leptin.
tissue, PAI-1 mRNA levels were observed to be higher in the stromal than in the adipocyte fractions. It could be hypothesized that the remaining PAI-1 found in the adipocyte fraction was due to contaminating stromal cells. Indeed, we never obtained a complete depletion of the stromal cells from the adipocyte fraction, because vWF mRNA was detected in both fractions (data not shown), but a production by adipocytes lower than the limit of sensitivity of the methods we have used cannot be excluded. Thus, PAI-1 antigen and mRNA were mainly found in the stroma of human adipose tissue. This result is original, inasmuch as most of the groups interested in studying PAI-1 synthesis by adipose tissue have focused on adipocyte rather than stromal cells.\textsuperscript{21,27,28}

Visceral fat was observed to express almost 5-fold more PAI-1 mRNA than did abdominal subcutaneous adipose tissue by use of real-time PCR TaqMan. This method is very accurate compared with the semiquantitative method that we used previously, which did not allow demonstration of such a difference.\textsuperscript{11} Furthermore, in situ hybridization showed that the number of PAI-1–expressing cells was higher in visceral than in subcutaneous fat. These results confirm previous reports showing a higher PAI-1 antigen secretion by visceral adipose tissue explants compared with subcutaneous explants.\textsuperscript{20,21,28} All these data are in favor of a high PAI-1 synthesis capacity of the visceral fat. These data are in contrast to the results published by Eriksson et al\textsuperscript{12} who, after normalizing the results by the number of fat cells, found a higher level of PAI-1 expression in subcutaneous than in visceral tissue. In the present study, the PAI-1 expression levels were normalized by the number of fat cells. The absence or low contribution of adipocytes in PAI-1 production could probably explain these results. Our results contrasted with the lack of difference between the adipose tissue PAI-1 antigen content of subcutaneous and visceral fats that we reported previously.\textsuperscript{11} This could be attributed to a retention of PAI-1 antigen in extracellular matrix, which could impair the demonstration of a difference because the quantification of surfaces labeled for PAI-1 antigen did not differ between territories (data not shown).

A complete identification of the stromal cells involved in PAI-1 synthesis could not be undertaken. However, the colocalization of CD14 and PAI-1 revealed that some of these cells could be of monocyte origin. The similar pattern of PAI-1 antigen and \(\alpha\)-actin is also in favor of a contribution of smooth muscle cells, as proposed by Samad et al.\textsuperscript{10} Nevertheless, a contribution of other stromal cells as preadipocytes cannot be excluded. To analyze this point, we have focused on the localization of PAI-1 synthesis during human adipocyte differentiation. In situ hybridization experiments localized PAI-1 mRNA in small cells free of lipids. This result was in contrast to the PAI-1 immunostaining, which revealed the presence of PAI-1 antigen in few lipid-filled cells. This phenomenon is not fully understood but could be due to a diffusion of the PAI-1 protein to the adhesion surface of the adipocyte unless, as underlined by Cousin et al,\textsuperscript{29} it reflects a phagocytic activity of these cells. Several groups,\textsuperscript{14,16} together with ours,\textsuperscript{20} have demonstrated that murine cell lines subjected to differentiation produce PAI-1. Few data have been published regarding human adipocyte cell culture. Crandall et al\textsuperscript{17} observed synthesis and secretion of PAI-1 after a 24-hour incubation of isolated stromal cells in medium containing 10% serum. This was in agreement with a PAI-1 synthesis by preadipocytes or nonadipocyte cells. More recently, Birgel et al\textsuperscript{18} demonstrated, like ourselves, that PAI-1 protein secretion increased 1.9-fold during the course of adipocyte differentiation, but no localization of PAI-1 mRNA was performed. The present results demonstrate that in human adipose tissue, stromal cells appear to be the main cells involved in PAI-1 production. Although mature adipocytes have not been found to produce PAI-1, a contribution of preadipocytes cannot be excluded. PAI-1 expression is almost 5-fold higher in the visceral than in the subcutaneous fat because of the increase in stromal PAI-1–producing cells. These results could explain the strong relationship observed between circulating PAI-1 levels and the accumulation of visceral fat and suggest that visceral fat is subjected to a particular environment that stimulates PAI-1 synthesis.

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