Inflammatory Cytokines Interleukin-6 and Oncostatin M Induce Plasminogen Activator Inhibitor-1 in Human Adipose Tissue

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Background—Adipose tissue is a prominent source of plasminogen activator inhibitor-1 (PAI-1), the primary physiological inhibitor of plasminogen activation. Increased PAI-1 expression acts as a cardiovascular risk factor, and plasma levels of PAI-1 strongly correlate with body mass index (BMI). Elevated serum levels of interleukin-6 (IL-6), an inflammatory cytokine and a member of the glycoprotein 130 (gp130) ligand family, are found in obese patients and might indicate low-grade systemic inflammation. Another gp130 ligand, oncostatin M (OSM), upregulates PAI-1 in cardiac myocytes, astrocytes, and endothelial cells. We used tissue explants and primary cultures of preadipocytes and adipocytes from human subcutaneous and visceral adipose tissue to investigate whether IL-6 and OSM affect PAI-1 expression in fat.

Methods and Results—Human subcutaneous and visceral adipose tissue responded to treatment with IL-6 and OSM with a significant increase in PAI-1 production. Human preadipocytes were isolated from subcutaneous and visceral adipose tissue. Adipocyte differentiation was induced by hormone supplementation. All cell types expressed receptors for IL-6 and OSM and produced up to 12-fold increased levels of PAI-1 protein and up to 9-fold increased levels of PAI-1 mRNA on stimulation with IL-6 and OSM. AG-490, a janus kinase/signal transducer and activator of transcription inhibitor, abolished the OSM-dependent PAI-1 induction almost completely.

Conclusions—We have for the first time established a link between the gp130 ligands, the proinflammatory mediators IL-6 and OSM, and the expression of PAI-1 in human adipose tissue. Thus, we speculate that IL-6 and OSM, by upregulating PAI-1 in adipose tissue, can contribute to the increased cardiovascular risk of obese patients. (Circulation. 2005;111:1938-1945.)

Key Words: obesity ■ inflammation ■ cardiovascular diseases ■ plasminogen ■ fibrinolysis
pleiotropic cytokine that plays an important role in a variety of physiological and pathophysiological events, including inflammation, hematopoiesis, tissue remodeling, and cell growth.\textsuperscript{19,20} IL-6, another well-known ligand of the gp130 receptor family, is a core feature of inflammatory processes and the acute-phase response.\textsuperscript{21} In addition, serum levels of IL-6 are significantly higher in obese patients, are strongly correlated with BMI, and might indicate low-grade systemic inflammation in these patients.\textsuperscript{22} Bastard et al\textsuperscript{23} could show in a recent study that IL-6 values are more strongly correlated with obesity and insulin resistance parameters than tumor necrosis factor-\(\alpha\) (TNF-\(\alpha\)) or leptin. Recent evidence suggests that by producing proinflammatory cytokines such as TNF-\(\alpha\) and IL-6, adipose tissue contributes to the development and progression of atherosclerosis.\textsuperscript{24,25} In the present study, we used tissue explants and primary cultures of preadipocytes and adipocytes from human subcutaneous and visceral adipose tissue to investigate whether gp130 ligands regulate PAI-1 expression in adipose tissue and thus could contribute to the elevated levels of PAI-1 found in obesity and the IRS.

\textbf{Methods}

\textbf{Origin of Adipose Tissue}

Subcutaneous adipose tissue was obtained from patients undergoing surgical mammary reduction or abdominoplasty and omental adipose tissue was obtained from patients undergoing open abdominal surgery. All subjects were of Caucasian origin and did not suffer with regard to presence of diabetes mellitus, acute infection, cancer, or any other consuming disease. For the present study, we included 26 donors (aged 45\textpm{}20 years, BMI 26\textpm{}4.8 kg/m\textsuperscript{2}). All human material was obtained and processed according to the recommendations of the hospital’s Ethics Committee and Security Board, which included obtaining informed consent.

\textbf{Ex Vivo Culture of Adipose Tissue}

Subcutaneous and visceral adipose tissue, obtained as above, was dissected from skin, visible blood vessels, and fibrous material, minced into small pieces (5 mm, \textless{}=40 mg), washed once with Hanks’ balanced salt solution, and incubated in Dulbecco’s modified Eagle’s/Ham F-12 medium (DMEM/F-12, Sigma) for 24 hours before further experiments.

\textbf{Isolation, Characterization, and Cultivation of Human Preadipocytes}

Primary cultures of human preadipocytes were prepared from adipose tissue of subcutaneous and visceral origin obtained as described above. Tissue was dissected from skin, visible blood vessels, and fibrous material, minced into small pieces (1 to 2 mm), and digested in Hanks’ balanced salt solution containing 0.1% collagenase type IV (both from Sigma) for 60 minutes at 37°C in a shaking incubator. After they were filtered through a 70-\(\mu\)m nylon mesh (BD Falcon), cells were centrifuged for 10 minutes at 1000 rpm, washed once with DMEM/F-12 containing 20% fetal calf serum (Biochrom), and centrifuged for 5 minutes at 1500 rpm. The isolated sedimented cell fraction was incubated with erythrocyte lysis buffer containing 154 mmol/L NH\textsubscript{4}Cl, 10 mmol/L KHCO\textsubscript{3}, and 0.1 mmol/L EDTA (all from Merck) for 10 minutes at room temperature. After centrifugation for 5 minutes at 1500 rpm, the cells were resuspended in DMEM/F-12 containing 20% fetal calf serum and seeded into 24-well plates and maintained at 37°C in a humidified atmosphere of 5% CO\textsubscript{2}, 95% air. After cell adhesion for 24 hours, preadipocytes exhibited the characteristic fibroblast-like shape (Figure 1A). Medium was replaced every other day. Aliquots of the respective preparations were analyzed by fluorescence-activated cell sorter and immunostaining for the presence of contaminating endothelial cells (CD31\textsuperscript{+}), mature fibroblasts (CD34\textsuperscript{−}/CD90\textsuperscript{+}), monocytes (CD34\textsuperscript{−}/CD14\textsuperscript{+}), macrophages (CD68\textsuperscript{+}), and smooth muscle cells (\(\alpha\)-smooth muscle cell actin). In all preparations of subcutaneous and visceral preadipocytes used for the experiments presented, <2% of the cells were CD14\textsuperscript{+}, <2% were CD34\textsuperscript{−}/CD90\textsuperscript{+}, <0.05% were CD34\textsuperscript{−}/CD14\textsuperscript{+}, <0.5% were CD68\textsuperscript{+}, and <1% stained positive for \(\alpha\)-smooth muscle cell actin. Mouse anti-human CD14, CD31, and CD34 antibodies were obtained from Immunotech, Beckman Coulter; mouse anti-human CD90 antibody was from Pharmingen, BD Biosciences; mouse anti-human CD68 antibody was from DakoCytomation; and mouse anti-human \(\alpha\)-smooth muscle actin antibody was from Sigma.

\textbf{Adipocyte Differentiation and Characterization}

After adhesion of preadipocytes for 24 hours, adipocyte differentiation was induced with serum-free DMEM/F-12 containing 33 mmol/L biotin, 17 mmol/L pantothenate, 1 mmol/L triiodothyronine, 100 mmol/L dexamethasone, 500 mmol/L insulin, 1 mmol/L pioglitazone, and, for the first 3 days, 0.25 mmol/L isobutyl-methylxanthine (all from Sigma). Half of the medium was replaced every other day; differentiation was verified by staining with Sudan III, in which differentiated adipocytes were defined as cells whose cytoplasm was filled completely with lipid droplets (Figure 1B). Only cultures with >90% adipocytes were used for further adipocyte experiments. All cell culture media and buffers contained 100 U/mL penicillin, 100 U/mL streptomycin, 0.25 \(\mu\)g/mL fungizone, and 2 mmol/L L-glutamine (all from Cambrex).

\textbf{Treatment of Human Preadipocytes, Adipocytes, and Ex Vivo Cultures With gp130 Ligands}

Preadipocytes, adipocytes, and ex vivo cultures were incubated in serum-free DMEM/F-12 for 24 hours before treatment with gp130

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure1}
\caption{Phase contrast micrographs of human subcutaneous preadipocytes and adipocytes. Human preadipocytes (A) and adipocytes (B) were prepared from subcutaneous adipose tissue as described in Methods. Adipocytes were stained with Sudan III.}
\end{figure}
ligands. Thereafter, the medium was replaced with fresh DMEM/F-12, and recombinant human (rh) OSM (R&D Systems), rhIL-6 (Biotrend), rhLIF (leukemia inhibitory factor; Biotrend), or rhCT-1 (cardiotrophin-1; Calbiochem/Merck), respectively, was added at the concentrations indicated. After incubation, the culture supernatants were collected after removal of cell debris by centrifugation and used immediately or stored at −80°C. In some experiments, cells were lysed for mRNA isolation. Tissue pieces from ex vivo cultures were weighed.

Quantification of PAI-1, Tissue Plasminogen Activator, and Urokinase Plasminogen Activator Protein
PAI-1 antigen in conditioned media was determined by a specific ELISA that measured free, complexed, and latent PAI-1 with monoclonal antibodies. Tissue plasminogen activator (tPA) and urokinase plasminogen activator (uPA) antigen in conditioned media was quantified with specific ELISAs that measured free and complexed plasminogen activators with monoclonal antibodies (all from Technoclone).

mRNA Purification
Cells were stimulated as described above, supernatant was removed, and mRNA was isolated with the QuickPrep Micro mRNA Purification Kit (Amersham Biosciences) according to the manufacturer’s instructions. Real-Time Polymerase Chain Reaction
Real-time polymerase chain reaction (PCR) was performed with LightCycler-RNA Master SYBR Green I (Roche) according to the manufacturer’s instructions. Primers were designed with LightCycler Probe Design software version 1.0 (Roche) and Primer3 software (http://frodo.wi.mit.edu/), as follows (annealing temperature in parentheses and corresponding position in brackets): PAI-1 (65°C), forward primer: 5'-gatcgaggtgaacgagagtg-3' [1814–1833], reverse primer: 5'-ggctgtggacagaaggatgt-3' [1119–1138]; tPA (69°C), forward primer: 5'-gacttttgaggagtcgggt-3' [1512–1493]; uPA (65°C), forward primer: 5'-gttcgatatgtaggtgggt-3' [1923–1904]; uPA (65°C), forward primer: 5'-acactcctctcccagggtctc-3' [890–869], reverse primer: 5'-cccagctcacaattccagtc-3' [1265–1246]; GAPDH (65°C), forward primer: 5'-ggcttggtgagggtaggtgg-3' [681–702], reverse primer: 5'-gctgcttgcttcaccacctt-3' [809–890]. The amplification conditions consisted of an initial incubation at 61°C for 20 minutes, followed by incubation at 95°C for 30 seconds, 50 cycles of 95°C for 1 second, the respective annealing temperature for 10 seconds and 72°C for 10 seconds, a melting step from 45°C to 95°C with increases of 0.1°C per second, and a final cooling to 40°C. Data were analyzed with LightCycler software version 3.5 (Roche).

Statistical Analysis
Data were compared statistically by ANOVA. Values of P<0.05 were considered significant.

Results
Effects of gp130 Ligands on PAI-1 Production by Ex Vivo Culture of Adipose Tissue
Human adipose tissue fragments of subcutaneous and visceral fat were treated with the gp130 ligands OSM (100 ng/mL), IL-6 (100 ng/mL), LIF (10^4 U/mL), and CT-1 (100 ng/mL), respectively, for 48 hours. In subcutaneous and visceral adipose tissue, OSM and IL-6 significantly increased the production of PAI-1 up to 7.5-fold and 6-fold (OSM) and up to 3.5-fold (IL-6), respectively. LIF led to a significant increase of PAI-1 by 2.5-fold in subcutaneous and 2-fold in visceral adipose tissue, whereas CT-1 increased PAI-1 production up to 3-fold in both tissue types (Figures 2A and 2B).

Preadipocytes and Adipocytes Express Receptors for gp130 Ligands
As determined by real-time PCR, performed as described by us recently, human preadipocytes and human adipocytes express mRNA specific for gp130, IL-6 receptor, LIF receptor, and OSM receptor (data not shown).26

Effects of gp130 Ligands on PAI-1 Production by Preadipocytes and Adipocytes
Preadipocytes of subcutaneous and visceral adipose tissue were incubated with OSM (100 ng/mL), IL-6 (100 ng/mL), LIF (10^4 U/mL), and CT-1 (100 ng/mL), respectively, for 48 hours. OSM and IL-6 induced a significant increase of PAI-1 production in both subcutaneous and visceral adipose cell cultures up to 8-fold and 5-fold (OSM) and up to 9.5-fold and 11.5-fold (IL-6). LIF led to an increase of PAI-1 by 3.5-fold in subcutaneous and up to 3-fold in visceral preadipocytes. CT-1 also significantly increased PAI-1 secretion in subcutaneous and visceral preadipocytes up to 4-fold.

To investigate a possible cell-dependent effect within the adipose tissue, we stimulated fully differentiated adipocytes of subcutaneous and visceral adipose tissue under the same conditions described above. OSM and IL-6 increased PAI-1 in subcutaneous adipose tissue up to 13-fold and 9.5-fold, respectively, and in visceral adipose tissue up to 12-fold and...
10-fold. CT-1 induced an increase of PAI-1 up to 4.5-fold in both tissue types. LIF also induced a significant increase up to 3.5-fold in subcutaneous adipocytes but had no effect in visceral adipocytes (Figures 3A through 3D). Neither tPA nor uPA antigen was detectable in conditioned media from preadipocytes and adipocytes isolated from subcutaneous or visceral adipose tissue incubated in the presence and absence of 100 ng/mL OSM (data not shown).

Dose-Dependent Effect of OSM and IL-6
When preadipocytes and fully differentiated adipocytes of subcutaneous and visceral fat were treated with increasing concentrations of OSM (0.1 to 100 ng/mL) or IL-6 (0.1 to 100 ng/mL) for 48 hours, PAI-1 protein release into the culture medium was stimulated in a dose-dependent manner. The maximum response to OSM and IL-6 was observed at a concentration of 100 ng/mL, respectively, in both cell types (Figures 4A through 4D and Figures 5A and 5B).

PAI-1 Upregulation by OSM in Adipocytes Is Independent of IL-6
To investigate a possible autocrine effect of IL-6 produced by adipocytes on OSM-induced PAI-1 secretion, subcutaneous adipocytes were stimulated for 24 hours with OSM (100 ng/mL) in the absence or presence of an IL-6-neutralizing polyclonal goat antibody (4 μg/mL, R&D Systems). As shown in Figure 6, increased PAI-1 production by OSM (100 ng/mL) in subcutaneous adipocytes was not abolished by neutralizing IL-6.

Effects of gp130 Ligands on PAI-1 mRNA
Specific PAI-1 mRNA was prepared from preadipocytes and adipocytes of subcutaneous and visceral adipose tissue, respectively, after incubation for the indicated period with OSM (100 ng/mL), IL-6 (100 ng/mL), LIF (10^4 U/mL), and CT-1 (100 ng/mL), respectively, and was assayed by use of quantitative real-time PCR. As demonstrated in the Table, an appreciable increase of PAI-1 mRNA was seen under these conditions after treatment with OSM and IL-6 in all cell types. PAI-1 mRNA also increased in subcutaneous and visceral preadipocytes and in subcutaneous adipocytes treated with LIF and CT-1, respectively, whereas no increase was observed in PAI-1 mRNA in visceral adipocytes treated with LIF for either 8 or 24 hours or treated with CT-1 for 8 hours. OSM did not affect tPA or uPA mRNA in any of the cells tested.

JAK/STAT Inhibitor AG-490 Interferes With OSM-Induced PAI-1 Expression in Adipose Tissue Cells
It is known that gp130–130 ligand interaction activates janus kinase/signal transducer and activator of transcription (JAK/STAT), mitogen-activated protein kinase (MAPK), and the phosphatidylinositol 3 kinase (PI3 K)-Akt pathways.26–28 It was also shown recently that OSM induced PAI-1 expression in astrocytes via the JAK/STAT pathway.15–18 To investigate which of these pathways was involved in OSM-induced PAI-1 expression in adipose tissue, we preincubated adipose
tissue cells for 60 minutes with a JAK/STAT inhibitor (AG-490), a PI3K inhibitor (LY294002), an MEK-1 inhibitor (PD98059), and an inhibitor of p38MAPK (SB202190; all at 30 μmol/L, Calbiochem/Merck). Thereafter, medium was removed, and cells were treated with fresh DMEM/F-12 in

the presence or absence of OSM at a concentration of 100 ng/mL for 24 hours. The subsequent ELISA showed that OSM-mediated induction of PAI-1 was highly dependent on the JAK/STAT pathway. The OSM-mediated increase of PAI-1 was reduced by 76% on treatment with AG-490 in

Figure 4. OSM increases PAI-1 production in human subcutaneous and visceral preadipocytes and adipocytes dose dependently. Human preadipocytes (A and C) and adipocytes (B and D) were prepared from subcutaneous (A and B) and visceral (C and D) adipose tissue as described in Methods and were incubated for 48 hours in absence or presence of rhOSM at indicated concentrations. Conditioned media were collected, and PAI-1 was determined as described in Methods. Values represent mean ± SD of 3 independent determinations. Experiments were performed 3 times for each cell type prepared from adipose tissue obtained from 3 different donors, respectively, with similar results. Representative experiment is shown. *P < 0.05, **P < 0.005, ***P < 0.0001 (A); *P < 0.005, **P < 0.0005 (B); *P < 0.005, ***P < 0.0001 (C); *P < 0.05, **P < 0.005, ***P < 0.0001 (D). n.s. indicates not significant.

Figure 5. IL-6 increases PAI-1 production in human subcutaneous and visceral preadipocytes dose dependently. Human subcutaneous (A) and visceral (B) preadipocytes were prepared from subcutaneous and visceral adipose tissue as described in Methods and were incubated for 48 hours in absence or presence of rhIL-6 at indicated concentrations. Conditioned media were collected, and PAI-1 was determined as described in Methods. Values represent mean ± SD of 3 independent determinations. Experiments were performed 3 times for each cell type prepared from adipose tissue obtained from 3 different donors, respectively, with similar results. Representative experiment is shown. *P < 0.01, **P < 0.0001 (A); *P < 0.01, **P < 0.005, ***P < 0.0005 (B). n.s. indicates not significant.
visceral preadipocytes, whereas LY294002 reduced PAI-1 secretion by 32%, PD98059 by 26%, and SB202190 by 22% under these conditions. No evidence of a toxic effect of the inhibitors was observed during the incubation, as assessed by morphology and by measurement of lactate dehydrogenase leakage as described previously.29 Similar results were seen when subcutaneous fully differentiated adipocytes were treated with OSM and different inhibitors as described above (Figures 7A and 7B).

**Discussion**

Sawdey and Loskutoff30 described for the first time that adipose tissue is a potent source of PAI-1, and several other studies31–33 supported this finding in vitro, ex vivo, and in vivo. The expression of PAI-1 in adipose tissue is highly regulated by a variety of modulators, such as TNF-α, IL-1, transforming growth factor-β, lipopolysaccharide, and angiotensin II.34–37

Here, we show that ex vivo explants of subcutaneous and visceral adipose tissue produce PAI-1 and that PAI-1 production in these explants is increased significantly by the gp130 ligands IL-6, OSM, LIF, and CT-1. Furthermore, we demonstrate that primary human preadipocytes and adipocytes isolated from visceral and subcutaneous adipose tissue constitutively produce PAI-1. The contribution of adipocytes to PAI-1 production within the adipose tissue is still controversially discussed;38 however, the present results are in line with other studies implicating these cells in the production of PAI-1 by adipose tissue.31,39,40

Moreover, we provide evidence that human preadipocytes and adipocytes isolated from subcutaneous and visceral adipose tissue express gp130, the common receptor unit for all gp130 ligands, and the specific receptor subunits IL-6 receptor, LIF receptor, and OSM receptor, and that similar to results obtained for IL-1β, TNF-α, and transforming growth factor-β, the gp130 ligands IL-6, OSM, CT-1, and LIF significantly upregulate PAI-1 production in all cell types studied.34,39 In particular, IL-6 and OSM induced a substantial (10-fold) increase in PAI-1. The smaller response in PAI-1 secretion after treatment of the respective cells with LIF and CT-1 could be due to differential threshold signaling caused by receptor multisite phosphorylation that determines duration and relative levels of activated STAT 1/3, which in turn would affect target gene activation differently.41 In correlation with data obtained on the level of protein, IL-6 and OSM also induced PAI-1-specific mRNA in cells isolated from subcutaneous and visceral adipose tissue, as demonstrated by real-time PCR. In contrast to other cell types, OSM did not affect the expression of mRNA specific for tPA or uPA in human preadipocytes and adipocytes.16,17,28 However, PAI-1 mRNA increased in subcutaneous and visceral preadipocytes and in subcutaneous adipocytes incubated with LIF and CT-1,
respectively, whereas no increase was observed in PAI-1 mRNA in visceral adipocytes treated with LIF for either 8 or 24 hours or treated with CT-1 for 8 hours. We could also show that the effect of OSM on PAI-1 is independent of autocrine IL-6 production in these cells, because it was not affected by neutralizing anti-IL-6 antibodies. In addition, AG-490, a JAK/STAT inhibitor, abolished the OSM-dependent PAI-1 induction almost completely. Thus, assuming specificity of AG-490 as a JAK/STAT inhibitor, we provide evidence that the effect was mediated mainly via activation of the JAK/STAT pathway. The MEK 1/2 inhibitor PD98059 showed only a minor effect. Similar to our observations, PAI-1 induction by OSM in human astrocytes is also regulated via the JAK/STAT pathway, whereas in a lung carcinoma cell line, OSM-mediated induction of PAI-1 largely depended on activation of the MEK 1/2 pathway, with the JAK/STAT pathway only playing a secondary role.18,28

As discussed above, growing evidence implicates inflammation as a key event in the pathophysiology of atherosclerosis and the IRS. In line with this notion, systemic low-grade inflammation, with elevated markers of inflammation such as IL-6, is found in patients with type 2 diabetes mellitus and IRS.42 Recently, OSM, derived from activated T lymphocytes and macrophages and originally described as a protein that modulates leukocyte adhesion, an early event in the development of adhesion molecules in endothelial cells and thereby contributes to the presence of inflammatory mediators in fat by producing TNF-α and IL-6. On the basis of their observations, the authors of these studies suggest that cytokines secreted by adipose tissue macrophages are involved in inflammatory activation of preadipocytes and adipocytes in obesity, which eventually might result in systemic insulin resistance.46,47 Because OSM is also, as mentioned above, a product of macrophages, we hypothesize that this cytokine expressed by macrophages accumulating in adipose tissue could contribute to the upregulation of PAI-1 seen in obese patients.

In conclusion, the present results for the first time establish a link between gp130 ligands and PAI-1 expression in human adipose tissue. We demonstrate that selected gp130 ligands, in particular the proinflammatory mediators IL-6 and OSM, significantly upregulate PAI-1 production in cultured human preadipocytes and adipocytes of subcutaneous and visceral origin. Thus, we speculate that IL-6 and OSM, by upregulating PAI-1 in adipose tissue, might contribute to the increased cardiovascular risk of patients with obesity and IRS.

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