Role of Cytokines in the Regulation of Plasminogen Activator Inhibitor-1 Expression and Secretion in Newly Differentiated Subcutaneous Human Adipocytes

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Abstract—Elevated levels of plasminogen activator inhibitor-1 (PAI-1) are characteristic for obesity and are associated with increased risk of thromboembolic complications. PAI-1 recently was reported to be expressed and secreted by human adipocytes, but little is known about regulation of PAI-1 in human adipose tissue. Therefore, we examined the effects of selected cytokines present in adipose tissue on expression and secretion of PAI-1 in in vitro, differentiated subcutaneous human adipocytes. Transforming growth factor-β1 (TGF-β1) increased PAI-1 secretion in a dose- and time-dependent manner. PAI-1 protein increased by 3.2-fold and PAI-1 mRNA by 1.9-fold after a 6-hour exposure to 400 pmol/L TGF-β1. This effect is probably mediated by TGF-β1 type 2 and 3 receptors, which were found to be expressed in cultured human adipocytes. Moreover, TNF-α and interleukin-1β (IL-1β) also exerted a stimulatory effect on PAI-1 release and increased PAI-1 mRNA levels. As assessed by a semiquantitative reverse transcription—polymerase chain reaction technique, TGF-β1 mRNA is expressed by differentiation of human preadipocytes and is moderately upregulated by TNF-α and IL-1β. In conclusion, our results clearly indicate that TGF-β1 is a potent inducer of PAI-1 production in subcutaneous human adipocytes. In addition, data suggest that TNF-α and IL-1β also have stimulatory effects on PAI-1 protein secretion and may contribute to the elevated PAI-1 levels observed in obesity.

Key Words: obesity ■ tumor necrosis factor ■ growth factors ■ interleukin ■ plasminogen activator inhibitor

Obesity is associated with an elevated risk of developing coronary heart disease. Among the multiple mechanisms that may explain the relationship between obesity and cardiovascular disease, disorders of the fibrinolytic system seem to play an important role.

Growing evidence indicates that increased levels of plasminogen activator inhibitor-1 (PAI-1) favor development of thromboembolic complications. PAI-1 is a member of the family of serine protease inhibitors (serpins) and is the main regulator of the endogenous fibrinolytic system (it inhibits tissue plasminogen activator).

Elevated plasma levels of PAI-1 were found in obese subjects in many clinical and epidemiological studies, and these appear to be closely correlated with an abdominal pattern of adipose tissue distribution in both men and women but are also positively associated with other components of insulin-resistance syndrome. The mechanisms responsible for elevated PAI-1 plasma levels in this syndrome are not yet completely understood. In vitro studies have demonstrated that a variety of cell types, including endothelial and mesothelial cells, are able to produce PAI-1.

In 1991, Sawdey and Loskutoff demonstrated for the first time the expression of PAI-1 in mouse adipose tissue. Subsequent studies detected PAI-1 mRNA and protein in adipocytes from other sources as well as in differentiated 3T3-L1 adipocytes. Recently, expression and secretion of PAI-1 was also demonstrated in adipose tissue from humans. Little currently is known about regulation of PAI-1 in adipose tissue. According to animal data, transforming growth factor-β1 (TGF-β1) appears to be a major inducer of PAI-1 synthesis in adipose tissue. A stimulatory effect of TGF-β1 on PAI-1 production has recently been described in human adipose tissue explants.

Studies in murine adipocytes and 3T3-L1 cells demonstrated a stimulating effect of tumor necrosis factor-α (TNF-α) on PAI-1 expression and secretion, whereas first experiments in human adipose tissue gave contradictory results. TNF-α is expressed and secreted by human adipocytes, and researchers have hypothesized that TNF-α is the main mediator of insulin resistance in the obese state. Another interesting cytokine is interleukin-1β (IL-1β), which, like TNF-α, is well known to suppress adipose differentiation and lipoprotein lipase expression and activity. A few studies reported that IL-1β is able to stimulate PAI-1 production in human and rat endothelial cells, but nothing is currently known about its effects on PAI-1 synthesis in human adipocytes.

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The aim of this study was to further characterize regulation of PAI-1 production in a model of in vitro differentiated human adipocytes. We were particularly interested to investigate the effects of TGF-β1, TNF-α, and IL-1β on PAI-1 mRNA expression and secretion.

**Methods**

**Materials**

Collagenase CLS type 1 was obtained from Worthington; human insulin and cortisol were kindly donated by Hoechst; human TGF-β1, IL-1β, and gentamicin were purchased from Sigma, and TNF-α from Pepro Tech Inc. The PAI-1 ELISA kit was from WAK Chemie; the TGF-β1 ELISA kit was from R&D; and Trizol, reverse transcriptase (RT) Superscript RT, random hexamers, Taq polymerase, and oligonucleotides were obtained from Gibco/BRL. For sequencing of transcripts, we used the ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit from Perkin Elmer; the gel-extraction kit was obtained from Clontech. Immunohistochemistry was performed with an LSAB-kit from Dako; polyclonal antibody against PAI-1 was obtained from Chemicon; and all other chemicals were from Boehringer or Merck. Sterile plastic ware for tissue culture was purchased from Flow Laboratories.

**Cell Isolation and Culture**

Subcutaneous adipose tissue samples (20 to 80 g) were obtained from the mammary adipose tissue of young, normal-weight women (body mass index, <26 kg/m²; age range, 19 to 36 years) undergoing surgical mammary reduction. Stromal cell fraction was isolated by collagenase digestion as described previously.30 Samples were dissected from fibrous material and visible blood vessels minced into small pieces (10 mg) and digested in 10 mmol/L PBS containing 0.5 mg/mL crude collagenase and 20 mg/mL BSA, pH 7.4, for 90 minutes. The stromal cell fraction obtained after a short centrifugation at 200g was incubated with erythrocyte-lysing buffer that contained 154 mmol/L NH₄Cl, 5.7 mmol/L K₂HPO₄, and 0.1 mmol/L EDTA to eliminate contaminating erythrocytes. Cells were repeatedly washed and resuspended in Dulbecco’s Eagle’s/Ham’s F-12 medium (vol/vol, 50:50) supplemented with 10% fetal calf serum. After cell adhesion for 16 hours, cells were cultured under serum-free hormone-supplemented conditions to allow adipose differentiation.30

The inoculated cell fraction did not contain adipocytes as assessed by morphological criteria. In vitro differentiated adipocytes were defined as cells, the cytoplasm of which was completely filled with lipid droplets. In addition, GPDH activity was used as a differentiation marker and determined by an established method.31 In undifferentiated cultures, GPDH activity was below detection levels (<20 mU/mg protein) and increased to an average level of 486±73 mU/mg protein on day 16 of culture under standard adipogenic conditions.

**Incubation With TNF-α, TGF-β1, and IL-1β**

During the 16-day culture period, 40% to 70% of the preadipocytes underwent adipose differentiation. Cultures were then incubated with TNF-α (0.1 to 5 nmol/L), TGF-β1 (4 to 400 pmol/L) and IL-1β (0.2 to 20 ng/mL) for the time periods indicated. Medium for the determination of PAI-1 protein was stored at −20°C.

**RNA Preparation**

RNA was prepared according to the RNA isolation technique described by Chomczynski and Sacchi.32 Cells were harvested in Trizol and 200 μL of chloroform was added. After centrifugation, the aqueous phase was mixed with an equal volume of isopropyl alcohol. After 12 hours at −20°C, RNA was pelletted for 15 minutes at 10,000 g, redissolved in LiCl, washed twice with 70% ethanol, dried, and redissolved in H₂O. The purity of the RNA yield was verified by electrophoresis in a formaldehyde-containing agarose gel.

**cDNA Generation and Polymerase Chain Reaction**

Total RNA was diluted to 0.2 μg/μL in H₂O and 1st strand cDNA prepared with 5 μL of RNA, superscript RT, and random hexamers (both Gibco/BRL) according to the instructions of the manufacturer. Two and one-half microliters of cDNA and a polymerase chain reaction (PCR) primer mix that contained 2 U Taq polymerase in PCR buffer, 200 μmol/L each of dCTP, dGTP, dTTP, and dATP, and 500 pmol/L of each primer in a 50 μL volume with 50 μL of mineral oil. PCR conditions were a denaturing step at 95°C for 1 minute, followed by 30 cycles of 94°C, 45 seconds; 57°C, 45 seconds; and 72°C, 45 seconds for the detection of PAI-1 mRNA and by 26 cycles of 94°C, 45 seconds; 61°C, 45 seconds; and 72°C, 45 seconds for the detection of TGF-β1 mRNA. PCR products were transferred onto a 2% Tris-Borate/EDTA–agarose gel, stained with ethidium bromide, and analyzed with the LumiAnalyst system from Boehringer.

For semiquantitative analysis, 2 primer sets were used simultaneously in the same tube: 1 was specific for PAI-1 and TGF-β1 cDNA and the other for transcription factor Sp1, which is ubiquitously expressed and was used as an internal standard.33 PAI-1–

![Figure 1. Immunohistochemical detection of PAI-1 protein in cultured human preadipocytes, including (A) Undifferentiated cells on day 6 and (B) fully developed adipocytes on day 16 in the absence and (C) in the presence of 400 pmol/L TGF-β1 for 24 hours. Arrows indicate examples of immunopositive cells. Final magnification, ×100.](http://atvb.ahajournals.org/)

specific primers (301 bp) were 5′- GTG TTT CAG CAG GTG GCG C-3′ sense (19 nucleotides) and 5′- CGG GAA CAG CCT GAA GAA GTG-3′ antisense (21 nucleotides). TGF-β1–specific primers (288 bp) were 5′- AAC CGG CCT TTC CTG CTT CTC A-3′ sense (21 nucleotides) and 5′- CGC GGG GGT TAT TGC GGT GTC-3′ antisense (22 nucleotides). Sp1–specific primers (231 bp) were 5′- CGC CCG GGT TAT GCT GGT TGT A-3′ sense and 5′- CGT TTA ACG CGG TAG CAG TAG AA-3′ antisense (19 nucleotides) and 5′- GAG AGT GGC TCA CAG CCT GTC-3′ antisense (21 nucleotides).

To determine the presence of mRNA of TGF-β1 receptor type 2 and 3, we performed a qualitative RT-PCR with specific primers for these products: TGF-β1 receptor 2 specific primers (324 bp), 5′- TGG CTG TAT GGA AAG AGT AGC-3′ sense and 5′- CGG TTA ACG CGG TAG CAG TAG AA-3′, and TGF-β1 receptor 3 specific primers (382 bp), 5′- CCC GCA AGC TGA CAT GGA TAA GA-3′ sense and 5′- ACA AGG CCC CCG TCA GGA GTG-3′ antisense. Sequences of the transcripts obtained were analyzed by use of ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit according to instructions of the manufacturer. Numbers of PCR cycles were chosen to ensure that amplification of PCR products was within exponential range.

Measurement of PAI-1 and TGF-β1 Protein
PAI-1 and TGF-β1 proteins were measured in culture medium by use of commercially available specific ELISA kits. The interassay and intra-assay variations of both ELISAs were <10%.

Immunochemistry Staining of PAI-1 in Newly Differentiated Adipocytes
Adipocytes were cultured in cell-chamber slides (Becton Dickinson) for 16 days. After being washed with PBS, newly differentiated adipocytes were fixed with ethanol that contained 10% methanol. Cells were subjected to the protocol of Dako (LSAB kit). Polyclonal antibody directed against PAI-1 was used. Cultures were counterstained with hematoxylin for 10 seconds, rinsed with tap water, and mounted in Entellan synthetic resin.

Statistical Analysis
Results are expressed as mean±SEM. Differences between groups were tested with Student’s t test for paired data. Differences with *P<0.05 were regarded as significant.

Results
Detection of PAI-1 Protein
In the first set of experiments, basal characterization of PAI-1 secretion into the culture medium was performed. PAI-1 protein secretion was low in the undifferentiated state and increased 1.9-fold during the course of differentiation: 8- and 16-day-old cultures of adipocytes secreted 14.6±2.5 and 27.9±2.1 ng/mL per 24 hours, respectively. Release of PAI-1 into the culture medium on day 16 was closely dependent on the percentage of differentiating cells (correlation coefficient r=0.81; n=6). To directly address the question of whether PAI-1 was produced by adipocytes, an immunohistochemical staining technique was applied. By use of a specific polyclonal antibody against human PAI-1, we determined that preadipocytes after 6 days of culture under adipogenic conditions did not reveal a significant number of positive cells, whereas after 16 days of culture, a positive signal was detected in many fully developed adipocytes (Figures 1A and 1B).

Effects of TGF-β, TNF-α, and IL-1 on PAI-1 Release Into the Culture Medium From Newly Developed Human Adipocytes
In the primary culture model of human preadipocytes, a high percentage of cells underwent differentiation and developed biochemical and morphological characteristics of human adipocytes within 16 days. These cultures were incubated with cytokines of interest and for the time periods indicated. As shown in Figure 2, TGF-β1 increased accumulation of PAI-1 in a dose- and time-dependent manner. Four, 40, and 400 pmol/L TGF-β1 increased the amount of PAI-1 in the medium after 48 hours by 1.4-fold, 2.7-fold, and 7.4-fold, respectively, to a maximum concentration of 431±40 ng/mL at 400 pmol/L. A significant stimulatory effect of 400 pmol/L TGF-β1 on PAI-1 protein was already seen after an incubation time of 6 hours (31.9±2.7 versus 9.7±2.7 ng/mL in control cultures; P<0.05). When TGF-β1–treated adipocytes were immunostained with specific polyclonal antibody against human PAI-1, the percentage of immunopositive adipocytes was not significantly different from control cultures at the same antibody titer, which indicated that TGF-β1 increased PAI-1 synthesis in each cell (Figure 1C).

As demonstrated in Figure 3, both TNF-α and IL-1β also increased accumulation of PAI-1 in culture medium. Incubation of newly differentiated human adipocytes with 5 nmol/L TNF-α for 24 hours stimulated PAI-1 accumulation by 3.1-fold (173.9±17.6 versus 55.4±20.4 ng/mL; P<0.05; Figure 3).

Effects of TGF-β, TNF-α, and IL-1 on PAI-1 mRNA in Newly Differentiated Human Adipocytes
Specific PAI-1 mRNA was assayed after a 24-hour incubation of newly differentiated human adipocytes with TGF-β1,
TNF-α, and IL-1β, respectively, by use of a semiquantitative RT-PCR method. As demonstrated in Figure 4, TGF-β1 led to an increase of PAI-1 mRNA by $\geq 2.3$-fold at the highest concentration, 400 pmol/L ($P<0.05$). TNF-α at a maximal concentration of 5 nmol/L increased the steady-state level of PAI-1 mRNA by 1.7-fold ($P = NS$). IL-1β stimulated PAI-1 mRNA 3.4-fold at 0.2 ng/mL and 3-fold at the maximum concentration of 20 ng/mL.

Expression of TGF-β1 Receptors in Cultured Human Adipocytes

Because TGF-β1 was found to be a potent stimulator of PAI-1 production and release, we were interested to study expression of the 2 TGF-β1 receptor subtypes that exert the biological effects of this cytokine. By use of specific primers for TGF-β1 receptor 2 and 3, we determined that both subtypes are expressed at the mRNA level in newly differentiated adipocytes (Figure 5). In contrast, TGF-β1 receptor subtype 1 was not detectable (data not shown).

Effects of TNF-α and IL-1 on TGF-β mRNA in Newly Differentiated Human Adipocytes

Samad et al recently postulated that the effect of TNF-α on PAI-1 production may be mediated by an increased production of TGF-β1 by adipocytes. To address this aspect, we studied the effects of TNF-α and IL-1β on the expression of TGF-β1 mRNA. As shown in Figure 6, both TNF-α and IL-1β only moderately increased TGF-β1 mRNA after 24 hours. TNF-α 5 nmol/L increased TGF-β1 mRNA levels by 37% ($P<0.05$); IL-1β 2 ng/mL, by 44% ($P<0.05$). We also determined TGF-β1 protein in the culture medium by highly sensitive ELISA. However, TGF-β1 protein was not detectable either under basal conditions or in the presence of TNF-α or IL-1β (data not shown).

Discussion

In the present study, we used a primary culture model of in vitro differentiated human adipocytes to investigate regulation of PAI-1 production in human adipose tissue. PAI-1 protein was undetectable by immunohistochemistry in 6-day-old human preadipocytes, which do not yet contain significant amounts of lipids and have not yet developed the gene
expression pattern characteristic of mature adipocytes. However, after morphological differentiation into adipocytes, many of these cells exhibited specific immunostaining for PAI-1. Concomitantly, secretion of PAI-1 into culture medium increased in a differentiation-dependent manner. These data clearly indicate that the bulk of PAI-1 is exclusively produced by adipocytes, thereby confirming similar findings in cultured adipocytes from rodents and 3T3-L1 cells. This observation may help to explain the clinical finding that PAI-1 levels are elevated in the obese state. In a recent study, PAI-1 protein concentrations in plasma were 7-fold and adipose tissue mRNA levels were 2-fold elevated in severely obese versus lean adults. This clinical observation together with our in vitro data provide strong evidence that adipose tissue is a significant source of circulating PAI-1 in humans.

To elucidate regulation of PAI-1 in human adipocytes we focused our interest on the role of TGF-β1 and other cytokines. TGF-β1 is a pleiotropic cytokine that has been implicated in numerous biological processes, which include wound repair and tissue remodeling. We now can provide extended evidence that TGF-β1 is a strong inducer of PAI-1 mRNA synthesis and PAI-1 release by human adipocytes. At the highest concentration of TGF-β1 used in our experiments, a 7.4-fold increase in PAI-1 protein and a 2.3-fold increase in PAI-1 mRNA levels were observed. These findings point to a direct and specific effect of TGF-β1 on PAI-1 synthesis and secretion that is mediated by PAI-1 mRNA expression. This effect was confined to the p55 TNF receptor. However, we were unable to confirm the observation that TNF receptor A was downregulated in human adipocytes in the presence of TGF-β1. This may be due to the use of 3T3-L1 clonal cell lines, which are likely to be less responsive to TGF-β1 compared to primary human adipocytes.

Apart from TGF-β1, 2 other ubiquitously detectable cytokines, TNF-α and IL-1β, also seem to play a physiological role in the regulation of PAI-1. To date, TNF-α is known to be effective for stimulation of PAI-1 release in many cell types, especially endothelial cells. We now report that human adipocytes in primary culture also respond to TNF-α with enhancement of PAI-1 release and elevation of PAI-1 mRNA. These data are in contrast to a recent report by Alessi et al., who could not find an effect of TNF-α on PAI-1 production in cultured human adipose tissue pieces, whereas Cigolini et al., recently showed an increase of PAI-1 production after TNF-α incubation. Samad et al., originally reported that mice treated with TNF-α exhibit elevated expression of PAI-1 mRNA in adipose tissue. In further experiments, these authors also were able to demonstrate that 3T3-L1 adipocytes in culture respond to TNF-α treatment with upregulation of PAI-1 mRNA expression. However, this latter finding could not be confirmed in a study in differentiated murine 3T3-L1 cells.

The effect of TNF-α on PAI-1 synthesis and secretion is of particular interest, because TNF-α is well established to be expressed in human adipose tissue. Animal studies also suggest that TNF-α could play an important role in the development of obesity-linked insulin resistance. In addition, the 2 TNF-α receptor subtypes are upregulated in human obesity. Therefore, upregulation of the TNF-α system could contribute to elevated levels of PAI-1 protein in obesity and, furthermore, link insulin resistance with impaired fibrinolysis. This was recently further substantiated by a study of Samad et al. in ob/ob mice; these authors reported that neutralization of TNF-α results in significantly reduced levels of plasma PAI-1 and adipose tissue PAI-1.

Detection of TGF-β1 mRNA by RT-PCR in human adipocyte cultures represents the first preliminary observation that TGF-β1 is produced at the local level and may contribute to the regulation of PAI-1 production and release in an autocrine and paracrine function. However, we were unable to measure significant amounts of TGF-β1 protein in the culture medium. Detection of TGF-β1 mRNA in human adipose tissue is not sufficient to prove production of substantial amounts of functional TGF-β1 protein. Therefore, further studies are required to demonstrate the secretion of TGF-β1 protein by adipocytes.

In our experiments, a modest effect of TNF-α exposure on TGF-β1 mRNA levels occurred, thereby confirming the original observation by Samad et al. in obese mice. In the recent study from the same group in ob/ob mice, neutralization of TNF-α or deletion of both TNF receptors resulted in reduced adipose tissue TGF-β1 mRNA expression. This effect was exclusively mediated by means of the p55 TNF receptor. Further studies are required to demonstrate clearly the expression of TGF-β1 by human adipocytes and to answer the question of whether TNF-α and IL-1β stimulate PAI-1 production by means of stimulation of local TGF-β1 expression.

Concerning a possible role of IL-1β, IL-1β is known to increase PAI-1 expression in human endothelial cells and rat cardiac microvascular endothelial cells. Our data unequivocally show that IL-1β is able to increase both PAI-1 protein and PAI-1 mRNA levels in differentiated human adipocytes in primary culture. A specific stimulatory effect was already seen at 0.2 ng/mL IL-1β, whereas rat endothelial cells showed an increase in PAI-1 production only at a 10-fold higher concentration. Again, this effect of IL-1β in human adipocytes may be mediated by an increase in expression of TGF-β1.

In conclusion, our present studies indicate that human adipocytes are an important source of PAI-1 production. TGF-β1 was found to be a strong inducer of PAI-1 release and PAI-1 mRNA expression in cultured human adipocytes. Likewise, TNF-α and IL-1β stimulate PAI-1 production in the same fat cell model, which indicates that PAI-1 production is controlled by a variety of locally synthesized cytokines.
or cytokines from other sources. These findings may lead to a better understanding of why PAI-1 concentrations are upregulated in human obesity.

References


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