0021-972X/99/$03.00/0
The Journal of Clinical Endocrinology & Metabolism

Vol. 84, No. 9
Printed in U.S.A.

Synthesis and Secretion of Plasminogen Activator Inhibitor-1 by Human Preadipocytes

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ABSTRACT

To further investigate the role of plasminogen activator inhibitor-1 (PAI-1) in adipose tissue physiology, the production and regulation of PAI-1 was determined in primary cultures of human preadipocytes. When expressed as production per cell and cultured under identical conditions, human preadipocytes from both visceral (omental) and sc depots of lean and obese individuals released significant, yet similar, amounts of PAI-1 protein into the conditioned medium. High steady-state PAI-1 messenger RNA (mRNA) concentrations were observed in visceral and sc preadipocytes, with the relative level of expression equivalent to β-actin mRNA. Tumor necrosis factor α significantly decreased PAI-1 production in a concentration-dependent manner in both visceral and sc cultures, whereas transforming growth factor β significantly elevated PAI-1 production, but only in sc preadipocytes from obese individuals. Addition of insulin had no effect on antigen levels in conditioned medium of preadipocyte cultures. Stimulation of the preadipocyte cultures with a defined medium resulted in differentiation to the adipocyte phenotype, as determined by flow cytometric analysis, verifying the cultures as human preadipocyte. These studies are the first to observe significant PAI-1 mRNA expression and protein production in primary cultures of a human adipose tissue cellular component, and they suggest that nascent adipocytes contribute significantly to the elevated plasma PAI-1 observed in obesity. (*J Clin Endocrinol Metab* **84:** 3222–3227, 1999)

PLASMINOGEN activator inhibitor-1 (PAI-1), a member of the serine protease inhibitor family, inhibits both tissue type plasminogen and urokinase type plasminogen activators. PAI-1 inactivation of the fibrinolytic system prevents fibrin degradation, and elevated circulating PAI-1 increases the risk of thrombosis (1, 2). Elevated plasma PAI-1 is observed in noninsulin-dependent diabetes mellitus and obesity, where the PAI-1 inhibition of fibrinolysis has been suggested to significantly contribute to atherothrombosis (3). Increased plasma PAI-1 correlates to the degree of upper body adipose tissue distribution and is normalized by weight reduction (4, 5). Elevated circulating PAI-1 in the cardiovascular-metabolic syndrome is directly related to increased serum insulin and triglycerides (6). Initial investigations into the pathologic basis of this interrelationship focused on the role of insulin in stimulating synthesis of hepatic PAI-1 (7). However, acute administration of insulin failed to augment plasma PAI-1 in normal individuals, suggesting that additional mechanisms must also be involved (8).

While elevated plasma PAI-1 is consistently observed in obesity, the cellular sources of the protein remain under investigation. PAI-1 has been shown to be actively synthesized by several tissues, including hepatic, vascular, and adipose (9). Considerable interest was generated by the identification of PAI-1 messenger RNA (mRNA) in murine adipose tissue, possibly providing a link between the increased body fat of obesity and elevated plasma PAI-1 (9). For example, in cultured murine 3T3 cells, development from the preadipocyte to adipocyte was associated with increased PAI-1 mRNA and release of PAI-1 into the medium (10). Importantly, however, the molecular or biochemical aspects of PAI-1 synthesis were not investigated in the preadipocyte phenotype, a prerequisite developmental step preceding differentiation to the adipocyte. Evidence for PAI-1 in human adipose tissue has been shown in explant studies examining mRNA expression and protein levels, but the relative contribution of the adipocyte and stromal-vascular fraction to total adipose tissue PAI-1 remains unclear (11, 12).

In addition to a regulatory role in vascular fibrinolysis, PAI-1 has been shown to effect cell migration by competing for a specific integrin binding site for vitronectin (13). PAI-1 may have an important role in the pericellular proteolysis, central to tissue remodeling, including the growth of adipose tissue during the development of obesity. The current study therefore investigates the contribution of preadipocytes to PAI-1 production by human adipose tissue. Primary cultures of preadipocytes were examined for their relative expression of PAI-1 message and the regulation of PAI-1 protein production.

Materials and Methods

Culture of human preadipocytes

Human preadipocytes were cultured as previously described in detail (14). Briefly, samples from knife biopsies of adipose tissue from abdominal operations and samples from liposuction were subjected to enzymatic digestion in Krebs-Ringer-bicarbonate buffer (pH 7.4) containing 6 mmol/L glucose and 2 mg/mL collagenase. After this initial enzymatic digestion, the contents of the flask were passed through a

Received February 19, 1999. Revision received April 28, 1999. Accepted June 1, 1999.

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sterile, 230-micron stainless steel tissue sieve (Cellector, Bellco Glass Inc., Vineland, NJ) into a 50-mL sterile, plastic test tube. Undigested stromal-vascular tissue trapped on the sieve was discarded, while the infranatant containing the preadipocyte fraction was collected, passed into another sterile tube, and the collagenase neutralized with an equal volume of growth medium containing Medium 199, 10% heat-inactivated FCS, and 1% antibiotic-antimycotic (Life Technologies, Grand Island, NY). After centrifugation, the pellet was resuspended in growth medium, filtered, transferred to a sterile tissue culture flask, and maintained in an incubator at 37 C, 5% CO_2. Cell attachment was allowed for 16–20 h, after which floating cells were removed by aspiration, followed by addition of fresh growth medium. To increase the variety of preadipocyte cultures representing different body mass indices (BMIs), additional primary cultures of human preadipocytes were obtained from Zen-Bio, Research Triangle Park, N.C. According to the supplier's specifications, these cells stained negative for factor VIII and do not express vascular endothelial growth factor receptor by Northern analysis.

In these studies of conditioned medium, cells were plated in 24-well plates. Growth medium was added with or without appropriate hormones or cytokines, and cells were incubated for a maximum of 24 h. Conditioned medium was removed, spun at 3000 $\times$ *g* for 10 min, and either analyzed immediately or frozen at −20 C and analyzed within 1 week. For quantitation of cell number, cells were trypsinized and counted using a Coulter counter, Model ZM (Beckman-Coulter, Inc., Hialeah, FL).

Quantification of PAI-1 protein

Primary cultures of preadipocytes were maintained in 24-well plates containing 1 mL of medium. The concentration of PAI-1 protein in conditioned medium was determined using an enzyme-linked immunosorbent assay, which measures both free and complexed human PAI-1 (American Diagnostica, Greenwich, CT). A standard curve of human PAI-1 was generated for each assay, with a correlation coefficient of $r > 0.950$ for concentrations ranging from 1–20 ng/mL. Samples of conditioned medium were diluted 50× with PBS containing 1% BSA before analysis. Calculations for total PAI-1 per well were then performed and were corrected for the number of cells. In all experiments, the final PAI-1 production was expressed per 10^6 cells. Additional reagents used included bovine insulin (Sigma Chemical Co., St. Louis, MO), recombinant human tumor necrosis factor α (TNF-α) and human transforming growth factor (TGF-β; Upstate Biotechnology Inc., Lake Placid, NY). Each was diluted to the appropriate concentration in culture medium before use.

RNA Isolation, probes, and solution hybridization

Total RNA from human preadipocyte cell cultures was prepared using an adaptation of the guanidinium thiocyanate extraction method (RNeasy; Qiagen, Chatsworth, CA). Total cellular RNA was quantitated by absorbance at 260 nm, and RNA integrity was assessed by nondenaturing agarose gel analysis before quantitation of specific PAI-1 mRNA by ribonuclease (RNase) protection analysis. To generate a probe for this purpose, a 336-bp complementary DNA (15) fragment of PAI-1 (nucleotides 883-1218 of Genebank accession no. M16006) was obtained by PCR amplification using human EST clone no. 83741 as a template (Genome Systems, St. Louis, MO), a 5′ primer, CTCTCTGCCCTCACCAAC, and a 3′ primer containing the T7 promoter, TAATACGACTCACTATAGGGAGGAAGGGTCTGTCCATGAT.

An appropriate amplicon size of 357 nucleotides was confirmed by agarose gel electrophoresis. An antisense riboprobe was synthesized by *in vitro* transcription (Strategene, La Jolla, CA) using T7 polymerase. A single protected fragment of 336-nt size results from hybridization of either PAI-1 mRNA variant with this probe.

PAI-1 mRNA abundance was quantitated by solution hybridization RNase analysis using previously described methods (16). Briefly, ^{33}P-labeled riboprobes in molar excess (1–3 $\times$ 10^5 cpm) were hybridized to 2–10 μg of total cellular RNA at 48 C for 16–20 h, followed by digestion with RNase T2 at 30 C for 2 h. Samples were analyzed by denaturing PAGE in 6% urea sequencing gels, and protected fragments were visualized and quantitated by PhosphorImager analysis (Molecular Dynamics, Inc., Sunnyvale, CA). Human β-actin riboprobe (Ambion, Inc., Austin, TX) was included as an internal standard to normalize the relative abundance of PAI-1 transcripts.

Assessment of differentiation

To confirm the population of cells as human preadipocyte, confluent cultures were stimulated to differentiate into adipocytes by supplementing the standard growth medium with 0.5 mmol/L isobutylmethylxanthine, 1×10^{-7} mol/L hydrocortisone, and 1×10^{-8} mol/L insulin for the first 2 days, and with growth medium, hydrocortisone, and insulin through the next 5 days (17). Differentiation was verified by assessing cytosolic triglyceride using fluorescent activated cell sorting for estimation of cellular triglyceride accumulation. Flow cytometric analysis was performed on a FACSCalibur flow cytometer (Becton Dickinson and Co., San Jose, CA). A 1-mg/mL stock solution of Nile Red (Molecular Probes, Inc., Eugene, OR) was prepared in dimethylsulfoxide and further diluted 100× in PBS to yield a working stock of 10 μg/mL. Preadipocytes and adipocytes were harvested by trypsinization, resuspended in 3 mL M199/10% FCS, and spun at 300 $\times$ *g* for 10 min. The supernatant was decanted, and the cells were resuspended in a final solution of M199/10% FCS containing 1% working stock of Nile Red at a concentration of 1×10^6 cells/mL. After incubation at room temperature for 10 min, the cells were immediately subjected to flow cytometric analysis, with 5000 events collected per sample. The 488-nm excitation light was used to generate forward scatter and right angle scatter signals. Nile Red fluorescence was detected at 530 nm and used to assess intracellular lipid accumulation.

Statistical analysis

Statistical analysis was performed using the Kruskal-Wallis analysis for multiple groups. Group means ± the SEM were considered significantly different at $P < 0.05$.

Results

To determine the potential for PAI-1 production by human preadipocytes, a total of 11 cultures were examined, each representing a different individual. Both visceral (omental) and sc adipose tissue depots were sampled. The sampled women ranged in age from 26–58 yr, with BMI from 18–57. Cells were analyzed in the fibroblast-like preadipocyte stage for both PAI-1 protein production and PAI-1 mRNA abundance.

PAI-1 production by human preadipocytes

Initial studies were performed to determine whether human preadipocytes expressed PAI-1 protein, followed by experiments determining the influence of the culture conditions on preadipocyte PAI-1. As shown in Table 1 and Fig. 1, PAI-1 protein accumulated in the medium of both sc and visceral preadipocyte cultures throughout a 24-h period. Regardless of anatomic site and BMI, similar quantities of PAI-1 were observed, when normalized for cell number. Cell num-

TABLE 1. Effect of depot and BMI on PAI-1 production by preadipocytes

Depot	BMI	PAI-1 production (μg/10^6 cells)
sc	19–24	7.6 ± 2.7
sc	36–43	7.4 ± 1.4
Omental	43–53	7.9 ± 3.5

Values are mean ± SEM of n = 3–4 separate cultures from different individuals within each group. Preadipocytes were plated at equivalent density, and PAI-1 antigen was determined 24 h after addition of fresh medium. No significant differences are apparent between cell types.

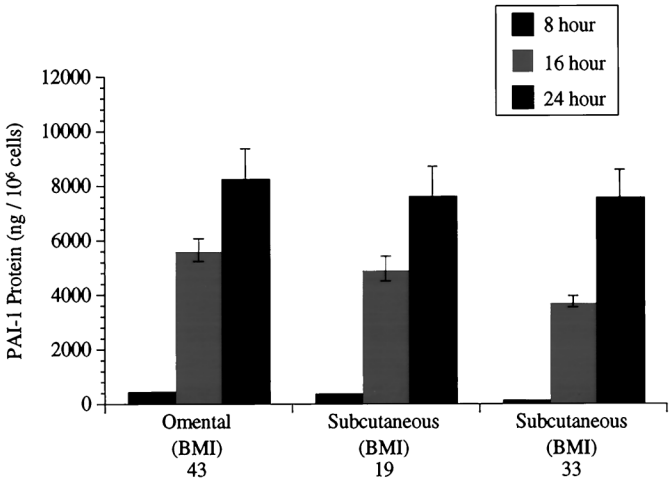

FIG. 1. Chronology of release of PAI-1 into medium of three preadipocyte cultures representing different depots and BMIs. Data represent mean ± SEM for triplicate determinations.

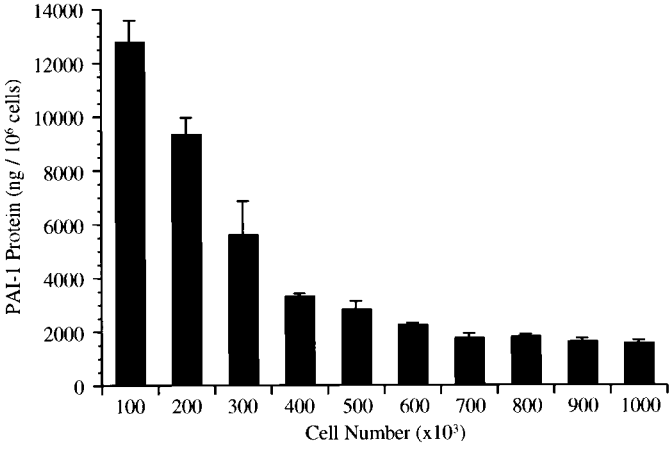

FIG. 2. The effect of cell density on PAI-1 production by human preadipocytes. sc preadipocytes from four different individuals with BMIs of 18–24 were plated in passages 2–4, at various densities, in 6-well plates, and PAI-1 antigen was assessed 24 h later. Values represent mean ± SEM. The correlation coefficient for the effect of density upon antigen concentration between 100,000 and 700,000 cells, where confluence is reached, is $r = -0.926$.

ber both within and between samples was constant throughout the 24-h period, and PAI-1 was not detected in the medium alone.

Effect of cell density

Significant effects of cell density on production of PAI-1 were observed. As shown in Fig. 2, actively proliferating preadipocytes exhibit an inverse relationship between cell number and PAI-1 concentration in the conditioned medium after 24 h of incubation. The correlation between density and PAI-1 concentration in the range of 100,000–700,000 preadipocytes is $r = -0.926$. Once confluence is reached, at approximately 700,000 cells, production becomes relatively constant, with an approximate 7.5× reduction in PAI-1, compared with cells plated at the lowest density. Based on the results of these experiments, cell number was strictly controlled throughout all studies.

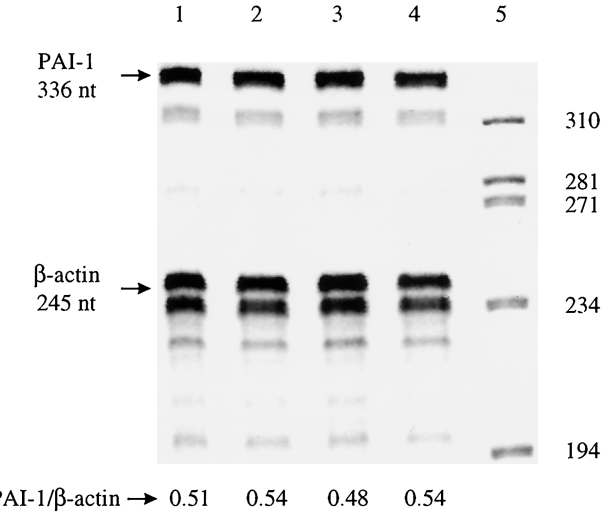

FIG. 3. RNase protection analysis of cultured human preadipocyte PAI-1 and β-actin mRNA. Radiolabeled probes were hybridized to 5 μg total RNA, and relative intensity of each band was determined by PhosphorImager analysis. PAI-1 mRNA, normalized to β-actin, indicated no differences between depots. Lane 1, omental, 47-yr-old female, BMI = 48; lane 2, sc, 26-yr-old female, BMI = 42; lane 3, visceral, 45-yr-old female, BMI = 60; lane 4, sc, 36-yr-old female, BMI = 22; lane 5, radiolabeled DNA size markers.

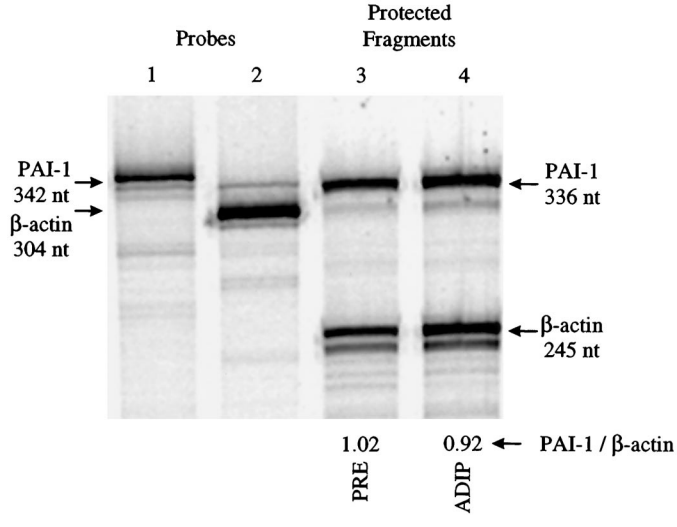

FIG. 4. Effect of differentiation on PAI-1 mRNA expression. Human sc preadipocyte (PRE) culture from a lean female (BMI = 19; age = 30 yr) was subjected to differentiation to the adipocyte (ADIP) phenotype, as described in *Materials and Methods*. Lanes 1–2, Radiolabeled probes (1×10^4 cpm); lanes 3–4, hybridization of 2 μg total RNA isolated from preadipocytes or adipocytes, respectively.

PAI-1 mRNA Expression

Identification of PAI-1 mRNA from preadipocyte cultures of 1 lean and 3 grossly obese women is shown in Fig. 3. PAI-1 mRNA is expressed in each culture and, when corrected for β-actin mRNA expression, is similar between individuals. In addition, these data indicate the high level of expression of PAI-1 by human preadipocytes, because PAI-1 mRNA represents a similar level of expression as the mRNA of the cytoskeletal protein β-actin. Differentiation to the adipocyte

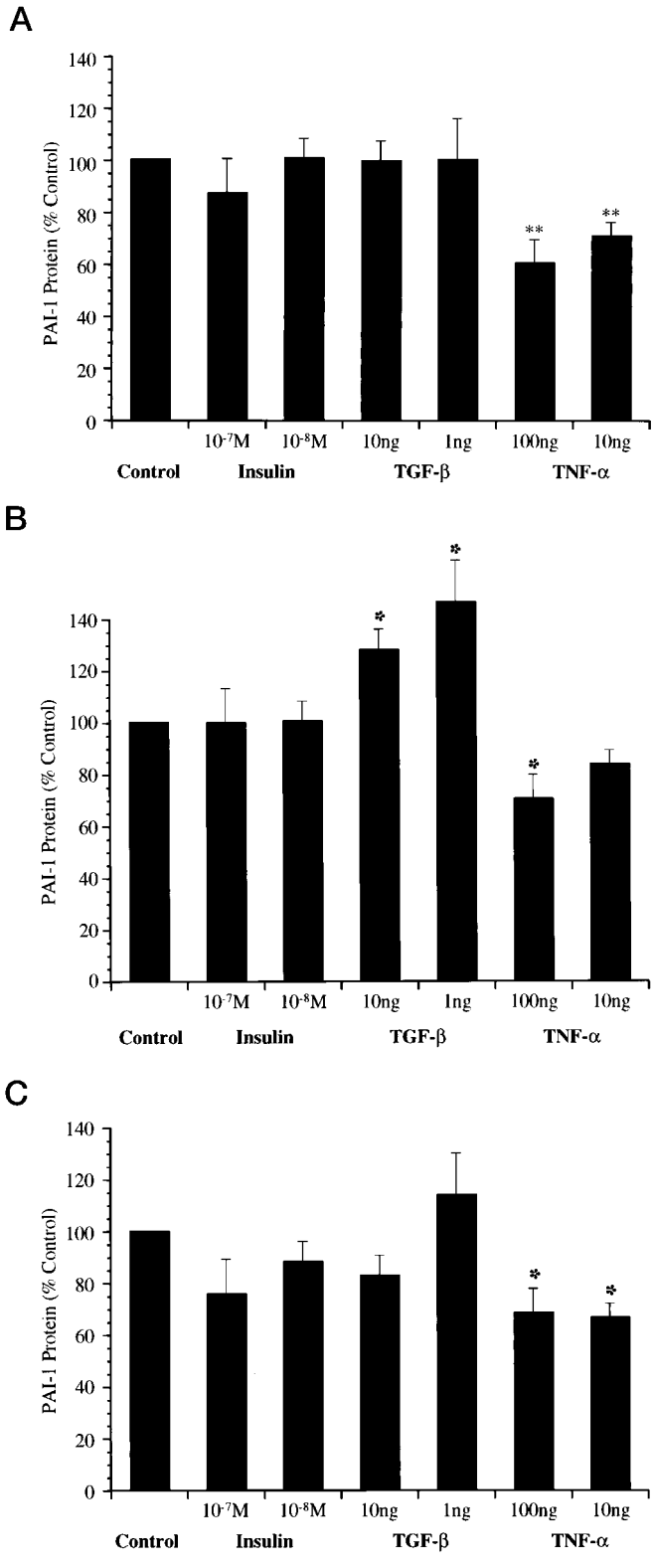

FIG. 5. Effect of insulin (mol/L), TGF-β (ng/mL), and TNF-α (ng/mL) on PAI-1 production by human preadipocytes from different anatomic sites and BMIs. Data were obtained from cultures of 11 different individuals and are expressed as percent change from control. A, Lean sc; B, obese sc; C, obese omental. Values represent mean ± SEM of n = 3–4 individuals for each site and BMI. Significantly different from control: *, $P < 0.05$; **, $P < 0.01$.

phenotype by culturing the cells in a defined medium did not significantly affect the pattern of mRNA expression (Fig. 4).

Effect of insulin, TGF-β, and TNF-α

Preadipocytes were assessed for PAI-1 production after incubation with either insulin, TGF-β, or TNF-α for 24 h. As shown in Fig. 5, insulin had no effect on PAI-1 production in any of the cultures examined. The effect of TGF-β on PAI-1 production in human preadipocytes was depot dependent. TGF-β had no effect on PAI-1 production in cultures from lean individuals, but it significantly elevated PAI-1 production in sc preadipocytes from obese patients. TNF-α significantly ($P < 0.05$) reduced PAI-1 concentrations in each of the depots examined, however, and in a dose-dependent manner.

Adipocyte differentiation

Confluent preadipocyte cultures were stimulated to differentiate to the adipocyte phenotype in the presence of isobutylmethylxanthine, hydrocortisone, and insulin (17). After 7 days of culturing in the differentiation medium, flow cytometric analysis of Nile Red fluorescence indicated both increased intracellular lipid and altered cellular morphology indicative of differentiated adipocytes (Fig. 6). In addition, differentiation under these conditions has been shown to result in the expression of leptin mRNA (14).

Discussion

Adipose tissue was first identified as a potential site of PAI-1 production by Sawdey and Loskutoff (9). This group examined a variety of tissues from mice, using RNase protection analysis, observing abundant PAI-1 mRNA expression in adipose tissue, which was further elevated by pretreatment with inflammatory cytokines. Subsequent cell culture studies in 3T3 cells found a correlation between TGF-β-stimulated elevations in PAI-1 mRNA and release of PAI-1 protein into the conditioned medium (10). More recently,

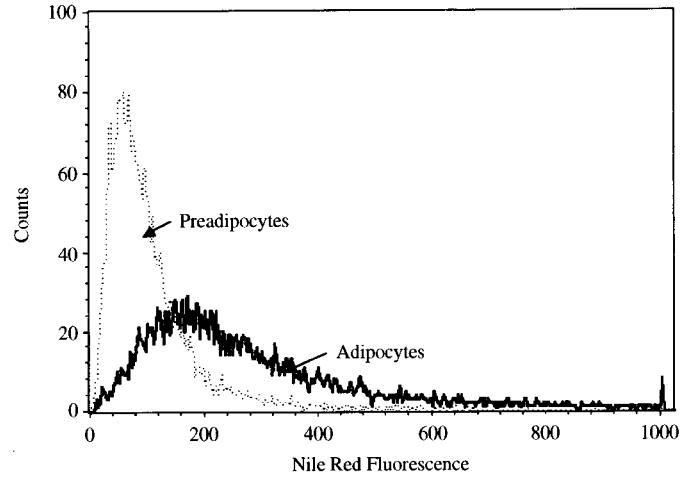

FIG. 6. Flow cytometric analysis of differentiation of preadipocytes to adipocytes. Adipocytes represent those cells maintained in a defined differentiation medium, as described in *Materials and Methods*. Nile Red staining indicates that differentiation was associated with increased cytosolic lipid.

PAI-1 mRNA has been identified in human adipose tissue explants (11, 12). Our experiments are the first to identify PAI-1 in primary culture of any human adipose tissue cellular component, and the first to identify preadipocytes as a source of adipose tissue PAI-1. The observation of both PAI-1 mRNA expression and PAI-1 protein further substantiates adipose tissue as a source of this serpin.

The pattern of production and regulation of PAI-1 we observed in human preadipocytes is comparatively different from that previously reported. Though detection of PAI-1 mRNA and protein has been investigated predominantly after differentiation to the adipocyte phenotype in murine cells (10, 18), we observed significant PAI-1 production in cultured human preadipocytes. In addition, in murine adipose tissue, TNF-α, TGF-β, and lipopolysaccharide, either injected *in vivo* or added to cultured adipocytes, elevates PAI-1 mRNA expression (9, 18). We did observe an elevation in PAI-1 antigen in response to TGF-β in preadipocytes from the sc depot of obese individuals. However, a significant reduction in PAI-1 protein production by human cells occurred in the presence of TNF-α, and this response was dose-dependent, suggesting a potential species variability in the regulation of adipose tissue PAI-1. These disparate results may be influenced by cell type and experimental design, because the initial observation of a stimulatory effect of TNF-α in culture used serum-deprived bovine aortic endothelial cells (19), and it reported mRNA expression but not PAI-1 protein production. In murine 3T3 cells, there are conflicting results of the response to TNF-α. Samad *et al.* (18) reported a stimulatory effect of 5 ng/mL of TNF-α on PAI-1 mRNA expression in 3T3-L1 cells but did not report the effect on PAI-1 antigen levels in the conditioned medium. Conversely, Lundgren *et al.* (10) observed no effect of TNF-α on either PAI-1 mRNA or protein in 3T3-L1 murine adipocytes at concentrations of the cytokine as high as 50 ng/mL. TNF-α is known to stimulate dedifferentiation of adipocytes; and in human adipocyte cultures, concentrations of TNF-α similar to those used in our experiments suppress mRNA for lipoprotein lipase, stimulate lipolysis, and abolish insulin-stimulated glucose transport (20). Our observations of a reduction in PAI-1 release in human preadipocytes treated with TNF-α is therefore consistent with those studies identifying a reversal of key cellular events of normal adipocyte metabolism (21).

Because of clinical observations associating insulin resistance with elevated plasma PAI-1 (3), the effect of insulin on PAI-1 expression has been studied in a variety of cultured cells. In primary human hepatocyte cultures, insulin stimulation results in a modest, 150% elevation in PAI-1 production, compared with control, untreated cells (22). In murine adipocytes, insulin has not been studied, and in our experiments, insulin had no effect. Our results are in close agreement with those of Klassen *et al.* (23), who performed similar analysis of PAI-1 production in human vascular endothelial cells and observed either no effect or a slight reduction in PAI-1 accumulation in the conditioned medium in response to insulin. Other studies, too, suggest that insulin alone does not directly stimulate PAI-1 production, including: human studies in which acute administration of exogenous insulin to normal individuals did not elevate circulating PAI-1 (8); and an epidemiologic analysis which failed to establish insulin sensitivity as an independent determinant of plasma PAI-1 (24). In addition, hyperinsulinemic euglycemic clamp studies, comparing the fasting plasma PAI-1 in obese nondiabetic and obese diabetic patients, found similar responses in each group (25). With the identification of glucose-responsive regions in the PAI-1 promoter of vascular smooth muscle cells, hyperglycemia may be responsible for the elevated plasma PAI-1 in diabetes (26). Similar experiments, examining PAI-1 expression after elevation of cellular glucose in the presence of insulin, need to be performed to better understand this process, and cultured human adipocytes would be an ideal cell type for these studies, potentially identifying new pathways of molecular regulation of PAI-1 in adipose tissue.

The current study has established that human preadipocytes synthesize PAI-1 and that this synthesis is regulated by the density of cells in culture and specific cytokines and growth factors, yet the physiologic role of PAI-1 in adipose tissue has yet to be elucidated. In addition to its function as an inhibitor of plasminogen-induced fibrinolysis, PAI-1 has recently been recognized in regulating cell migration, albeit in cells other than adipocytes (13, 27–29). In obesity, the growth of adipose tissue necessarily involves extracellular matrix degradation and local tissue remodeling, but the signals responsible for this process remain largely unknown (30). Before reaching confluence, the concentration of PAI-1 in the conditioned medium was dependent on the parameters of cell culture, with an inverse relationship between protein concentration and cell density. This observation agrees with similar cell density studies performed using microvascular endothelial cells (27). In smooth muscle cells, the active conformation of PAI-1 blocks migration by inhibiting cell attachment to vitronectin (13). Our observed reduction in PAI-1 synthesis per cell with increased density suggests that with cell-to-cell contact PAI-synthesis is also reduced, as the active process of obtaining confluence has been achieved. Further insight into the understanding of this process will require additional investigations, however, including identification and quantitation of other regulatory components of cell migration in human preadipocytes. Adipose tissue is one of the most highly vascularized tissues (30), and adipocytes are beginning to be recognized as a source of vascular growth factors (31), suggesting an autocrine regulation of the events necessary for tissue expansion, including angiogenesis. With the recent report that PAI-1 is required for tumor angiogenesis (32), PAI-1 production in human preadipocytes may contribute to adipose tissue growth through PAI-1-mediated proteolysis and vascularization. The production of significant levels of a molecule capable of regulating these processes by human adipose tissue suggests a novel mechanism for adipose tissue growth that warrants further investigation.

Acknowledgments

We appreciate the technical assistance of Dan Longhi.

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