

Plasminogen Activator Inhibitor 1, Transforming Growth Factor- β_1 , and BMI Are Closely Associated in Human Adipose Tissue During Morbid Obesity

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In adipose tissue from both obese mice and humans, plasminogen activator inhibitor 1 (PAI-1) expression has been reported to be upregulated to levels of increased plasma PAI-1. This elevated expression has been shown to be partly controlled by tumor necrosis factor (TNF)- α in mice. In humans, increased PAI-1 expression is associated with insulin resistance characterized by visceral fat accumulation. Therefore, the aim of this study was to investigate the expression pattern of PAI-1 and TNF- α (antigen and mRNA) in visceral human adipose fat in comparison with subcutaneous (SC) fat. Because transforming growth factor (TGF)- β_1 is a potent inducer of PAI-1 synthesis and has been shown to influence adipocyte metabolism, this work was extended to TGF- β_1 quantification. A total of 32 obese individuals (BMI 42 ± 6.8 kg/m²) were investigated. Freshly collected visceral adipose tissue did not exhibit a higher content of PAI-1 or TGF- β_1 than did SC tissue. Although most of the TNF- α values were at the detection limit of the methods, TNF- α antigen was 3-fold higher and TNF- α mRNA was 1.2-fold higher in visceral fat. The levels of tissue TGF- β_1 antigen correlated well with those of PAI-1 antigen, regardless of the fat depot studied (SC tissue: $n = 21$, $r = 0.72$, $P = 0.0006$; visceral tissue: $n = 20$, $r = 0.49$, $P < 0.03$), and they were both significantly associated with BMI. Conversely, no relationship was observed between the levels of TNF- α and PAI-1 or TNF- α and BMI. Tissue PAI-1 levels were also significantly correlated with those of circulating PAI-1. These results describe, in severe obesity, a proportional increase in tissue PAI-1 and TGF- β_1 in visceral and SC tissues. This increased PAI-1 expression could be the result of tissue cytokine disturbances, such as elevated TGF- β_1 expression. *Diabetes* 49:1374–1380, 2000

Elevated levels of plasminogen activator inhibitor 1 (PAI-1) are found in subjects with a history of myocardial infarction or angina pectoris (1,2). Prospective cohort studies conducted in atherosclerotic patients have indicated that elevated PAI-1 plasma concentrations are associated with increased risk for future coronary events (3–5).

Circulating levels of PAI-1 have been shown to be an inducible target controlled for insulin resistance with obesity (6,7). The relationship observed between high PAI-1 levels and the occurrence of myocardial infarction disappeared after controlling for the variables of the insulin resistance syndrome (4). Thus, it could be speculated that increased PAI-1 expression contributes to the increased susceptibility to atherogenesis described in insulin-resistant patients (8).

An individual's risk of cardiovascular disease relates closely to the inheritance of central obesity (9). In clinical studies, the relationship between PAI-1 levels and fat mass was emphasized because it persists after controlling for the other biological variables of the insulin resistance syndrome (10). Weight loss secondary to calorie restriction or surgery is associated with reduced PAI-1 activity (the changes are related to weight loss rather than reductions in the biological insulin resistance level) (11). Interestingly, waist-to-hip ratio, a reflection of central fat accumulation, has been found in women to be the only independent predictor of circulating PAI-1 activity (12). In individuals subjected to a calorie-restricted diet, PAI-1 levels were more closely related to changes in the central fat depot than in the subcutaneous (SC) fat depot (13). Thus, the visceral fat depot may be of importance for the occurrence of increased plasma PAI-1 levels.

Experimental data have confirmed the contribution of adipose tissue in controlling plasma PAI-1 levels. Interesting observations came from animal models. Obese mice exhibit increased plasma PAI-1 levels associated with elevated tissue PAI-1 expression compared with lean mice, particularly in adipose tissue, suggesting a specific contribution of this tissue in increasing plasma PAI-1 levels (14). In a model of obesity in rats, Shimomura et al. (15) have detected PAI-1 mRNA in both types of fat tissue, but its expression increased only in the visceral fat during the development of obesity. In humans, we have shown that freshly collected adipose tissue expressed PAI-1 mainly in the stromal cell compartment (16). When maintained in culture, adipose tissue explants produced PAI-1

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PAI-1, plasminogen activator inhibitor 1; RT-PCR, reverse transcriptase-polymerase chain reaction; SC, subcutaneous; TGF, transforming growth factor; TNF, tumor necrosis factor.

at a level higher in the visceral than in the SC territory (16), even though SC adipose PAI-1 mRNA levels and PAI-1 secretion have been shown to be increased in obese individuals to the same extent as circulating PAI-1 levels (17).

The PAI-1 synthesis is inducible. Thus, it could be speculated that obesity with insulin resistance represents a favorable condition for expression of the inducers of PAI-1 synthesis. Among them, tumor necrosis factor (TNF)- α and transforming growth factor (TGF)- β_1 have been shown to be potent inducers of PAI-1 synthesis in several cell systems (18,19) and in human adipose tissue explants (16). In *ob/ob* mice, neutralization of TNF- α or deletion of TNF receptors I and II results in significantly reduced levels of plasma PAI-1 antigen and adipose tissue PAI-1 (20). TNF- α is also a potent inducer of TGF- β_1 in murine adipose tissue (20) and contributes to the elevated TGF- β_1 expression demonstrated in the adipose tissue of obese mice (21). Moreover, TGF- β_1 administered in mice increased PAI-1 activity in plasma and PAI-1 mRNA expression in adipose tissue (22). In humans, a positive correlation was observed between the production of PAI-1 by incubated SC adipose tissue explants and those of TNF- α or TGF- β_1 (23).

Because visceral fat appears to be of particular importance in explaining the relationships observed between plasma PAI-1 and insulin resistance, we have evaluated the level of expression of PAI-1 in freshly collected human visceral and SC fats. These levels were then compared with those of TNF- α and TGF- β_1 . The results show a parallel evolution of PAI-1 and TGF- β_1 tissue concentration in visceral and SC adipose tissues. Both were positively associated with BMI. A contribution of TGF- β_1 to the elevated PAI-1 expression observed during insulin resistance is proposed.

RESEARCH DESIGN AND METHODS

Population and tissue collection. SC and visceral adipose tissues were obtained during gastropasty from 32 patients (27 women and 5 men) with a mean BMI of 42 ± 6.8 kg/m² and a mean age of 39 ± 12 years. Fasting insulinemia was 8.9 ± 7.7 μ U/ml. Of the study subjects, 22% exhibited insulinemia >15 μ U/ml. The mean fasting triglyceride level was 1.52 ± 0.59 g/l. Of the study subjects, 38% had values >1.5 g/l. Informed consent was obtained from each patient. All subjects were of Caucasian origin and did not suffer from any ongoing disease (i.e., infection or 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. In a subpopulation of 21 individuals, tissues were also collected in Hank's balanced salt solution (Gibco) for a 19-h incubation of adipose tissue explants as previously described (24).

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 to obtain platelet free plasma, and stored at -80°C until used.

PAI-1, TNF- α , and TGF- β_1 antigen determinations. Tissue and supernatant PAI-1 antigens were assayed using enzyme-linked immunosorbent assays specific for human PAI-1, as previously described (25). TNF- α and total TGF- β_1 antigens were assayed with commercially available kits from Immunotech and R&D Systems, respectively. All measurements were performed in duplicate. Results were expressed as nanograms per microgram of protein or DNA. Plasma PAI-1 antigen and PAI-1 activity were assayed using commercially available kits, Asserachrom PAI-1 (Diagnostica Stago) and Chromolize (Biopool), respectively.

Protein, RNA, and DNA extraction. Tissue proteins were extracted using a denaturing buffer (150 mmol/l NaCl, 10 mmol/l sodium phosphate, 1% Triton X 100, 0.1% SDS, 0.5% sodium deoxycholate, 0.2% sodium azide, 0.8 mmol/l Pefabloc [Interchim], and 100 μ l/100 mg tissue) under permanent stirring for 12 h at 4°C . Tubes were then centrifuged 10 min at 10,000g. Infranatants were collected and frozen at -80°C until used. Proteins were assayed according to the specifications of the Pierce bicinchoninic acid protein kit.

Total RNA was extracted using Trizol (Gibco). The integrity of the RNA was confirmed by electrophoresis in ethidium bromide containing agarose gels, and the RNA concentration was determined spectrophotometrically.

DNA was extracted as proposed by Maniatis et al. (26), except 2 chloroform extractions were performed instead of 1. The DNA concentration was determined spectrophotometrically. Each tissue was subjected to 3 different extractions. The mean level obtained was used for calculation.

Semiquantitative reverse transcriptase-polymerase chain reaction. The levels of PAI-1, TNF- α , and TGF- β_1 mRNA were determined by semiquantitative reverse transcriptase-polymerase chain reaction (RT-PCR). eEF1- α was preferred over actin as a housekeeping gene. Reverse transcription was performed using hexarandom primers (0.5 μ g/ μ g RNA) (Amersham) in a final volume of 20 μ l containing 1X First-Strand Buffer (Gibco), 2 mmol/l dNTPs (Gibco), 20 U rRNasin (Promega), 10 mmol/l dithiothreitol (Gibco), 100 U SuperScript II (Gibco). cDNA was synthesized at 37°C for 1 h and then for 10 min at 75°C . Amplification was carried out in 25- μ l samples (Gene Amp PCR System 2400; Perkin Elmer). Each sample contained 1 μ l cDNA in 1X Taq polymerase buffer (BioTaq; Quantum), 160 μ mol/l dNTPs, 0.35 U DNA polymerase (BioTaq; Quantum), and 0.4 μ mol/l of the corresponding primers (Gibco). The sequence of the upstream primers was as follows: PAI-1, 5'-ACATGACCAGGCTGCCCGC-3'; TNF- α , 5'-ATGGCAGAGAGGAGGTTGAC-3'; TGF- β_1 , 5'-ACCACTATTGCTTCAGTCCA-3'; and EF1- α , 5'-ACTGTGCTGCTGATTGTTC-3'. The sequence of the downstream corresponding primers was as follows: 5'-TGGGGTTGTCCGGACCACAA-3'; 5'-GCCTGTAGCCCATGTTGTAG-3'; 5'-TGCGGCCACGTTAGTACAC-3'; and 5'-GCACTTGCTCCAGCCATTGTT-3'. Different numbers of cycles were run to ensure that amplification of both fragments was within the linear range of the PCR. A first denaturation at 95°C for 2 min was followed by 30 cycles for EF1- α , 35 cycles for PAI-1 and TGF- β_1 , and 45 cycles for TNF- α amplification at 58°C for 1 min (annealing), 72°C for 1 min (extension), and 94°C for 45 s (denaturation). Each amplification sample was loaded on an ethidium bromide-stained 1.5% agarose gel. Amplification products were visualized under ultraviolet irradiation (Fig. 1). Quantitative analysis was performed using the Visiolab 100 software for image capture and the Phoretix software for calculation (Biocom, les Ulis, France). Three determinations were performed for each sample. The results were expressed as the ratios of PAI-1 to EF1- α , TNF- α to EF1- α , and TGF- β_1 to EF1- α . The between-assay variability of EF1- α determination calculated on the entire population was 7.95%. The within-assay variation coefficient of EF1- α measured from 3 determinations was 6.04 ± 2.73 (range 0.94–13.7).

Statistical analysis. Results were expressed as means \pm SD. The value n represents the number of tissue samples. In the study aimed to compare visceral and SC expression, the between-group comparison was tested using the nonparametric paired Wilcoxon's test. The nonparametric correlation coefficient (Spearman's) was used to examine the relations among study variables. Significance was defined as $P \leq 0.05$.

RESULTS

Comparison of DNA and protein contents of the visceral and SC fat depots. Visceral fat exhibited a higher DNA content than SC fat. The values for visceral and SC tissues

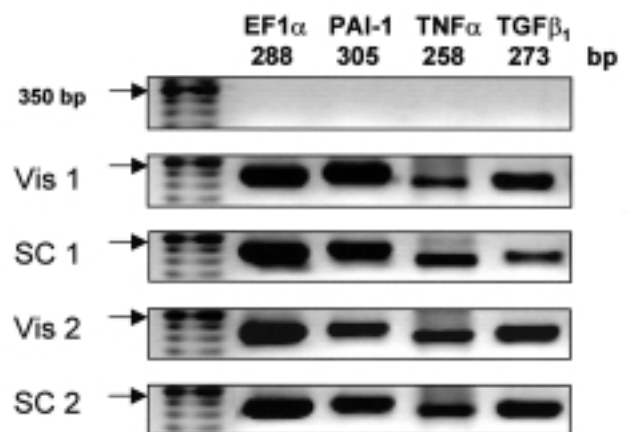


FIG. 1. Analysis by RT-PCR of the mRNA levels of EF1- α , PAI-1, TNF- α , and TGF- β_1 . The figure illustrates the results obtained with visceral (Vis) and SC adipose tissues from 2 individuals (1 and 2). The first line represents a control experiment in which PCR was performed without cDNA matrix.

were $0.21 \pm 0.08 \mu\text{g/g}$ (range 0.06–0.42) and $0.14 \pm 0.05 \mu\text{g/g}$ (range 0.05–0.35) tissue, respectively ($n = 30$, $P < 0.0001$). By contrast, no difference was observed for total proteins: $4.1 \pm 1.1 \text{ mg/g}$ tissue (range 2.3–6.7) and $3.9 \pm 1.1 \text{ mg/g}$ tissue (range 1.8–8.1). Nevertheless, a positive correlation was observed between the DNA and the protein content, regardless of the fat depot tested (SC tissue: $n = 29$, $r = 0.58$, $P = 0.0009$; visceral tissue: $n = 28$, $r = 0.54$, $P = 0.003$). Results were thus expressed per microgram of DNA.

Comparison of PAI-1, TNF- α , and TGF- β_1 expression between visceral and SC fat depots. The amounts of PAI-1 and TGF- β_1 from freshly collected visceral adipose tissue did not differ from those of SC tissue (Fig. 2A and C). The level of expression of TNF- α was significantly higher in visceral than in SC fat for either mRNA ($50 \pm 19\%$ [range 9.5–111] vs. $41 \pm 15\%$ [range 6.2–69], visceral vs. SC, respectively [$n = 30$, $P = 0.008$]) or antigen ($0.048 \pm 0.042 \text{ pg}/\mu\text{g DNA}$ [range 0–0.12] vs. $0.016 \pm 0.019 \text{ pg}/\mu\text{g DNA}$ [0–0.05], visceral vs. SC, respectively [$n = 18$, $P = 0.044$]) (Fig. 2B). It is noteworthy that in 37% of the cases TNF- α mRNA content from the visceral territory was lower than that of the SC territory. Moreover, most of the TNF- α antigen values were at the detection limit of the method.

When the secretion rate of PAI-1 was evaluated during a 19-h incubation period, PAI-1 antigen level produced in the conditioned medium from visceral fat was higher than that secreted from SC tissue ($6.1 \pm 4.1 \text{ ng}/\mu\text{g DNA}$ [range 1.1–18.3] vs. $3.7 \pm 4.5 \text{ ng}/\mu\text{g DNA}$ [range 1.1–22], visceral vs. SC, respectively [$n = 21$, $P = 0.005$]).

Relation among the levels of PAI-1, TNF- α , and TGF- β_1 expression in adipose tissue. Among all of the parameters evaluated, only tissue TGF- β_1 antigen levels correlated well with those of tissue PAI-1. This relationship was observed regardless of the fat depot studied (SC fat: $n = 21$, $r = 0.72$, $P = 0.0006$; visceral fat: $n = 20$, $r = 0.49$, $P < 0.03$) (Fig. 3A). In the SC territory only, a significant relationship was also observed between TGF- β_1 antigen and the PAI-1 mRNA content ($n = 19$, $r = 0.52$, $P = 0.02$). By contrast, no relationship between the levels of TNF- α and PAI-1 expression was observed (Fig. 3B). Interestingly, the TGF- β_1 mRNA content of the visceral tissue was significantly associated with that of TNF- α ($n = 30$, $r = 0.54$, $P = 0.002$).

Relation among the levels of PAI-1, TNF- α , and TGF- β_1 expression in adipose tissue and BMI. This analysis was performed in a subpopulation of 18 individuals, from which all of the parameters studied above have been collected. The levels of tissue PAI-1 antigen and TGF- β_1 antigen were both positively correlated with BMI, regardless of the fat depot tested (SC fat: $r = 0.56$, $P = 0.01$, and $r = 0.54$, $P = 0.02$, respectively; visceral fat: $r = 0.43$, $P < 0.05$, and $r = 0.57$, $P = 0.01$, respectively) (Fig. 4A and B). Neither tissue TNF- α antigen nor mRNA levels were associated with BMI.

Relation between the level of PAI-1 expression in adipose tissue and circulating PAI-1. Circulating active PAI-1 was significantly correlated with the PAI-1 antigen content of the SC tissue ($n = 27$, $r = 0.49$, $P = 0.01$) but not with that of the visceral tissue. It was also significantly associated with the PAI-1 mRNA content of the visceral tissue ($n = 29$, $r = 0.51$, $P = 0.004$) but not with that of the SC tissue (Fig. 5). A similar trend was observed with circulating PAI-1 antigen (SC tissue PAI-1 antigen: $n = 27$, $r = 0.39$, $P = 0.044$; visceral PAI-1 mRNA: $n = 29$, $r = 0.32$, $P = 0.09$). These results were not modified after restricting the analysis to the female population (data not shown).

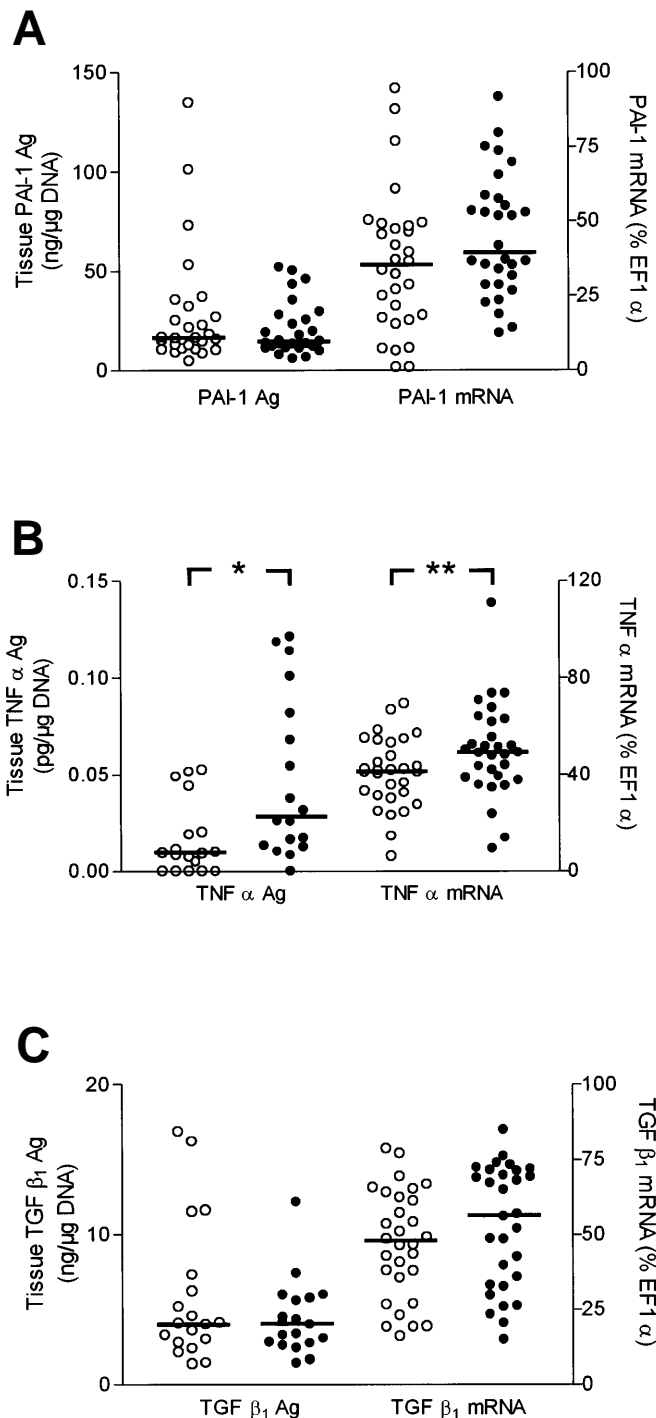


FIG. 2. The antigen (Ag) and the mRNA contents of PAI-1 (A), TNF- α (B), and TGF- β_1 (C) in SC adipose tissue (\circ) were compared with those quantified in the visceral adipose tissue (\bullet). * $P < 0.05$; ** $P < 0.01$.

DISCUSSION

The elevated plasma PAI-1 concentration observed during central obesity and the positive correlation observed between PAI-1 levels and visceral fat accumulation have led us to characterize visceral fat as a primary PAI-1 synthesis place. Using freshly collected tissues from severely obese patients, our results did not exhibit differences in PAI-1 expression between visceral and SC fats. Thus, the increase in PAI-1 levels observed in obese subjects does not reflect

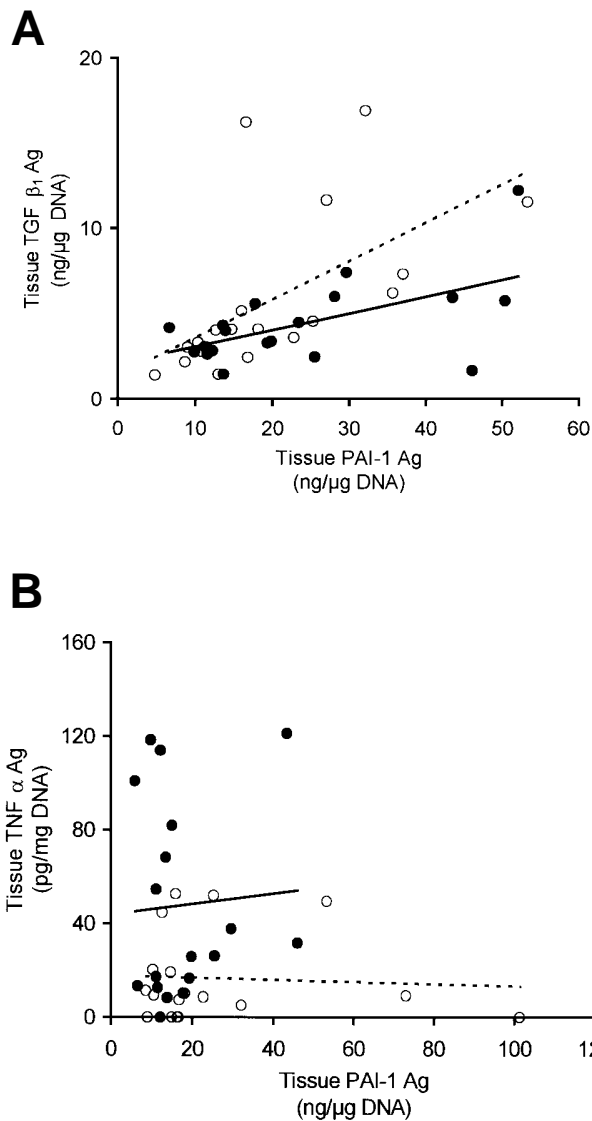


FIG. 3. Correlation between (A) tissue PAI-1 and TGF- β_1 antigens and between (B) tissue PAI-1 and TNF- α antigens contents in SC (\circ , ---) ($n = 21$) and visceral (\bullet , —) ($n = 20$) tissues. Significant associations were observed between tissue PAI-1 and TGF- β_1 (SC fat: $r = 0.72$, $P = 0.0006$; visceral fat: $r = 0.49$, $P < 0.03$).

production restricted to visceral fat. This agrees with the work of Samad and Loskutoff (14), who described in genetically obese mice, by comparison with their lean counterparts, an increase in PAI-1 expression in several tissues, the greatest of which was observed in adipose tissue without a strong difference between SC and epididymal fat (5). Whereas it is well established in mice that the PAI-1 adipose tissue content increases with the level of obesity, only one study has addressed this point in humans, showing that the SC adipose tissue secretion of PAI-1 correlated significantly with BMI and more strongly with the volume of adipocytes (17). Our results confirm this data and additionally show that the same relationship was obtained with visceral instead of SC adipose tissue. The significant relationship observed between plasma PAI levels and the SC adipose tissue PAI-1 antigen underscores the contribution of the SC tissue in determining plasma PAI-1 levels.

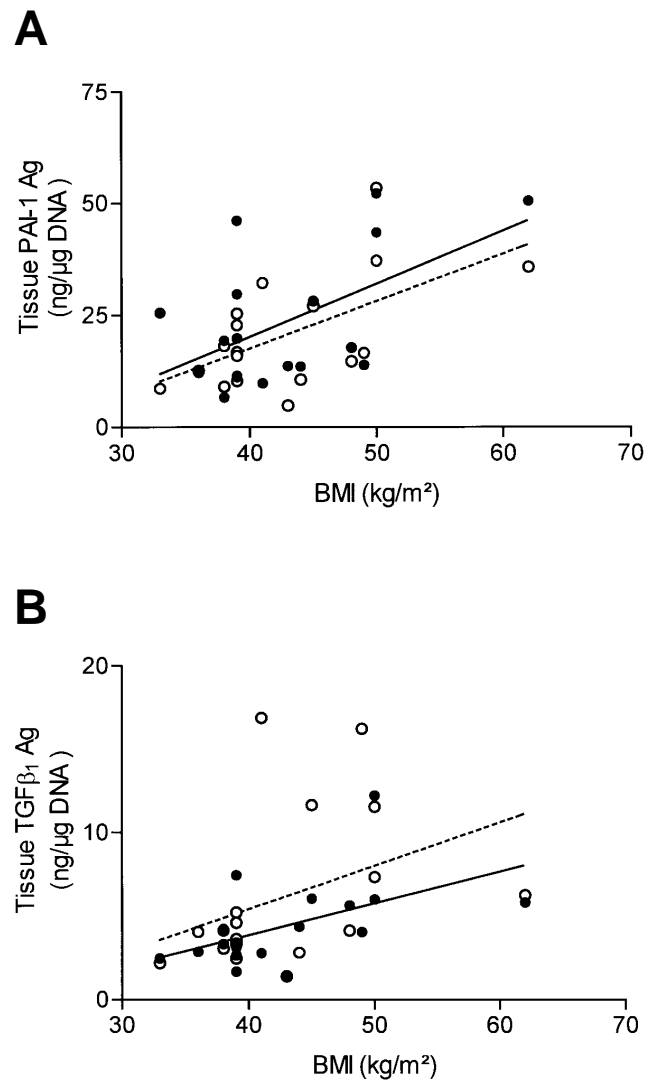


FIG. 4. Correlation between (A) tissue PAI-1 antigen and BMI and (B) tissue TGF- β_1 antigen and BMI in SC (\circ , ---) and visceral (\bullet , —) tissues ($n = 18$). Significant associations were observed between tissue PAI-1 and BMI (SC fat: $r = 0.56$, $P = 0.01$; visceral fat: $r = 0.43$, $P < 0.05$) and between tissue TGF- β_1 and BMI (SC fat: $r = 0.54$, $P = 0.02$; visceral fat: $r = 0.57$, $P = 0.01$).

PAI-1 synthesis is mainly inducible. One could expect that the increase in PAI-1 observed during central obesity with insulin resistance is due to the presence of a specific inducer. The absence of PAI-1 upregulation in patients with peripheral (gynoid) obesity known to be free of insulin resistance favors this hypothesis (27). Among the inducers of PAI-1, TNF- α and TGF- β_1 occupy a place of choice. They have been shown to stimulate PAI-1 synthesis in numerous cell systems as well as in adipose tissue either in vivo or ex vivo (16,22,28,29). The relations described among TNF- α , obesity, and insulin resistance suggest a contribution of TNF- α in explaining the PAI-1 increase observed during obesity. TNF- α has been demonstrated to interfere with insulin signaling, thereby contributing to insulin resistance (30,31). Moreover, higher plasma TNF- α concentration and bioactivity were found in patients with android obesity compared with patients with peripheral obesity (32,33). This latter group of patients had the same

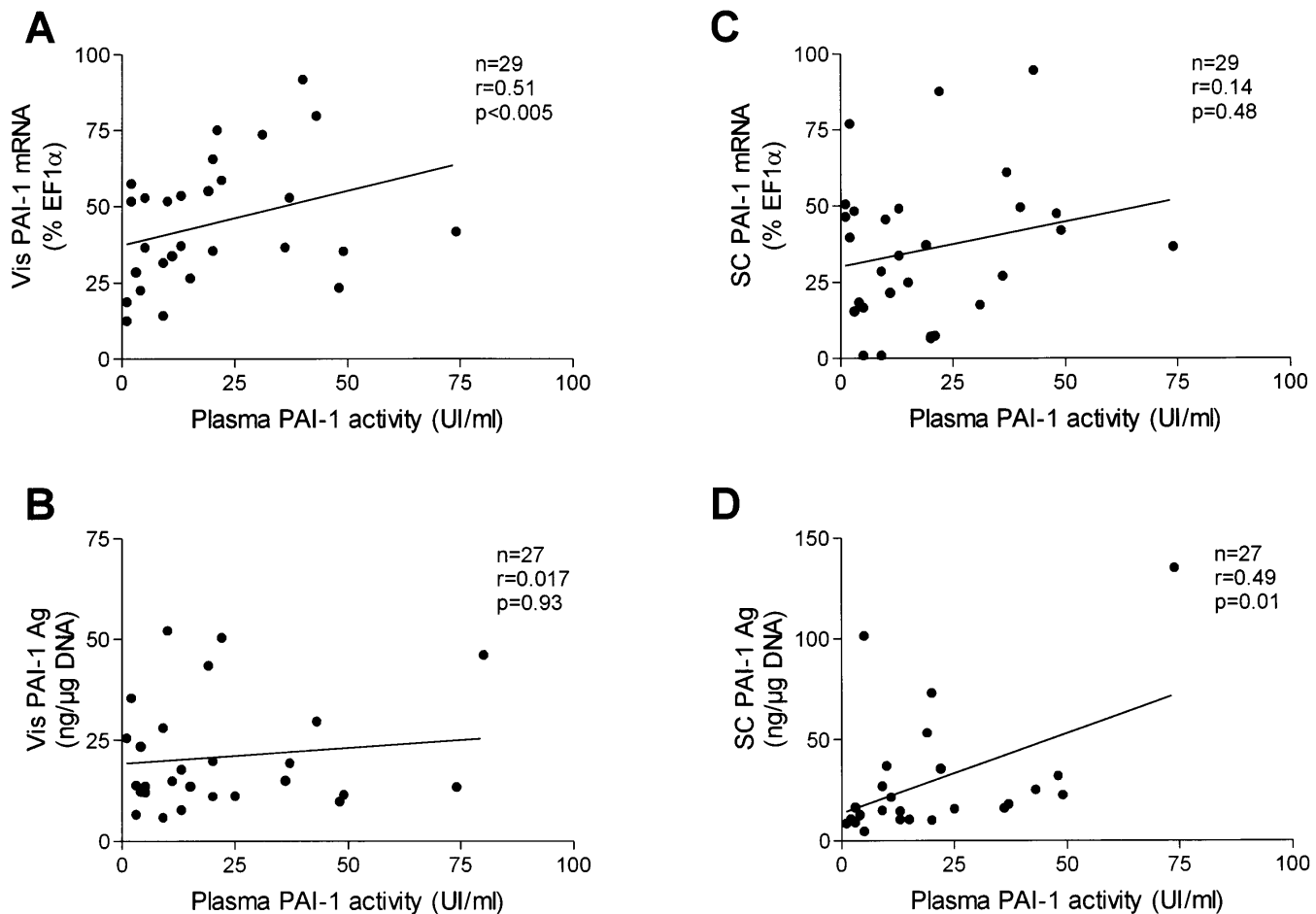


FIG. 5. Scatterplots showing the correlations between plasma PAI-1 activity and the levels of tissue PAI-1 expression. **A:** Visceral adipose tissue PAI-1 mRNA. **B:** Visceral PAI-1 antigen. **C:** SC PAI-1 mRNA. **D:** SC PAI-1 antigen. Vis, visceral.

level of TNF- α concentration as lean control subjects. Serum levels of TNF- α have been shown to be significantly and positively correlated with the area of the visceral fat (34). Neutralization of TNF- α or deletion of both TNF- α receptors in mice results in significantly reduced levels of plasma PAI-1 and adipose tissue PAI-1 (20), suggesting that TNF- α is a common link between insulin resistance and elevated PAI-1. In this study, we were surprised by the low adipose tissue content of TNF- α for either antigen or mRNA. The mean TNF- α protein level was 10-fold lower than that previously obtained by Kern et al. (35). This result could be attributed to the severely obese population we have studied. Indeed, it has been previously reported that TNF- α mRNA tended to decrease in obese patients with BMI >45 (35). Therefore, a decrease in TNF- α expression could affect the severity of obesity. Indeed TNF- α is known to inhibit fat-cell development *in vitro* (36). It may act at a very low level in an autocrine/paracrine manner. Moreover, its transmembrane form has been shown to be biologically active and capable of altering the adipogenic process in cultured adipocytes (36). This mechanism of action possibly explains why, in a mildly obese population, Mohammed-Ali et al. (37) did not observe arterio-venous differences in concentrations of TNF- α across an SC adipose tissue bed, which suggests an absence of TNF- α secretion. We found a significantly greater amount of TNF- α in the visceral than in the SC

adipose tissue. Contradictory results were found in literature. Hube et al. (38) showed a significantly higher expression of TNF- α mRNA in the SC than in the omental adipose tissue. In contrast, Montague et al. (39) did not demonstrate any difference in the TNF- α mRNA levels between SC and omental adipocytes, unlike Dusserre et al. (40), who observed such differences by using fat biopsies. We were not able to exclude the results concerning the low adipose tissue TNF- α content as reflections of mere chance. Moreover, we did not observe any relationship between the levels of PAI-1 and TNF- α within each tissue. Another possible explanation regarding the difficulty of assessing the contribution of TNF- α is that measurement of total TNF- α does not accurately reflect the amount of active TNF- α bound to membranes.

TGF- β_1 is a potent inducer of PAI-1 in many cell systems. It has been shown that TGF- β_1 interferes with adipose tissue development. It potentiates angiogenic activity, decreases lipid filling in SC adipocytes of fetal pigs (41), and inhibits the differentiation of both human adipocyte precursor cells in primary culture (42) and 3T3-L1 preadipose cells (43). Moreover, elevated plasma levels of TGF- β_1 were described in type 2 diabetes (44). Until now, no data have been published on TGF- β_1 expression by freshly collected human adipose tissue. Crandall et al. (45) demonstrated with primary cultures of human preadipocytes that TGF- β_1 significantly ele-

vated PAI-1 production, but only with SC preadipocytes from obese individuals. We have found that TGF- β_1 is strongly expressed by human adipose tissue without a difference between the visceral and the SC territories, and its antigen level increased proportionally to the degree of obesity. The adipose tissue content of PAI-1 antigen was found to be strongly correlated with that of TGF- β_1 , regardless of the territory tested. These results demonstrate an association between PAI-1 and TGF- β_1 but do not allow for speculation on a direct relationship between these 2 variables. It could be hypothesized that either TGF- β_1 triggers PAI-1 synthesis or that the increase in PAI-1 leads to a decrease in plasmin formation, thereby impairing active TGF- β_1 formation (46) and consequently delaying clearance of TGF- β_1 antigen. Samad et al. (21) have observed an increase in TGF- β_1 expression in obese mice after TNF- α administration (21). The same process could occur in humans, because a strong positive correlation was described between TNF- α and TGF- β_1 mRNA in the visceral territory only.

In conclusion, our results show that the entire fat mass contributes to plasma PAI-1 level in morbidly obese patients. The increase in adipose tissue PAI-1 could be the result of cytokine disturbance, which specifically accompanies insulin resistance. From our results, it could be speculated that TGF- β_1 is of particular relevance.

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