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Plasminogen activator inhibitor-1 and haemostasis in obesity

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The connection between obesity and disordered haemostasis is well established, but incompletely understood. There is a strong link between inhibition of fibrinolysis and obesity, and elevation of the plasma inhibitor, plasminogen activator inhibitor-1 (PAI-1), is regarded as a central factor. Here we explore the increased risk of atherothrombotic disorders in obese subjects, and the evidence for metabolic and genetic causes. There is a clear relationship between plasma PAI-1 and obesity, and adipose tissue synthesises PAI-1, as has been shown in mouse and rat models, and more recently in human material. This tissue also produces several effector molecules that can up regulate PAI-1. These molecules include transforming growth factor β, tumour necrosis factor α, angiotensin II and interleukin 6, all of which up regulate PAI-1 in various cell types. The issue of whether adipose tissue directly contributes to plasma PAI-1, or whether it primarily contributes indirectly, its products stimulating other cells to produce PAI-1 that feeds into the plasma pool, is not yet resolved. Finally, we briefly examine other proteins of haemostasis that are products of adipose tissue. Further studies are needed to define the regulation of these proteins, in adipose tissue itself and in other cells influenced by its products, in order to extend recent insights into the links between obesity and haemostasis.

Obesity: PAI-1: Thrombosis: Insulin resistance: Cardiovascular disease

Haemostasis describes the systems that prevent loss of blood from the organism by clotting at sites of injury. Many different components are active in this process, including the endothelium of the vessel wall, circulating blood cells, notably platelets and leucocytes, and plasma components. Two major protease cascades are involved, the coagulation and fibrinolytic pathways (Fig. 1), which consist primarily of inactive plasma precursors that are activated to become serine proteases.

Cell surfaces and/or fibrin provide sites for local activation of the haemostatic system; this process is not favoured in free solution. Coagulation is initiated primarily by cell surface expression of tissue factor, which acts as a focus for plasma coagulation factors, culminating in the formation of thrombin; it then converts fibrinogen to fibrin. Fibrinolysis depends on bringing together plasminogen, an inactive plasma protein, and its activators, tissue plasminogen activator and urokinase, on fibrin or cells, where plasmin is generated and degrades fibrin. The essential balance in plasma is between the proteolytic activities of tissue plasminogen activator and urokinase and the inhibitor, plasminogen activator inhibitor-1 (PAI-1). In general, PAI-1 is present in 4–5-fold excess over the activators, favouring the stabilization of fibrin. Fibrin formation is an essential defensive mechanism, protecting the body from bleeding. If it persists it can cause thrombosis. Deposition of excess fibrin in the vessel wall is also relevant to atherosclerosis. Obese subjects suffer many atherothrombotic diseases. The protein of the haemostatic system that is most disordered in obesity is PAI-1, an inhibitor of plasminogen activation, which stabilises fibrin (Fig. 1). The present review focuses on this inhibitor.

Plasminogen activator inhibitor-1

PAI-1 is produced by a variety of cells in culture and is widely distributed in tissues (Sawdey & Loskutoff, 1991; Simpson *et al.* 1991). In cultured cells PAI-1 production is stimulated by many agents, including thrombin, insulin, cytokines, lipoproteins, angiotensin II and bacterial lipopolysaccharide (Andreasen *et al.* 1990; Loskutoff, 1991). PAI-1 occurs at low concentrations in plasma, about 20 ng/ml or 400 pM, while platelets account for more than 90 % of blood PAI-1 (Booth, 1999). PAI-1 activity is unstable, but is protected in plasma by its interaction with vitronectin (Declerck *et al.* 1988). There is good agreement

Abbreviations: PAI-1, plasminogen activator inhibitor-1; RAS, renin–angiotensin system; TGFβ, transforming growth factor β; TNF-α, tumour necrosis factor α.

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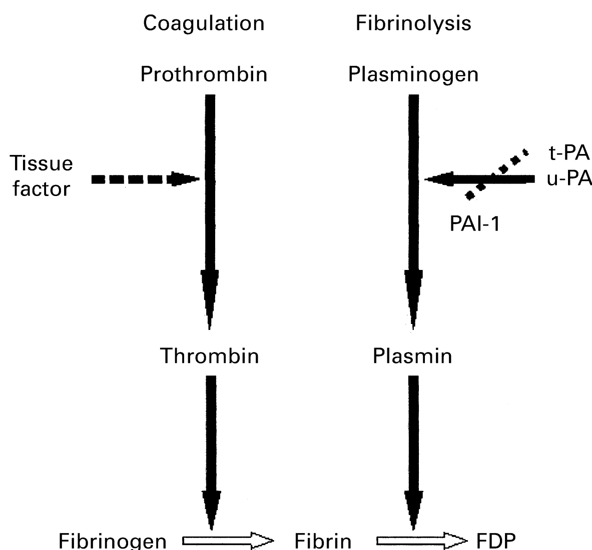

Fig. 1. A simple schematic diagram showing the two principal cascade systems of haemostasis. The final enzyme in the coagulation cascade, thrombin, is generated from its precursor prothrombin through a sequence of reactions triggered by exposure to tissue factor. Thrombin converts fibrinogen to fibrin to produce an insoluble network. The zymogen plasminogen is activated to plasmin by the plasminogen activators, tissue plasminogen activator (t-PA) or urokinase (u-PA), a process that is inhibited by the plasminogen activator inhibitor, PAI-1. Plasmin, which degrades fibrin to fibrin degradation products (FDP), is inhibited by α_2-antiplasmin.

between measurements of PAI-1 as activity or antigen, but antigen measurements can be influenced by platelet contamination. The platelet pool of PAI-1 explains the high local concentrations achieved in thrombi (Robbie *et al.* 1996*a*), correlating inversely with susceptibility to lysis *in vitro* (Potter van Loon *et al.* 1992). Similarly, PAI-1 mRNA and protein are elevated in atherosclerosis (Schneiderman *et al.* 1992; Robbie *et al.* 1996*b*), suggesting that local synthesis of PAI-1 in endothelial and smooth muscle cells is increased, stabilizing the fibrin that is associated with vessel wall disease (Smith, 1994). Deficiency in PAI-1 has been shown to be associated with bleeding after trauma in a human patient (Fay *et al.* 1992) and in knockout mice (Carmeliet & Collen, 1996). Conversely, transgenic mice overexpressing PAI-1 suffered frequent venous thrombosis (Erickson *et al.* 1990).

Plasma PAI-1 has been a focus of study for 20 years. It is not clear where plasma PAI-1 is synthesised, and two sources were originally proposed; endothelium and the liver. Another potential source that has emerged is adipose tissue, as will be discussed later (pp. 342–343). The range of plasma PAI-1 is rather variable, even among healthy individuals, and it shows distinct circadian variations (Kluft *et al.* 1988). Even against this variable background, elevation in plasma PAI-1 has been consistently observed in a range of diseases (Kruithof *et al.* 1988) and in patients with features that predispose them to cardiovascular disease, including advancing age, increased body mass, central fat distribution, raised blood pressure, and triacylglycerol level and increased plasma insulin level (Juhan-Vague & Alessi, 1993).

The early studies showed a coincidence between disease and elevations in plasma PAI-1. The possibility of a causal link between high PAI-1 and pathological conditions was then examined in large prospective studies, measuring either PAI-1 activity or antigen. These studies also sought a genetic basis for elevated plasma PAI-1. Several polymorphisms in the PAI-1 gene have been reported to be represented more frequently in patients than in healthy populations, but there are many conflicting reports, and no compelling data to connect the PAI-1 gene to disease (Lane & Grant, 2000). The problem of inconsistent results in these genetic studies has sometimes overshadowed the clear connection between elevated plasma PAI-1 and disease. PAI-1 in plasma is elevated in the insulin resistance syndrome (also termed syndrome X; Reaven, 1988), being associated strongly with plasma insulin, triacylgycerols and BMI (Juhan-Vague & Alessi, 1997). Once corrected for these other markers, the elevation in plasma PAI-1 becomes non-significant, leading to the conclusion that PAI-1 is a marker of the insulin resistance syndrome, and not an independent risk factor (Juhan-Vague *et al.* 1996). This lack of independence as a risk factor should not be regarded negatively; rather it signals the close connection between PAI-1 and metabolic disturbance, including obesity.

Obesity is an independent risk factor for atherosclerosis and cardiovascular disease, and a major contributor to mortality and morbidity (Rosenbaum *et al.* 1997). More than one mechanism may explain this increased risk, but impaired fibrinolysis has been strongly associated over several decades with obesity (Fearnley *et al.* 1963; Bennett *et al.* 1966). Increased plasma PAI-1 has emerged as the main cause of the decreased fibrinolytic activity (Vague *et al.* 1986, 1989; Landin *et al.* 1992; McGill *et al.* 1994). Importantly, energic weight loss led to a decrease in plasma PAI-1 (Sundell *et al.* 1989, 1991; Folsom *et al.* 1993; Marckmann *et al.* 1998), which rose again if weight was regained (Mavri *et al.* 1999). The same effect was achieved by surgical removal of fat (Primrose *et al.* 1992).

These clinical studies supported the possibility that adipose tissue contributed directly to elevated plasma PAI-1, a hypothesis that emerged strongly when it was observed that mouse adipose tissue expressed high levels of PAI-1 mRNA (Sawdey & Loskutoff, 1991). A seminal finding was made in *ob/ob* mice, which cannot produce leptin, as a result of which they develop obesity and insulin resistance. These mice had plasma PAI-1 levels 5-fold higher than their lean counterparts, with overexpression of the PAI-1 gene in adipose tissue (Samad & Loskutoff, 1996; Loskutoff *et al.* 2000). Expression of PAI-1 mRNA was also demonstrated in visceral and subcutaneous fat of obese rats (Shimomura *et al.* 1996). These findings and the emerging role of adipose tissue as an organ secreting proteins such as adipsin, leptin and adiponectin into blood (Spiegelman & Flier, 1996) suggested the importance of this tissue in the elevation of plasma PAI-1 (Fig. 2). The potentially large mass of adipose tissue in obese subjects would have a capacity to synthesise PAI-1 that could exceed that of other tissues.

PAI-1 activity in human plasma is correlated with adipsin, itself a product of adipose tissue (Alessi *et al.* 1995), and it was shown later that human adipose tissue produced PAI-1 (Alessi *et al.* 1997). PAI-1 mRNA was

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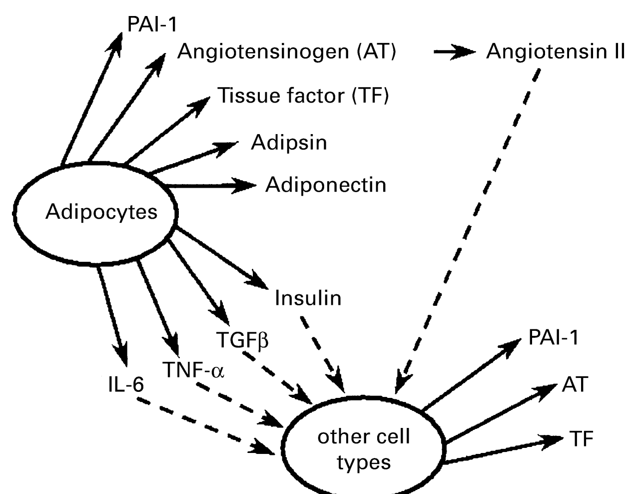

Fig. 2. A scheme showing the potential influences of adipocytes on synthesis of proteins of the haemostatic system. PAI-1, plasminogen activator inhibitor-1; IL-6, interleukin 6; TNF-α, tumour necrosis factor α; TGFβ, transforming growth factor β.

detected in freshly-isolated stromal and adipocyte fractions of human adipose tissue. The weak signal in fresh tissue was greatly increased on culture, and the adipocytes were identified as the main source of PAI-1. The illustration of PAI-1 mRNA in human adipose tissue raised interest in adipose tissue as a source of PAI-1, and several studies went on to analyse PAI-1 and related factors. Adipose tissue explants released PAI-1 after 2 h incubation with significantly more released antigen from obese subjects than from non-obese subjects ($P < 0.0001$), consistent with a 2-fold difference ($P < 0.05$) in the steady-state level of PAI-1 mRNA (Eriksson *et al.* 1998). These authors expressed PAI-1 secretion per adipose tissue fat cell, which revealed a larger difference between obese and non-obese controls. They also illustrated that PAI-1 mRNA levels and adipocyte PAI-1 secretion were related to adipocyte volume and lipid content.

Studies on human subjects have revealed differences in PAI-1 release from different fat depots. A direct link has been shown between plasma PAI-1 and visceral fat area in obese and non-obese children and adults (Cigolini *et al.* 1996; Ferguson *et al.* 1998). PAI-1 secretion by human adipocytes has been reported to be more pronounced in visceral fat than in subcutaneous fat (Alessi *et al.* 1997), a phenomenon also reported in animal models. Analysis of rat adipose tissue suggested that both visceral and subcutaneous fat could synthesize PAI-1, but that the increased expression associated with obesity is related only to increases in visceral fat PAI-1 expression (Shimomura *et al.* 1996). An interesting study on obese men and women, in which visceral fat was measured by magnetic resonance imaging, showed that visceral fat was directly correlated with plasma PAI-1 antigen and activity in men but not in women (Kockx *et al.* 1999). This finding is consistent with men having more visceral fat. Plasma PAI-1 correlated well with PAI-1 produced *in vitro* by cultured subcutaneous and visceral adipose tissue, and there was good correlation between the two sources, with visceral fat producing about twice as much PAI-1 as subcutaneous fat (Morange *et al.* 1999). Alessi *et al.* (2000) found that visceral and subcutaneous fat were comparable in their production of PAI-1, while Eriksson *et al.* (2000) found higher PAI-1 mRNA in subcutaneous fat compared with visceral fat, and a higher rate of synthesis of PAI-1 antigen. These authors also point out that the subcutaneous depot is the largest fat depot. Further studies are required to define the differences in PAI-1 production by these different fat sources, particularly in tissue of human origin, as animal body fat distribution differs from that in human subjects. Recent studies focus on the possible role of local depots of adipose tissue, such as the artery-associated fat, in the pathobiology of atherogenesis (Chaldakov *et al.* 2000).

PAI-1 is up regulated by a number of hormones, cytokines and metabolic factors (Andreasen *et al.* 1990; Loskutoff, 1991). It was speculated that the general up regulation of cytokines and effector molecules in obesity would lead to increased PAI-1 synthesis. To address the question of whether adipose tissue expression of PAI-1 contributed directly to plasma PAI-1, Yudkin *et al.* (1999) measured arterio–venous differences across subcutaneous adipose tissue. No net increase in PAI-1 activity or antigen was observed in healthy subjects, leading to the conclusion that subcutaneous adipose tissue does not significantly contribute to circulating PAI-1. They found significant arterio–venous differences in the concentration of interleukin 6 ($P < 0.001$) and some difference in tumour necrosis factor α (TNF-α), again consistent with the idea that the effects of adipose tissue may be mediated through cytokines and growth factors (Yudkin *et al.* 2000). This finding would predict that synthesis of PAI-1 in other tissues would be increased, and indeed Pandolfi *et al.* (2000) found increased expression in the liver, adipose tissue and aorta wall of diabetic rats that were chronically hyperglycaemic.

Cytokines, growth factors and other effector molecules

Two cytokines that up regulate PAI-1 synthesis by various cells are TNF-α and transforming growth factor β (TGFβ) (Andreasen *et al.* 1990; Loskutoff, 1991). The influence of these cytokines on PAI-1 synthesis in adipose tissue, and that of other effector molecules, insulin and angiotensin II, will be addressed.

TNF-α is expressed in adipose tissue of both rodents (Hotamisligil *et al.* 1993) and human subjects (Peraldi & Spiegelman, 1997). Intraperitoneal injection of TNF-α into lean mice caused up regulation and cellular expression of PAI-1 comparable with that observed in obese mice (Samad & Loskutoff, 1996; Samad *et al.* 1996). In *ob/ob* mice neutralization of TNF-α or deletion of the two TNF-α receptors (I and II) resulted in significantly decreased plasma PAI-1 antigen ($P < 0.02$) and adipose tissue PAI-1 ($P < 0.0001$; Samad *et al.* 1999). Similarly, treatment of human adipose tissue explants with TNF-α resulted in up regulation of PAI-1 mRNA, an effect that was abolished by antibodies to TNF-α (Cigolini *et al.* 1999). Adipose tissue PAI-1 and TNF-α mRNA levels produced by human adipocytes correlated well (Morange *et al.* 1999), suggesting a role for this cytokine in PAI-1 gene expression. However, a number of reports do not show a connection. Incubation of human adipose tissue with TNF-α failed to up regulate

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PAI-1 mRNA levels (Alessi *et al*. 1997) and no association was observed between PAI-1 antigen and TNF-α (Alessi *et al*. 2000). These results on human tissue are in agreement with those of Lundgren *et al*. (1996), who did not observe an effect of TNF-α in murine 3T3L1 differentiated adipocytes. There is no obvious reason for the discrepancies between studies, which appear not to reflect species differences. It may be that subtle differences in the cells, resulting from different experimental handling, affect their response to TNF-α. Further work is needed to clarify the relationship between TNF-α and PAI-1 synthesis by adipose tissue.

TNF-α is also a potent inducer of TGFβ in murine adipose tissue, and it contributes to the elevated TGFβ expression (Samad *et al*. 1999) that is observed in the adipose tissue of obese mice (Samad *et al*. 1997). Administration of TGFβ to mice resulted in an increase in plasma PAI-1 activity and PAI-1 mRNA expression in adipose tissue (Lundgren *et al*. 1996). Similarly, TGFβ treatment of human adipose tissue resulted in a 3-fold increase in PAI-1 antigen and a 2-fold increase in PAI-1 mRNA (Birgel *et al*. 2000). In agreement with these data, several reports have shown that PAI-1 mRNA is correlated with TGFβ mRNA (Morange *et al*. 1999) and PAI-1 antigen is correlated with TGFβ antigen in human adipose tissue explants (Alessi *et al*. 1997, 2000). Both PAI-1 mRNA and TGFβ mRNA were positively associated with BMI (Alessi *et al*. 2000). The data on TGFβ and PAI-1 in obesity suggest that the elevated plasma concentrations of PAI-1 may be due to the involvement of mediators in the regulation of PAI-1 gene expression, rather than a direct up regulation of adipose PAI-1. It may be that the effect of TNF-α can also be explained by its action on TGFβ and thus on PAI-1 synthesis.

Insulin has been shown to be correlated with PAI-1 in several studies, and indeed relationships between PAI-1 and BMI, triacyglycerol level and blood pressure are regarded as being secondary to this relationship (Juhan-Vague & Alessi, 1993). Efforts to explain this relationship by its up regulation of PAI-1 in cultured cells, including endothelial cells and hepatocytes, have shown inconsistent results (Kooistra *et al*. 1989; Schneider & Sobel, 1991; Anfosso *et al*. 1993). This inconsistency may reflect effects of insulin on both uptake of glucose and gene expression; by using insulin-resistant cells the up regulation is observed (Samad *et al*. 2000). In whole animals the effect is clearer. Administration of exogenous insulin to rabbits (Nordt *et al*. 1995), mice (Samad & Loskutoff, 1997) and human subjects (Carmassi *et al*. 1999) resulted in elevated plasma PAI-1 concentrations. This effect may be a complex one, involving direct and indirect effects, and more than one cell type.

The haemostatic system protease cascades can be affected by the products of other cascades such as the renin–angiotensin system (RAS), which plays an important role in the regulation of vascular and local fibrinolytic balance (Vaughan, 1998). Angiotensinogen is converted to angiotensin I by renin and to the potent vasoactive peptide angiotensin II by angiotensin converting enzyme. Angiotensin II stimulates PAI-1 production in a number of cells including endothelial cells and smooth muscle cells (Feener *et al*. 1995), mesangial cells in the glomerulus (Wilson *et al*. 1997) and in brain astrocytes (Olson *et al*. 1991). In human subjects infusion of physiological concentrations of angiotensin II exerted a rapid dose-dependent increase in plasma PAI-1 release (Ridker *et al*. 1993). The RAS system regulates neointimal PAI-1 expression, and angiotensin converting enzyme inhibitors decreased PAI-1 in the vessel wall *in vivo* (Hamdan *et al*. 1996). In a separate but complementary pathway angiotensin converting enzyme degrades bradykinin, resulting in decreased tissue plasminogen activator production (Brown *et al*. 1997).

Adipose tissue is an important source of angiotensinogen, and these cells can also process it to angiotensin II (Karlsson *et al*. 1998). Angiotensinogen expression in adipocytes is stimulated by a high-fat diet concurrent with enlargement of fat mass associated with insulin resistance (Zorad *et al*. 1995). In obese patients the involvement of angiotensin II as a consequence of increased plasma angiotensinogen secreted from adipose tissue has been proposed in the development of hypertension. The RAS is present both as a circulating hormone and as a local system. Recent evidence shows that a local RAS is present in human adipose tissue and may act as a distinct system from plasma RAS (Schling *et al*. 1999). Adipose tissue also has receptors for angiotensin II that are regulated by age and fat mass (Zorad *et al*. 1995). It is likely that angiotensin II stimulates PAI-1 synthesis in adipocytes, as it does in other cell types.

Locally-produced angiotensin II has been suggested to participate in the control of tissue growth and development. In adipose tissue angiotensin II stimulates the production of prostacyclin, which in turn converts preadipocytes to adipocytes and increases lipid synthesis and storage in adipocytes (Ailhaud, 1999). Thus, angiotensinogen-derived peptides produced in adipose tissue itself may affect adipogenesis and play a role in the pathogenesis of obesity.

An interesting report by Morange *et al*. (2000) investigated the possibility that PAI-1 is not only a marker of obesity, but also contributes to the development and modification of adipose tissue. Using the PAI-1 null mouse they showed that the absence of PAI-1 resulted in faster weight gain in the early weeks of life when animals were fed on a high-fat diet. They suggest that increased expression of PAI-1 in obesity could reflect effects of this protein in adipose development.

Other haemostatic proteins in obesity

Most of the present review has focused on PAI-1, but there are other proteins of the haemostatic system that are products of adipose tissue. Tissue factor, the initiator of the clotting cascade, is up regulated in obese mice (Samad *et al*. 1998). Elevated tissue factor mRNA was found in the adipose tissue of *ob/ob* mice compared with matched lean controls. The level of expression increased with both age and degree of obesity. Preliminary data suggest that this mechanism could also be regulated by TGFβ (Samad *et al*. 1997), which up regulates PAI-1 gene expression. It will be of interest to see if the data on tissue factor mRNA hold also at the protein level. Tissue factor expression in rabbit atheroma was decreased by lowering the lipid content of the diet. This effect was functional, in that it resulted in reduced cellular binding of coagulation factors and consequent fibrin deposition (Aikawa *et al*. 1999).

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Adiponectin, another secreted product of adipose tissue, is an adhesion molecule that affects binding of monocytes to endothelial cells. It is found in plasma at high concentrations and seems to have a protective effect against atherogenesis, being decreased in obesity and in diabetes (Hotta *et al.* 2000).

Conclusion

Recent studies on the involvement of adipose tissue in haemostasis are beginning to dissect potential mechanisms for the long-established correlations between defective fibrinolysis and obesity. Adipose tissue is emerging as a secretory organ, and PAI-1 is among its products. The question of whether this tissue contributes directly to circulating PAI-1 is still not settled. Even if it does not, there is little doubt that its synthesis of cytokines and growth factors, which in turn up regulate PAI-1, contributes to local, and probably to circulating PAI-1 (Fig. 2). Questions remain on the depots that are most important for PAI-1 synthesis and on the subtleties of its regulation. Increasing efforts to study human adipose tissue are extending and corroborating the research in mouse models, which has been so important in opening up this field of study.

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