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DIABETES MELLITUS AND FIBRINOLYSIS

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Stellingen

1. Bij het "syndroom X" dient eveneens een verhoogd plasma-gehalte van plasminogeen activator inhibitor type 1 (PAI-1) gerekend te worden.

2. Tussen de plasmaspiegel van plasminogeen activator inhibitor type 1 (PAI-1) enerzijds en die van insuline anderzijds bestaat geen causaal verband.

3. Diabetische hardlopers zijn geen "doodlopers".

4. In vitro onderzoek rechtvaardigt niet de klinische overweging dat streptokinase alleen bij verse thromboembolische processen werkzaam is.

5. De invulling van het Geneesmiddelen Vergoedingen Systeem (GVS) doet afbreuk aan de taak opgedragen aan het College ter Beoordeling van Geneesmiddelen.

6. Bij van insuline-afhankelijke diabetes mellitus kan pancreastransplantatie de progressie van neuropathie tot stilstand brengen.

7. De onderdrukking van de thyreotropinesecretie is de sleutel tot succes bij de medicamenteuze behandeling van de ziekte van Graves.

8. "Niet-producerende" hypofysetumoren produceren niet wat wij zoeken, maar wat wij niet zoeken.

9. Bij de conservatieve behandeling van cholelithiasis verdient Temoe Lawak een grotere plaats.
(J. Potter van Loon, Utrecht, 1933)

10. Bij de beschrijving van het stadium van HIV-infectie dient bij de categorie CDC IV C1 te worden toegevoegd: gegeneraliseerde Penicillium marneffei infectie.

11. Elke bedlegerige bejaarde patient verdient de aandacht van een fysiotherapeut.

12. Wiskunde is de moeder der wetenschappen. Wiskunde A is haar onecht kind.

13. Het werken als klinisch beoordelaar bij het College ter Beoordeling van Geneesmiddelen ligt op één lijn met het schrijven van een proefschrift.

14. Als terugdringing van het verbruik van benzine, tabak, of alcohol een feit is, is de overheid het eerste slachtoffer.

Leiden, 12 December 1991

B.J. Potter van Loon

DIABETES MELLITUS AND FIBRINOLYSIS

PROEFSCHRIFT

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The investigations described in this thesis were performed in the Departments of Endocrinology and Metabolic Diseases and of Internal Medicine, University Hospital, Leiden, The Netherlands; in the Gaubius Institute TNO, Leiden (head: Prof. Dr. P. Brakman), now part of the TNO Institute for Ageing and Vascular Research located at the Gaubius Laboratorium, Leiden, The Netherlands; and in the Department of Internal Medicine, Westeinde Hospital, The Hague, The Netherlands.

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CHAPTER 1

GENERAL INTRODUCTION AND OUTLINE OF INVESTIGATIONS

INTRODUCTION

Diabetes mellitus is one of the most frequent chronic diseases in industrialized countries: the prevalence is commonly estimated at 2 % of the general population [1]. This means that in the Netherlands over a quarter of a million of people are suffering from diabetes. Although effective treatments are available to prevent ketosis and to reduce plasma glucose levels, many patients with diabetes mellitus will ultimately develop "long-term" diabetic sequelae. These consist of neuropathy, renal disease, retinal disease and early atherosclerotic disease. The renal and retinal complications of diabetes are called "microvascular" complications, addressing the lesions seen in capillaries and small vessels. The manifestations of early atherosclerosis, in turn, are referrred to as "macrovascular" disease. In fact, diabetes mellitus is considered a "major" risk factor for acquiring cardiovascular disease, together with smoking, hypercholesterolaemia and hypertension [2]. The increase in cadiovascular risk is more prominent in women than in men, probably because of the more favourable risk profile of non-diabetic women compared to nondiabetic men [3]. The mechanisms leading to increased cardiovascular risk in diabetics have not yet been elucidated. Indeed, diabetes and hypertension are co-related [4] and abnormal plasma lipid patterns are frequently encountered in diabetics [5]. However, even after correction for these confounding associations the risk for cardiovascular events in diabetes mellitus remains markedly elevated, compared with the nondiabetic population [6-8]: a two- to threefold risk for acute myocardial infarction, a three- to fivefold risk for cerebrovascular events and a five- to ninefold risk for acquiring peripheral arterial disease. Lower limb amputations are fortyfold more common in diabetics than in nondiabetics [9].

Factors contributing to the increased atherogenesis in diabetes mellitus may include increased glycosylation of proteins [10], alterations in lipoprotein composition [5], decreased intracellular myo-inositol [11], endothelial dysfunction [12], altered platelet function [12,13], and abnormalities in haemostasis and/or fibrinolysis [12-15]. Although overall function tests of coagulation (e.g prothrombin time) are normal in

diabetes, increased levels of fibrinogen [16,17], factor VII [18], factor VIII [17,19] and Von Willebrand Factor [18-20] have been demonstrated. In addition, platelet aggregability is commonly elevated in diabetes mellitus with signs of an increased platelet activation and a reduced platelet survival [21]. All of the above-mentioned features promote clotting of blood ("procoagulant state") and therefore, theoretically, may result in early atherosclerosis and occlusion of blood vessels.

The body possesses a defense mechanism to counteract thrombus formation: the fibrinolytic system. Especially in cases of (proposed) enhancement of coagulation activity and thrombocyte activation/aggregation, i.e. in a procoagulant state, such as occurs in diabetes mellitus, a properly functioning fibrinolytic system is essential.

The fibrinolytic system consists of a series of proteins interacting with one another and finally resulting in the conversion of plasminogen to plasmin, which degrades fibrin (see **figure** 1 on page 13). In diabetes mellitus the activity of the fibrinolytic system has been reported enhanced [19,22-24], reduced [16,18,25-29], and normal [30-35]. Explanations for these inconsistencies include the type of diabetes studied (insulin dependent [Type I] versus non-insulin dependent [Type II]) diabetes mellitus), the degree of long-term vascular diabetic complications, the presence or absence of concurrent disease, the existence of differences in the degree of glycaemic control and the use of different kinds of assays for estimating fibrinolytic activity or components of the fibrinolytic system. However, a large body of evidence favours a reduced plasma fibrinolytic activity (a "hypofibrinolytic state") in non-insulin dependent diabetes mellitus (NIDDM) patients, secondary to high levels of the fibrinolysis inhibitor plasminogen activator inhibitor type 1 (PAI-1)[25,34-37]. In insulin-dependent diabetes mellitus (IDDM) patients the situation is more complicated.

Studies on fibrinolysis in diabetics have usually included both IDDM and NIDDM. Of the recently published papers addressing either IDDM or NIDDM, some have stratified patient groups only according to the presence of retinopathy, whereas others have stratified only according to the degree of urinary albumin excretion, which is an early marker of renal damage. However, no study has addressed a homogeneous group of diabetic patients without long-term micro- or macrovascular sequelae. This precludes the relationship between diabetes mellitus and fibrinolysis, i.e. one cannot distinguish whether the abnormalities found are associated with diabetes or whether they are the result of vascular sequelae often complicating diabetes. Furthermore, in the past scanty attention has been paid to the impact of glycaemic control on fibrinolysis. We therefore studied this issue in IDDM (**Chapter 2**) and compared IDDM without significant micro-or macrovascular complications with nondiabetic subjects (**Chapter 3**).

With regard to NIDDM, we have focussed on PAI-1. In epidemiological studies, a close correlation has been demonstrated between PAI-1 and fasting plasma insulin levels in various groups of patients [25,36,38-41] and it has been suggested that insulin, by stimulating hepatocytic PAI-1 synthesis [42], increases PAI-1. The resulting "hypofibrinolytic" state has been suggested as a (partial) explanation for the early occurrence of atherosclerotic lesions in diabetes mellitus and the increased cardiovascular morbidity and mortality in insulin-resistant subjects in general [43]. The relationship between insulin resistance and PAI-1, however, has not directly been studied in man. We studied both PAI-1 and insulin levels and, in addition, measured insulin action in order to directly elucidate the relationships between the three (**Chapters 4 & 5**).

Finally, we have to realize that the regulation of fibrinolysis at the relevant site of action in vivo, the fibrin surface or thrombus, may differ from that found in plasma. On this issue, to our knowledge, no studies have been presented in diabetics. Even without respect to diabetes mellitus there are no quantitative data on composition and lysibility of human thrombi. To address this issue, we studied randomly selected human thrombi ex vivo. A few thrombi were from diabetic subjects whereas the majority was derived from nondiabetic subjects. The composition, histological age and lysibility of the thrombi were studied and reported in **Chapters 6 & 7**. A summary of the data presented in this thesis is given in **Chapter 8**, in which the data obtained are placed into perspective. A more detailed discussion on what is known of the fibrinolytic system in diabetes mellitus will be given below and finally (page 20) we will discuss more in detail the aim of the present studies and the rationale of the chosen study designs.

THE FIBRINOLYTIC SYSTEM

A scheme of the fibrinolytic system is depicted in **Figure 1**. The final pathway leading to fibrinolysis is the conversion of plasminogen to plasmin, which digests fibrin. The lack of specificity of plasmin for its substrate is compensated for in the circulation by rapid binding to α2-antiplasmin of free plasmin [44]. The plasmin - α2-antiplasmin complexes thus formed are inactive, rendering the formed plasmin harmless to circulating plasma components. On fibrin surfaces plasmin is in closer contact with its substrate and although some inactivation by α2-antiplasmin occurs at this site, activation of fibrin-associated plasminogen results in rapid fibrinolysis [45].

Apart from the inactivation by α2-antiplasmin, plasmin activity can be regulated at

two other levels. First, binding of plasminogen to histidine rich glycoprotein (HRG) [46] reduces the amount of free plasminogen available for binding to fibrin. Second, and probably the most important under physiological circumstances, is the variation of plasminogen activator activity.

Plasminogen activators

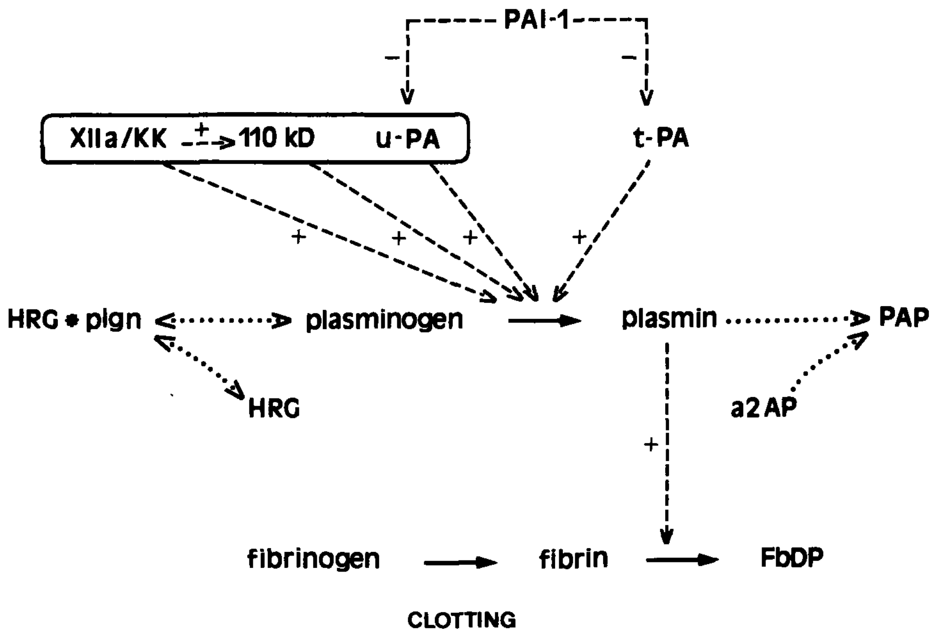
A variety of plasminogen activators are capable of catalysing the conversion of plasminogen to plasmin. Generally, a distinction is made between the intrinsic and extrinsic routes of plasminogen activation. The intrinsic route consists of three pathways. First, a pathway which is dependent on activation of coagulation factor XII and kallikrein (XIIa/KK) [47]: the contact-activation pathway. Second, a contact-activation dependent pathway involving a recently described 110 kD polypeptide [48]. The third intrinsic pathway is by means of urokinase-type plasminogen activator (u-PA), a 50 kD glycoprotein serine protease [49]. The direct contribution of contact-activation pathway to overall plasma fibrinolytic activity in vivo is probably of limited importance [47]. In contrast, u-PA is responsible for about 40 % of intrinsic plasma fibrinolytic activity. Furthermore, administration of exogenous u-PA can result in rapid lysis of reperfusion of occluded vessels [50]. u-PA exists in a single-chain (scu-PA or pro-urokinase) and in a two-chain form (tcu-PA). scu-PA is a zymogen and has only little activity. It is converted to the active form (tcu-PA) by plasmin. The site of synthesis of u-PA is unknown at present: endothelial as well as hepatic synthesis have been suggested. Originally it was thought that the kidney was the site of synthesis, but u-PA activity was found in normal amounts in anephric patients [51]. u-PA is cleared from the circulation with a half-life of only 7 minutes, presumably in the liver [52].

The extrinsic plasminogen activating system consists of tissue-type plasminogen activator (t-PA), formerly described as extrinsic plasminogen activator (EPA). t-PA is a 70 kD glycoprotein serine protease. It is released in parallel with Von Willebrand Factor (vWF) from endothelial stores, where it is probably synthesised as well [53]. t-PA specifically binds to fibrin (in contrast to other plasminogen activators) and is 100 times more potent on a fibrin surface than in solution [54]. Therefore t-PA is considered "fibrin-specific". In the circulation t-PA is rapidly bound to PAI-1, which results in inactivation. Therefore t-PA antigen in plasma does not necessarily parallel t-PA activity (vide infra).

Besides the naturally occurring intrinsic and extrinsic plasminogen activators, plasminogen can be converted to plasmin by a number of exogenously applied

proteins including streptokinase (which forms a complex with plasminogen), urokinase, recombinant t-PA (rt-PA) (both one-chain and two-chain forms), recombinant single-chain (rscu-PA) and two-chain u-PA (rtcu-PA) and anisolyated plasminogen streptokinase complex (APSAC). Each of these proteins has been used in the treatment of vasoocclusive disease (mainly acute myocardial infarction) in the present years [50,55].

Figure 1 A simplified scheme of the fibrinolytic system



XIIa/KK and 110 kD denote contact-activation and 110 kD polypeptide-dependent intrinsic pathway activation of plasminogen repectively. α2AP, PAP and FbDP represent α2-antiplasmin, plasmin - α2-antiplasmin complex and fibrin degradation products respectively. The other abbreviations used are clarified in the text. A broken line represents a stimulating (+) or an inhibitory (-) action, whereas a solid line denotes a conversion. A dotted line represents binding of two proteins. A circle includes the components of the intrinsic pathway of fibrinolysis.

Table 1 Characteristics of the major proteins involved in fibrinolysis

	type of protein	M_r	site of synthesis	T½	eli	concen-tration	function
Plgn	1cG SP	90	Hep			1.5 μM	digests fibrin
t-PA	1/2cG SP	70	EC?	2-5'	Hep	5-300 pM	activates plgn
u-PA	1/2cG SP	50	Hep?	7'	Hep?	100 pM	activates plgn
PAI-1	1cG PI	50	EC,SMC Hep, Tr*	10'	Hep	500 pM	inactivates t-PA and u-PA
HRG	1cG	80	Hep	3 d		1.8 μM	binds plgn
α2-AP	1cG	67	Hep	2.6 d		1.5 nM	inactivates plasmin

plgn denotes plasminogen and α2-AP represents α2-antiplasmin. The abbreviations t-PA, u-PA, PAI-1 and HRG are clarified in the text.
M_r denotes molecular weight (kD), T½ represents plasma half-life, and eli represents elimination site.
1(/2)cG = 1(/2) chain glycoprotein; SP = serine protease; PI = protease inhibitor.
EC = endothelial cell; Hep = liver; SMC = smooth muscle cell;
Tr* = present in large amounts in platelets but synthesised elsewhere.

Plasminogen activator inhibitors

Among the proteins that inhibit fibrinolysis there at least are three types of plasminogen activator inhibitors in plasma: PAI type 1 (PAI-1), PAI type 2 (PAI-2) and PAI type 3 (PAI-3) [56,57]. All are single chain glycoproteins of ± 50 kD. PAI-1 (plasma concentration approximately 10 ng/ml) is the most important biologically in fibrinolysis, since this is the major binding protein of both t-PA and u-PA [58]. PAI-1 is produced in liver and in endothelium [53]. PAI-1 is present in large amounts in platelets accounting for 80 % of the total pool [56] in blood. The amount in platelets is fairly constant [59] and independent of the plasma PAI-1 pool, which can show great variations. Stimuli for secretion of PAI-1 in vitro are insulin [42,60], endotoxin, interleukin-1 (IL-1) and tumor necrosis factor (TNF) [53], while in vivo only the latter and endotoxin [61,62] have elicited substantial increments of plasma levels. PAI-2, the placental type, is only found in blood during pregnancy. PAI-3 is the most abundant inhibitor (plasma concentration approximately 2 μg/ml). However, as the affinity for plasminogen activators is several orders of magnitude less than that of PAI-1, the

biological importance of PAI-3 is rather limited in this respect [57]. The major function of PAI-3 is inactivation of (activated) protein C [57]. Protein C is a vitamin K dependent protein, which, when activated, inhibits clotting factors V and VIII and enhances fibrinolysis, presumably by inactivating PAI-1 [63].

Plasminogen activator activity in plasma is dependent on the concentration of free plasminogen activator [64]. As plasminogen activators are rapidly bound to inhibitors in plasma, the amount of "free" plasminogen activator normally is limited in unstimulated conditions. It is only after the excess of inhibitors is overcome that the amount of free plasminogen activator in plasma rises substantially. Therefore, one can state that the level of inhibitors normally determines plasminogen activator activity and therefore is the major determinant of plasma fibrinolytic activity. In this respect it should be realized that t-PA activity does not necessarily parallel the t-PA antigen level: whereas t-PA activity represents free t-PA, t-PA antigen includes both free t-PA and t-PA bound to PAI-1: generally, the higher the concentration of PAI-1 in plasma, the more t-PA is bound and the less active t-PA is left.

Physiology

Basal

In the unstimulated condition excess PAI-1 is present relative to the amounts of t-PA and u-PA, resulting in low levels of free plasminogen activators and therefore low plasminogen activator activity. The factor XII/kallikrein dependent intrinsic pathway of plasminogen activation is thought to be of limited importance in vivo and will further be excluded from the discussion. Likewise,binding of plasminogen to HRG and the binding of plasmin to α2-antiplasmin in the circulation are not considered to be rate-limiting (for plasma fibrinolytic activity) under basal conditions.

Stimulated

The classical stimuli of plasma fibrinolytic activity are venous occlusion, physical exercise and infusion of DDAVP. All three stimuli result in increased levels of t-PA. In addition, exercise and DDAVP result in increased u-PA levels [65,66]. Several mechanisms have been proposed by which these stimuli result in enhancement of plasminogen activator levels: endothelial release [46] either by a direct effect or

following activation of adrenergic nerve endings [67], diminished total body clearance of t-PA [68] and, following venous occlusion, diminished clearance within a compartment of the body resulting in elevated local levels of t-PA [69]. Different stimuli may act synergistically, suggesting different mechanisms of action [70]. In addition to the above-mentioned stimuli, thrombin, bradykinin and activated protein C all can induce t-PA release [71]. Recently the injection of endotoxin as well as the injection of tumor necrosis factor (TNF) in healthy volunteers has been shown to transiently stimulate fibrinolysis by inducing rises in t-PA and u-PA [61,62,72]. The transient rise, however, was followed by a rise in PAI-1 resulting in a profound inhibition of fibrinolysis.

Prolonged stimulation

Of the stimuli for plasminogen activator release, physical exercise is probably the one most operational in everyday life. Moreover, exercise a physiological stimulus and can be "applied" for a longer period. Following prolonged exercise, i.e. marathon running [73,74] and triathlon [75], fibrinolytic activity and t-PA are elevated. Repeated exercise [76], like repeated venous occlusions [77], results in a dampened fibrinolytic response. To explain this phenomenon, it has been suggested that endothelial stores become depleted of t-PA [77]. Ex vivo, however, t-PA can be released maximally from endothelial cells for several hours without signs of depletion, suggesting release from an endothelial storage pool [78]. However, this issue has never been addressed directly in humans.

Links with the coagulation cascade

Surprisingly, activation of clotting factor XII results in both clotting (by further activating the intrinsic coagulation cascade) and activation of the intrinsic pathway of fibrinolysis (see above). Besides this common pathway several other links exist between the coagulation and fibrinolytic systems. Amongst others, thrombin enhances release of t-PA by endothelium (see above) and inactivates scu-PA [79], protein C inactivates coagulation factors V and VIII and stimulates fibrinolysis [80], thrombin decreases fibrinolysis [63] and PAI-3 inactivates protein C [57].
It is difficult at present to determine the clinical relevance of each of these links.

Local fibrinolysis

Extrapolation of data from in vitro studies with fibrin clots suggests that fibrinolysis at the site of a thrombus or fibrin clot follows different kinetics from fibrinolysis in a buffer or plasma milieu. As stated earlier, catalytic activity of t-PA is increased hundredfold at the fibrin surface. Second, the plasmin formed is less susceptible to inactivation by α2-antiplasmin, as it is in close approximation with fibrin. The partial degradation of fibrin further enhances binding of plasminogen and t-PA to the clot [81]. Third, the relatively high concentrations of active plasminogen activators and the presence of active plasmin facilitate synergism between u-PA and t-PA, as has been demonstrated in vitro [82] and in vivo [83].
Finally, the cellular components of a thrombus or blood clot may play a significant role in local fibrinolysis, either by release of non-plasmin proteases from leucocytes [84] or PAI-1 from platelets [56], or by entrapping plasminogen and its activators in the thrombus by binding of these proteins to specific receptors on the cell surface [85].
It should be recognized, however, that most of our knowledge on fibrinolysis is derived from studies with purified systems in vitro. Studies on thrombi are sparse and on in vivo formed human thrombi are rare.
In conclusion, our knowledge about the regulation of fibrinolysis at its target site is still limited at present.

THE FIBRINOLYTIC SYSTEM IN DIABETES MELLITUS

In the past, many studies have addressed fibrinolysis in diabetes mellitus. Conflicting results have emerged as to whether fibrinolysis is "enhanced", "normal", or "depressed" in patients with "diabetes". The different biological meanings of performed tests to evaluate fibrinolysis, the lack of differentiation of type of diabetes (IDDM or NIDD-M), the lack of referral to the degree of metabolic control, and the pooling of data on patients with and without macro- and microvascular complications all may have contributed to the diverging findings.
In the following paragraphs a summary will be given of what is known of fibrinolysis in diabetics, with respect to the above-mentioned aspects.

Insulin dependent diabetes mellitus

Basal plasma fibrinolytic activity in IDDM, studied by means of euglobulin lysis times or areas, has been reported increased [19,22] or similar to that of healthy control subjects [25,28,31]. One study [16] has reported decreased basal fibrinolytic activity in IDDM only in females. More specific measurements of components of the fibrinolytic system have been performed in studies in recent years, mainly including PAI-1 (both antigen and activity) and t-PA (antigen and activity).

PAI-1 levels in IDDM are normal or depressed [31,86-88], although diabetes duration, metabolic control, and the presence diabetic nephropathy may influence the results [31,34,87]. Auwerx et al. [25] have reported increased PAI-1 in IDDM (means 1.8 vs 1.5 ng/ml).

Basal t-PA antigen levels in IDDM have been reported depressed [87,88], increased [25] or normal [31,34], although differences can be seen between groups with and without microvascular complications [87,88].

Stimulated values of t-PA antigen [28,34,87] and t-PA activity [28,87] are often reduced in IDDM and this is believed to reflect endothelial damage occurring in diabetes mellitus [87]. However, some categories of patients have a normal response of t-PA (both antigen and activity determinations) to stimulation [87,89]

At present, no data on u-PA in IDDM have been reported yet.

In summary, most of the evidence favours normal basal PAI-1 and t-PA levels in IDDM and a normal plasma fibrinolytic activity. The t-PA release in response to stimuli may be reduced, especially in patients with microvascular complications.

Non-insulin dependent diabetes mellitus

In 1979 a large epidemiological survey [16] first drew attention to the differences between IDDM and NIDDM regarding plasma fibrinolytic activity: the lysis times of euglobulin precipitates of plasma were markedly prolonged in NIDDM compared with healthy controls, indicating a reduced plasma fibrinolytic activity in the NIDDM subjects. This was not the case in IDDM. Subsequently the finding of impaired overall plasma fibrinolytic activity in NIDDM has been confirmed by several authors [25,26] in Caucasians, although a recent report in Chinese patients with NIDDM found normal overall fibrinolysis in NIDDM [35]. A possible explanation may be offered by differences between the western and the chinese diet [90], but racial differences

cannot be excluded.

The impairment of basal plasma fibrinolytic activity in NIDDM parallels an increase of PAI-1 activity [25,34]. Elevated levels of PAI-1 activity have been found in most studies in NIDDM [25,34,36,37]. PAI-1 was normal in the above-mentioned chinese NIDDM study [35]. Rydzewski et al. [91] found that PAI-1 antigen levels in NIDDM differed with the severity of diabetic retinopathy. Thus, the absence or presence of diabetic vascular complications may influence the results. One should bear in mind, however, that the "normal range" of PAI-1 is extremely wide (both activity [25,37,71] and antigen [56]) and despite the increased mean values in NIDDM, a substantial number of diabetics (70 %) have PAI-1 activity levels within the normal range [37,92].

Lately, NIDDM has been identified within syndrome X [93], which includes impaired glucose tolerance, hyperinsulinaemia, elevated levels of VLDL triglycerides, decreased levels of HDL-cholesterol, hypertension and abdominal type of obesity. The common denominator of this syndrome is resistance to the action(s) of insulin [93]. Each of the features of syndrome X has been associated with high levels of PAI-1 activity [25,36,38-41,94-97]. The elevated PAI-1 levels in NIDDM are believed to be secondary to the accompanying hyperinsulinaemia [98], which is present in the majority of NIDDM [99].

Basal t-PA antigen levels in NIDDM are either elevated [25,35,100] or normal [34,91].

Stimulated t-PA antigen levels in NIDDM are normal [34,89,100] or may be elevated compared with healthy controls [35].

u-PA has only been studied in NIDDM by Rydzewski et al [91]. These authors suggest that u-PA production may be impaired in NIDDM.

In summary, most of the evidence favors elevated PAI-1 in NIDDM with resultant depression of t-PA activity and plasma fibrinolytic activity, whether or not the t-PA antigen level is normal. Most of the evidence indicates that in NIDDM t-PA release in response to stimuli is normal. However, in the case of elevated levels of PAI-1 a normal rise in t-PA may not be sufficient to "neutralize" the abundant PAI-1 and the resultant may be an impaired fibrinolytic response to stimulation [101].

Glycosylation and metabolic control

Despite the differences in IDDM and NIDDM regarding fibrinolysis, the two conditions have in common the increased glycosylation of proteins as a result of hyperglycaemia.

Fibrinogen is glycosylated to a greater degree in diabetic subjects than in healthy controls [102]. The fibrin formed from this fibrinogen has an increased susceptibility to fibrinolysis. This is possibly due to impaired α-crosslinking of fibrin strands during clotting in diabetes [103]. Following nonenzymatic glycosylation of fibrinogen in vitro, other authors have found resistance of fibrin to plasmin-mediated lysis [104,105].

In poorly controlled diabetic patients plasminogen activation by t-PA was impaired [106]. Following improvemant of glycaemic control in these patients the defect disappeared. It was suggested that the impaired plasminogen activation by t-PA in these patients could be attributed to enhanced nonenzymatic glycosylation of plasminogen [106]. However, the degree of glycosylation of plasminogen was not measured in that study.

Several studies have addressed the relationship between the degree of metabolic control (glycosylated hemoglobin or fasting blood glucose levels) on one hand and overall fibrinolysis or individual components of the fibrinolytic system on the other [25,27,37,87,89,92,100,107]. Van Wersch et al. [91] have claimed a relationship between PAI-1 and glycosylated hemoglobin, but in fact their data do not support this view. Nilsson et al. [107] found a positive correlation between glycosylate hemoglogin and t-PA activity following venous occlusion, suggesting enhancement of fibrinolysis with poor metabolic control.

AIM OF THE PRESENT STUDIES

The impact of metabolic control on fibrinolysis is an important issue, which has only been studied in cross-sectional surveys [25,27,37,87,89,92,100,107] or during intervention studies of short duration [17,31]. One study has been published on patients with NIDDM in poor metabolic control, who were switched to insulin [108]. In this study three months of insulin failed to significantly reduce glycosylated hemoglobin, but plasma fibrinolysis appeared to be increased following the insulin treatment. We studied both cross-sectionally and longitudinally the effect of metabolic control on the principal determinants of plasma fibrinolytic activity: PAI-1 and t-PA

(basal and following stimulation) fibrinolytic system. This study is presented in **Chapter 2.**

The studies in IDDM have commonly included patients with microvascular complications (i.e. retinopathy more than background and/or microalbuminuria) or have not adequately addressed the degree of microvascular complications. This precludes the elucidation of the mechanisms of altered fibrinolysis in diabetics, as the change in fibrinolysis may either precede (and possibly cause) vascular complications or, alternatively, may result from the vascular complications. Only one study has been performed in IDDM without microvascular complications [87]. In this study plasminogen activator release was lower than that of healthy controls, but the difference did not appear significant. However, the data are suggestive of a distinct impairment of plasminogen activator release in IDDM without microvascular complications. To maximize the chance of demonstrating an impairment of plasminogen activator release in IDDM without vascular complications, we stimulated fibrinolysis continuously for a prolonged period speculating that prolonged stimulation of the endothelium would ultimately show differences between IDDM and healthy controls. This study is presented in **Chapter 3.**

The decreased fibrinolysis in NIDDM appears to be predominantly the result of elevated PAI-1 levels. Hyperinsulinaemia has been suggested as the cause of elevated PAI-1 levels in NIDDM [99] and indeed in vitro insulin stimulates PAI-1 synthesis. However, in man exogenous hyperinsulinaemia of short duration does not elevate PAI-1 levels [109]. Furthermore, the elevation of PAI-1 in other insulin-resistant conditions suggests that insulin resistance rather than insulin level may predominate in the association with elevated PAI-1 levels. To examine whether PAI-1 is directly related to insulin action we measured insulin action (by means of sequential hyperinsulinaemic euglycaemic glucose clamp studies) in addition to insulin levels and PAI-1 in both obese nondiabetics and obese NIDDM. The results of this study are presented in **Chapter 4.**

The use of sequential hyperinsulinaemic euglycaemic glucose clamp studies gave us the opportunity to follow PAI-1 levels during prolonged (severe) exogenous hyperinsulinaemia. These results are presented in **Chapter 5.**

Despite the importance of the determination of plasma fibrinolytic activity of the plasma concentrations of t-PA and PAI-1, one should keep in mind that the target site of fibrinolysis is the fibrin in a thrombus or clot. Cellular elements and altered

flow characteristics may profoundly influence fibrinolysis kinetics at the fibrin surface. In vitro studies have suggested a regulatory function for local t-PA and plasminogen (see above). However, it has recently been recognised that platelets, which are a major constituent of thrombi and clots, are the most prominent reservoir of PAI-1 in blood [56]. The role of PAI-1 in thrombi or clots is still unclear. We determined the composition of human thrombi, i.e. the amounts of PAI-1, t-PA and plasminogen in the thrombi and related these amounts to the lysibilty of the same thrombi. This is described in **Chapter 6**.

Furthermore, in **Chapter 7** we histologically determined the thrombus age (of the same thrombi mentioned in Chapter 6) and related ageing of thrombi to composition and lysibility.

REFERENCES

1. Harris M.I., Hadden W.C., Knowler W.C., et al: Prevalence of diabetes and impaired glucose tolerance and plasma glucose levels in US population ages 20 - 74 years. Diabetes 1987; 36: 523-534
2. Kannel W.B., Schatzkin W.B.: Risk factor analysis. Progress in Cardiovascular Diseases 1983; 26: 309-332
3. Barrett-Connor E.L., Cohn B.A., Wingard D.L., Edelstein S.L.: Why is diabetes mellitus a stronger risk factor for fatal ischaemic heart disease in women than in men ? The Rancho Bernardo Study. JAMA 1991; 265: 627-631
4. Ferrannini E., DeFronzo R.A.: The association of hypertension,diabetes and obesity: a review. J Nephrol 1989; 1: 3-15
5. Kostner G.M., Karadi I.: Lipoprotein alterations in diabetes mellitus. Diabetologia 1988; 31: 717-722
6. Kannel W.B., McGee D.L.: Diabetes and cardiovascular disease. The Framingham Study. JAMA 1979; 241: 2035-2038
7. Uusitupa M.I.J., Niskanen L.K., Siitonen O., Voutilainen E., Pyörälä K.: 5-year incidence of atherosclerotic vascular disease in relation to general risk factors, insulin level, and abnormalities in lipoprotein composition in non-insulin-dependent diabetic and nondiabetic subjects. Circulation 1990; 82: 27-36
8. Consensus statement: Role of cardiovascular risk factors in prevention and treatment of macrovascular disease in diabetes. Diabetes Care 1989; 12: 573-579
9. Marble A., Krall L.P., Bradley R.F., Christlieb A.R., Soeldner J.S. (Eds.) : Joslin's Diabetes Mellitus, 12 Ed. Philadelphia, Lea & Febiger, 1985
10. Steiner G.: Diabetes and atherosclerosis. Metabolic links. Drugs 1988; 36 (Supp 3): 22-26
11. Van Gerven J.M.A.: Aldose reductase inhibitor treatment in diabetic complications. Thesis, Leiden, 1990. p.36-37
12. Colwell J.A., Winocour W.D., Lopes-Virella M.F., Halushka B.V.: New concepts about the pathogenesis of atherosclerosis in diabetes mellitus. Am J Med 1983; 75 (Supp 5B): 67-80

13. Ganda M.P.: Pathogenesis of macrovascular disease in the human diabetic. Diabetes 1980; 29: 931-942
14. Colwell J.A., Lopes-Virella M.F.: A review of the development of large-vessel disease in diabetes mellitus. Am J Med 1988; 85 (Supp 5A): 113-118
15. Juhan-Vague I., Collen D.: Hypofibrinolysis and atherothrombosis. Diabet Metab 1988; 14: 479-483
16. Fuller J.H., Keen H., Jarrett R.J., Omer T., Meade T.W., Chakrabarti R., North W.S., Stirling Y.: Haemostatic variables associated with diabetes and its complications. Br Med J 1979: ii: 964-966
17. Borsey D.Q., Prowse C.V., Gray R.S., Dawes J., James K., Elton R.A., Clarke B.F.: Platelet and coagulation factors in proliferative diabetic retinopathy. J Clin Pathol 1984; 37: 659-664
18. Christe M., Fritschi J., Lämmle B., Tran T.H., Marbet G.A., Berger W., Duckert F.: Fifteen coagulation and fibrinolysis parameters in diabetes mellitus and in patients with vasculopathy. Thromb Haemostas 1984; 52: 138-143
19. Bern M.M., Cassani M.P., Horton J., Rand L., Davis G.: Changes of fibrinolysis and factor VIII coagulant, antigen, and ristocetin cofactor in diabetes mellitus and atherosclerosis. Thromb Res 1980; 19: 831-839
20. Jensen T.: Increased plasma concentration of von Willebrand factor in insulin dependent diabetics with incipient nephropathy. Br Med J 1989; 298: 27-28
21. Ostermann H., van de Loo J.: Factors of the hemostatic system in diabetic patients. A survey of controlled studies. Haemostasis 1986; 16: 386-416
22. Sharma S.C.: Platelet adhesiveness, plasma fibrinogen, and fibrinolytic activity in juvenile-onset and maturity-onset diabetes mellitus. J Clin Pathol 1981; 34: 501-503
23. Elder G.E., Mayne E.E., Daly J.G., Kenndy A.L., Hadden D.R., Montgomery D.A.D., Weaver J.A.: Antithrombin III activity and other coagulation changes in proliferative diabetic retinopathy. Haemostasis 1980; 9: 288-296
24. Grignani G., Gamba G., Geroldi D., Pacchiarini L., Solerte B., Ferrari E., Ascari E.: Enhanced antithrombotic mechanism in patients with maturity-onset diabetes mellitus without thromboembolic complications. Thromb Haemostas 1981; 46: 648-651
25. Auwerx J., Bouillon R., Collen D., Geboers J.: Tissue-type plasminogen activator antigen and plasminogen activator inhibitor in diabetes mellitus. Arteriosclerosis 1988; 8: 68-72
26. Schneider S.H., Kim H.C., Khachadurian A.K., Ruderman N.B.: Impaired fibrinolytic response to exercise in type II diabetes: effects of exercise and physical training. Metabolism 1988; 37: 924-929
27. Haitas B., Barnes A.J., Cederholm-Williams S.A., Moore J., Shogry M.E.C., Turner R.C.: Abnormal endothelial release of fibrinolytic activity and fibronectin in diabetic microangiopathy. Diabetologia 1984; 27: 493-496
28. Cederholm-Williams S.A., Dornan T.L., Turner R.C.: The metabolism of fibrinogen and plasminogen related to diabetic retinopathy in man. Eur J Clin Invest 1981; 11: 133-138
29. Almèr L.O., Pandolfi M.: Fibrinolysis and diabetic retinopathy. Diabetes 1976; 25 (Supp 2): 807-810
30. Dornan T.L., Rhymes I.L., Cederholm-Williams S.A., Rizza C.R., Pepys M.B., Bron A.J., Turner R.C.: Plasma haemostatic factors and diabetic retinopathy. Eur J Clin Invest 1983; 13: 231-235
31. Juhan-Vague I., Vague P., Poisson C., Aillaud M.F., Mendez C., Collen D.: Effect of 24 hours of normoglycaemia on tissue-type plasminogen activator plasma levels in insulin-dependent diabetes. Thromb Haemostas 1984; 51: 97-98
32. Van der Knaap J.H., de Boer A.C., Pannebakker M.A.G., van Weerde W., den Ottolander G.J.H.: The effect of diabetes regulation on platelet release, fibrinolysis and coagulation tests, before and after stimulation with DDAVP. Thromb Haemostas 1985; 53: 118-121
33. Juhan-Vague I., Rijken D.C., De Cock F., Mendez C., Collen D.: Extrinsic plasminogen activator levels in clinical plasma samples. In: J.F. Davidson, F. Bachmann, C.A. Bouvier, E.K.O. Kruithof, eds. Progress in fibrinolysis vol. VI. Churchill Livingstone, Edinburgh, 1983. pp 65-69

34. Almèr L.O.: Fibrinolytic disorders in diabetes mellitus. Diabet Metab 1988; 14: 519-522
35. Ho C.H., Jap T.S.: Fibrinolytic activity in chinese patients with diabetes or hyperlipidaemia in comparison with healthy controls. Thromb Haemostas 1991; 65: 3-6
36. Juhan-Vague I., Roul C., Alessi M.C., Ardissone J.P., Heim M., Vague P.: Increased plasminogen activator inhibitor activity in non insulin dependent diabetic patients - relationship with plasma insulin. Thromb Haemostas 1989; 61: 370-373
37. Small M., Kluft C., MacCuish A.C., Lowe G.D.O.: Tissue plasminogen activator inhibition in diabetes mellitus. Diabetes Care 1989; 12: 655-658
38. Vague P., Juhan-Vague I., Aillaud M.F., Badier C., Viard R., Alessi M.C., Collen D.: Correlation between blood fibrinolytic activity, plasminogen activator inhibitor level, plasma insulin level, and relative body weight in normal and obese subjects. Metabolism 1986; 35: 250-253.
39. Landin K., Stigendahl L., Eriksson E., Krotkiewski M., Risberg B., Tengborn L., Smith U.: Abdominal obesity is associated with an impaired fibrinolytic activity and elevated plasminogen activator inhibitor-1. Metabolism 1990; 39: 1044-1048.
40. Van Gaal L., Vansant G., Van der Plancken M., De Leeuw I.: fat distribution and plasminogen activator inhibitor activity in obese and non-obese type 2 diabetics. Diabetologia 1990; 33: A 52
41. Juhan-Vague I., Vague P., Alessi M.C., Badier C., Valadier J., Aillaud M.F., Atlan C.: Relationship between plasma insulin, triglyceride, body mass index and plasminogen activator inhibitor 1. Diab Metab 1987; 13: 331-336
42. Alessi M.C., Juhan-Vague I., Kooistra T., Declerck P.J., Collen D.: Insulin stimulates the synthesis of plasminogen activator inhibitor 1 by the human hepatocellular cell line Hep G2. Thromb Haemostas 1988; 60: 491-494
43. Juhan-Vague I., Vague P.: Increased plasma plasminogen activator 1 levels. A possible link between insulin resistance and atherothrombosis. Diabetologia 1991; 34: 457-462
44. Aoki N., Moroi M., Matsuda M., Tachiya K.: The behaviour of α2-plasmin inhibitor in fibrinolytic states. J Clin Invest 1977; 60: 361-369
45. Wiman B., Collen D.: Molecular mechanism of physiological fibrinolysis. Nature 1978; 272: 549-550
46. Emeis J.J., Brommer E.J.P., Kluft C., Brakman P.: Progress in fibrinolysis. In: Recent advances in blood coagulation, vol 4. L. Poller, ed. Churchill Livingstone, Edinburgh, 1985
47. Kluft C., Dooijewaard G., Emeis J.J.: Role of the contact system in fibrinolysis. Semin Thromb Haemostas 1987; 13: 50-68
48. Binnema D.J., Dooijewaard G., van Iersel J.J.L., Turion P.N.C., Kluft C.: The contact-system dependent plasminogen activator from human plasma: identification and characterisation. Thromb Haemostas 1990; 64: 390-397
49. Binnema D.J., Dooijewaard G., de Jong Y.F.: Characterisation of type I-urokinase related plasminogen activator activities in activated plasma. Thromb Haemostas 1985; 54: 103
50. Diefenbach C., Erbel R., Pop T., Mathey D., Schofer J., Hamm C., Ostermann H., Schmitz-Hübner U., Bleifeld W., Meyer J.: Recombinant single-chain urokinase-type plasminogen activator during acute myocardial infarction. Am J Cardiol 1988; 61: 966-970
51. Brommer E.J.P., Schicht I., Verheijen J., Rijken D.C.: Fibrinolytic activators and inhibitors in terminal renal insufficiency and in anephric patients. Thromb Haemostas 1984; 52: 311-314
52. Bachmann F.: the role of the human liver in the regulation of thombolytic activity. J Lab Clin Med 1966; 68: 854-855
53. Kooistra T.: The use of cultured human endothelial cells and hepatocytes as an in vitro model system to study modulation of endogenous fibrinolysis. Fibrinolysis 1990; 4 (Supp.2): 33-39
54. Hoylaerts M., Rijken D.C., Lijnen H.R., Collen D.: Kinetics of the activation of plasminogen by human tissue-type plasminogen activator. Role of fibrin. J Biol Chem 1982; 257: 2912-2919

55. Sherry S.: Dose-response and patency in coronary thrombolysis. In: Controversies in coronary thrombolysis. S.Sherry, R. Schröder, C. Kluft, A.J. Six, K.L. Mettinger, eds. Current Medical Literature, London, 1989, pp 1-14
56. Sprengers E.D., Kluft C.: Plasminogen activator inhibitors. Blood 1987; 69: 381-387
57. Geiger M.: Protein C Inhibitor/Plasminogen Activator Inhibitor 3. Fibrinolysis 1988; 2: 183-188
58. Kruithof E.K.O., Tran-Thang C., Bachmann F.: The fast-acting inhibitor of tissue-type plasminogen activator in plasma is also the primary plasma inhibitor of urokinase. Thromb Haemostas 1986; 55: 65-69
59. Simpson A.J., Booth N.A., Moore N.R., Bennett B.: The platelet and plasma pools of plasminogen activator inhibitor (PAI-1) vary independently in disease. Br J Haematol 1990; 75: 543-548
60. Kooistra T., Bosma P.J., Töns H.A.M., van den Berg A.P., Meyer P., Princen H.M.G.: Plasminogen activator inhibitor 1: Biosynthesis and mRNA are increased by insulin in cultured human hepatocytes. Thromb Haemostas 1989; 62: 723-728
61. Van Hinsbergh V.A., Bauer K., Kooistra T., Kluft C., Dooijewaard G., Sherman M.L., Nieuwenhuizen W.: Progress of fibrinolysis during tumor necrosis factor infusions in humans. Concomitant increase in tissue-type plasminogen activator, plasminogen activator inhibitor type 1, and fibrin(ogen) degradation products. Blood 1990; 76: 2284-2289
62. Suffredini A.F., Harpel P.C., Parrillo J.E.: Promotion and inhibition of plasminogen activation after administration of intravenous endotoxin to normal subjects. N Engl J Med 1989; 320: 1165-1172
63. De Fouw N.J.: Activated protein C and fibrinolysis. Thesis, Leiden, 1988
64. Kooistra T., Bosma P.J., Jespersen J., Kluft C.: Studies on the mechanism of oral contraceptives with regard to fibrinolytic variables. Am J Obstet Gynaecol 1990; 163: 404-412
65. Levi M., Ten Cate J.W., Dooijewaard G., Sturk A., Brommer E.J.P., Agnelli G.: DDAVP induces systemic release of urokinase-type plasminogen activator. Thromb Haemostas 1989; 62: 686-689
66. Dooijewaard G., De Boer A., Turion P.N.C., Cohen A.F., Breimer D.D., Kluft C.: Physical exercise induces enhancement of urokinase type plasminogen activator (u-PA) levels in plasma . Thromb Haemostas 1991; 65: 82-86
67. Colwell J.A.: Effects of exercise on platelet function, coagulation, and fibrinolysis. Diab Metab Rev 1986; 1: 501-512
68. De Boer A., Kluft C., Cohen A.F.: Life style and blood fibrinolytic activity: a comment on clearance of plasminogen activators. Fibrinolysis 1990; 4 (Supp 2): 61-63
69. Keber D., Blinc A., Fettich J.: Increase in tissue plasminogen activator in limbs during venous occlusion: A simple haemodynamic model. Thromb Haemostas 1990; 64: 433-437
70. Keber D.: Mechanism of tissue plasminogen activator release during venous occlusion. Fibrinolysis 1988; 2 (Supp 2): 96-103
71. Engesser L.: Thrombophilia. Disorders of blood coagulation and fibrinolysis. Thesis, Leiden, 1988
72. Van der Poll T., Levi M., Büller H.R., van Deventer S.J.H., de Boer J.P., Hack C.E., ten Cate J.W.: Rapid stimulation and subsequent inhibition of the fibrinolytic system after administration of tumor necrosis factor to normal subjects. Comparison with procoagulant effects. Presentation at 1991 Dutch internists Meeting, Veldhoven, NL. (abstract No. 35)
73. Mandalaki T., Dessypris A., Louizou C., Panayotopoulou C., Dimitriadou C.: Marathon Run III: Effects on coagulation, fibrinolysis, platelet aggregation and serum cortisol levels. A 3-year study. Thromb Haemostas 1980; 43:49-52
74. Taenzer M., Lill H., Drygas W.K., Altenkirch H.U., Heyduck B., Röcker L.: The role of endogenous plasminogen activator (t-PA) after marathon running. Int J Sports Med 1988; 9: 370 (abstr)
75. Arai M., Yorifuji H., Ikematsu S., Nagasawa H., Fujimaki M., Fukutake K., Katsumura T., Ishii T., Iwane H.: Influences of strenuous exercise (triathlon) on blood coagulation and fibrinolytic system. Thromb Res 1990; 57: 465-471

76. Rosing D.R., Brakman P., Redwood D.R., Goldstein R.E., Beiser G.D., Astrup T., Epstein S.E.: Blood fibrinolytic activity in man. Diurnal variations and the response to varying intensities of exercise. Circulation Res 1970; 27: 171-184
77. Keber D., Stegnar M.: Exhaustion of arm fibrinolytic potential after repeated venous occlusions and local exercise. Thromb Res 1982; 28: 693-704
78. Tranquille N., Emeis J.J.: Protein synthesis inhibition by cycloheximide does not affect the acute relase of tissue-type plasminogen activator. Thromb Haemostas 1989; 61: 442-447
79. De Munck G.A.W., Rijken D.C: Fibrinolytic properties of single-chain urokinase-type plasminogen activator (pro-urokinase). Fibrinolysis 1990; 4: 1-9
80. Van Hinsbergh V.W.M., Bertina R.M., Van Wijngaarden A., Van Tilburg N.H., Emeis J.J., haverkate F.: Activated protein C decreases plasminogen activator inhibitor activity in endothelial cell-conditioned medium. Blood 1985; 66: 1452-1455
81. Suenson E., Lützen O., Thorsen S.: initial plasmin-degradation of fibrin as the basis of a positive feed-back mechanism in fibrinolysis. Eur J Biochem 1984; 140: 513-522
82. Pannell R., Black J., Gurewich V.: Complementary modes of action of tissue-type plasminogen activator and pro-urokinase by which their synergistic effect on clot lysis may be explained. J Clin Invest 1988; 81: 853-859
83. Collen D., Stassen J.M., De Cock F.: Synergistic effect on thrombolysis of sequential infusion of tissue-type plasminogen activator (t-PA), single-chain urokinase-type plasminogen activator (scu-PA) and urokinase in the rabbit jugular vein thrombosis model. Thromb Haemostas 1987; 58: 943-946
84. Plow E.F., Edgington T.S.: An alternative pathway for fibrinolysis. I. The cleavage of fibrinogen by leucocyte proteases at physiologic pH. J Clin Invest 1975; 56: 30-38 (of: Moroz; Semin Thromb Haemost 1984 10:80)
85. Miles L.A., Plow E.F.: Receptor mediated binding of the fibrinolytic components, plasminogen and urokinase, to peripheral blood cells. Thromb Haemostas 1987; 58: 936-942
86. Takahashi H., Tsuda A., Tatewaki W., Wada K., Niwane H., Shibata A.: Activation of blood coagulation and fibrinolysis in diabetes mellitus; evaluation by plasma levels of thrombin-antithrombin III complex and plasmin-alpha 2-plasmin inhibitor complex. Thromb Res 1989; 55: 727-735
87. Jensen T., Bjerre-Knudsen J., Feldt-Rasmussen B., Deckert T.: Features of endothelial dysfunction in early diabetic nephropathy. Lancet 1989; i: 461-463
88. Wilson S., Jennings P.E., Mclaren M., Jung R.T., Newton R.W., Belch J.J.F.: Relationship of microcirculatory bnlood flow and biochemical parameters of haemostasis in diabetic microangiopathy. Diabetologia 1988; 32: 537 A
89. Grant M.B., Guay C., Lottenberg R.: Desmopressin stimulates parallel tissue plasminogen activator release in normal subjects and patients with diabetes mellitus. Thromb Haemostas 1988; 59: 269-272 (normal response to stimulation in some categories of IDDM)
90. Snapper I.: Chinese lessons to Western Medicine. A contribution ti geographical medicine from the clinics of Peiping Union Medical College, 2nd ed. Grune & Stratton, New York, 1965
91. Rydzewski A., Kawamura H., Watanabe I., Takada Y., Takada A.: Plasminogen activators and plasminogen activator inhibitors (PAI-1) in type II diabetesa mellitus. Fibrinolysis 1990; 4: 183-188
92. Van Wersch J.W.J., Westerhuis L.W.J.J.M., Venekamp W.J.R.R.: Glycometabolic control and fibrinolysis in diabetic patients. Haemostasis 1990; 20: 241-250
93. Reaven G.M.: Role of insulin resistance in human disease. Diabetes 1988; 37: 1595-1607.
94. Heinrich J., Nguyên T., Kokott R., Epping P.H., Schulte H., Assmann G.: Interrelationships between coronary risk factors and the fibrinolytic system. Fibrinolysis 1990; 4 (Suppl 1): 37 (abstr)
95. Hamsten A., Wiman B., De Faire U., Blombäck M.: Increased plasma levels of a rapid inhibitor of tissue plasminogen activator in young survivors of myocardial infarction. N Engl J Med 1985; 313: 1557-1563

96. Hamsten A., Walldius G., Szamosi A., Blömback M., De Faire U., Dahlén G., Landou C., Wiman B.: Plasminogen activator inhibitor in plasma: risk factor for recurrent myocardial infarction. Lancet 1987; ii: 3-9
97. Landin K., Tengborn L., Smith U.: Elevated fibrinogen and plasminogen activator inhibitor (PAI-1) in hypertension are related to metabolic risk factors for cardiovascular disease. J Int Med 1990; 227: 273-278.
98. Juhan-Vague I., Vague Ph.: Interrelationships between carbohydrates, lipids, and the hemostatic system in relation to the risk of thrombotic and cardiovascular disease. Am J Obstet Gynecol 1990; 163: 313-315
99. DeFronzo RA, Ferrannini E, Simonson DC: Fasting hyperglycaemia in non-insulin-dependent diabetes mellitus: contributions of excessive hepatic glucose production and impaired tissue glucose uptake. Metabolism 38 : 387 - 395 , 1989
100. Grant M.B., Fitzgerald C., Guay C., Lottenberg R.: Fibrinolytic capacity following stimulation with desmopressin acetate in patients with diabetes mellitus. Metabolism 1989; 38: 901-907
101. Brommer E.J.P., Verheijen J.H., Chang G.T.C., Rijken D.C.: Masking of fibrinolytic response to stimulation by an inhibitor of tissue-type plasminogen activator in plasma. Thromb Haemostas 1984; 52: 154-156
102. Lütjens A., te Velde A.A., van der Veen E.A., van der Meer J.: Glycosylation of human fibrinogen in vivo. Diabetologia 1985; 28: 87-89
103. Lütjens A., Jonkhoff-Slok T.W., Sandkuijl C., van der Veen E.A., van der Meer J.: Polymerisation and crosslinking of fibrin monomers in diabetes mellitus. Diabetologia 1988; 31: 825-830
104. Brownlee M., Vlassara H., Cerami A.: Nonenzymatic glycosylation reduces the susceptibility of fibrin to degradation by plasmin. Diabetes 1983; 32: 680-684
105. Mirshahi M., Soria J., Soria C., Bertrand O., Mirshahi M., Basdevant A.: Glycosylation of human fibrinogen and fibrin in vitro. Its consequences on the properties of fibrinogen. Thromb Res 1987; 48: 279-289
106. Geiger M., Binder B.R.: Nonenzymatic glucosylation as a contributing factor to defective fibrinolysis in diabetes mellitus. Haemostasis 1986; 16: 439-446
107. Nilsson T.K., Lithner F.: Glycaemic control, smoking habits and diabetes duration affect the extrinsic fibrinolytic system in type I diabetic patients but microangiopathy does not. Acta Med Scand 1988; 224: 123-129
108. Small M.,Paterson K.R., Flannigan P., Lowe G.D.O., Forbes C.D., MacCyuish A.C.: Enhancement of fibrinolysis after insulin administration. Diabetes Care 1986; 9: 216-217
109. Landin K., Tengborn L., Chmielewska J., Von Schenck H., Smith U.: The acute effect of insulin on tissue plasminogen activator and plasminogen activator inhibitor in man. Thromb Haemostas 1991; 65: 130-133

CHAPTER 2

THE IMPACT OF GLYCAEMIC CONTROL ON FIBRINOLYTIC PARAMETERS IN INSULIN-DEPENDENT DIABETES MELLITUS

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SUMMARY

Impaired fibrinolysis has been suggested to contribute to the increased atherogenesis in diabetes mellitus. We studied the impact of glycaemic control on fibrinolysis parameters. 8 insulin-dependent diabetes mellitus patients (IDDM) with adequate glycaemic control (group A; glycosylated haemoglobin (Hb_{A1c}) 7.3 ± 0.8 %) and 7 IDDM with poor glycaemic control (group P; Hb_{A1c} 9.7 ± 0.6 %) were studied on two occasions with a five month interval. In group P subjects treatment was intensified during the five month interval, resulting in marked improvement of glycaemic control (Hb_{A1c} 7.9 ± 0.7 %, $p<0.001$), and approximating the level of glycaemic control in group A (Hb_{A1c} 7.2 ± 0.8 %, N.S.). Plasminogen activator inhibitor type 1 (PAI-1) activity and basal and exercise-stimulated tissue-type plasminogen activator (t-PA) antigen levels in plasma were measured.
At the start of the study plasma PAI-1 in group P was lower than in group A (4.3 ± 1.1 vs. 6.2 ± 1.9 IU/ml, $p<0.05$). Improvement of glycaemic control in group P resulted in an increase of PAI-1 (6.0 ± 1.4 IU/ml, $p<0.05$), while PAI-1 remained unaltered in group A. Basal t-PA was reduced in group P subjects as compared to group A both before ($p<0.05$) and after ($p<0.05$) improvement of glycaemic control (1.6 ± 0.8 vs. 4.6 ± 3.1 ng/ml and 1.3 ± 1.0 vs. 5.1 ± 4.2 ng/ml respectively). Stimulated t-PA was lower in group P subjects only following improvement of glycaemic control (3.6 ± 1.6 vs. 8.1 ± 4.9 ng/ml, $p<0.05$).

These findings suggest that five months of improvement of glycaemic control in poorly controlled IDDM adversely affects the fibrinolytic system and could offer an explanation why a relationship between macrovascular disease and poor glycaemic control has not been established in IDDM. Studies of a longer duration are needed to determine whether on the long term improvement of glycaemic control enhances fibrinolysis.

INTRODUCTION

Evidence is accumulating that strict glycaemic control diminishes the risk of microvascular complications in insulin-dependent diabetes mellitus (IDDM) [1,2]. In contrast, a relationship between cardiovascular morbidity and degree of glycaemic control has not been found [3].

Recently cardiovascular risk markers have been identified including increased levels of plasminogen activator inhibitor type 1 (PAI-1) [4] and decreased plasma activity of tissue-type plasminogen activator (t-PA) [5]. Both are major determinants of plasma fibrinolytic activity by inhibiting (PAI-1)[6], or by stimulating (t-PA)[7] fibrinolysis. Impaired fibrinolysis has been encountered in IDDM [8-10] and may contribute to the pathogenesis of atherosclerotic disease in IDDM [11].

It is not clear whether a relationship exists between fibrinolysis and the degree of glycaemic control, and whether alteration of glycaemic control influences fibrinolysis. The relationship between glycosylated haemoglobin and several fibrinolytic parameters has been studied in several cross-sectional studies [9,12-17], with equivocal results. 24-hour improvement of glycaemic control decreases t-PA and plasma fibrinolytic activity [18]. At present, only two longitudinal studies [19,20] have been performed in which the fibrinolytic system has been studied in diabetic subjects with poor glycaemic control, both before and after improvement of glycaemic control. In one study [19], no change was observed in overall basal euglobulin fibrinolytic activity; no determinations were done of PAI-1 and of t-PA, and t-PA release following stimulation was not studied. The study by Vukovich et al. [20] demonstrated a decrease in fibrin degradation products in a subset of patients, but did not address plasma fibrinolytic activity or components of the fibrinolytic system.

Therefore, we studied basal levels of t-PA and PAI-1 and stimulated levels of t-PA in insulin-dependent diabetic subjects with poor and with adequate glycaemic control. Following that, we improved glycaemic control in the poorly controlled group and studied both groups on a second occasion, five months later.

PATIENTS AND METHODS

Patients

Eight IDDM with adequate glycaemic control (group A), i.e. glycosylated haemoglobin (Hb_{A1c}) less than 8.5 %, and seven IDDM with poor glycaemic control (group P) were studied. None of the subjects was ketotic. Patients were matched for age and duration of diabetes. All subjects were normotensive, normo-albuminuric, and had background retinopathy at most. None had clinically detectable peripheral neuropathy and 3 patients were smokers (group A: 1 ; group P: 2). Patient characteristics are given in Table 1. After the initial test (may - july) subjects with poor glycaemic control were subjected to a five-month period of intensified treatment (with frequent home determination of blood glucose levels), which resulted in improved glycaemic control (see Table 1). After this five month period all subjects underwent a second test (october - december). The test protocol had been approved by the local Ethics Board (Catharina Hospital).

Test procedure

Patients were tested in the morning between 9:00 and 11:00 a.m. On each occasion blood was collected for basal values 40 minutes following insertion of an intravenous cannula, while the subject remained supine. After a further 20 minutes, exercise was performed on a bicycle ergometer for 20 minutes (100 W, unless heart rate increased to more than 85 % of estimated age-adjusted maximal heart rate; in that case exercise load was diminished to 75 W). Immediately following exercise, blood was collected for determination of stimulated values of t-PA. Heart rate was recorded every five minutes during the exercise and the maximal heart rate is shown in Table 1.

Assays

PAI-1 activity was measured by the Verheijen method [21] in platelet-poor plasma (PPP) collected in 0.1 vol/vol citrate 3.8%, to which were added theofylline, adenosine and dipyradimole as anti-aggregating agents (CTAD). The variation coefficient of the determination was 12 %. Plasma was rendered platelet-poor by centrifugation at 4°C during 20 minutes at 2000 x g. Results were expressed in IU/ml. 1 ml of pooled plasma contains 7.6 IU of PAI-1 activity.

t-PA antigen was measured in platelet-poor citrate-plasma (0.1 vol/vol 3.8% citrate) by enzyme immunoassay (Imulyse™ t-PA, Biopool, Umeå, Sweden). The variation coefficient of the determination was 8 %.

Hb_{A1c} was determined by affinity chromatography. Ambient blood glucose was determined by Haemo-Glucotest 1-44R (Boehringer Mannheim, Almere, The Netherlands).

Table 1

Patient characteristics at the start (first test) and at the end (second test) of the study. Group A represents IDDM with adequate glycaemic control. Group P represents IDDM with poor glycaemic control, which was subsequently improved.

	Group A		Group P	
	start	end	start	end
Male : female	5 : 3		2 : 5	
Age (yrs)	32 ± 9		30 ± 10	
Weight (kg)	73 ± 12		69 ± 7	
duration (yrs)	12 ± 12		14 ± 7	
Dose (U/day)	39 ± 13	39 ± 13	54 ± 15 @	55 ± 12 @
Hb_{A1c} (%) §	7.5 ± 0.7	7.2 ± 0.8	9.7 ± 0.6 @	7.9 ± 0.7 *
Heart rate (+) (min^{-1})	141 ± 24	142 ± 16	131 ± 14	132 ± 16

§ normal values: 4.0-6.0 %
@ group P vs. group A: $p<0.05$
* change within group: $p<0.05$
+ during exercise

Statistics

All values are expressed as mean ± standard deviation (SD), except where specifically noted otherwise (Fig.2). Differences between the two groups were tested by the Mann- Whitney U-test. Changes within one group were tested by means of the Wilcoxon signed rank test. A probability level of 0.05 or less was considered significant.

RESULTS

Glycaemic control and exercise performance

Intensified treatment of the patients in group P resulted in a significant reduction of glycosylated haemoglobin from 9.7 ± 0.6 % to 7.9 ± 0.7 % ($p<0.05$), despite a similar final daily dose (see Table 1). Corresponding values in group A were 7.3 ± 0.8 % at the first ($p<0.05$ vs. group P) and 7.2 ± 0.8 % at the second test (not different from group P).

During the first exercise test ambient blood glucose levels were 10.9 ± 3.0 and 9.3 ± 5.0 mmol/l in groups A and P respectively (N.S.) Corresponding values during the second exercise test were 12.1 ± 6.9 and 7.7 ± 5.5 mmol/l respectively (N.S.). All patients in group A and 6 out of 7 in group P performed 100 W during the first exercise test (1 patient performed 75 W) and all patients completed 100 W the second test.

Plasma levels of PAI-1 and t-PA

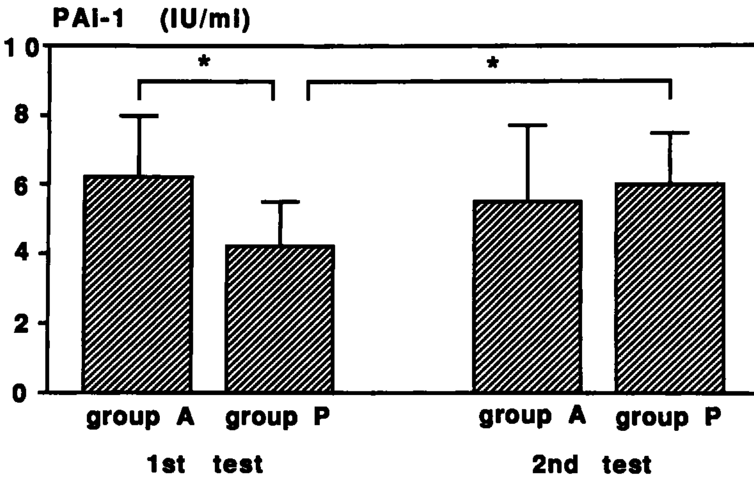
Plasma levels of PAI-1 activity were significantly less in group P patients, as compared to levels in group A: 4.3 ± 1.1 IU/ml vs. 6.2 ± 1.9 IU/ml ($p<0.05$). Improvement of glycaemic control in group P resulted in an increase of PAI-1 levels ($p<0.05$). At the time of the second exercise test, PAI-1 levels were similar in both groups: 5.4 ± 2.3 IU/ml in group A vs. 6.0 ± 1.4 IU/ml in group P (see Figure 1).

Basal plasma levels of t-PA, as shown in Figure 2, were significantly reduced in group P subjects in comparison with group A: 1.6 ± 0.8 ng/ml vs. 4.6 ± 3.1 ng/ml ($p<0.05$). Improvement of glycaemic control in group P subjects did not result in a significant change of basal t-PA and, as a result, group P continued to have reduced t-PA levels at the second test: 1.3 ± 1.0 vs. 5.1 ± 4.2 ng/ml ($p<0.05$).
The ratio of t-PA to PAI in group A was 0.71 ± 0.12 at the first and 0.88 ± 0.14 at the second test. Corresponding values for group P were 0.39 ± 0.07 ($p=0.05$ vs. group A) and 0.25 ± 0.08 ($p<0.01$ vs. group A).

Stimulated values of t-PA at the first test were 11.2 ± 5.1 and 5.8 ± 5.2 ng/ml in groups A and P, respectively (see Figure 2), whereas the comparative values at the second test were 8.1 ± 4.9 and 3.7 ± 1.6 ng/ml. The difference between groups was statistically significant only at the second test ($p<0.05$). The increase in t-PA following

exercise during the first test was 6.6 ± 4.0 ng/ml in group A and 4.2 ± 5.7 ng/ml in group P; the corresponding values during the second test were 2.9 ± 2.5 and 2.3 ± 1.4 ng/ml. These values do not differ significantly between groups or within one group in time.

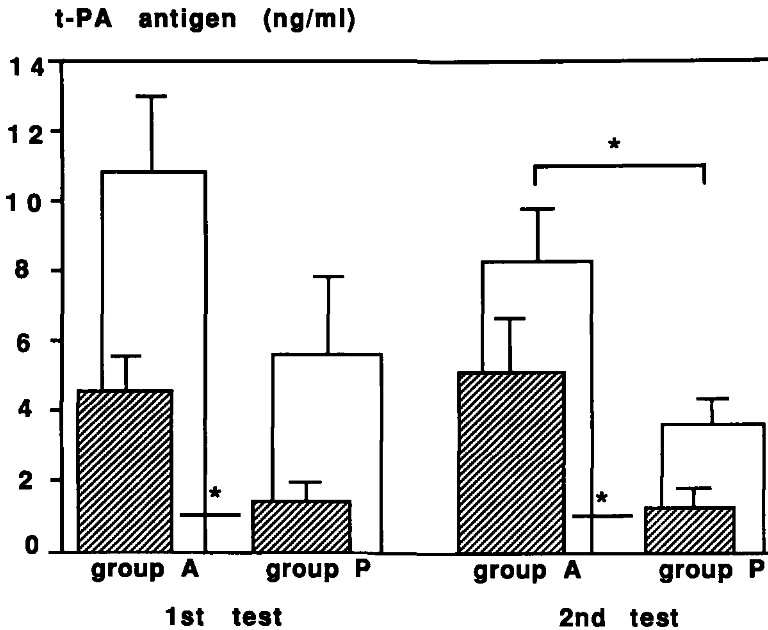
Figure 1 PAI-1 in relation to glycaemic control in IDDM.



Plasma PAI-1 activity in group A (adequately controlled) and group P (poorly controlled) IDDM at the first and at the second test. Between the first and second tests glycaemic control in group P subjects was improved to a level similar to that of group A. At the first test, PAI-1 is lower in group P. After improvement of glycaemic control in group P, PAI-1 increases significantly and the groups no longer differ. Bars represent mean ± SD. Normal values for PAI-1 are 2-21 IU/ml.

* $p<0.05$

Figure 2 Basal and stimulated t-PA in relation to glycaemic control in IDDM.



Basal (hatched bar) plasma t-PA antigen is reduced in group P (poor glycaemic control) as compared to group A (adequate glycaemic control), both before and after improvement of glycaemic control in group P. Stimulated t-PA (blank bar) is significantly lower in group P only after improvement of glycaemic control. Bars represent mean ± SEM.
* $p<0.05$

DISCUSSION

The present study was undertaken to study the impact of glycaemic control on fibrinolytic parameters, and to evaluate whether improvement of glycaemic control influences fibrinolysis. Basal levels of PAI-1 activity and of t-PA antigen were significantly lower in the group with poor glycaemic control (group P) in comparison with adequately controlled subjects (group A). Improvement of glycaemic control in poorly controlled diabetic patients resulted in a significant increase of PAI-1 activity

(Figure 1). However, improvement of glycaemic control in group P failed to increase basal t-PA levels. A previous study by Juhan-Vague [18] has demonstrated a reduction of t-PA and a reduction of basal plasma fibrinolytic activity in poorly controlled diabetic subjects rendered normoglycaemic for 24 hours. PAI-1 was not measured in these studies. Recently Tengborn et al. [22] reported that treatment of normoglycaemic hypertensive men with metformin resulted in an increase of PAI-1 activity, which parallelled a decrease in fasting blood glucose.

In our study, stimulated t-PA levels were lower in the poorly controlled subjects than in the adequately controlled patients. The difference was significant only after improvement of glycaemic control (see Figure 2). This finding suggests that five months improvement of glycaemic control has an adverse effect on exercise-induced t-PA release. To our knowledge, only four studies of t-PA levels in diabetic patients following exercise have been published [13,23-25]. None of these was longitudinal. Jensen et al. found decreased levels of basal t-PA antigen in IDDM patients compared to healthy controls, and an impaired t-PA response to exercise in diabetic subjects with microalbuminuria and overt proteinuria [13], but not in normoalbuminuric patients. However, glycaemic control was slightly superior in the normoalbuminuric patients (glycosylated haemoglobin 7.1 %, vs. 7.6 and 7.6 % for the microalbuminuric and proteinuric patients, respectively) and therefore may have accounted for the differences. Hornsby et al. [23] and Schneider et al. [24] studied NIDDM subjects, which are quite distinct from IDDM regarding fibrinolysis [10,12]. Sundkvist et al. [25] studied t-PA levels following exercise in IDDM subjects with or without retinopathy, but did not address the degree of glycaemic control.

The fact that basal and stimulated t-PA differed between the two groups after improvement of glycaemic control in the poorly controlled subjects suggests the existence of a - yet unidentified - difference between the two studied groups, in addition to the difference in the level of glycaemic control which was present at onset. The female preponderance among the poorly controlled subjects could explain the persisting differences in t-PA; women are reported to have lower plasma t-PA levels than men [26], although not all authors agree [7]. Another possibility, the persistent difference in daily insulin dose, is unlikely since insulin dose is not related with t-PA levels in IDDM [12]. Finally, the level of glycaemic control (Hb_{A1c} 7.9 %, vs. 7.2 % in group A) as well as the duration of improvement of glycaemic control may have been insufficient to "restore" plasma t-PA levels.

The mechanism underlying the impaired t-PA response to exercise in group P patients is not clear. None of the subjects had clinical signs of significant micro- or macroangiopathy. Possible explanations include either endothelial dysfunction

secondary to prolonged inadequate glycaemic control, or a temporary imbalance of fibrinolysis *secondary to an increase in* PAI-1 *without concurrent increase in* t-PA. It is tempting to speculate that such an imbalance may contribute to the deterioration of diabetic retinopathy seen in some patients following improvement of glycaemic control [27], although we did not observe this in this study.

In summary, improvement of glycaemic control in poorly controlled insulin-dependent diabetic patients resulted in an increase of PAI-1 activity, failed to restore reduced basal t-PA levels relative to adequately controlled IDDM, and finally did not result in increase of exercise-induced t-PA release. In other words: improvement of glycaemic control during five months in poorly controlled diabetics has an unfavourable effect on the fibrinolytic system. This could offer an explanation why an association between macrovascular disease and poor glycaemic control has not been established in IDDM. Longitudinal studies of a longer duration are needed in diabetes to elucidate the impact of glycaemic control on the fibrinolytic system, and, ultimately, on cardiovascular risk.

REFERENCES

1. Lauritzen T., Frost-Larsen H-W., Deckert T., Steno Study Group: Effect of 1 year near-normal blood glucose levels on retinopathy in insulin-dependent diabetics. Lancet 1983; i: 200-204
2. Dahl-Jørgensen K., Brinchmann-Hansen O., Hanssen K.F., Ganet T., Kierulf P., Smeland E., Sandvik L., Aagenæs ø: Effect of near normoglycaemia for two years on progression of early diabetic retinopathy, nephropathy, and neuropathy: The Oslo Study. Br Med J 1986; 293: 1195-1199.
3. American Diabetes Association: Role of cardiovascular risk factors in prevention and treatment of macrovascular disease in diabetes. Consensus Statement. Diabetes Care 1989; 12: 573-579.
4. Hamsten A., de Faire U., Walldius G., Dahlén G., Szamosi A., Landou C., Blombäck M., Wiman B.: Plasminogen activator inhibitor in plasma: risk factor for recurrent myocardial infarction. Lancet 1987; ii: 3-9
5. Gram J., Jespersen J.: A selective depression of tissue plasminogen activator (t-PA) activity in euglobulin characterises a risk group among survivors of acute myocardial infarction. Thromb Haemostas 1987; 57: 137-139
6. Brommer E.J.P., Verheijen J.H., Chang G.T.C., Rijken D.C.: Masking of fibrinolytic response to stimulation by an inhibitor of tissue-type plasminogen activator in plasma. Thromb Haemostas 1984; 52: 154-156
7. Nicoloso G., Hauert J., Kruithof E.K.O., Van Melle G., Bachmann F.: Fibrinolysis in normal subjects - Comparison between plasminogen activator inhibitor and other components of the fibrinolytic system. Thromb Haemostas 1988; 59: 299-303
8. Cash J.D., McGill R.C.: Fibrinolytic response to moderate exercise in young male diabetics and non-diabetics. J Clin Pathol 1969; 22: 32-35

9. Haitas B., Barnes A.J., Cederholm-Williams S.A., Moore J., Shogry M.E.C., Turner R.C.: Abnormal endothelial release of fibrinolytic activity and fibronectin in diabetic microangiopathy. Diabetologia 1984; 27: 493-496
10. Almér L.-O.: Fibrinolytic disorders in diabetes mellitus. Diab Metab 1988; 14: 519-522
11. Colwell J.A., Winocour P.D., Lopes-Veiella M., Halushka V.: New concepts about the pathogenesis of atherosclerosis in diabetes mellitus. Am J Med 1983; 81, Supp:5B, 67-80
12. Auwerx J., Bouillon R., Collen D., Geboers J.: Tissue-type plasminogen activator antigen and plasminogen activator inhibitor in diabetes mellitus. Arteriosclerosis 1988; 8: 68-72
13. Jensen T., Feldt-Rasmussen B., Bjerre-Knudsen J., Deckert T.: Features of endothelial dysfunction in early diabetic nephropathy. Lancet 1989; I: 461-463
14. Small M., Kluft C., MacCuish A.C., Lowe G.D.O.: Tissue plasminogen activator inhibition in diabetes mellitus. Diabetes Care 1989; 12: 655-658
15. Grant M.B., Fitzgerald C., Guay C., Lottenberg R.: Fibrinolytic capacity following stimulation with desmopressin acetate in patients with diabetes mellitus. Metabolism 1989; 38: 901-907
16. Van Wersch J.W.J., Westerhuis L.W.J.J.M., Venekamp W.J.R.R.: Glycometabolic control and fibrinolysis in diabetic patients. Haemostasis 1990; 20: 241-250
17. Nilsson T.K., Lithner F.: Glycaemic control, smoking habits and diabetes duration affect the extrinsic fibrinolytic system in type I diabetic patients but microangiopathy does not. Acta Med Scand 1988; 224: 123-129
18. Juhan-Vague I., vague P., Poisson C., Aillaud M.F., Mendez C., Collen D.: effect of 24 hours of normoglycaemia on tissue-type plasminogen activator plasma levels in insulin-dependent diabetes. Thromb Haemostas 1984; 51: 97-98
19. Van der Knaap J.H., De Boer A.C., Pannebakker M.A.G., Van Weerde W., Den Ottolander G.J.H.: The effect of diabetes regulation on platelet release, fibrinolysis and coagulation tests, before and after stimulation with DDAVP. Thromb Haemostas 1985; 53: 118-121
20. Vukovich T., Schernthaner G., Knöbi P.N., Hay U.: The effect of near-normoglyemic control on plasma Factor VIII/ von Willebrand Factor and fibrin degradation products in insulin-dependent diabetic patients. J Clin Endocrinol Metab 1989; 69: 84-89
21. Verheijen J.H., Chang G.T.C., Kluft C.: Evidence for the occurrence of a fast-acting inhibitor for tissue-type plasminogen activator in human plasma. Thromb Haemostas 1984; 51: 392-395
22. Tengborn L., Landin K., Andersson I., Chmielewska J., Smith U.: The influence of metformin on the fibrinolytic system and insulin resistance in patients with hypertension. Fibrinolysis 1990; 4, Supp:2, 91-92
23. Hornby W.G., Boggess K.A., Lyons T.J., Barnwell W.H., Lazarchick J., Colwell J.A.: Hemostatic alterations with exercise conditioning in NIDDM. Diabetes Care 1990; 13: 87-92
24. Schneider S.H., Kim H.C., Khachadurian A.K., Ruderman N.B.: Impaired fibrinolytic response to exercise in type II diabetes: effects of exercise and physical training. Metabolism 1988; 37: 924-929
25. Sundkvist G., Almér L.-O., Lilja B., Pandolfi M.: Growth hormone and endothelial function during exercise in diabetics with and without retinopathy. Acta Med Scand 1984; 215: 55-61
26. Takada Y., Takada A.: Plasma levels of t-PA, free PAI-1 and a complex of t-PA with PAI-1 in human males and females at various ages. Thromb Res 1989; 55: 601-609
27. Dahl-Jørgensen K., Brinchmann-Hanssen O., Hanssen K.F., Sandvik L., Aagenæs ø, Aker Diabetes Group: Rapid tightening of blood glucose control leads to transient deterioration of retinopathy in insulin dependent diabetes mellitus: the Oslo study.
Br Med J 1985; 290: 811-815

CHAPTER 3

THE FIBRINOLYTIC SYSTEM DURING LONG-DISTANCE RUNNING IN PATIENTS WITH TYPE I (INSULIN-DEPENDENT) DIABETES MELLITUS AND IN HEALTHY SUBJECTS.

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SUMMARY

Endurance exercise has been advocated in diabetes mellitus both to improve metabolic control and to prevent atherosclerotic complications. The response of the fibrinolytic system during prolonged exercise has not been studied in diabetes.
In 7 male marathon runners with type I (insulin-dependent) diabetes mellitus (IDDM) and 8 healthy male controls , matched for age and degree of training, we studied fibrinolytic and coagulation parameters during a 3 hr, 32 km outdoor running session. Measurements included tissue-type plasminogen activator (t-PA), urokinase-type plasminogen activator (u-PA), plasminogen activator inhibitor type 1 (PAI-1), plasmin - α2 antiplasmin complexes (PAP) as a measure of in vivo activation of fibrinolysis, fibrin degradation products (FbDP), intact fibrinogen (Fgn), von Willebrand Factor (vWF) and coagulation factor VIII.
In both IDDM and controls, levels of t-PA, u-PA, PAP, vWF and VIII continued to rise throughout the exercise, whereas PAI-1 showed a similar decline during the daytime. FbDP and Fgn did not change during exercise in either group. t-PA levels during exercise correlated closely with exercise intensity. These findings indicate that

continued stimulation by exercise does not deplete endothelial plasminogen activator stores. Differences between IDDM and controls were seen only for t-PA, vWF and u-PA. The area under the curve during exercise (AUCex) of t-PA in IDDM was insignificantly lower than in controls (53 ± 19 vs. 67 ± 31 ng/ml*hr), but the ratio of t-PA to exercise intensity was lower in the IDDM (0.24 ± 0.11 vs. 0.31 ± 0.13, $p<0.05$). The AUCex of vWF was lower in IDDM than in controls (569 ± 268 vs 880 ± 265 %*hr, $p<0.05$). The AUCex of u-PA was higher in IDDM than in controls (15.1 ± 3.5 vs 11.2 ± 1.8 ng/ml*hr, $p<0.05$).
In conclusion, despite a defect in the exercise-induced endothelial release of vWF and of t-PA, the overall potential to activate fibrinolysis is intact in IDDM, possibly by enhancement of u-PA following exercise. Our data suggest that in IDDM, like in nondiabetics, long distance running may slow the progression of atherosclerosis by stimulating fibrinolysis.

INTRODUCTION

Patients with diabetes mellitus are reported to have a twofold higher incidence of coronary artery disease and a threefold higher incidence of peripheral arterial disease, as compared to nondiabetic subjects matched for other cardiovascular risk factors [1]. A decrease in plasma fibrinolytic activity may play a role in the pathogenesis of atherosclerotic complications [2-3], by reducing degradation of fibrin.
Plasma fibrinolytic activity is determined by the balance between plasminogen activators and their inhibitors. In the basal state, activators are mostly bound to plasminogen activator inhibitor type 1 (PAI-1) and the resultant fibrinolytic activity is low [4]. Following stimuli like exercise, venous occlusion, or infusion with 1-desamino-8-D-arginine vasopressin (DDAVP), fibrinolytic activity increases due to the endothelial release of both tissue-type plasminogen activator (t-PA) and urokinase-type plasminogen activator (u-PA) [5-7]. Studies in IDDM have demonstrated an impaired t-PA response to exercise in subjects with microalbuminuria and proteinuria, but not in normoalbuminuric patients [8]. However, the stimulus for t-PA release was only of short duration.
Endurance exercise has been advocated in the treatment of diabetes both for improvement of metabolic control [9,10] and for prevention of atherosclerotic complications [11]. We have previously reported on the metabolic effects of prolonged exercise in insulin-dependent diabetic subjects [12]. Repeated bouts of exercise may result in a blunted t-PA response [13] and it has been suggested that the endothelium

becomes depleted of t-PA following repeated stimulation [14,15]. Like this prolonged exercise might adversely affect fibrinolysis. Furthermore, the finding of a blunted response to repeated exercise suggests that prolonged exercise may be a sensitive test to uncover defects in the release of plasminogen activators. In diabetes mellitus however, prolonged, continuous, stimulation of the fibrinolytic system has not been studied.
We had the oppurtunity to study 7 well trained IDDM marathon runners without micro- or macroalbuminuria. Here we report on the response of the fibrinolytic system to prolonged exercise, in these IDDM and in nondiabetic control subjects, during a 32 km, 3 hour outdoor running session.

SUBJECTS

7 insulin-dependent (type I) diabetic men (IDDM) with adequate metabolic control, and 8 nondiabetic healthy men matched for age and degree of training participated in the study after having given informed consent. The study protocol had been approved by the Ethics Committee of the University Hospital, Leiden. All subjects were able to run a complete marathon within 3.5 hrs. Diabetic subjects all were C-peptide negative, had grade 0-1 retinopathy at most, and had a normal albumin excretion (albumin/creatinine ratio in morning urine < 2.5 mg/mmol). None had evidence of macrovascular disease or of peripheral neuropathy. Mean insulin dose was 34 U/24 hrs (range 24-46).

METHODS

Prior to the study, IDDM subjects were asked to withhold the morning insulin dose. A normal breakfast was taken. After arrival at the study center (9:00 to 9:30 a.m.) a forearm vein was cannulated (Venflon 17 G, Viggo Products, Sweden) and baseline measurements were performed. The cannula was subsequently flushed with 2 ml of a heparin solution (50 U/ml) after each procedure. The first 5 ml of blood at each collection was discarded, and in the collected blood no heparin could be demonstrated by activated partial thromboplastin time. Immediately before the start of the exercise, at 10:00 a.m., baseline blood (0 hrs) was collected. Subjects subsequently performed a 3-hour 32 km outdoor running session, in which a diabetic subject was always accompanied by a nondiabetic runner. Blood was collected after 0.5, 1, 2 and 3 hrs of

running (see Figure 1). Delay in blood collection at each interruption of the running session was less than 5 minutes in all cases and averaged 2.2 minutes. Heart rate was continually measured telemetrically (Sport-tester PE 3000, Polar Electro Inc., Finland) and expressed as a percentage of estimated maximal heart frequency (%EMHR). EMHR was considered 220 - age [16]. Body weight and fluid intake (water) were registered.

Table 1 Characteristics of IDDM and of nondiabetic subjects

	Insulin-dependent diabetic subjects		Nondiabetic subjects		
Age, years	42.7 (5.3)		37.1 (9.0)		
training, km/week	64 (19)		58 (25)		
diabetes duration, yrs	12.0 (5.7)				
Hb_{A1c}, %	9.4 (1.4)		5.1 (0.6)		*
urinary albumin, mg/mmol creatinine	0.4 (0.9)		0.3 (0.4)		
during run:					
Glucose, mmol/l					
pre	15.9 (3.6)	-$	4.4 (0.4)	-$	*
post	9.2 (9.2)	-	4.0 (0.8)	-	*
Exercise level, % EMHR	84.2 (7.4)		80.9 (6.9)		
H_2O consumption, ml	1315 (400)		1075 (490)		
weight loss, g	1615 (420)		1625 (660)		

Values represent means (SD). An asterisk (*) denotes a statistically significant difference ($p<0.05$) between groups. A syphon (-$-) denotes a statistically significant difference between measurements within a group.
Normal values of Hb_{A1c} are 4.4-6.7 %

In addition to fibrinolytic parameters, von Willebrand factor antigen (vWF) and factor VIII (VIII:C) were determined, as the levels of these proteins also increase following exercise. vWF is derived from endothelium [17], whereas VIII:C probably is of hepatic origin [18].

Blood was collected at room temperature in 0.1 vol sodium citrate (0.11 M) to which platelet inactivators were added (theofylline 15 mM, adenosine 3.7 mM, dipyridamole 0.198 mM)(CTAD, Boehringer Mannheim, Almere, NL), for determination of PAI-1. For determination of t-PA, u-PA, fibrin degradation products (FbDP), intact fibrinogen (Fgn), plasmin-antiplasmin complexes (PAP), von Willebrand factor antigen (vWF) and clotting factor VIII (VIII:C) blood was collected in chilled tubes containing 0.1 vol sodium citrate 0.129 M. Platelet poor plasma was prepared by centrifugation at 2000 x g during 20 minutes at 4°C and aliquots were stored at -70 °C until processing.

Figure 1 Scheme of the exercise performance and blood collections.

time	10:00	10:30	11:00	12:00	13:00	13:45
km	0	5	10	21	32	
	*	*	*	*	*	
	$				$	

* = determination of
-glucose
-tissue-type plasminogen activator antigen (t-PA)
-urokinase-type plasminogen activator antigen (u-PA)
-von Willebrand Factor antigen (vWF)
-factor VIII activity (VIII:C)
-fibrin degradation products (FbDP)
-intact fibrinogen (Fgn)
-plasmin - $\alpha 2$ antiplasmin complexes (PAP)
$ = determination of
-PAI-1 Antigen

Analytical procedures

Venous whole blood glucose was determined on a SMAC II analyzer (Technicon, Tarrytown, NY). Hb_{A1c} was determined by HPLC. PAI-antigen was measured by enzyme immunoassay (Tint Elize PAI-1, lot number 210220, Biopool AB, Umeå, Sweden). t-PA antigen was measured by enzyme immunoassay (Imulyse t-PA, Biopool AB). u-PA was measured by ELISA [19]. vWF antigen and VIII:C were measured as described before [20]. FbDP [21] and Fgn [22] were measured by enzyme immunoassay. PAP was measured by RIA [23].

Statistical analysis and calculations

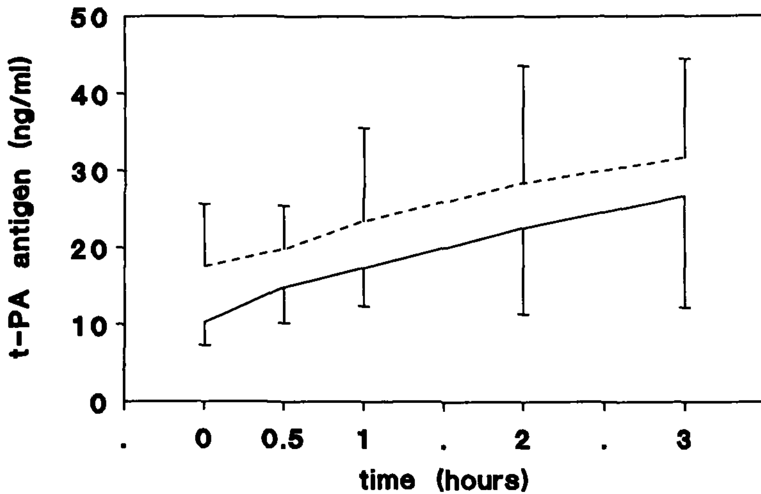
Results are expressed as mean ± standard deviation (SD). In order to eliminate the possible influence of group differences in baseline values, areas under the curve during exercise (AUCex) were calculated from the data at 0.5 - 3 hours. Unpaired and paired t-tests were performed. Nonparametric tests were performed in case of non-normal distribution. Linear regression analysis of t-PA and of u-PA vs. exercise intensity was performed on the pooled data of 0.5 - 3 hrs. All calculations were done with Number Cruncher Statistical System (NCSS).

RESULTS

All subjects completed the 32 km run in 3 hours without difficulties. IDDM and control subjects perfomed the exercise equally well regarding to fitness at the end of the run and fluid loss during the run (see Table 1). All subjects achieved submaximal exercise levels during the entire run, i.e. the heart rate was at least 70 % of age-corrected maximal heart rate. Exercise level ranged from 70 to 98 % of EMHR and increased towards the end of the run in both IDDM and control subjects (data not shown). Exercise intensity was slightly higher in the IDDM group, but the differences were not statistically significant (see Table 1). Blood glucose during the exercise decreased in all IDDM subjects except one, in whom an increase was seen from 23.6 to 28.8 mmol/l. This patient however did not develop ketosis.

PAI-1 antigen decreased between 10:00 and 13:00 hrs in both IDDM (7.7 ± 3.2 to 6.7 ± 2.7 ng/ml, N.S.) and control (10.7 ± 7.2 to 8.2 ± 3.1 ng/ml, N.S.) subjects. No differences were noted between IDDM and control subjects. Most probably this can be attributed to the physiological decline during this time of the day [24]. Results of t-PA antigen, u-PA antigen, PAP, vWF, VIII:C, intact fibrinogen and FbDP are given in Table 2. Basal levels of t-PA antigen were highest in the control subjects (p=N.S.). During exercise t-PA antigen increased in both groups (see Figure 2). The AUCex of t-PA antigen was lower in IDDM than in controls (52.7 ± 19.5 vs. 66.9 ± 31.0 ng/ml*hr), although the difference did not reach statistical significance (95 % confidence interval -43 to +15 ng/ml*hr).

Figure 2 t-PA antigen during exercise in IDDM and nondiabetics.

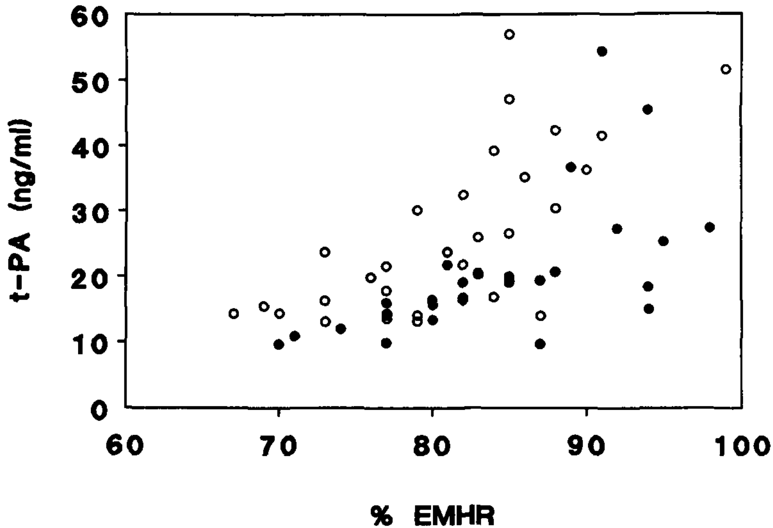


t-PA antigen levels (mean ± SD) continue to rise in both IDDM (solid line) and nondiabetics (dotted line) during a 3 hour, 32 km outdoor run. Although levels are lower in IDDM, the difference is not statistically significant.

In both IDDM and control subjects t-PA antigen during exercise correlated with exercise intensity (%EMHR)(IDDM: $r=0.60$, $p<0.001$; controls: $r=0.71$, $p<0.001$)(see Figure 3). The ratio of t-PA to exercise intensity (t-PA / %EMHR) was 0.24 ± 0.11 in IDDM and 0.31 ± 0.13 in controls ($p<0.05$), *indicating* a *defective response* of t-PA to exercise. Likewise, the regression line of t-PA vs. %EMHR in Figure 3 is shifted downwards in IDDM subjects.

u-PA antigen levels during exercise (see Figure 4) were higher in IDDM (AUCex 15.1 ± 3.5 ng/ml*hr) than in controls (11.2 ± 1.8 ng/ml*hr, $p<0.05$). No correlation was seen between u-PA levels and exercise intensity.

Figure 3   The relationship between exercise intensity and t-PA antigen.



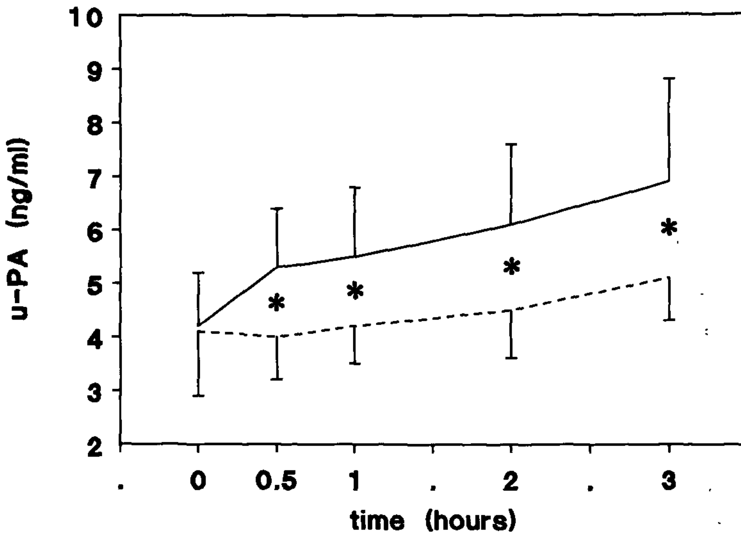
t-PA antigen levels increase in response to increasing exercise intensity in both IDDM (•) and nondiabetics (o). Exercise intensity (% EMHR) is expressed as the percentage of age-adjusted estimated maximal heart rate. The regression line in IDDM (y=0.85 x - 51) is displaced ($p<0.05$) compared to the regression line in nondiabetics (y=1.26 x - 76) in controls. Results are from pooled data of 5 measurements in 15 subjects.

PAP levels were similar in both groups at baseline (see Table 2). During exercise PAP rose progressively in both IDDM and control subjects (see Figure 5) and the AUCex were not different (49.2 ± 17.1 vs. 41.6 ± 19.5 nmol/l*hr, N.S.).

Both vWF and VIII:C increased in IDDM as well as in control subjects. The increase of vWF parallelled the increase of t-PA (see Table 2). The ratio of t-PA to vWF was similar in IDDM (0.104 ± 0.021) and controls (0.088 ± 0.020) and did not change during the exercise. In contrast, u-PA di not increase in parallel with vWF. Instead, u-PA increased in paralllel with VIII:C. The ratio of u-PA to VIII:C was similar in IDDM (0.019 ± 0.003) and controls (0.019 ± 0.004) and did not change during the

exercise.
FbDP as well as fibrinogen levels were similar in both IDDM and nondiabetic subjects (see Table 2) and remained stable throughout the exercise.
No correlation was found between pre-running blood glucose or glycosylated hemoglobin on the one hand, and basal or stimulated values of t-PA, u-PA, PAP, vWF or VIII:C on the other hand.

Figure 4 u-PA antigen during exercise in IDDM and nondiabetics.



u-PA antigen levels (mean ± SD) rise in both IDDM (solid line) and nondiabetics (dotted line) during a 3 hour, 32 km outdoor run. Levels at 0.5, 1, 2 and 3 hours are significantly higher in IDDM.
* $p<0.05$

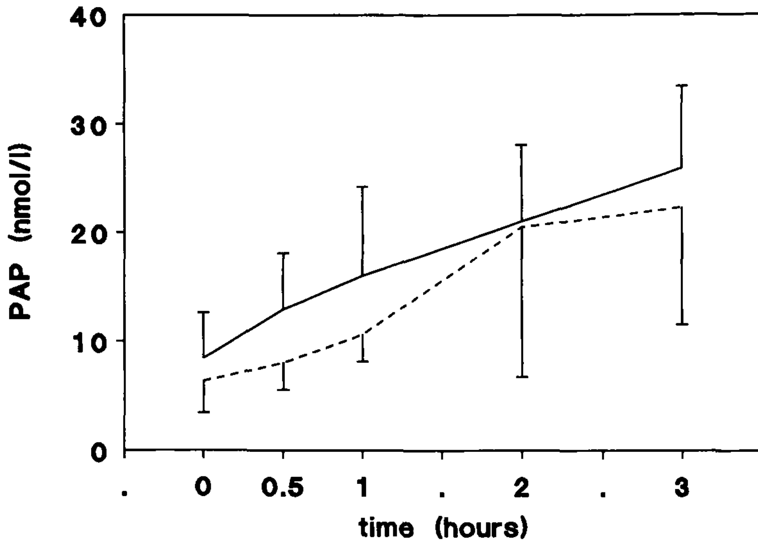
Table 2 Haemostatic variables in IDDM and in nondiabetic subjects during a 3 hr outdoor running session.

time (hrs)	0	0.5	1	2	3
t-PA Ag, ng/ml					
IDDM	10.3(3.0)	14.9(4.7)	17.4(5.0)	22.6(11.2)	26.7(14.5)
control	17.6(8.1)	19.9(5.6)	23.5(12.1)	28.5(15.2)	31.7(12.8)
u-PA Ag, ng/ml		*	*	*	*
IDDM	4.2(1.0)	5.3(1.1)	5.5(1.3)	6.1(1.5)	6.9(1.9)
control	4.1(1.2)	4.0(0.8)	4.2(0.7)	4.5(0.9)	5.1(0.8)
vWF, %	*	*	*	*	
IDDM	129(48)	169(65)	194(87)	222(113)	317(178)
control	228(98)	274(107)	303(103)	357(101)	452(161)
PAP, nmol/l		*			
IDDM	8.4(4.2)	12.9(5.2)	16.0(8.2)	21.0(7.1)	25.9(7.6)
contol	6.3(2.9)	8.0(2.5)	10.6(2.5)	20.5(13.8)	22.3(10.0)
VIII:C, %					
IDDM	236(39)	257(28)	280(30)	333(58)	431(107)
control	265(148)	278(156)	299(155)	298(116)	356(129)
FbDP, μg/l					
IDDM	224(32)	320(133)	277(74)	276(84)	283(96)
control	249(33)	267(42)	271(33)	299(91)	286(48)
Fgn, g/l					
IDDM	2.76(.51)	2.83(.56)	2.80(.46)	2.75(.45)	2.79(.37)
control	2.33(.22)	2.39(.29)	2.46(.45)	2.45(.26)	2.40(.25)

Values represent mean (SD). Normal morning basal values: tissue-tpe plasminogen activator antigen (t-PA): 5-15 ng/ml; urokinase-type plasminogen activator antigen (u-PA): 1-6 ng/ml; plasmin - α2 antiplasmin complexes (PAP): < 8 nmol/l; Von Willebrand Factor antigen (vWF): 31-260 %; Factor VIII activity (VIII:C): 47-217 %; fibrin degradation products (FbDP): < 1000 μg/l; intact fibrinogen (Fgn): 2-4 g/l.

*: IDDM vs. control $p<0.05$

Figure 5 Plasmin - α2 antiplasmin complexes during exercise in IDDM and nondiabetics



Levels of plasmin - α2 antiplasmin complexes (mean ± SD) continue to rise in both IDDM (solid line) and in nondiabetics (dotted line) during a 3 hour, 32 km outdoor run. There is no difference between IDDM and nondiabetics.

DISCUSSION

Release of plasminogen activators in IDDM has been studied following stimuli of short duration [8,25,26]. In this study, we followed plasminogen activator levels and in vivo fibrinolysis during prolonged simulation by means of submaximal exercise. The purpose of this study design was dual: first, to detect subtle abnormalities in plasminogen activator release in normoalbuminuric IDDM. The second purpose was to evaluate whether in IDDM exhaustion of plasminogen activator release occurs, as this would adversely affect the haemostatic balance and the consequence would be to discourage prolonged submaximal exercise in IDDM.

We have demonstrated that, in both type I diabetic and in healthy nondiabetic subjects, prolonged submaximal exercise results in a sustained increase in the plasma

levels of the plasminogen activators t-PA and u-PA. The progressive rise of these activators during exercise indicates continued secretion and demonstrates that depletion of t-PA and of u-PA stores does not occur in the measured time span of 3 hours. This is in contrast with the findings of Rosing et al. [13] and of Keber et al. [14,15], but confirms the data of Arai et al. who demonstrated enhanced fibrinolytic activity following a triathlon [27]. Whether a decreased clearance of plasminogen activators during exercise contibutes to the increases in plasma levels cannot be told from our study. However, the continued increase over 3 hours argues against accumulation due to a reduction in clearance as has been suggested by de Boer et al. [28]. Since the normal half-lives of t-PA and u-PA are 5 and 7 minutes respectively [29], a reduction in clearance secondary to exercise would have resulted in stable levels within half an hour [30].

The response in t-PA was somewhat lower in the IDDM subjects (see Figure 2), and in relation to the attained level of exercise appeared to be significantly decreased in IDDM in comparison with healthy control subjects (see Figure 3). These findings are in contrast with those of Jensen et al. [8], who found no significant differences in t-PA release between normoalbuminuric IDDM and controls. However, the exercise in that study was of shorter duration and the workload in relation to maximal exercise intensity was not given.

It has been suggested that age-adjusted heart rate is not an adequate measure of exercise intensity in diabetes [31]: due to autonomic nerve dysfunction in diabetes maximal aerobic exercise is achieved at lower heart rates than in controls. In our study however, the mean heart rate was highest in the IDDM. This demonstrates that the IDDM subjects performed the exercise at an exercise-intensity level at least as high or even higher than the control subjects.

The defective response of t-PA to exercise in IDDM, which was accompanied by an impaired response of vWF levels to exercise, suggests an endothelial defect in the studied IDDM [8,17]. To our knowledge, this is the first report demonstrating a defective response of t-PA and vWF to exercise in IDDM without apparent long-term sequelae.

In contrast to the defective responses of t-PA and vWF to exercise, IDDM individuals demonstrated a significantly larger increase in u-PA than the control individuals (see figure 4). The u-PA response to exercise was rather poor in our control subjects, as Dooijewaard et al. recently showed a 100 % rise in u-PA antigen following a 12 minute bout of exhaustive exercise in healthy male volunteers [7]. Although u-PA antigen did not correlate with exercise intensity in our study (data not shown), we cannot exclude the possibility that the submaximal level of exercise

intensity was insufficient to elicit a substantial rise of u-PA levels in the control subjects. The u-PA response of the IDDM subjects to exercise seems adequate and may compensate for the defective t-PA response [32], as u-PA and t-PA potentiate the effects of each other [33,34].

The origin of u-PA following exercise is unknown [7]. In both IDDM and controls the u-PA response to exercise parallelled VIII:C (believed to be of hepatic origin) but not t-PA or vWF (endothelial origin). Our findings suggest that the mechanism causing exercise-induced rise of u-PA is distinct from that of the t-PA and vWF response and point to hepatic rather than to endothelial origin of u-PA.

The net result of the impaired t-PA response and the increased u-PA response to exercise was a similar degree of plasminogen activation in IDDM and controls, as measured by plasminogen - α2 antiplasmin complexes (see Figure 5). Moreover, fibrin degradation products were similar in both groups and neither IDDM nor controls experienced changes in intact fibrinogen.

Basal values of t-PA and vWF appeared somewhat higher in the control subjects. This is strange, since levels of vWF are elevated rather than suppressed in diabetes mellitus [8,35]. We have no explanation for this finding except that the control subjects appeared to be more stressed than the IDDM subjects at the collection of blood for basal values.

A relation between fibrinolytic variables and degree of glycaemic control was looked for, but could not be demonstrated. As the numbers in this study are small, this does not exclude a relationship between fibrinolysis and the degree of metabolic control.

In conclusion, prolonged exercise in IDDM results in a sustained increase in plasma levels of the plasminogen activators t-PA and u-PA. Although the t-PA response to exercise is impaired, the enhanced u-PA response may act as a counterbalance. Given these findings, one can safely speculate that in IDDM without micro- or macrovascular complications prolonged exercise stimulates fibrinolysis and thereby contributes to the prevention of vascular disease.

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REFERENCES

1. Kannel W.B., McGee D.L.: Diabetes and cardiovascular disease - the Framingham Study. JAMA 1979; 241: 2035-2038
2. Juhan-Vague I., Collen D.: Hypofibrinolysis and atherothrombosis. Diabet Metab 1988; 14: 479-483
3. Hamsten A., Walldius G., Szamosi A., Blömback M., De Faire U., Dahlén G., Landou C., Wiman B.: Plasminogen activator inhibitor in plasma: risk factor for recurrent myocardial infarction. Lancet 1987; ii: 3-9
4. Kruithof E.K.O., Tran-Thang C., Bachmann F.: The fast-acting inhibitor of tissue-type plasminogen activator in plasma is also the primary plasma inhibitor of urokinase. Thromb Haemostas 1986; 55: 65-69
5. Prowse C.V., Farrugia A., Boulton F.E., Tucker J., Ludlam C.A., McLaren M., Belch J.J.F., Prentice C.R.M., Daews J., MacGregor I.R.: A comparative study using immunological and biological assays of the haemostatic responses to DDAVP infusion, venous occlusion and exercise in normal men. Thromb Haemostas 1984; 51: 110-114
6. Levi M., Ten Cate J.W., Dooijewaard G., Sturk A., Brommer E.J.P., Agnelli G.: DDAVP induces systemic release of urokinase-type plasminogen activator. Thrombos Haemostas 1989; 62: 686-689
7. Dooijewaard G., De Boer A., Turion P.N.C., Cohen A.F., Breimer D.D., Kluft C.: Physical exercise induces enhancement of urokinase-type plasminogen activator (u-PA) levels in plasma. Thromb Haemostas 1991; 65: 82-86
8. Jensen T., Bjerre-Knudsen J., Feldt-Rasmussen B., Deckert T.: Features of endothelial dysfunction in early diabetic nephropathy. Lancet 1989; i: 461-463
9. Rogers M.A., Yamamoto C., King D.S., Hagberg J.M., Ehsani A.A., Hollszy J.O.: Improvement in glucose tolerance after 1 week of exercise in patients with mild NIDDM. Diabetes Care 1988; 11: 613-618
10. Krotkiewski M., Lönnroth P., Mandroukas K., Wrolebki Z., Rebuffé-Scrive M., Holm G., Smith U., Björntorp P.: The effects of physical training on insulin secretion and effectiveness and on glucose metabolism in obesity and type 2 (non-insulin-dependent) diabetes mellitus. Diabetologia 1985; 28: 881-890
11. Vignati L., Cunningham L.N.: Exercise and diabetes. In: Joslin's Diabetes Mellitus, 12th edn. A. Marble, L.P. Krall, R.F. Bradley, A.R. Christlieb, J.S. Soeldner, eds. Lea & Febiger, Philadelphia, 1985. pp 453-464.
12. Meinders A.E., Willekens F.L., Heere L.P.: Metabolic and hormonal changes in IDDM during long-distance run. Diabetes Care 1988; 11: 1-7
13. Rosing D.R., Brakman P., Redwood D.R., Goldstein R.E., Beiser G.D., Astrup T., Epstein S.E.: Blood fibrinolytic activity in man. Diurnal variation and the response to varying intensities of exercise. Circul Res 1970; 27: 171-184
14. Keber D., Stegnar M., Keber I., Accetto B.: Influence of moderate and strenuous daily physical activity on fibrinolytic activity of blood: possibility of plasminogen activator stores depletion. Thrombos Haemostas 1979; 41: 745-755
15. Keber D., Stegnar M.: Exhaustion of arm fibrinolytic potential after repeated venous occlusions and local exercise. Thromb Res 1982; 28: 693-704
16. Astrand P.O., Rodahl K. textbook of work physiology. McGraw-Hill, Philadelphia, 1970
17. Porta M., La Selva M., Molinatti P.A.: Von Willebrand Factor and endothelial abnormalities in diabetic microangiopathy. Diabetes Care 1991; 14, Suppl. 1: 167-172
18. Zimmerman T.S., Meyer D.: Structure and function of factor VIII / von Willebrand factor. In: Haemostasis and thrombosis. A.L. Bloom and D.P. Thomas, eds. Churchill Livingstone, Edinburgh, 1981. pp 111-123
19. Binnema D.J., van Iersel J.J.L., Dooijewaard G.: Quantitation of urokinase antigen in plasma and culture media by use of an ELISA. Thromb Res 1986; 43: 569-577

20. Bröcker-Vriends A.H.J.T., Briët E., Quadt R. et al.: Genotype assignment of haemophilia A by use of intragenic and extragenic restriction length polymorphisms. Thromb Haemostas 1987; 57: 131-136
21. Koppert P.W., Hoegee-de Nobel E., Nieuwenhuizen W.: A monoclonal antibody-based enzyme immunoassay for fibrin degradation products in plasma. Thromb Haemostas 1988; 59: 310-315.
22. Hoegee-de Nobel E., Voskuilen M., Briët E., Brommer E.J.P., Nieuwenhuizen W.: A monoclonal antibody-based quantitative enzyme immunoassay for the determination of plasma fibrinogen concentrations. Thromb Haemostas 1988; 60: 415-418
23. Van Deventer S.J.H., Büller H.R., ten Cate J.W., Aarden L.A., Hack C.E., Sturk A.: Experimental endotoxaemia in humans: analysis of cytokine release and coagulation, fibrinolytic, and complement pathways. Blood 1990; 76: 2520-2526
24. Andreotti F., Davies G.J., Hackett D., et al.: Circadian variation of fibrinolytic factors in normal human plasma. Fibrinolysis 1988; 2 (Suppl 2): 90-92
25. Cash J.D., McGill R.C.: Fibrinolytic response to moderate exercise in young male diabetics and non-diabetics. J Clin Pathol 1969; 22: 32-35
26. Sundkvist G., Almér L.-O., Lilja B., Pandolfi M.: Growth hormone and endothelial function during exercise in diabetics with and without retinopathy. Acta Med Scand 1984; 215: 55-61
27. Arai M., Yorifuji H., Ikematsu S. et al.: Influences of strenuous exercise (triathlon) on blood coagulation and fibrinolytic system. Thromb Res 1990; 57: 465-471
28. A. de Boer: Drug interaction studies with anticoagulant, antiplatelet and thrombolytic drugs in healthy subjects. Chapter 12. Ph.D. thesis, Leiden, 1990.
29. Marder V.J., Sherry S.: Thrombolytic therapy: current status. N Engl J Med 1988; 318: 1514-1520, 1585-1595
30. Benet L.Z., Sheiner L.B.: Pharmacokinetics: the dynamics of drug absorption, distribution, and elimination. In: Goodman and Gilman's The pharmacologic basis of therapeutics, 7th ed. A.Goodman Gillman, L.S. Goodman, T.W. Rall, F. Murad, eds. MacMillan, New York, 1985. pp 3-34.
31. Fujita Y., Kawaji K., Kanamori A. et al.: Relationship between age-adjusted heart rate and anaerobic threshold in estimating exercise intensity in diabetics. Diabetes Res Clin Pract 1990; 8: 69-74
32. Huisveld I.A., Kluft C., Hospers A.J.H., Bernink M.J.E., Erich W.B.M., Bouma B.N.: Effect of exercise and oral contraceptive agents on fibrinolytic potential in trained females. J Appl Physiol: Respirat Environ Exercise Physiol 1984; 56: 906-913
33. Pannell R., Black J., Gurewich V.: Complementary modes of action of tissue-type plasminogen activator and pro-urokinase by which their synergistic effect on clot lysis may be explained. J Clin Invest 1988; 81: 853-859
34. Collen D., Stassen J.M., Stump D.C., Verstraete M.: Synergism of thrombolytic agents in vivo. Circulation 1986; 74: 838-842
35. Ostermann H., Van de Loo J.: Factors of the hemostatic system in diabetic patients. A survey of controlled studies. Haemostas 1986; 16: 386-416

CHAPTER 4

THE CARDIOVASCULAR RISK FACTOR PLASMINOGEN ACTIVATOR INHIBITOR TYPE 1 (PAI-1) IS RELATED TO INSULIN RESISTANCE.

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SUMMARY

The cardiovascular risk factor plasminogen activator inhibitor type 1 (PAI-1) has been associated with abdominal type obesity, hypertension, hypertriglyceridaemia, hyperinsulinaemia, glucose intolerance and type II diabetes, conditions known to be linked with insulin resistance. To determine whether PAI-1 is related to insulin resistance we studied 9 obese nondiabetics and 10 obese type II diabetics by means of a sequential hyperinsulinaemic euglycaemic clamp study. Plasma PAI-1 antigen (Ag) correlated significantly with peripheral insulin resistance, represented by the insulin level at which peripheral glucose uptake is half-maximal (ED_{50}pgu): $r=0.87$, $p<0.001$. Multiple regression analysis including indices of hepatic and peripheral insulin action, fasting plasma insulin levels, triglycerides, blood pressure, waist-hip ratio and body mass index disclosed ED_{50}pgu to account for 76% of the variance of PAI-1 Ag. We suggest that PAI-1 contributes to the increased cardiovascular risk encountered with insulin resistance.

INTRODUCTION

A constellation of cardiovascular risk markers, referred to as syndrome X [1], has been identified, consisting of abdominal obesity, hypertension, elevated fasting plasma levels of very low-density lipoprotein triglycerides and reduced levels of high-density lipoprotein cholesterol, hyperinsulinaemia, glucose intolerance and type II diabetes mellitus. The common denominator of this syndrome is insulin resistance [1].

Plasminogen activator inhibitor type 1 (PAI-1) is a potent inhibitor of fibrinolysis, by binding to and rapidly inactivating both tissue-type plasminogen activator and urokinase-type plasminogen activator [2]. Increased levels of PAI-1 and decreased plasma fibrinolytic activity have been demonstrated in survivors of acute myocardial infarction [3-5] and in patients with coronary artery stenosis [6-8]. Furthermore, PAI-1 is of prognostic value in predicting recurrence of myocardial infarction [9].

A positive association between fasting plasma insulin levels and PAI-1 has been demonstrated by several authors [10-12]. In addition, PAI-1 has been linked to separate features of syndrome X, including abdominal obesity [13], hypertension [14-16], hypertriglyceridaemia [3,7,12,14,16], glucose intolerance and type II diabetes mellitus [11,14]. The associations of PAI-1 with the features of syndrome X suggest a link between PAI-1 and insulin resistance.

To elucidate whether such a relationship exists, we determined both plasma levels of PAI-1 antigen (Ag) and of insulin, and directly measured insulin action, by means of the hyperinsulinaemic euglycaemic glucose clamp technique.

PATIENTS AND METHODS

Nine obese nondiabetic (group I) and ten obese type II diabetic (group II) subjects participated in the study, after having given informed consent. The protocol had been approved by the Ethical Committee of the University Hospital Leiden. Clinical characteristics are given in Table 1. Nondiabetic subjects had no family history of diabetes and a normal glucose tolerance test [17]. Diabetic subjects were treatedwith diet only. None had clinical macrovascular disease, proteinuria or (pre)proliferative retinopathy.

All subjects had a systolic blood pressure 160 mmHg or less and a diastolic blood pressure 95 mm Hg or less, and none were treated with antihypertensive medication. Blood pressure (BP) was measured in sitting position after 5 minutes of rest. Korot-

koff phase V was recorded as diastolic blood pressure. Body mass index (BMI) was calculated as weight divided by height square (kg/m^2). Waist-hip ratio (WHR) was calculated according to Krotkiewski [18].

Table 1. Clinical characteristics of participating subjects

	NONDIABETIC	DIABETIC	
age (years)	47 ± 2	52 ± 2	N.S.
sex (m:f)	5 : 4	6 : 4	N.S.
WHR	0.96 ± 0.02	0.98 ± 0.02	N.S.
BMI (kg/m^2)	32.6 ± 1.1	33.8 ± 1.0	N.S.
BP (mean, mmHg)	138 / 81	144 / 84	N.S.
FBG (mmol/l)	4.7 ± 0.2	9.4 ± 1.4	$p<0.001$
HbA1c (%)	5.3 ± 0.1	7.7 ± 0.4	$p<0.001$
Triglycerides (mmol/l)	2.5 ± 0.84	3.5 ± 1.1	N.S.

WHR: waist-hip ratio
BMI: body mass index
BP: blood pressure
FBG: fasting blood glucose
Hb_{A1c}: reference values 4.4 - 6.7 %

Clamp studies

Insulin action was measured by means of the sequential hyperinsulinaemic euglycaemic glucose clamp technique [19], in combination with a 3-^{3}H-glucose labelled tracer infusion [20]. Subjects with diabetes were rendered normoglycaemic by means of an overnight variable insulin infusion prior to the clamp study. Glucose was "clamped" at 5 mmol/l by infusion of 20 % dextrose at a variable rate, which was adjusted every 5 minutes to the corresponding plasma glucose level (Glucose Analyzer, Beckman Instruments, Palo Alto, CA, USA). Insulin (Humuline Regular, Eli Lilly, Indianapolis, Ind, USA) was infused during four consecutive two-hour periods at the following rates: "basal" - 0.75 - 1.5 - 10 mU/kg/min, following bolus injections of 0, 4, 8 and 100 mU/kg at the start of each two-hour period. "Basal" equals zero in nondiabetics and 0.31 ± 0.06 mU/kg/min in subjects with diabetes, i.e. the dose necessary to obtain a fasting plasma glucose of 5 mmol/l without the need for concurrent dextrose infusion.

EDTA-anticoagulated plasma was examined half-hourly and, during the last 30 minutes of each two-hour period, at 10-minute intervals for determination of the momentary insulin level and plasma 3-^{3}H-glucose specific activity [20]. Peripheral glucose uptake (PGU) was calculated as either the quotient of 3-^{3}H-glucose infusion rate and plasma 3-^{3}H-glucose specific activity, or as the rate of infusion of exogenous glucose, whichever was the highest. Hepatic glucose production (HGP) was calculated as the difference between PGU and exogenous infusion rate. A dose-response curve was constructed relating the momentary insulin level to PGU. The dose-response curve can be characterized by the maximal PGU (V_{max}pgu), representing peripheral insulin responsiveness, and by the insulin level at which PGU is half-maximal (ED_{50}p-gu), representing peripheral insulin sensitivity [21]. In the case of a decrease in insulin sensitivity, i.e. insulin resistance, the curve is shifted to the right, which is represented by an increase in ED_{50}pgu. Similarly, hepatic insulin sensitivity is represented by the insulin level at which HGP is half-maximally suppressed (ED_{50}hgp) [21].

PAI-1 Antigen

PAI-1 Ag follows a diurnal rhythm with a peak in the morning hours [22,23]. Therefore, blood was collected, at 9:00 a.m. just prior to the clamp study, in 3.8 % sodium citrate . To validate the results in the diabetics on a day without prior overnight insulin infusion, blood was collected on a control day within six weeks of the clamp at 9:00 a.m. for determination of PAI-1 Ag.

The collected blood was centrifuged at 2000 x g for 20 minutes at 4° C to obtain platelet free plasma for determination of PAI-1 Ag (TintElize lot number 210220, Biopool, Umeå, Sweden). All assays were carried out in duplicate, the interassay coefficient of variation for PAI-1 Ag was 8 % and the intra-assay coefficient 8 %.

Fasting plasma insulin, proinsulin and triglycerides

Because the diabetics had received insulin during the night prior to the clamp, plasma levels of insulin, proinsulin and triglycerides in the diabetics were suppressed on the day of the clamp. Serum and plasma, therefore, were collected in both the diabetics and the nondiabetics after an overnight fast on two different occasions within six weeks of the clamp study for determination of fasting plasma immunoreactive insulin [24], fasting plasma proinsulin [25] and triglycerides [26]. The mean of each pair of values, obtained in every person, was taken.

Statistics

Results are expressed as mean ± SEM. Statistical analysis was performed by bivariate and multivariate regression analysis and by t-testing. In cases of non-normal group distribution, nonparametric tests were used. A probability level (two-tailed test) below 0.05 was considered significant.

RESULTS

Clamp studies

Plasma glucose averaged 5.06 ± 0.04 mmol/l (Coëfficient of variation 3.3 %) during the clamp. Insulin levels were 16 ± 3 and 38 ± 8 mU/l during the first 2-hr period in groups I and II respectively. Subsequent periods yielded insulin levels of 96 ± 5, 199 ± 11 and 4548 ± 226 mU/l and were similar in both groups. ED_{50}pgu ranged from 62 to 394 mU/l (group I: 127 ± 21 mU/l; group II: 206 ± 34 mU/l, N.S.). V_{max}pgu ranged from 25.8 to 81.6 μmol/kg/min (group I: 59.2 ± 3.5 μmol/kg/min; group II: 42.3 ± 3.6 μmol/kg/min, $p<0.01$). Basal HGP ranged from 7.8 to 12.2 μmol/kg/min (group I: 9.2 ± 0.4 μmol/kg/min; group II: 10.3 ± 0.4 μmol/kg/min, N.S.). ED_{50}hgp ranged from 14 to 114 mU/l (group I: 28 ± 4; group II: 66 ± 11 mU/l, N.S.).

PAI-1 Antigen

On the day of the clamp at 9:00 a.m. PAI-1 Ag ranged from 15 to 91 ng/ml (group I: 34 ± 8 ng/ml; group II: 44 ± 7 ng/ml, N.S.). PAI-1 Ag on the control day in the diabetics was 49 ± 7 ng/ml.

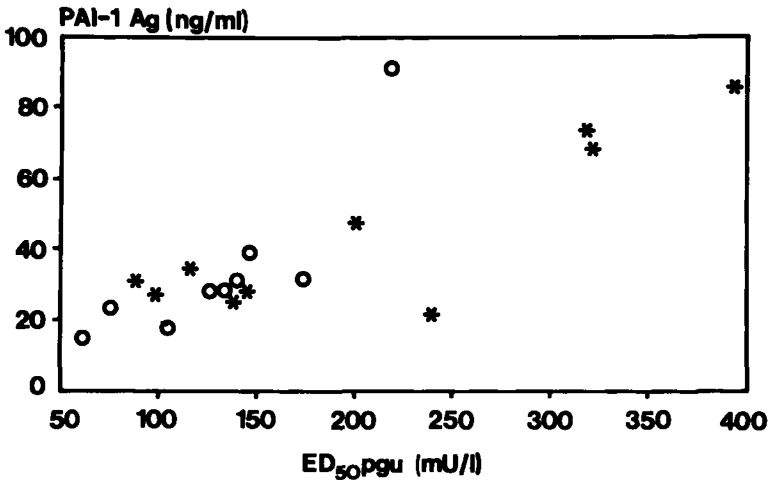
Fasting plasma insulin, proinsulin and triglycerides

Fasting plasma insulin ranged from 7 to 44 mU/l (group I: 14 ± 2 mU/l; group II: 22 ± 3 mU/l, N.S.). Fasting plasma proinsulin ranged from 107 to 607 ng/l (group I: 160 ± 11 ng/l; group II: 354 ± 54 ng/l, $p<0.001$). Triglycerides ranged from 0.9 to 11.8 mmol/l (group I: 2.1 ± 0.5; group II: 3.3 ± 1.0 mmol/l, N.S.).

Correlations

A significant correlation was found between PAI-1 Ag on the clamp day and ED_{50}pgu in both group I ($r=0.98$, $p<0.001$) and group II ($r=0.87$, $p<0.01$).

Figure 1 The relation between peripheral insulin sensitivity and plasma plasminogen activator inhibitor type 1 antigen (PAI-1 Ag).



Plasma levels of PAI-1 Ag correlate strongly ($r=0.87$, $p<0.001$) with the insulin level at which peripheral glucose uptake is half-maximal (ED_{50}pgu), which represents the degree of resistance to the action of insulin on peripheral tissues. In both nondiabetic (o) subjects ($r=0.98$, $p<0.001$) and in type II diabetic (*) subjects ($r=0.87$, $p<0.01$) this correlation is significant.

Further correlation studies were performed on the combined groups, after excluding significant differences in regression lines of the individual groups. Figure 1 demonstrates the correlation between PAI-1 Ag and ED_{50}pgu ($r=0.87$, $p<0.001$). In addition PAI-1 Ag correlated with V_{max}pgu ($r=-0.51$, $p<0.05$) and with ED_{50}hgp ($r=0.67$, $p<0.01$). As expected, PAI-1 Ag correlated with fasting plasma insulin levels ($r=0.65$, $p<0.01$). Furthermore, the previously-described correlations between PAI-1

on the one hand, and BMI, WHR, triglycerides, systolic and diastolic blood pressure on the other, were not significant in our small number of subjects.

PAI-1 Ag on the day of the clamp was not significantly related to fasting plasma proinsulin (r=0.37).

Multiple regression analysis including the parameters of insulin action (ED_{50}pgu, V_{max}pgu, basal HGP, ED_{50}hgp) and the factors known to be associated with PAI-1 [10,12-16] (fasting plasma insulin, BMI, WHR, triglycerides, systolic and diastolic blood pressure), identified ED_{50}pgu, V_{max}pgu, diastolic blood pressure, BMI and WHR to be the only variables significantly related to PAI-1 Ag ($F=24.4$, $p<0.001$), explaining 90 % of the variance of PAI-1 Ag (see Table 2). 76 % of the variance of PAI-1 Ag could be explained by ED_{50}pgu, each of the remaining four parameters accounting for 1 - 9 % of the variance. Fasting plasma insulin was not independently associated with PAI-1 Ag.

Table 2 Multivariate regression analysis of plasminogen activator inhibitor type 1 antigen in nondiabetics and type II diabetics.

$F=24.4$, $p<0.001$, $R^2=0.90$, adjusted $R^2=0.87$

	ß ± SE	t-value	Sequential R^2
ED_{50}pgu	.34 ± .04	8.9	76 %
V_{max}pgu	.65 ± .22	2.9	79 %
DBP	-.80 ± .32	-2.5	80 %
Body mass index	1.72 ± .67	2.6	81 %
Waist hip ratio	-145 ± 42	-3.5	90 %

The independent variables included in the regression model were: ED_{50}pgu, V_{max}pgu, HGP, ED_{50}hgp, fasting plasma insulin level, body mass index (BMI), waist-hip ratio (WHR), fasting plasma triglycerides, sytolic blood pressure and diastolic blood pressure (DBP).

ß denotes the regression coefficient, ED_{50}pgu denotes the insulin level at which peripheral glucose uptake is half-maximal, V_{max}pgu represents maximal peripheral glucose uptake.

Substitution of the diabetics' PAI-1 Ag values on the clamp day with values obtained on the control day without prior insulin infusion essentially resulted in a similar outcome of the regression analysis ($F=12.4$, $p<0.001$): $ED_{50}pgu$ accounted for 54 % of the variance, $V_{max}pgu$ for 4 % and BMI for 13 %, whereas fasting plasma insulin was not independently related to PAI-1 Ag.

DISCUSSION

In this study we have shown a strong correlation between peripheral insulin resistance, as expressed by $ED_{50}pgu$ derived from euglycaemic clamp data, and morning plasma levels of PAI-1 Ag (see Figure 1). The correlation between $ED_{50}pgu$ and levels of PAI-1 Ag appeared dominant in a multivariate regression analysis including parameters of insulin action and parameters known to be associated with PAI-1. To validate the relationship between insulin resistance and PAI-1 Ag, we also determined PAI-1 Ag in the diabetics on a control day, on which they had not received prior overnight insulin. Substitution of the original value of PAI-1 Ag in the multivariate regression analysis with this alternative confirmed the results.

Our results seem to contrast with previously-published data, which emphasise the relationship between levels of PAI-1 and insulin [10-13,15,16]. Nevertheless, like these authors we too found a significant correlation between fasting plasma insulin levels and PAI-1. However, as we directly measured peripheral and hepatic insulin action and included the results of these measurements in the multiple regression analysis, the relationship between fasting plasma insulin levels and PAI-1 Ag appeared secondary to the association of PAI-1 Ag and peripheral insulin resistance. Apparently, the link is not between insulin and PAI-1, but between peripheral insulin resistance and PAI-1.

To our knowledge, only Landin et al. have directly assessed the relation between PAI-1 and insulin action by clamp studies. Their results are in line with ours, demonstrating an inverse relationship between PAI-1 activity and glucose disposal in normotensive and in hypertensive men [16] and in obese and lean women [13]. These authors performed clamp studies with a single insulin level and measured PAI-1 activity rather than PAI-1 antigen. The association between glucose disposal and PAI-1 in their studies seems weaker than the associations of PAI-1 and insulin. Moreover, they did not perform a multivariate analysis. This, in combination with the differences in techniques may explain the apparent contradictions between their studies and ours.

It appears therefore, that PAI-1 is associated with insulin resistance, hypertensi-

on [14,16], hypertriglyceridaemia [3,7,8,12,14,16], abdominal obesity [13], hyperinsulinaemia [10-13,15,16] and type II diabetes mellitus [11,14,27]. As all the above features are co-related and all are associated with insulin resistance, this suggests that high PAI-1 antigen levels should be included in the syndrome of insulin resistance. However, caution is warranted, since we have only studied obese middle-aged normotensive nondiabetic and type II diabetic subjects.

It is not clear what is the mediator between insulin resistance and PAI-1. It has been suggested that insulin may be such a mediator [28]. In vitro both insulin and proinsulin promote the secretion of PAI-1 by cultured hepatocytes [29,30], but experimental hyperinsulinaemia does not elicit an increase in PAI-1 in humans [31,32]. Recent findings of increased fasting plasma levels of proinsulin in insulin-resistant states [33-35], as well as the occurrence of cardiovascular disease following proinsulin administration [36], have prompted us to investigate whether fasting plasma proinsulin levels are more closely associated with PAI-1 than is insulin sensitivity. However, the correlation between proinsulin and PAI-1 Ag appeared insignificant. In addition, had proinsulin been included in the multiple regression analysis (data not shown), it would have lowered PAI-1 Ag. Therefore it is unlikely that proinsulin is a mediator between insulin resistance and elevated PAI-1 Ag levels.

In conclusion, we have demonstrated that plasma levels of PAI-1 antigen are related to peripheral insulin resistance in middle-aged obese nondiabetic and obese type II diabetic subjects. The mechanisms of this relationship require further elucidation. We suggest that the increased cardiovascular risk in insulin-resistant conditions may be mediated in part by elevated levels of PAI-1.

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REFERENCES

1. Reaven G.M.: Role of insulin resistance in human disease. Diabetes 1988; 37: 1595-1607.
2. Sprengers E.D., Kluft C.: Plasminogen activator inhibitors. Blood 1987; 69: 381-387.
3. Hamsten A., Wiman B., de Faire U., Blombäck M.: Increased plasma levels of a rapid inhibitor of tissue plasminogen activator in young survivors of myocardial infarction. N Engl J Med 1985; 313: 1557-1563

4. Nilsson T.K., Johnsson O.: The extrinsic fibrinolytic system in survivors of myocardial infarction. Thromb Res 1987; 48: 621-630.
5. Almér L.O., Ohlin H.: Elevated levels of the rapid inhibitor of plasminogen activator (t-PAI) in acute myocardial infarction. Thromb Res 1987; 47: 335-339
6. Francis R.B., Kawanishi D., Baruch T., Mahrer P., Rahimtoola S., Feinstein D.I.: Impaired fibrinolysis in coronary artery disease. Am Heart J 1988; 115: 776-780
7. Aznar J., Estellés A., Tormo G., Sapena P., Tormo V., Blanch S., España F.: Plasminogen activator inhibitor activity and other fibrinolytic variables in patients with coronary artery disease. Br Heart J 1988; 59: 535-541
8. Olofsson B.O., Dahlén G., Nilsson T.K.: Evidence for increased levels of plasminogen activator inhibitor and tissue plasminogen activator in plasma of patients with angiographically verified coronary artery disease. Eur Heart J 1989; 10: 77-82
9. Hamsten A., deFaire U., Walldius G., Dahlén G., Szamosi A., Landou C., Blombäck M., Wiman B.: Plasminogen activator inhibitor in plasma: risk factor for recurrent myocardial infarction. Lancet 1987; ii: 3-9
10. Vague P., Juhan-Vague I., Aillaud M.F., Badier C., Viard R., Alessi M.C., Collen D.: Correlation between blood fibrinolytic activity, plasminogen activator inhibitor level, plasma insulin level, and relative body weight in normal and obese subjects. Metabolism 1986; 35: 250-253.
11. Juhan-Vague I., Roul C., Alessi M.C., Ardissone J.P., Heim M., Vague P.: Increased plasminogen activator inhibitor activity in non insulin dependent diabetic patients - relationship with plasma insulin. Thromb Haemostas 1989; 61: 370-373
12. Juhan-Vague I., Vague P., Alessi M.C., Badier C., Valadier J., Aillaud M.F., Atlan C.: Relationship between plasma insulin, triglyceride, body mass index and plasminogen activator inhibitor 1. Diab Metab 1987; 13: 331-336
13. Landin K., Stigendahl L., Eriksson E., Krotkiewski M., Risberg B., Tengborn L., Smith U.: Abdominal obesity is associated with an impaired fibrinolytic activity and elevated plasminogen activator inhibitor-1. Metabolism 1990; 39: 1044-1048.
14. Heinrich J., Nguyên T., Kokott R., Epping P.H., Schulte H., Assmann G.: Interrelationships between coronary risk factors and the fibrinolytic system. Fibrinolysis 1990; 4 (Suppl 1): 37 (abstr)
15. Auwerx J., Bouillon R., Collen D., Geboers J.: Tissue-type plasminogen activator antigen and plasminogen activator inhibitor in diabetes mellitus. Arteriosclerosis 1988; 8: 68-72.
16. Landin K., Tengborn L., Smith U.: Elevated fibrinogen and plasminogen activator inhibitor (PAI-1) in hypertension are related to metabolic risk factors for cardiovascular disease. J Int Med 1990; 227: 273-278.
17. National Diabetes Data Group: Classification and diagnosis of diabetes mellitus and other categories of glucose intolerance. Diabetes 1979; 28: 1039 - 1057
18. Krotkiewski M., Björntorp P., Sjöström L., Smith U.: Impact of obesity on metabolism in men and women. Importance of regional adipose tissue distribution. J Clin Invest 1983; 72: 1150-1162
19. DeFronzo R.A., Tobin J.D., Andres R.: Glucose clamp technique: a method for quantifying insulin secretion and resistance. Am J Pysiol 1979; 237: E214-223
20. Nankervis A., Proietto J., Aitken P., Harewood M., Alford F.: Differential effects of insulin therapy on hepatic and peripheral insulin sensitivity in Type II (Non-insulin-dependent) diabetes. Diabetologia 1982; 23: 320-325
21. Kahn C.R.: Insulin resistance, insulin insensitivity, and insulin unresponsiveness: a necessary distinction. Metabolism 1978; 27, Supp 2: 1893-1902
22. Kluft C., Jie A.F.H., Rijken D.C., Verheijen J.H.: Daytime fluctuations in blood of tissue-type plasminogen activator (t-PA) and its fast-acting inhibitor (PAI-1). Thromb. Haemostas. 1989; 59: 329-332.
23. Andreotti F., Davies G.J., Hackett D. et al.: Circadian variation of fibrinolytic factors in normal human plasma. Fibrinolysis 1988; 2 (suppl 2): 90-92

24. Kuzuya H., Blix P.M., Horwitz D.L., Steiner D.F., Rubenstein A.H.: Determination of free and total insulin and C-peptide in insulin-treated diabetics. Diabetes 1976; 26: 22-29
25. Huisman J., Oolbekkink M., Erkelens D.W., Bowsher R.R., Thijssen J.H.H.: Radioimmunoassay for human proinsulin. In: J. Huisman: Proinsulin; Influence of exogenous administration of rDNA proinsulin on carbohydrate and lipid metabolism. Ph.D. Thesis, Utrecht, The Netherlands, 1990. pp 47-65
26. Rantela G.S., Hall R.G., Bekie S.Z. et al.: A kinetic method for the rapid and automatic determination of triglycerides in biological fluids. Clin Chem 1976; 20: 476-481
27. Small M., Kluft C., MacCuish A.C., Lowe G.D.O.: Tissue plasminogen activator inhibition in diabetes mellitus. Diabetes Care 1989; 12: 655-658
28. Juhan-Vague I., Vague Ph.: Interrelationships between carbohydrates, lipids, and the hemostatic system in relation to the risk of thrombotic and cardiovascular disease. Am J Obstet Gynecol 1990; 163: 313-315
29. Alessi M.C., Juhan-Vague I., Kooistra T., Declerck P.J., Collen D.: Insulin stimulates the synthesis of plasminogen activator inhibitor 1 by the human hepatocellular cell line Hep G2. Thromb Haemostas 1988; 60: 491-494
30. Kooistra T., Bosma P.J., Töns H.A.M., van den Berg A.P., Meyer P., Princen H.M.G.: Plasminogen activator inhibitor 1: Biosynthesis and mRNA level are increased by insulin in cultured human hepatocytes. Thromb Haemostas 1989; 62: 723-728
31. Potter van Loon B.J., de Bart A.C.W., Radder J.K., Frölich M., Kluft C., Meinders A.E.: Acute exogenous hyper-insulinaemia does not result in elevation of plasma plasminogen activator inhibitor - 1 (PAI-1) in humans. Fibrinolysis 1990; 4, Supp 2: 93-94
32. Landin K., Tengborn L., Chmielewska J., von Schenck H., Smith U.: The acute effect of insulin on tissue plasminogen activator and plasminogen activator inhibitor in man. Thromb Haemostas 1991; 65: 130-133
33. Temple R.C., Carrington C.A., Luzio S.D., Owens D.R., Schneider A.E., Sobey W.J., Hales C.N.: Insulin deficiency in non-insulin-dependent diabetes. Lancet 1989; i: 293-295.
34. Porte D., Kahn S.E.: Hyperproinsulinaemia and amyloid in NIDDM. Clues to etiology of islet beta-cell dysfunction. Diabetes 1989; 38: 1333-1336.
35. Madsbad S., Tronier B., Dejgaard A., Andersen T.: Plasma proinsulin in obesity and type 2 (non-insulin-dependent) diabetes mellitus. Diabetologia 1990; 33 (Suppl): A212.
36. Spradlin C.T., Galloway J.A., Anderson J.H.: Apparent increase in coronary heart disease with human proinsulin (HPI) - randomization failure or HPI effect ? Diabetologia 1990; 33 (Suppl): A60.

CHAPTER 5

ACUTE EXOGENOUS HYPERINSULINAEMIA DOES NOT RESULT IN ELEVATION OF PLASMA PLASMINOGEN ACTIVATOR INHIBITOR-1 (PAI-1) IN HUMANS.

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SUMMARY

In vitro, insulin stimulates synthesis and release of PAI-1 by hepatocytes. To test whether insulin infusion results in elevation of PAI-1 levels in vivo, we studied PAI-1 levels in 9 obese nondiabetic and in 10 obese type II diabetic subjects during and after a sequential hyperinsulinaemic euglycaemic glucose clamp. Insulin levels up to ± 4500 mU/l failed to overrule diurnal changes of PAI-1. PAI-1 levels 6 hrs after the start of a supraphysiologic insulin dose were not different from control values at the same time of the day. These findings suggest that exogenous hyperinsulinaemia does not result in significant increases of PAI-1 in vivo.

INTRODUCTION

In vitro, insulin stimulates synthesis and release of plasminogen activator inhibitor-1 (PAI-1) by hepatocytes [1,2]. In vivo, elevated levels of PAI-1 are associated with type II diabetes [3,4] and hyperinsulinaemia [5,6], conditions known to result in an accelerated rate of atherosclerosis . On the other hand, elevation of PAI-1 by itself is associated with increased cardiovascular risk [7,8]. By reduction of fibrinolytic activity [9], elevated PAI-1 levels may be of pathogenetic significance for development of atherosclerotic lesions.

To examine whether administration of insulin results in increases of PAI-1, we studied PAI-1 levels in nine obese nondiabetic subjects and in ten obese type II diabetic patients during and after a sequential hyperinsulinaemic euglycaemic clamp.

METHODS

9 obese nondiabetic subjects (age 47 ± 2.5 yrs [mean ± SEM], body mass index(BMI) 32.6 ± 1.1 kg/m2) and 10 obese type II diabetic patients (age 52 ± 2.0 yrs, BMI 33.8 ± 1.0 kg/m2, Hb_{A1c} 7.7 ± 0.4 %), treated with diet only, were recruited. Normal reference values for Hb_{A1c} are 4.4 - 6.7 %.

Diabetic patients were rendered euglycaemic by an overnight variable insulin infusion prior to the clamp. In both the diabetic and the nondiabetic subjects a forearm vein was cannulated retrogradely and placed in a heated box to obtain arterialized blood for determinations of plasma glucose, immunoreactive insulin (IRI), and PAI-1 antigen. Insulin was infused in four consecutive two-hour periods in the contralateral arm vein. Insulin infusion rates were 0 (in nondiabetic subjects) and 0.31 ± 0.06 mU/kg/min (in diabetic patients) during the first two-hour period, and 0.75, 1.50 and 10 mU/kg/min in subsequent two-hour periods (see figure 1). At the beginning of the second to fourth two-hour periods a bolus was given (4, 8 and 100 mU/kg respectively). The plasma glucose was clamped at 5 mmol/l by a variable glucose infusion.

Blood was collected each 5 minutes for determination of plasma glucose, half-hourly for determination of IRI and two-hourly for determination of PAI-1 antigen during a period of 12 hours, from 9:00 to 21:00 hrs. As a control, blood was collected on another day at 21:00 hrs. Blood for determination of PAI-1 antigen was collected in siliconized tubes containing citrate, theofylline , adenosine and dipyramidole

(CTAD), placed on ice and centrifuged (20 minutes 2500 g) within 10 minutes at 4°C to obtain platelet poor plasma (PPP). Aliquots of PPP were snap frozen and stored at -70°C until processing.

Plasma glucose was determined by the glucose oxidase method on a Beckman analyzer within forty seconds, IRI was measured by radioimmunoassay, and PAI-1 antigen was measured by enzyme immunoassay (Tintelize, Biopool AB, Umeå, Sweden).

Results are expressed as mean ± SEM. Statistical analysis was performed using Friedman's test for repeated measurements and Wilcoxon's signed rank test for paired measurements within groups. Between groups, the Mann-Whitney U-test was applied. All calculations were made with Number Cruncher Statistical System (NCSS).

The protocol had been approved by the Medical Ethical Committee of the University Hospital, Leiden

RESULTS

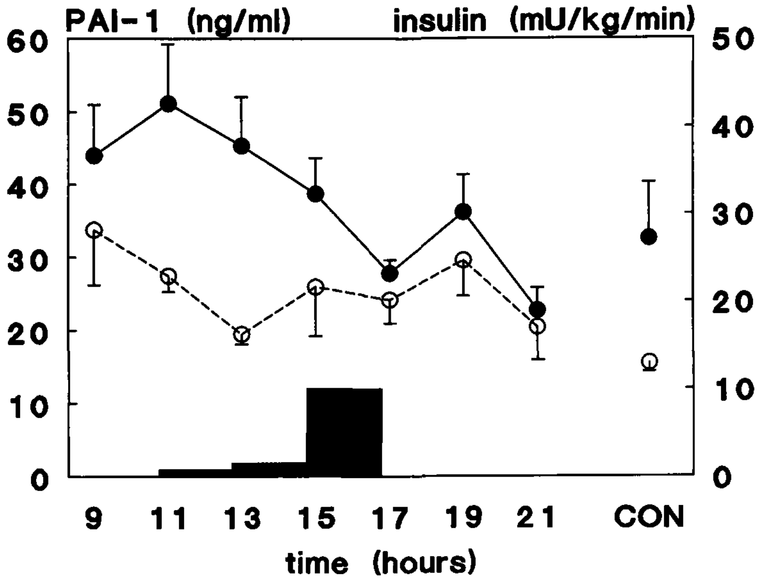
The insulin necessary to obtain and maintain euglycaemia in diabetics prior to the clamp amounted 0.29 ± 0.19 mU/kg/min (range 0.06 to 0.61). Insulin levels during the four consecutive two-hour periods were 16 ± 3, 94 ± 7, 209 ± 11 and 4773 ± 439 mU/l in the nondiabetic subjects and 38 ± 8, 98 ± 8, 190 ± 18 and 4368 ± 214 mU/l in the diabetic patients.

Levels of PAI-1 antigen were highest at the start of the clamp, i.e. when insulin levels were low, in both the nondiabetic subjects (33.8 ± 7.5 ng/ml at 9:00 hrs.) and the diabetic patients (51.2 ± 8.1 ng/ml at 11:00 hrs.) and showed a subsequent decline during the day (see figure 1), reaching nadir values after twelve hours, four hours after cessation of insulin infusion.

Levels of PAI-1 antigen after twelve hours, i.e. at 21:00 hrs. were not different from values at 21:00 hrs. on the control day in either nondiabetic subjects (20.4 ± 4.5 vs. 15.5 ± 1.2 ng/ml, N.S.) or in diabetic patients (22.7 ± 3.1 vs. 32.6 ± 7.7 ng/ml, N.S.).

Levels of PAI-1 were higher in the diabetic patients than in the nondiabetic subjects at 11:00 hrs.(51.2 ± 8.1 vs. 27.5 ± 2.2 ng/ml, p=0.028), at 13:00 hrs. (45.4 ± 6.7 ng/ml vs. 19.5 ± 1.4 ng/ml, p=0.003), at 15:00 (38.8 ± 4.9 ng/ml vs. 26.0 ± 6.8 ng/ml,p=0.041), and at 21:00 hrs. on the control day (32.6 ± 7.7 ng/ml vs. 15.5 ± 1.2 ng/ml, p=0.007)

Figure 1 PAI-1 levels during exogenous hyperinsulinaemia



Levels of PAI-1 antigen during and after insulin infusion in diabetic (——•——) and in nondiabetic subjects (---o---). Insulin dose is represented in bars. In both diabetic and in nondiabetic subjects a diurnal pattern is seen with highest values in the morning. Levels at 21:00 hrs. after insulin infusion are similar to levels at 21:00 hrs. on the control day.

DISCUSSION

In this study we have demonstrated that infusion of insulin resulting in physiologic and in supraphysiologic levels does not result in increased levels of PAI-1. As PAI-1 antigen levels show diurnal changes [10,11], it can be argued that a failure to increase PAI-1 levels may not exclude increased release of PAI-1. However, insulin infusion failed to abolish the diurnal changes of PAI-1, resulting in the highest levels in the morning in both the nondiabetic subjects ($p<0.05$) and in the diabetic patients ($p<0.01$). Thus any possible effect of insulin on PAI-1 antigen levels is negligible in comparision with "normal" diurnal changes.

It has been demonstrated in vitro [1,2] that insulin-induced release of PAI-1 by hepatocytes requires approximately six hours, i.e. the time necessary to go through the sequence of transscription from DNA to RNA to protein. Therefore, we continued to study the subjects until twelve hours, i.e. six hours after starting the last and supraphysiologic dose of insulin, which resulted in plasma insulin levels even higher than the levels acquired in the in vitro experiments [1,2]. Even six hours after the start of the supraphysiologic insulin infusion, we could not demonstrate an increase in PAI-1 antigen levels over the control day. Therefore, we believe that exogenous insulin administration has no significant effect on plasma PAI-1 antigen levels in vivo.

It may be argued that peripheral infusion of insulin, by resulting in peripheral rather than portal hyperinsulinaemia, may not be the correct route of administration to stimulate hepatocytes to produce and release PAI-1. However, the insulin levels achieved were such that supraphysiological portal hyperinsulinaemia must have been present during the final two hours of the clamp.

In conclusion, the persisting presence of diurnal changes during insulin infusion, and the absence of raised PAI-1 levels relative to a control day, six hours after start of a supraphysiologic insulin infusion, suggest that in vivo the effect of exogenous insulin on the release of PAI-1 antigen, if any, is negligible.

REFERENCES

1. Alessi M.C., Juhan-Vague I., Kooistra T., Declerck P.J., Collen D.: Insulin stimulates the synthesis of plasminogen activator inhibitor 1 by the human hepatocellular cell line Hep G2. Thromb Haemostas 1988; 60: 491-494
2. Kooistra T., Bosma P.J., Töns H.A.M., van den Berg A.P., Meyer P., Princen H.M.G.: Plasminogen activator inhibitor 1: Biosynthesis and mRNA level are increased by insulin in cultured human hepatocytes. Thromb Haemostas 1989; 62: 723-728
3. Small M., Kluft C., MacCuish A.C., Lowe G.D.O.: Tissue plasminogen activator inhibition in diabetes mellitus. Diabetes Care 1989; 12: 655-658
4. Juhan-Vague I., Roul C., Alessi M.C., Ardissone J.P., Heim M., Vague P.: Increased plasminogen activator inhibitor activity in non insulin dependent diabetic patients - relationship with plasma insulin. Thromb Haemostas 1989; 61: 370-373
5. Juhan-Vague I., Vague P., Alessi M.C., Badier C., Valadier J., Aillaud M.F., Atlan C.: Relationship betwwen plasma insulin, triglyceride, body mass index and plasminogen activator inhibitor 1. Diab. Metab. 1987; 13: 331-336
6. Stigendal L., Krotkiewski M., Tengborn L., Risberg B., Smith U.: Abdominal obesity is assocciated with impaired fibrinolysis. Fibrinolysis 1988; 2 (suppl 1): 129
7. Meade T.W., Brozovic M., Chakrabarti R. et al.: Haemostatic function in ischaemic heart disease - principal results of the Northwick Park Heart Study. Lancet 1986 ii: 533-537
8. Hamsten A., Wiman B., de Faire U., Blombäck M.: Increased plasma levels of a rapid inhibitor of tissue plasminogen activator in young survivors of myocardial infarction. N. Engl. J. Med. 1985; 313: 1557-1563

9. Brommer E.J.P., Verheijen J.H., Chang G.T.C., Rijken D.C.: Masking of fibrinolytic response to stimulation by an inhibitor of tissue-type plasminogen activator in plasma. Thromb. Haemostas. 1984; 52: 154-156.
10. Huber K., Beckmann R., Lang I. et al.: Circadian fluctuations in plasma levels of tissue plasminogen activator antigen and plasminogen activator inhibitor activity. Fibrinolysis 1989; 3 : 41-43
11. Andreotti F., Davies G.J., Hackett D. et al.: Circadian variation of fibrinolytic factors in normal human plasma. Fibrinolysis 1988; 2 (suppl 2): 90-92

CHAPTER 6

THE AMOUNT OF PLASMINOGEN, TISSUE-TYPE PLASMINOGEN ACTIVATOR AND PLASMINOGEN ACTIVATOR INHIBITOR TYPE 1 IN HUMAN THROMBI AND THE RELATION TO EX-VIVO LYSIBILITY.

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ABSTRACT

Thrombolytic therapy successfully reopens obstructed blood vessels in the majority of cases. However, it is not known why a substantial amount of thrombi are resistant to lysis by a fibrinolytic agent. In vitro studies have demonstrated that tissue-type plasminogen activator (t-PA) and plasminogen incorporated in the clot (during formation) increase lysibility. To test whether lysibility of in vivo formed human thrombi is related to their composition, we studied 25 venous thrombi obtained at autopsy and 21 arterial thrombi obtained during embolectomy.

Plasminogen activator inhibitor-1 (PAI-1) antigen was measured in a phosphate-buffered saline (PBS) extract of each thrombus; t-PA antigen and plasminogen antigen were determined in a 6M urea extract of the thrombus, representing bound proteins. Lysibility was measured as weight reduction during 8 hours of incubation in PBS containing streptokinase (SK) 100 U/ml, corrected for spontaneous lysis, reflected by weight loss in PBS without SK. In addition, lysibility in SK was compared with lysibility in urokinase (UK) 100 U/ml and in t-PA 200 U/ml.

Spontaneous lysis amounted to 29 ± 5 % (mean ± SEM) and 33 ± 5 % in venous and arterial thrombi, respectively, and inversely correlated with the PAI-1 content of thrombi ($r=-0.43$, $p<0.01$). Lysibility amounted to 76 ± 6 % in venous and 90 ± 4 % in arterial thrombi (venous vs. arterial: $p=0.051$). PAI-1-, plasminogen- and t-PA-

content of venous thrombi were 902 ± 129 ng, 34.3 ± 4.8 ug and 26.7 ± 3.0 ng per gram of wet thrombus respectively; for arterial thrombi these values were 2031 ± 401 ng/g ($p=0.011$), 64.1 ± 11.4 ug/g ($p=0.088$) and 62.2 ± 8.3 ng/g ($p=0.0001$), respectively. A correlation was found between t-PA and plasminogen ($r=0.74$, $p<0.001$). Lysibility by SK related to plasminogen content in both venous ($r=0.60$, $p<0.002$) and arterial ($r=0.44$, $p<0.05$) thrombi; PAI-1 and t-PA did not correlate with lysibility. Lysibility in the chosen concentrations of SK, UK and t-PA were similar.

We conclude that spontaneous lysis of thrombi in saline is dependent on PAI-1 content and that susceptibility of thrombi to lysis by SK ex vivo is dependent on the plasminogen content.

INTRODUCTION

Despite numerous approaches to optimize the dosage and duration of thrombolytic agents in venous and arterial thromboembolism, a certain percentage of thrombi remains resistant to thrombolytic therapy. Resistance of thrombi to thrombolytic therapy is thought to be associated with clinical factors such as ageing of the thrombus, a distal localization, and a large extension of the thrombus [1-3]. However, several authors could not relate resistance to thrombolytic therapy to the abovementioned clinical parameters [4-9] and it has been suggested that the composition of a thrombus may influence its susceptibility to fibrinolytic agents [1]. This has not been studied systematically.

It has been shown in studies with in vitro formed plasma clots, that fibrinolysis depends on the amount of plasminogen (Plg) [10-12] and on the amount of tissue-type plasminogen activator (t-PA) bound to fibrin [13,14]. Whether these findings can be extended to pathological thrombi is unknown.

Apart from transient increases in fibrinolytic activity due to infusion of thrombolytic agents or to spontaneous release of t-PA, the fibrinolytic activity in plasma is determined mainly by the level of plasminogen activator inhibitor - 1 (PAI-1)[15,16]. About 90 % of the total blood PAI-1 is present in platelets [17]. It is unknown to what extent PAI-1 is present in human thrombi and whether it influences susceptibility to lysis by fibrinolytic agents.

To our knowledge the composition of ex vivo human thrombi has not previously been studied in detail. In the present study we examined the amounts of PAI-1, plasminogen and t-PA in human thrombi and whether a relationship exists between

susceptibility to fibrinolytic agents on the one hand and thrombus composition on the other.

MATERIALS AND METHODS

Thrombi

21 arterial thrombi were collected from patients undergoing
surgical embolectomy for peripheral arterial occlusion, mostly of a femoral or popliteal artery. 25 venous thrombi, including 19 pulmonary emboli, were collected at autopsy from patients with various causes of death. Most of these patients appeared to have clinically-unsuspected venous thromboembolism. The thrombi were rinsed in phosphate-buffered saline (PBS), pH 7.4, containing 0.2% sodium azide. Each thrombus was cut into several pieces of 100 to 300 mg weight. After selecting representative pieces for routine histological examination, half of the remaining pieces was stored at -70°C for lysibility studies, whereas the other half was stored for determination of thrombus composition. Care was taken to obtain macroscopically identical pieces of each thrombus for the different studies.

Lysibility

Each thrombus was thawed and cut into pieces weighing approximately 20-40 mg. Three to six pieces were placed in
tubes containing buffer (PBS, pH 7.4, 0.02% sodium azide), buffer with streptokinase (SK) 100 U/ml (Kabikinase, Kabi Vitrum, Stockholm, Sweden), buffer with urokinase (UK) 100 IU/ml (Urokinase Medac, Kabi Vitrum, Stockholm, Sweden) or buffer with two-chain t-PA 200 IU/ml (Wellcome, Beckenham, United Kingdom). Before, and after 8 hours of incubation at 37°C in a rotating water bath, the weight of the thrombus pieces in each tube was measured after expressing the fluid from the thrombus parts by placing them between filter papers during 10 minutes and applying a pressure of 1.5 g/cm^2.

Significant weight reduction occurred during incubation of the thrombus pieces in buffer without a thrombolytic agent. Because pilot studies had demonstrated both the occurrence of fibrin degradation products after incubation of pathological human thrombi in PBS (data not shown) and a close relationship between weight reduction

and the loss of ^{125}I-labelled fibrin in artifical human thrombi (r=0.97), we regarded the weight reduction in PBS as the result of spontaneous lysis. Spontaneous lysis (SL) was recorded as % weight reduction after 8 hrs. of incubation in PBS.

Lysibility by a fibrinolytic agent was expressed as % weight loss during 8 hours of incubation in the corresponding agent (FL), corrected for the observed spontaneous lysis. The formula used is as follows:

$$\text{Lysibility (in \%)} = 100 * (\% \text{ FL} - \% \text{ SL}) / (100 \% - \% \text{ SL})$$

Extraction procedures of PAI-1, plasminogen and t-PA

An amount of 206 - 3876 (mean 1324) mg of each thrombus was thawed and homogenized by pottering in 2 - 5 ml of PBS, pH 7.4, containing 0.05 % Tween 80, at 4°C. After centrifugation (10 min., 20000 g) the supernatant was frozen and stored at -20°C until further processing, while the pellet underwent the same procedure two more times, in order to extract PAI-1 and to eliminate unbound Plg and t-PA as well as possible. After the third PBS extraction virtually no Plg or t-PA could be extracted with PBS. In order to extract the remaining, fibrin-bound Plg and t-PA, the pellet (31.1 ± 1.6 % of the original thrombus weight) was subsequently homogenized twice in 2 - 4 ml of 6 M urea solution in PBS containing 0.05 % Tween 80, pH 7.4, and incubated at 4°C for 4 and for 10 hrs respectively. Pilot experiments had revealed that with this procedure approximately 90 % of fibrin-bound plasminogen and t-PA is eluted. After centrifugation both supernatants were dialyzed against PBS, pH 7.4, and stored at -20°C until further processing.

Assays of PAI-1, plasminogen and t-PA

PAI-1 antigen (Ag) was measured by enzyme immuno assay (Monozyme, Charlottenlund, Denmark). Plasminogen Ag was measured by radial immunodiffusion technique with polyclonal goat anti-human Plg serum (Nordic Laboratories, Tilburg, Netherlands). t-PA Ag was measured in serial dilutions by an enzyme immuno assay (Imulyse t-PA, Biopool AB, Umea, Sweden). Results were expressed as ng (PAI-1), µg (Plg) or as ng (t-PA) per gram of wet thrombus.

PAI-1, Plg and t-PA measured in the three PBS extracts were considered predominantly unbound. Plasminogen and t-PA measured in the dialyzed urea extracts were considered fibrin-bound.

Statistical analysis

Data are presented as mean ± SEM. In case of non-normal distribution, log-transformation of the data was performed. Analysis of variance (ANOVA) was used for evaluating differences in lysibility in different thrombolytic agents. Comparisons between venous and arterial thrombi were made by the unpaired Student-t test. The Mann-Whitney U-test was employed in case of persisting unequal group variance after log-transformation. Linear regression analysis was used to determine correlations between measured variables. A probability level of 0.05 or less was considered significant.

RESULTS

Lysibility

Spontaneous lysis amounted to 29 ± 5 % (range 0 - 97 %) in venous thrombi and 33 ± 5 % (range 0 - 79 %) in arterial thrombi.

Lysibility in SK, UK or t-PA was 76 ± 6 % (n=25), 76 ± 6 % (n=24) and 69 ± 7 % (n=25), respectively, for venous thrombi and 90 ± 4 % (n=21), 97 ± 2 % (n=14) and 87 ± 6 % (n=16), respectively, for arterial thrombi. Arterial thrombi thus lysed to a greater extent ex vivo than venous thrombi in SK (p=0.051), UK (p=0.037) and in t-PA (p=0.020). Lysibility of the 24 venous and 14 arterial thrombi tested in all three fibrinolytic agents was similar in SK, UK and t-PA at the chosen concentrations in both venous (ANOVA: F=0.53, p=0.59) and in arterial thrombi (ANOVA: F=0.50, p=0.61), respectively, with only one thrombus showing a clear difference in lysibility in different fibrinolytic agents (Fig. 1).

PAI-1, plasminogen and t-PA

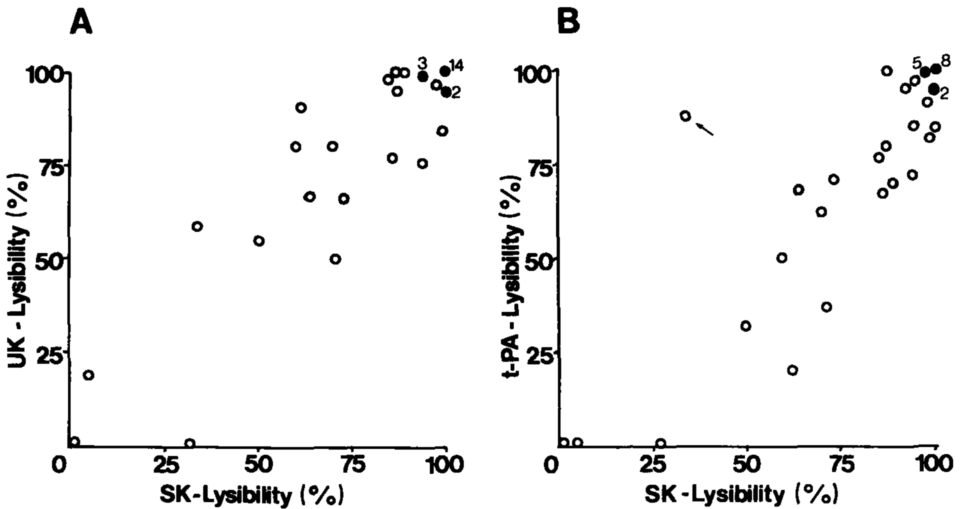
In the three PBS extracts a total of 902 ± 129 ng/g (range 70 - 3345) of PAI-1 was recovered from venous thrombi, as compared with 2031 ± 401 ng/g (range 187 - 8005) from arterial thrombi (p=0.011). The data are summarized in Table 1.

A total of 34.6 ± 5.3 μg/g (range 0.01 - 144) of Plg was recovered from the PBS extracts of venous thrombi and 51.7 ± 8.1 μg/g (range 13.5 - 145) from arterial thrombi (p=0.048). In the urea extracts a total of 34.3 ± 4.8 μg/g (range 0.4 - 104) of

Plg was obtained, compared with 64.1 ± 11.4 μg/g (range 1.4 - 184) in the case of arterial thrombi (p=0.088).

The amounts of t-PA removed by PBS were 29.0 ± 4.0 ng/g (range 5.9 - 81.6) and 43.1 ± 6.7 ng/g (range 6.9 - 135.8) respectively for venous and for arterial thrombi (p=0.080). t-PA in the urea extracts amounted to 26.7 ± 3.0 ng/g (range 7.7 -70.7) in venous thrombi as compared with 62.2 ± 8.3 ng/g (range 13.7 - 146.8) in arterial thrombi (p=0.0001).

Figure 1 The lysibility of thrombi ex vivo in SK, UK and t-PA



Lysibility by streptokinase (SK) is represented on the abcissa, whereas lysibility by urokinase (UK) (A) and two-chain tissue-type plasminogen activator (t-PA) (B) are represented on the ordinates. The closed circles represent more than one thrombus with the same coordinates, the number of thrombi given in the figure. Lysibility by SK correlates closely with both lysibility by UK (r=0.91) and lysibility by t-PA (r=0.87). Of 38 thrombi tested in all three agents, only one thrombus (arrow) appeared lysible in t-PA and resistant to lysis in SK.

Table 1 Amounts of plasminogen activator inhibitor 1 (PAI-1), plasminogen (Plg) and tissue-type plasminogen activator (t-PA), recovered from venous (n=25) and arterial (n=21) human thrombi. The PBS extract represents predominantly unbound proteins, whereas the plasminogen and t-PA recovered in the urea extract are considered fibrin-bound.

	PBS extract			urea extract		
	venous	arterial		venous	arterial	
PAI-1 (ng/g)				902 ± 129	2031 ± 401	*
Plg (μg/g)	34.6 ± 5.3	51.7 ± 8.1	*	34.3 ± 4.8	64.1 ± 11.4	
t-PA (ng/g)	29.0 ± 4.0	43.1 ± 6.7		26.7 ± 3.0	62.2 ± 8.3	*

*: venous vs. arterial $p<0.05$

Correlation studies

A significant, positive correlation (Fig. 2) was found between the amount of fibrin-bound t-PA and the amount of fibrin-bound Plg ($r=0.74$, $p<0.001$). This correlation proved significant within arterial thrombi ($r=0.77$, $p<0.001$), but not within venous thrombi ($r=0.36$, N.S.).

Spontaneous lysis inversely correlated with (the log-transformed) PAI-1 content of the thrombus: $r=-0.43$ ($p<0.01$) (see Fig. 3). This relationship was observed in both venous ($r=-0.44$, $p<0.05$) and in arterial thrombi ($r=-0.57$, $p<0.01$). Spontaneous lysis inversely correlated with (log-transformed) fibrin-bound plasminogen ($r=-0.44$, $p<0.01$), but did not correlate with fibrin-bound t-PA.

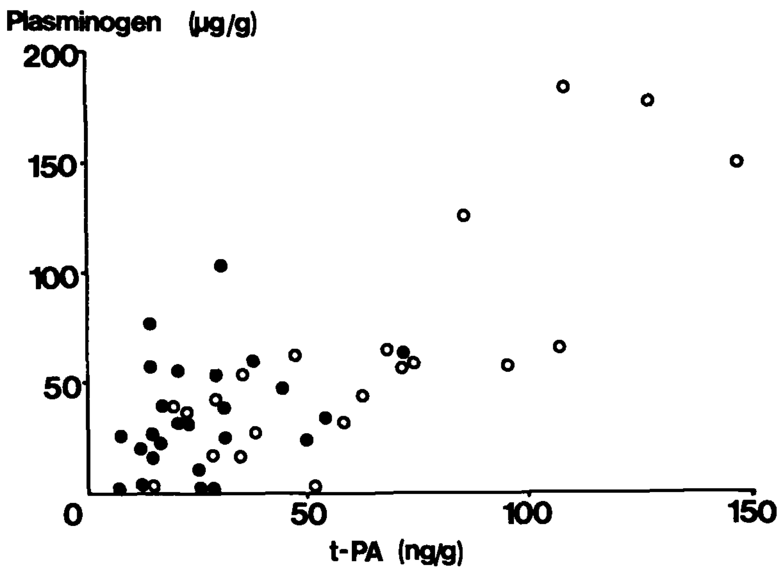
Since only part of the thrombi were incubated in UK or t-PA, whereas all thrombi were incubated in SK, lysibility in SK was further studied in relation to PAI-1 and to fibrin-bound Plg and t-PA. As depicted in Fig. 4, a significant correlation exists between lysibility and (the logarithm of) fibrin-bound Plg ($r=0.57$, $p<0.001$), which is more pronounced for venous ($r=0.60$, $p<0.002$) than for arterial thrombi ($r=0.44$,

p<0.05).

No relation was found between lysibility and either PAI-1 or fibrin-bound t-PA in both venous and arterial thrombi (data not shown).

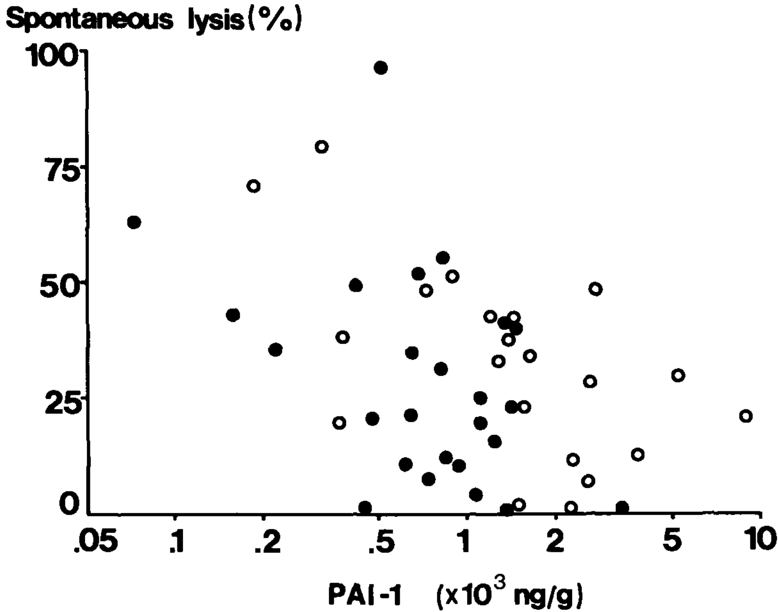
Unbound Plg correlated closely with fibrin-bound Plg (venous: $r= 0.76$, $p<0.001$; arterial: $r=0.80$, $p<0.001$), but not with lysibility by SK. Unbound t-PA was related to fibrin-bound t-PA only in venous thrombi ($r=0.79$, $p<0.001$) and did not correlate with lysibility by SK.

Figure 2 The relation between fibrin-bound t-PA and fibrin-bound plasminogen.



The amount of fibrin-bound plasminogen correlates with the amount of fibrin-bound tissue-type plasminogen activator ($r=0.74$) in the thrombus. The individual correlation coefficients for arterial (o) and venous (•) thrombi are 0.77 and 0.36 respectively.

Figure 3 The relation between thrombus plasminogen activator inhibitor type 1 and spontaneous lysis.



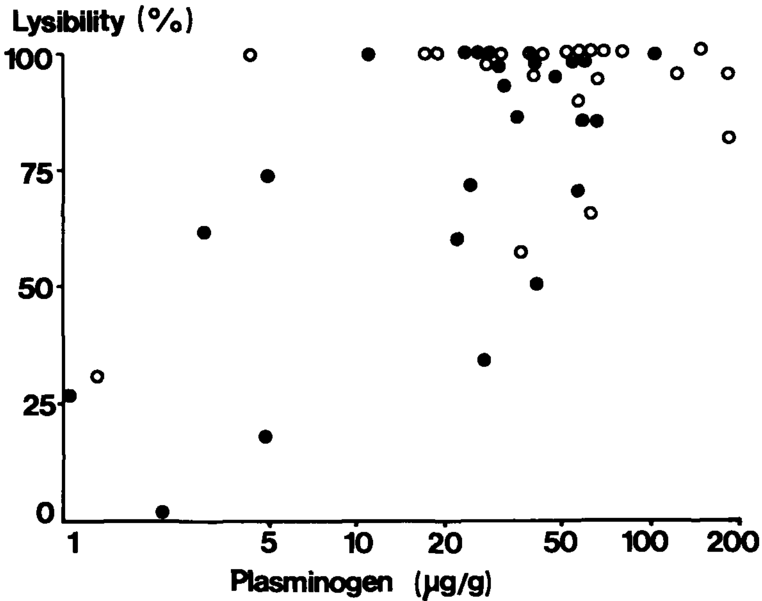
Spontaneous lysis correlates inversely with the logarithm of the amount of plasminogen activator inhibitor type 1 in the thrombus (r=-0.43). The individual correlation coefficients for arterial (o) and venous (•) thrombi are -0.57 and -0.44 respectively.

DISCUSSION

By incubating human thrombi in fibrinolytic agents ex vivo, and by measuring PAI-1, plasminogen and t-PA in these same thrombi we have demonstrated that the rate of lysis shows a broad range and that thrombolysis by SK depends on the amount of fibrin-bound plasminogen in the thrombus (Fig. 4). In addition, spontaneous lysis of thrombi in buffer was inversely related to the amount of PAI-1 in the thrombus (Fig. 3). Thus, as Marder and Sherry stated [1], the lysibility of a thrombus is determined by its intrinsic properties. One of these is the amount of fibrin-bound plasminogen.

This amount is small, being 25 - 50 % of the plasma concentration (comparing 1 g of thrombus with 1 ml of plasma) with a comparable fraction unbound in the meshes of the fibrin network.

Figure 4 The relation between fibrin-bound plasminogen and lysibility by SK.



Lysibility by streptokinase correlates with the logarithm of the amount of fibrin-bound plasminogen present in the thrombus (r=0.57). The individual correlation coefficients for venous (•) and arterial (o) thrombi are 0.60 and 0.44 respectively.

During clotting, plasminogen is incorporated in the clot [18, 19, 20]. The amount of entrapped plasminogen decreases with retraction of the thrombus [4, 21] to a nadir after 24-48 hrs. and subsequently increases [4]. Small amounts of plasminogen have been recovered from thrombi by Hedner et al.[18], who reported the amount of plasminogen present in human thrombi to be 7 % of the plasma concentration. In addition, Ogston et al.[19] found a plasminogen content of 18 % of the blood

concentration in artificially-formed thrombi. Several authors have suggested that plasminogen binding to clots enhances susceptibility to lysis [10-12]. Recently the existence of such a relationship has been demonstrated for plasma clots and retracted whole blood clots by Sabovic et al. [22]. The present study has extended this relationship to in vivo formed human thrombi.

Mean values of fibrin-bound t-PA in the venous and arterial human thrombi were 27 and 62 ng/g respectively, indicating an accumulation of t-PA in the thrombi relative to plasma (average basal plasma concentration 10 ng/ml). These findings can be explained by the specific affinity of t-PA for fibrin. In vitro, binding of t-PA to plasma clots exerts a great influence on lysibility [13,14], both by a direct action and by increasing the plasminogen binding to the clot [10]. Binding of t-PA to fibrin has been shown to result in partial fibrin degradation, exposing carboxy-terminal lysine binding sites [23] and thereby increasing the binding of plasminogen to fibrin. We could not demonstrate a direct relationship between t-PA and lysibility. Instead, our data support only an indirect effect of fibrin-bound t-PA by mediating the binding of plasminogen to fibrin (Fig. 2).

The accumulation of PAI-1 in thrombi was even more pronounced than the accumulation of t-PA: venous and arterial thrombi respectively contain on average 60 and 150 times the amount present in the equivalent volume of platelet-poor plasma, and 4 and 10 times the amount in platelet-rich plasma respectively. We suppose these large amounts of PAI-1 in thrombi are secondary to the accumulation of platelets. Lysibility by SK was not related to the amount of PAI-1 in the thrombus, but spontaneous lysis in buffer correlated inversely with thrombus PAI-1 content. This is in line with previous findings that spontaneous lysis is inhibited by PAI-1 both in vitro and in vivo [24,25]. The inverse relationship between PAI-1 and spontaneous lysis suggests that the small proportion (3 - 5 %) of active PAI-1 in platelets [17] apparently is overcome by the large amount of PAI-1 present in thrombi, whereas the the amount of active PAI-1 in thrombi probably is too small to inhibit the more rapid thrombolysis induced by fibrinolytic agents. Surprisingly, spontaneous lysis inversely correlated with the amount of fibrin-bound plasminogen in the thrombus. We cannot explain this finding. However, it is unlikely that plasminogen inhibits spontaneous lysis.

Interestingly, arterial thrombi contained more fibrin-bound t-PA and more PAI-1 than did venous thrombi, and, in addition, were more sensitive to thrombolytic agents. There were no differences between the two groups in thrombus age, fluid content, macroscopical or histological appearance or proportion of "white", "red" and "mixed" thrombi. Arterial endothelial lining is known to contain less plasminogen activator than venous endothelium [26], which makes increased diffusion of t-PA from

the arterial wall into the thrombus an unlikely explanation. The present t-PA antigen assay does not distinguish between t-PA and the t-PA.PAI-1 complex [27]; thus the measured t-PA might reflect t-PA.PAI-1 complex to a great extent.

Finally, we have demonstrated that human thrombi, ex vivo, lyse equally well in the chosen concentrations of SK, UK and two-chain t-PA (Fig.1), only one of 38 thrombi lysing far better in t-PA than in SK. No gross differences in lysibility were noted between SK and UK and thus, at least in buffer in vitro, a thrombus sensitive to lysis by SK will be sensitive to UK and t-PA as well. Conversely, a thrombus resistant to SK will be resistant to UK and (in most cases) to t-PA as well. These findings are supported by the results obtained by Keber et al.[28].

The use of postmortem obtained venous thrombi, as well as freezing and thawing thrombi may have influenced the results. Major postmortem changes are unlikely since no significant correlation was found between the time elapsed before autopsy on the one hand, and lysis rate, PAI-1, fibrin-bound Plg, fibrin- bound t-PA and spontaneous lysis on the other hand. Concerning the effect of freezing and thawing, a pilot study revealed that both the lysibility by SK and spontaneous lysis of parts of human thrombus studied immediately were similar to those of the parts which were first frozen and thawed (data not shown, n=6). Additionally a pilot study of plasma and whole blood clots (n=4 and 2, respectively, data not shown) demonstrated similar lysibility and similar amounts of fibrin-bound Plg and fibrin-bound t-PA in fresh and in frozen and thawed parts.

In conclusion, considerable spontaneous thrombolysis occurs in buffer in vitro in human thrombi and this is inversely related to the amount of PAI-1 present in the thrombus. Lysibility by streptokinase is determined by the amount of fibrin-bound plasminogen, which, in turn, is dependent on the amount of fibrin-bound t-PA. Finally, lysibility was similar by streptokinase, urokinase and (two-chain) t-PA in buffer milieu in vitro.

Acknowledgements

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REFERENCES

1. Marder VJ, Sherry S: Thrombolytic Therapy: Current Status. N Engl J Med 1988; 318: 1512-1521; 1585-1595
2. Chesterman CN: Thrombolytic therapy. In: AL Bloom, DP Thomas (eds): Haemostasis and Thrombosis, 2nd ed. Churchill Livingstone, London, 1987, pp 829-848
3. Hess H, Ingrisch H, Mietaschk A, Rath H: Local low-dose thrombolytic therapy of peripheral arterial occlusions. N. Engl. J. Med. 1982; 307: 1627-1630
4. Gottlob R: Plasminogen and plasma Plasmin Inhibitors in arterial and venous thrombi of various ages. In: JF Davidson, MM Samama, PC Desnoyers (eds): Progress in chemical fibrinolysis and thrombolysis, vol. 1. Raven Press, New York, 1975, pp 23-36
5. Tibbutt DA, Williams EW, Walker MW, Chesterman CN, Holt JM, Sharp AA: Controlled trial of Ancrod and Streptokinase in the treatment of deep vein thrombosis of the lower limb. Br. J. Haem. 1974; 27: 407-414
6. Arnesen H, Heilo A, Jakobsen E, Ly B, Skaga E: A prospective study of streptokinase in the treatment of deep vein thrombosis. Acta Med Scand 1978; 203: 457-463
7. Weimar W, Stibbe J, van Seyen AJ, Billiau A, De Somer P, Collen D: Specific lysis of an ileofemoral thrombus by administration of extrinsic (tissue-type) Plasminogen Activator. Lancet 1981: ii: 1018-1020
8. Luomanmäki K, Halttunen PK, Hekali P, Valle M, Heikkilä: Experience with streptokinase treatment of major pulmonary embolism. Ann Clin Res 1983; 15: 21-25
9. Robinson JA, Carty GA, Burch BH: Factors predicting the outcome of regional streptokinase therapy for peripheral arterial occlusions. Surg Gynaecol Obstet 1985; 161: 453-456
10. Tran-Thang C, Kruithof EKO, Bachmann F: Tissue-type Plasminogen Activator increases the binding of Glu-Plasminogen to clots. J Clin Invest 1984; 74: 2009-2016
11. Zamarron C, Albert A: Adsorbed Plasminogen and fibrinolysis rate of recent and aged plasma clots Haemostasis 1981; 10: 59 (abstr)
12. Chevalet-Prandini MH, Chapel A, Suscillon A: The fibrinolytic action of flufenamate and its influence on Plasminogen binding to fibrin. Thromb. Res. 1985; 38: 481-489
13. Brommer EJP: The level of extrinsic Plasminogen Activator (t-PA) during clotting determines the rate of fibrinolysis; inefficiency of activators added afterwards. Thromb. Res. 1984; 34: 109-115
14. Tran-Thang C, Kruithof EKO, Bachmann F: The mechanism of in vitro clot lysis induced by vascular Plasminogen Activator. Blood 1984; 63: 1331-1337
15. Brommer EJP, Verheijen JH, Chang GTC, Rijken DC: Masking of fibrinolytic response to stimulation by an inhibitor of tissue-type plasminogen activator in plasma. Thromb. Haemostas. 1984; 52: 154-156
16. Sprengers ED, Kluft C: Plasminogen Activator Inhibitors. Blood 1987; 69: 381-387
17. Booth NA, Croll A, Bennett B: The activity of Plasminogen Activator Inhibitor-1 (PAI-1) of human platelet. Fibrinolysis 1990; 4, suppl. 2: 138-140
18. Hedner U, Nilsson IM, Robertson B: Determination of Plasminogen in clots and thrombi. Thromb. Diath. Haem. 1966; 15: 220-230
19. Ogston D, Ogston CM, Fullerton HW: The Plasminogen content of thrombi. Thromb. Diath. Haem. 1966; 16: 38-50
20. Alkjaersig N, Fletcher AP, Sherry S: The mechanism of clot dissolution by Plasmin. J. Clin. Invest. 1959; 38: 1086-1095
21. Sabovic M, Lijnen HR, Keber D, Collen D: Effect of retraction on the lysis of human clots with fibrin spacific and non-fibrin specific plasminogen activators. Thromb. Haemostas. 1989; 62: 1083-1087
22. Sabovic M, Lijnen HR, Keber D, Collen D: Correlation between progressive adsorption of plasminogen to blood clots and their sensitivity to lysis. Thromb. Haemostas. 1990; 64: 450-454
23. Suenson E, Lutzen O, Thorsen S: Initial plasmin-degradation of fibrin as the basis of a positive feed-back mechanism in fibrinolysis. Eur. J. Biochem. 1984; 140: 513-522

24. Brommer E.J.P., Boks A.L., Koopman J., Haverkate F.: Protraction of whole blood and plasma clot lysis in patients with high levels of an inhibitor of tissue-type plasminogen activator. Thromb. Res. 1985; 39: 271-280
25. Knabb R.M., Chiu A.T., Reilly T.M.: Effects of recombinant plasminogen activator inhibitor type 1 on fibrinolysis in vitro and in vivo. Thromb. Res. 1990; 59: 309-317
26. Noordhoek Hegt V: Distribution and variation of fibrinolytic activity in the walls of human arteries and veins. In: Histochemical studies on vascular fibrinolysis. Haemostasis 1976; 5: 355-372
27. Rånby M., Nguyen G., Scarabin P.Y., Samama M.: Immunoreactivity of tissue plasminogen activator and of its inhibitor complexes. Thrombos Haemostas 1989; 61: 409-414
28. Keber D, Sabovic M, Stendler M, Stegnar M: Dissolution of "ex vivo" thrombi by tissue Plasminogen Activator, Streptokinase and Urokinase. Thromb. Res. 1986; suppl. VI: 109

CHAPTER 7

PLASMINOGEN CONTENT AND (EX VIVO) LYSIBILITY OF HUMAN THROMBI IN RELATION TO THROMBUS AGE

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SUMMARY

Ageing of thrombi is commonly believed to render fibrinolytic therapy less effective. We studied the ex vivo lysibility in streptokinase of 25 venous and of 21 arterial human thrombi, in relation to thrombus age. Thrombus age was defined by histological criteria as either fresh (group I, i.e. less than 2 days), recent (group II, i.e. 2-10 days) or aged (group III, i.e. more than 10 days). In addition, plasminogen activator inhibitor type 1 (PAI-1) in the thrombi was measured and thrombus-bound tissue type plasminogen activator (t-PA) and plasminogen (plg) were determined.
Lysibility of venous thrombi amounted to (mean ± SEM) 61 ± 15, 78 ± 9 and 87 ± 6 % in groups I, II and III, respectively. Lysibility of arterial thrombi was 79 ± 9, 99 ± 1 and 94 ± 5 %, respectively. Plg in venous thrombi amounted to 18 ± 5, 33 ± 6 and 53 ± 11 μg per gram of thrombus in groups I, II and III, respectively (Kruskall-Wallis: p=0.024), and in arterial thrombi to 40 ± 9, 55 ± 11 and 132 ± 38 μg/g, respectively (Kruskall-Wallis: N.S.). Plasminogen content was significantly greater in group III than in group I venous thrombi (p=0.013). Likewise, if venous and arterial thrombi are taken together, group III thrombi contained more plg than group I thrombi (p=0.01-8). PAI-1 and t-PA in thrombi were similar in the three age groups.
In conclusion, aged thrombi contain more thrombus-bound plasminogen and are more or at least as susceptible to ex vivo lysis by streptokinase as are fresh thrombi.

INTRODUCTION

It is commonly assumed that older thrombi are more resistant to thrombolytic therapy than fresh thrombi, in venous thrombosis, pulmonary embolism, peripheral arterial occlusion and in acute coronary artery occlusion [1-4]. However, it is difficult to determine the age of a thrombus exactly.

While the duration of symptoms can be recorded with an accuracy to the hour in coronary artery occlusion, the duration and clinical manifestations of the other mentioned conditions are characterized to a lesser degree: in only half of the patients with deep venous thrombosis (DVT), signs and symptoms alert the clinician to this diagnosis, while the diagnosis of DVT is rejected after further examination in 50 % of the clinically suspected cases [5]. When the age of the thrombus is judged by duration of signs and symptoms [6-12], even in studies, in which the diagnosis is confirmed by venography, inaccuracies should be envisaged.

In pulmonary embolism, the diagnosis is usually made by ventilation / perfusion scanning [13,14], a technique known to have a low specificity [15]. Furthermore, emboli are usually multiple [13,16], significant embolisation having taken place before the onset of clinical signs. Additionally, emboli may arise from different parts of a thrombus, e.g. older or fresh parts. All these factors may contribute to errors in estimating the age of the occluding clot, if one depends on clinical criteria.

In peripheral arterial disease the -clinical- judgement of the age of the thrombus may not be accurate due to symptoms of severe preexisting arterial stenosis masking the acute thrombotic occlusion , by appearance of collateral circulation, and by the fact that it usually is not known if the occlusion is due to an acute thrombus or due to embolisation, possible sources being heart, arteries and veins [17,18].

With regard to the above-mentioned pitfalls in determining thrombus age, efforts to relate efficacy of thrombolytic therapy to clinically defined thrombus age could lead to erroneous conclusions. However, with histological examination the composition of a thrombus is disclosed and parts of different age can be recognized. Although an exact estimation of thrombus age is not possible, a reliable differentiation into several age groups (e.g. fresh, recent and aged) is possible [19-21].

In a previous study [22] we have demonstrated that susceptibility of in vivo formed human thrombi to -ex vivo- lysis by streptokinase (SK), i.e.lysibility, correlates with the amount of plasminogen (Plg) bound to the thrombus. Here we report on an extension of this study, in which we determined thrombus age by histological criteria. We related thrombus age to lysibility and to Plg, tissue-type plasminogen activator (t-PA) and plasminogen activator inhibitor type 1 (PAI-1) content of the thrombus.

MATERIALS and METHODS

25 venous thrombi and pulmonary emboli were obtained at autopsy. In addition, 21 arterial thrombi were obtained from patients undergoing embolectomy because of acute peripheral arterial occlusion. Whether or not a clinical diagnosis of thrombosis had been made was recorded, and, if present, the duration of clinical signs and symptoms suggesting thromboembolism (expressed in days). Representative parts were taken from macroscopically different parts of each thrombus for histologic axamination. The remainder of each thrombus was divided into two macroscopically equivalent portions, which were snap-frozen and stored at -70°C until proceeding. One portion served for testing of ex vivo lysibility in streptokinase, while in the other part PAI-1 and thrombus-bound plasminogen and t-PA were determined.

Determination of thrombus age

The cut slices of thrombus were fixed in formaldehyde 10%, embedded in paraffin and stained by haematoxylin and eosin (HE) and by phosphotungstic acid and haematoxylin (PTAH) for visualisation of fibrin. The thrombi were divided into three categories of age, i.e. fresh (i.e. less than 2 days), recent (i.e. 2-10 days) and aged (i.e. more than 10 days). Criteria for scoring age were as follows:

Group I, fresh thrombi:	intact erythrocytes and fine threaded fibrin structure. (macroscopical appearance most red, soft and often surrounded by a hide)
Group II, recent thrombi:	thrombi, not fulfilling criteria of either Group I or Group III. (macroscopical appearance sometimes soft and red, sometimes elastic pink and white parts, and sometimes firm)
Group III, aged thrombi:	recanalization or organization, hyalinization and infiltration by mononuclear cells. (macroscopical appearance most firm, sometimes crumbly, and often white - yellowish)

In cases in which the thrombus was composed of parts of different ages, the age of the part containing over half of the thrombus was chosen. If the thrombus consisted of equal amounts of fresh, recent or aged parts, the mean of the ages was chosen.

Lysibility ex vivo in streptokinase

A detailed description has been given elsewhere (submitted). Briefly, lysibility of the thrombus was determined ex vivo by means of weight reduction (% loss of initial weight) during 8 hours of incubation at 37°C in a solution of phosphate buffered saline (PBS) containing 100 U/ml of streptokinase (SK). The weight reduction in SK was corrected for "spontaneous" lysis, i.e. weight loss in PBS without SK. Results are expressed as % lysis.

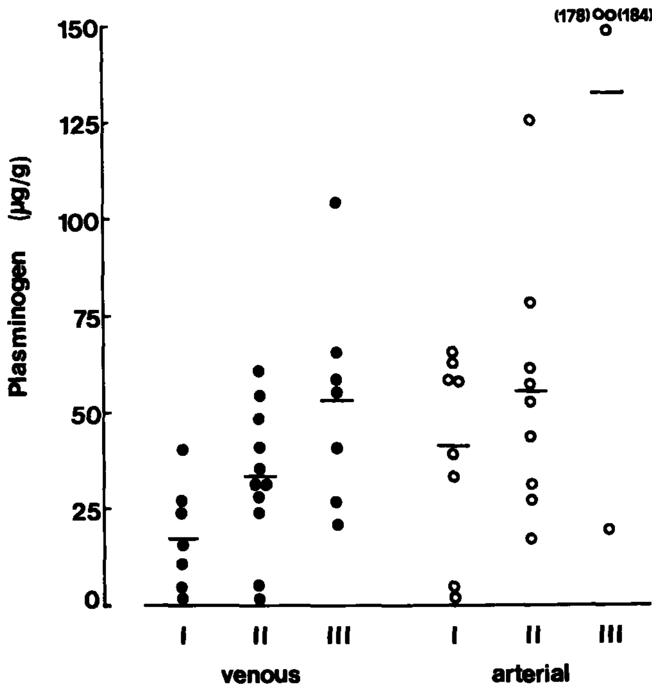
Determination of PAI-1 and of t-PA and plasminogen bound to the thrombus

Each thrombus was homogenized, washed three times at 4°C by adding approximately 3 to 4 volumes of PBS pH 7.4 with 0.05 % Tween 80, centrifuging for 10 minutes at 20,000 x g at 4°C, and removing the supernatant. All supernatants were stored at -20°C for determinatiuon of PAI-1. After the third washing procedure virtually no t-PA, plasminogen or PAI-1 could be extracted from the pellet by PBS. The remaining t-PA and plasminogen in the pellet were considered thrombus-bound. The pellet was subsequently homogenised twice in a 6 M Urea/Tween 0.05 % solution and incubated at 4°C during 4 and during 10 hrs respectively, to extract the remaining t-PA and plasminogen from the thrombus. After centrifugation both supernatants were dialyzed against PBS pH 7.4 at 4°C and stored at -20°C for determination of Plg and t-PA. PAI-1 antigen was measured by an enzyme immunoassay (Monozyme, Charlottenlund, Denmark). Plasminogen antigen was measured by radial immunodiffusion (Goat ani-human Plg, Nordic Laboratories, Tilburg, The Netherlands). t-PA antigen was measured by an enzyme immunoassay (Imulyse t-PA, Biopool AB, Umeå, Sweden). Results for PAI-1, Plg and t-PA are expressed as ng (PAI-1), μg (Plg), and ng (t-PA) per g of wet thrombus respectively.

Statistical methods

All values are represented as mean ± SEM. If necessary, the data were log transformed. Comparisons between groups of thrombi were performed by one-way analysis of variance (ANOVA), followed by Student's t- test. In certain cases of non-normal group distribution or of small numbers the Kruskall-Wallis (K.W.) and Mann-Whitney U (M.W.U.)-tests were used (noted in the text). A probability level of 0.05 or less was considered significant. Statistical evaluation was performed with Number Cruncher Statistical System (NCSS).

Figure 1 Plasminogen content in relation to thrombus age.



Venous (•) and arterial (o) thrombi of various ages contain different amounts of thrombus-bound plasminogen. Groups I, II and III represent fresh, recent and aged thrombi respectively. Individual values and means are given.

RESULTS

Clinical parameters and pathological - anatomical determination of thrombus age

In all of the arterial thromboses, but in only 13 of 25 cases of venous thromboembolism the diagnosis was made clinically. Moreover, in only 9 out of 21 arterial thromboses and in 8 out of 13 clinically suspected cases of venous thromboembolism scrutiny of the case history allowed to record the duration of clinical signs and symptoms. In 7 out of 9 (78 %) arterial thromboses and in 5 out of 8 (63 %) cases of venous thrombosis, in which thrombus age had been determined clinically, the clinical estimation of thrombus age was in line with the histological determination of thrombus age. Overall, 7 out of 21 (33 %) arterial and 5 out of 25 (20 %) venous thrombi were adequately dated by clinical grounds alone.
On histological grading of the arterial thrombi 8 were considered fresh, whereas 9 and 4 were considered recent and aged, respectively. For venous thrombi and pulmonary emboli, these numbers were 7, 11 and 7, respectively.

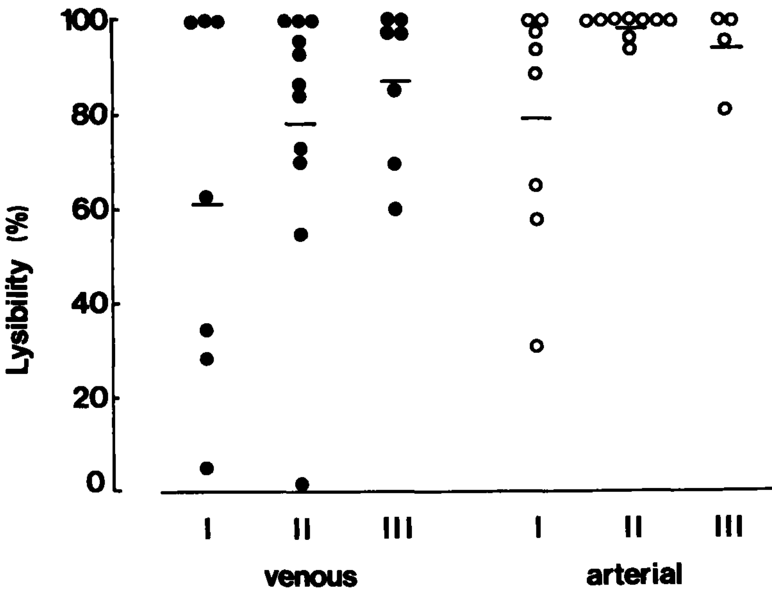
PAI-1, t-PA and plasminogen

PAI-1 amounted to 902 ± 129 ng/g (range 70 - 3345) in venous thrombi and pulmonary emboli, as compared to 2031 ± 401 ng/g (range 187 -8005) in arterial thrombi. In fresh, recent and aged venous thrombi the amount of PAI-1 was 581 ± 161, 1027 ± 254 and 1026 ± 141 ng/g (ANOVA: F=1.22, N.S.), whereas the corresponding figures for arterial thrombi were 2346 ± 897, 1922 ± 500 and 1650 ± 498 ng/g, respectively (ANOVA: F=0.20, N.S.).

In venous thrombi, thrombus-bound t-PA amounted to 26.7 + 3.0 ng/g (range 7.7 - 70.7 ng/g). In arterial thrombi thrombus-bound t-PA amounted to 62.2 ± 8.3 ng/g (range 13.7 - 146.8 ng/g). The amount of t-PA in thrombi was similar in all age groups of venous thrombi: 27 ± 5, 26 ± 4 and 28 ± 8 ng/g in fresh, recent and aged thrombi, respectively (ANOVA: F=0.01, N.S.). The coresponding figures for arterial thrombi are 61 ± 12, 45 ± 8 and 103 ± 26 ng/g, respectively (K.W.: N.S.)

Plasminogen amounted to 34.3 ± 4.8 μg/g (range 0.4 - 104 μg/g) in venous and to 64.1 ± 11.4 μg/g (range 1.4 - 184 μg/g) in arterial thrombi. In both venous and in arterial thrombi a similar pattern was seen with aged thrombi containing more thrombus-bound Plg than fresh or recent thrombi (Figure 1). Figures for fresh, recent and aged venous thrombi are 18 ± 5, 33 ± 6 and 53 ± 11 μg/g, respectively (K.W.: p=0.024). For arterial thrombi corresponding amounts were 40 ± 9, 55 ± 11 and 132 ± 38 μg/g respectively (K.W.: N.S.). Aged venous thrombi contained significantly more Plg than fresh ones (p=0.013) and the difference between aged and fresh thrombi similarly was significant if the venous and arterial thrombi were taken together (M.W.U.: p=0.018).

Figure 2 Lysibility in relation to thrombus age.



Venous (•) and arterial (o) thrombi of various ages are similarly susceptible to lysis by Streptokinase. Groups I, II and III represent fresh, recent and aged thrombi respectively. Individual values and means are given.

Lysibility

Figure 2 demonstrates the lysibilties of thrombi of different ages in buffer containing SK, for venous as well as for arterial thrombi. Lysibility of fresh, recent and aged venous thrombi was 61 ± 15, 78 ± 9 and 87 ± 6 % respectively (K.W.: N.S.). The corresponding values for arterial thrombi were 79 ± 9, 99 ± 1 and 94 ± 5 % respectively (K.W.: N.S.). In both venous and in arterial thrombosis, the aged thrombi were at least as susceptible to lysis by SK as the fresh thrombi.

DISCUSSION

In this study we have demonstrated that aged human thrombi are not more resistant to lysis by streptokinase ex vivo than are recent or fresh thrombi (Fig. 2). In contrast, in venous as well as in arterial thromboembolism aged thrombi tended to be more susceptible to SK than fresh ones.

Extrapolation of these findings to the in vivo situation may not be justified, since in vivo additional factors play a role, e.g. access of fibrinolytic agents [28], or plasminogen [29] to a thrombus.

In our study a clinical diagnosis of thromboembolism was made only in half of the cases of venous thromboembolism. Clinical estimation of thrombus age was possible only in 24 % of cases of venous and in 52 % of cases of arterial thromboembolism. Overall, only 20 % of venous and 33 % of arterial thrombi were adequately dated by clinical criteria.

Histological determination of thrombus age fails to differentiate beween hours or one day, but lacks the fallacy of classifying a thrombus fresh when in fact it is aged and vice versa. However, a new problem is introduced with histological determination of thrombus age as parts of several ages can be recognized within one thrombus.

In this study we took efforts to select representative parts from several segments of each thrombus for the histological examination, for the lysibility studies and for the determinations of PAI-1, Plg and t-PA. During the lysibility studies we rarely noted resistance to lysis of one part of thrombus and susceptibility of others. As each thrombus was homogenised for determination of PAI-1, Plg and t-PA, inhomogeneity of a thrombus could not have affected the outcome of these proteins.

At a first glance our findings seem incompatible with the results of various studies in acute myocardial infarction [3,4,30,32], which, in contrast, suggest that very fresh thrombi are more susceptible to thrombolytic therapy than are older thrombi. In

those studies, the decrease in reperfusion rate following thrombolytic therapy, which was associated with a longer duration of symptoms, occurred in the first 6 hours. Our clinical records included a detailed description of the timing of symptoms in 7 of the 14 cases of fresh thromboembolism. In all these 7 cases clinical symptoms had been present for at least one day. Although we cannot exclude that the remaining fresh thrombi were indeed less than 6 hrs old, it is more likely that, overall, the age of a substantial proportion of fresh thrombi was beyond the 6-hour time range of the mentioned studies. We therefore believe that our findings do not contradict the findings in acute myocardial infarction.

In contrast to acute myocardial infarction, the duration of signs and symptoms is usually expressed in terms of days or even weeks in cases of venous thrombosis, pulmonary embolism and of peripheral arterial disease [6-12,23-27]. Studies with thrombolytic agents in these conditions show either no relationship between thrombus age and lysibility [6,7,10,11, 23-27] or have not addressed the question [8,9]. In addition, several studies have demonstrated the efficacy of fibrinolytic agents in longstanding vascular occlusions [12,25,26,32].

Gottlob [33] has shown that very fresh thrombi and unretracted blood clots (i.e. up to 30 minutes of age) contain plasminogen and lyse excellently. Subsequently plasminogen content and lysibility decrease to a nadir at 24-48 hours, as the thrombus retracts. Thereafter, both plasminogen content and lysibility increase to become impaired only in very old thrombi (i.e. several months of age), in which plasminogen was virtually absent. Gottlob related the changing pattern of lysibility to the immuno-histochemically determined plasminogen content of the thrombus. In the present study, we similarly demonstrated a relationship between thrombus age on the one hand and plasminogen content (Fig.1) and lysibility (Fig.2) on the other hand. However, in contrast to Gottlob, we determined quantitavely and specifically fibrin-bound plasminogen. In addition, we quantitvely measured PAI-1 and fibrin-bound t-PA in thrombi and found no relation with thrombus age. Previously, we have reported that lysibility in streptokinase is not related to either t-PA and PAI-1 [22].

Recently, the findings of Gottlob have been supported by Sabovic et al. [29] who demonstrated poor lysibility of retracted blood clots, but not of unretracted blood clots. It was shown that enrichment of the blood clots with plasminogen increased lysibility [34].

In aged thrombi, spontaneous plasmin-mediated fibrinolysis can be demonstrated [35]. It has been demonstrated by Suenson et al. [36] that initial plasmin-mediated cleavage of fibrin discloses new Glu-plasminogen binding loci on fibrin, with resultant increase of binding of Glu-plasminogen to fibrin. Thus, by continuous spontaneous

plasmin-mediated lysis binding of plasminogen to the thrombus may be enhanced, explaining the increased plasminogen content of aged thrombi.

In conclusion, aged thrombi are at least as susceptible to (ex vivo) lysis by SK as are fresh thrombi. The increase in thrombus-bound plasminogen, which is associated with ageing of thrombi, may render aged thrombi susceptible to lysis.

REFERENCES

1. Marder VJ, Sherry S: Thrombolytic Therapy: Current Status. N Engl J Med 318: 1512-1521; 1585-1595, 1988
2. Chesterman CN: Thrombolytic therapy. In: AL Bloom, DP Thomas (eds): Haemostasis and Thrombosis, 2nd ed., London, Churchill Livingstone, 1987, pp 829-848
3. Chesebro JH, Katterud G, Roberts R, Borer J, Cohen LS et. al.: Thrombolysis in myocardial infarction (TIMI) Trial, Phase 1: a comparison between intravenous tissue plasminogen activator and intravenous streptokinase. Clinical findings through hospital discharge. Circulation 76: 142-154, 1987
4. Gruppo Italiano per lo studoio della streptochinasi nell'infarto miocardico (GISSI): Effectiveness of intravenous thrombolytic treatment in acute myocardial infarction. Lancet 1986: i: 397-401
5. Wheeler HB, Anderson FA Jr: Diagnostic approaches for deep vein thrombosis. Chest: 89 suppl: 407S - 412S, 1986
6. Tibutt DA , Chesterman CN, Williams EW, Faulkner T, Sharp AA: Controlled trial of the sequential use of streptokinase and ancrod in the treatment of deep vein thrombosis of lower limb. Thromb Haemost: 37: 222-231, 1977
7. Gallus AS, Hirsch J, Cade JF, Turpie AGG, Walker IR, Gent M: Thrombolysis with a combination of small doses of streptokinase and full doses of heparin. Semin Thromb Haemost 2: 14-32, 1975
8. Arnesen H, Heilo A, Jakobsen E, Ly B, Skaga E: A prospective study of streptokinase in the treatment of deep vein thrombosis. Acta Med Scand 203: 457-463, 1978
9. Davies JA, Merrick MV, Sharp AA, Holt JM: Controlled trial of ancrod and heparin in treatment of deep-vein thrombosis of lower limb. Lancet 1972: i: 113-115
10. Tibbutt DA, Williams EW, Walker MW, Chesterman CN, Holt JM, Sharp AA: Controlled trial of Ancrod and Streptokinase in the treatment of deep vein thrombosis of the lower limb. Br J Haem 27:407-414, 1974
11. Duckert F, Müller G, Nyman D, Benz A, Prisender S, Madar G, Da Silva MA, Widmer LK, Schmitt HE: Treatment of deep vein thrombosis with Streptokinase. Br Med J 1975: i:479-481
12. Trübestein G: Can thrombolytics prevent post-phlebitic syndrome and thrombo-embolic disease? Haemostas 16 (suppl 3): 38-50, 1986
13. Rosenow EC III, Osmundson PJ, Brown ML: Pulmonary embolism. Mayo Clin Proc: 56: 161-178, 1981
14. Terrin M, Goldhaeber SZ, Thompson B and the TIPE-investigators: Selection of patients with acute pulmonary embolism for thrombolytic therapy. Thrombolysis in Pulmonary Embolism (TIPE) Patient Survey. Chest 95: 279S - 281S, 1989
15. PIOPED Investigators: Value of the ventilation/perfusion scan in acute pulmonary embolism. Results of the prospective investigation of pulmonary embolism diagnosis (PIOPED). J Am Med Assoc 263: 2753-2759, 1990
16. Tibbutt DA, Chesterman CN: Pulmonary embolism: current therapeutic concepts. Drugs11: 161-192, 1976

17. Johannessen K-A, Nordrehaug JE, von der Lippe G, Vollset SE: Risk factors for embolisation in patients with left ventricular thrombi and acute myocardial infarction. Br Heart J 60: 104-110, 1988
18. Lechat P, Mas JL, Lascault G, Loron P, Theard M, Klimczac M, Drobinski G, Thomas D, Grosgogeat Y: Prevalence of patent foramen ovale in patients with stroke. N Engl J Med: 318: 1148-1152, 1988
19. Hume M, Sevitt S, Thomas DP: Venous thrombosis and pulmonary embolism. Cambridge, Harvard University Press, 1970, pp 25-53
20. Freiman DG: The structure of thrombi. In: RW Colman, J Hirsch, VJ Marder, EW Salzman (eds): Haemostasis and thrombosis. Basic principles and clinical practice. Philadelphia, Lippincott, 1982, pp 766-780
21. Woolf N: Thrombosis and atherosclerosis. In: AL Bloom, DP Thomas (eds): Haemostasis and thrombosis. London, Churchill Livingstone, 1981, pp 527-553
22. BJ Potter van Loon, DC Rijken, EJP Brommer: Composition and lysibility of human thrombi (ex vivo). Fibrinolysis 4 (Supp 1): 7, 1990 (abstr)
23. Urokinase Pulmonary Embolism Trial (UPET), Phase 1 Results. J Am Med Assoc: 214: 2163-2172, 1970
24. Luomanmäki K, Halttunen PK, Hekali P, Valle M, Heikkilä: Experience with streptokinase treatment of major pulmonary embolism. Ann Clin Res: 15: 21-25, 1983
25. Hess H, Ingrisch H, Mietaschk A, Rath H: Local low-dose thrombolytic therapy of peripheral arterial occlusions. N EngJ Med 307: 1627-1630, 1982
26. Robinson JA, Carty GA, Burch BH: Factors predicting the outcome of regional streptokinase therapy for prripheral arterial occlusions. Surg Gynaecol Obstet 161: 453-456, 1985
27. Collen D, Stassen JM, Verstraete M: Thrombolysis with human extrinsic (tissue-type) plasminogen activator in rabbits with experimental jugular vein thrombosis. J Clin Invest 71: 368-376, 1983
28. Blinc A, Schara M, Koren M: One-dimensional electron spin resonance imaging. Fibrinolysis 4 (Supp 1): 28, 1990 (abstr)
29. Sabovic M, Lijnen HR, Keber D, Collen D: Effect of retraction on the lysis of human clots with fibrin specific and non-fibrin specific plasminogen activators. Thromb Haemostas 62: 1083-1087, 1989
30. ISIS-2 (Second International Study of Infarct Survival) Collaborative Group: Randomised trial of intravenous streptokinase, oral aspirin, both or neither among 17,187 cases of suspected acute myocardial infarction. Lancet 1988: ii: 349-360
31. Kent RS, Batson AG, Littlejohn JK: Thrombolytic efficacy of tissue-type plasminogen activator. In: S Sherry, R Schröder, C Kluft, AJ Six, KL Mettinger (eds): Controversies in Coronary Thrombolysis. London, Current Medical Literature, 1989, pp 3-14
32. Weimar W, Stibbe J, van Seyen AJ, Billiau A, De Somer P, Collen D: Specific lysis of an iliofemoral thrombus by administration of extrinsic (tissue-typ0e) plasminogen activator. Lancet 1981: *ii: 1018-1020*
33. Gottlob R: Plasminogen and plasma plasmin inhibitors in arterial and venous thrombi of various ages. In: JF Davidson, MM Samama, PC Desnoyers (eds): Progress in Chemical Fibrinolysis and Thrombolysis, Vol 1. New York, Raven Press, 1975, pp 23-35
34. Sabovic M, Lijnen HR, Keber D, Collen D: Correlation between progressive adsorption of plasminogen to blood clots immersed in a plasma milieu and clot sensitivity to lysis. Fibrinolysis 4 (Supp 1): 33, 1990 (abstr)
35. Francis CW, Markham RE, Marder VJ: Demonstration of in situ fibrin degradation in pathologic thrombi. Blood 63: 1216-1224, 1984
36. Suenson E, Lützen O, Thorsen S: Initial plasmin-degradation of fibrin as the basis of a positive feed-back mechanism in fibrinolysis. Eur J Biochem 140: 513-522, 1984

CHAPTER 8

SUMMARY AND CONCLUDING REMARKS

In this thesis several studies are reported, dealing with pathophysiological mechanisms resulting in aberrant fibrinolysis. Particular interest has been given to fibrinolysis in relation to diabetes mellitus, and to regulation of fibrinolysis at its target's site, i.e. the thrombus.

Chapter 1 gives an overview of what is known of fibrinolysis in relation to diabetes mellitus. Large-scale studies have demonstrated a depressed basal plasma fibrinolytic activity in non-insulin dependent diabetes [1]. The depression of fibrinolysis is mainly due to elevated levels of plasminogen activator inhibitor type 1 (PAI-1), a common finding in NIDDM [2,3]. Both elevated levels of PAI-1 and depressed plasma fibrinolytic activity have been associated with increased cardiovascular morbidity [4,5]. The mechanisms resulting in either elevated levels of PAI-1 or in depressed plasma fibrinolytic activity are unknown. Several investigators have found rather high correlations between PAI-1 (mainly activity determinations) and insulin levels. This, in combination with the finding that insulin promotes PAI-1 synthesis by hepatocytes in vitro, has lead to the hypothesis that hyperinsulinaemia results in elevated PAI-1 levels and depression of fibrinolysis.

In insulin-dependent diabetes mellitus (IDDM) basal plasma fibrinolytic activity is not depressed and PAI-1 levels are considered normal. However, fibrinolysis may be depressed in IDDM due to inadequate release of tissue-type plasminogen activator (t-PA). Defects in t-PA release are best studied following stimulation by venous occlusion, DDAVP, or exercise. The defect in t-PA secretion following stimulation is considered a marker of endothelial damage [6]. The role of glycaemic control in the regulation of fibrinolysis remains controversial: cross-sectional studies in IDDM report equivocal results [3,6,7]. A longitudinal study in which IDDM patients have been studied both during poor and during good glycaemic control has not yet been performed.

Chapter 2 concerns the relationship between glycaemic control and fibrinolysis in IDDM. PAI-1 activity and basal and exercise-stimulated t-PA antigen levels were studied in patients with poor glycaemic control and in patients with adequately

controlled IDDM on two occasions: first, during poor glycaemic control in the former group and adequate control in the latter. Second, after five months of intensified treatment had rendered the poorly controlled subjects into adequate control. At the first occasion, PAI-1, basal and stimulated t-PA were lower in the poorly controlled group, although the difference was significant only for PAI-1 and basal t-PA. At the second occasion PAI-1 did not differ between the two groups, whereas both basal and stimulated t-PA remained reduced in the poorly controlled group, despite five months of adequate metabolic control. These findings surprisingly imply that improvement of glycaemic control during a period of five months in poorly controlled IDDM patients has an unfavourable effect on fibrinolysis. We speculate that (temporary ?) impairment of fibrinolysis may offer a pathophysiologic mechanism of deteriorating retinopathy following improvement of glycaemic control. Furthermore, the adverse effect of adequate glycaemic control on fibrinolysis may offer an explanation for the lack of a relationship between glycaemic control and macrovascular disease in IDDM. Studies in larger patient groups and of a longer duration are needed to further resolve this important issue.

Chapter 3 addresses exercise-stimulated fibrinolysis in IDDM in comparison with healthy control subjects. For this purpose well-trained healthy control subjects and IDDM patients without micro- or macrovascular disease were studied during a 3-hour 32 km outdoor running session. t-PA antigen, urokinase-type plasminogen activator (u-PA) antigen and plasmin - α2-antiplasmin complexes (PAP) increased steadily throughout the exercise period, indicating that during this 3-hour run the potential to activate fibrinolysis does not become exhausted. In relation to the intensity of exercise, t-PA was lower during exercise in the IDDM as compared to the control subjects. t-PA levels parallelled von Willebrand Factor levels during exercise, suggesting a defective exercise-induced endothelial release of both proteins in IDDM.

In contrast to t-PA, the rise of u-PA was higher in the IDDM and overall activation of fibrinolysis, as measured by PAP and fibrin degradation products, was similar in the two groups. These findings suggest that, despite an endothelial defect in IDDM without apparent macro- or microvascular disease, overall activation of fibrinolysis is sufficient. The practical implication of these findings is that IDDM patients should not be discouraged to perform endurance exercise.

The issue of hyperinsulinaemia and elevated levels of PAI-1 is addressed in Chapters 4 and 5. Besides the associations between PAI-1 and insulin levels, associations exist between PAI-1 levels on the one hand and hypertension, hypertriglyceridaemia,

reduced levels of HDL-cholesterol and abdominal type of obesity. The latter conditions are all associated with insulin resistance, of which hyperinsulinaemia is a hallmark.

Chapter 4 describes a study to elucidate whether PAI-1 antigen levels are indeed linked to insulin resistance. Insulin action was measured by sequential hyperinsulinaemic euglycaemic glucose clamp studies in obese NIDDM and nondiabetic subjects. Like other authors we found a significant correlation between PAI-1 and insulin levels, but in addition we found a very strong correlation between PAI-1 and the degree of insulin resistance. This latter correlation ($r=0.87$) appeared dominant in a multivariate analysis: 76 % of the total variance of PAI-1 could be explained by the degree of peripheral insulin resistance (as measured by ED_{50}pgu, i.e. the insulin level at which peripheral glucose uptake is 50 % of maximal). In contrast, insulin levels did not appear to be independently related to PAI-1. These findings suggest that, rather than hyperinsulinaemia, insulin resistance is the pathophysiologic characteristic resulting in elevated levels of PAI-1. If insulin is not the mediator between insulin resistance and PAI-1, what is ? We have investigated the possibility of endogenous proinsulin being the mediator, but this seems unlikely.

Chapter 5 describes the response of PAI-1 to exogenous insulin infusion in the same subjects as Chapter 4, i.e. during and after a hyperinsulinaemic euglycaemic glucose clamp. Despite hyperinsulinaemia for over 8 hours, up to supraphysiological levels, PAI-1 levels followed the normal diurnal pattern of a decrease during the daytime. In addition, PAI-1 levels after the clamp were not different from PAI-1 levels at the same time of the day without insulin infusion. These results demonstrate that (exogenous) insulin does not result in elevation of PAI-1 levels. Since this study has been published subsequent investigators have confirmed these findings [8,9].

Clinical studies usually address plasma fibrinolytic activity, or, more recently, the components of the fibrinolytic system considered pivotal in determining fibrinolytic activity. However, in vitro and in vivo studies have demonstrated that the rate of fibrinolysis at the site of a clot [10-12] or thrombus [13,14] depends largely on the composition of the clot. Understanding why some clots or thrombi are resistant to fibrinolysis while others are susceptible may contribute to the understanding of increased atherogenesis in diabetes mellitus, since fibrin deposists are an essential component of advanced atheromatous lesions.

Chapter 6 describes the study of venous and arterial human thrombi ex vivo. Composition of the thrombi (PAI-1 antigen, t-PA antigen and plasminogen antigen) were studied, as was the lysibility in three different fibrinolytic agents (streptokinase [SK], urokinase [UK] and t-PA). PAI-1 had accumulated in the thrombi, arterial thrombi containing more (mean: 200 times the plasma concentration) than venous (mean: 90 times the plasma concentration). Presumably this PAI-1 is platelet-derived. t-PA also was present in thrombi to a high extent (6 and 3 times the plasma concentration for arterial and venous thrombi, respectively), about half of which was considered fibrin-bound. Most thrombi showed spontaneous lysis in buffer (mean about 30 %, range 0 - 97 %), which correlated inversely with the amount of PAI-1 antigen in the thrombus. Lysis in streptokinase was dependent on (fibrin-bound) plasminogen content, which, in turn, correlated strongly with (fibrin-bound) t-PA. These results demonstrate that the previously reported dependence of lysis on plasminogen content and on t-PA content of a clot [10-14] also is valid in the situation of human thrombi. In addition, we have demonstrated that susceptibilty to lysis of a given thrombus in SK is fairly similar to its susceptibilty in either UK or t-PA. In other words, if thrombolysis does not occur with one agent, don't expect success with another.

Chapter 7 is an extension of Chapter 6. In this study the lysibility of thrombi is related to thrombus age. Surprisingly, aged thrombi (i.e. 10 days or older, by histologic criteria) were at least as susceptible to lysis as fresh thrombi (i.e. 2 days or less). The older thrombi appeared to contain significantly more plasminogen than the fresh ones, possibly explaining the above-mentioned findings. The clinical implication of this finding is that, in cases of expected benefit of reperfusion, one should not restrict thrombolytic therapy to fresh thrombi.

A hypothesis

The studies in thesis, although addressing only part of the spectrum of fibrinolysis in diabetes mellitus, have given ample data to question existing hypotheses and to construct new ones. Based on chapter 3, it is likely that there is endothelial damage early in the course of IDDM, i.e. before the existence of microalbuminuria, as expressed by a defect in t-PA release. This defect in t-PA release is more pronounced in patients with poor metabolic control (Chapter 2), but improvement of glycaemic control does not restore this defect in t-PA release. In contrast, PAI-1 increases with improvent of control resulting in a (temporary ?) depression of fibrinolysis. This may

explain the lack of an association between glycaemic control and macrovascular disease in IDDM. In addition, a temporary depression of fibrinolysis may contribute to deterioration of retinopathy seen in the first months after improving glycaemic control.

Whereas the cardinal feature of IDDM is insulin deficiency, it is insulin resistance in the majority of NIDDM. Insulin resistance, in NIDDM, but also in other conditions, gives rise to elevated levels of PAI-1 (Chapter 4), which add to the risk of acquiring a cardiovascular event. Whether insulin resistance is the cause of elevated PAI-1 levels is unknown, as well as the mediator between insulin resistance and PAI-1. The most favourite candidate, insulin, has been excluded (Chapter 5) and another favourite candidate, proinsulin, is unlikely the mediator (Chapter 4). Either a plasma factor associated with insulin resistance is the mediator between the two or both insulin resistance and elevated levels of PAI-1 may be epiphenomena of yet another feature causal to both.

The defect in t-PA release in IDDM and the increase in PAI-1 in NIDDM both result in an imbalance between activators and inhibitors of fibrinolysis. This results in impaired plasma fibrinolysis. However, the site of the action in fibrinolysis is not plasma, but a thrombus, clot or fibrin deposition. Here too, PAI-1 inhibits (spontaneous) lysis and t-PA, by increasing the binding of plasminogen to fibrin, promotes lysis (Chapter 6). Ageing of thrombi increases the plasminogen content and therefore does not necessarily render them resistant to lysis (Chapter 7). Future studies should consider more the regulation of fibrinolysis at its target's site and whether medical intervention (e.g. directed at increasing plasma levels of plasminogen and/or t-PA, or at decreasing platelet hyperaggregability in diabetes mellitus) can alter this.

REFERENCES

1. Fuller J.H., Keen H., Jarrett R.J., Omer T., Meade T.W., Ckakrabarti R., North W.S., Stirling Y.: Haemostatic variables associated with diabetes and its complications. Br med J 1979: ii: 964-966
2. Almèr L.O.: Fibrinolytic disorders in diabetes mellitus. Diabet Metab 1988; 14: 519-522
3. Auwerx J., Bouillon R., Collen D., Geboers J.: Tissue-type plasminogen activator antigen and plasminogen activator inhibitor in diabetes mellitus. Arteriosclerosis 1988; 8: 68-72
4. Meade T.W., Mellows S., Brozovic M., et al.: Haemostatic function and ischaemic heart disease: principal results of the Northwick park Heart Study. Lancet 1986; ii: 533-537
5. Hamsten A., Walldius G., Szamosi A., Blömback M., De Faire U., Dahlén G., Landou C., Wiman B.: Plasminogen activator inhibitor in plasma: risk factor for recurrent myocardial infarction. Lancet 1987; ii: 3-9
6. Jensen T., Bjerre-Knudsen J., Feldt-Rasmussen B., Deckert T.: Features of endothelial dysfunction in early diabetic nephropathy. Lancet 1989; i: 461-463

7. Nilsson T.K., Lithner F.: Glycaemic control, smoking habits and diabetes duration affect the extrinsic fibrinolytic system in type I diabetic patients but microangiopathy does not. Acta Med Scand 1988; 224: 123-129
8. Grant P.J., Kruithof E.K.O., Felley C.P., Felber J.P., Bachmann F.: short-term infusions of insulin, triacylglycerol and glucose do not cause acute increases in plasminogen activator inhibitor-1 concentrations in man. Clinical Science 1990; 79: 513-516
9. Landin K., Tengborn L., Chmielewska J., Von Schenck H., Smith U.: The acute effect of insulin on tissue plasminogen activator and plasminogen activator inhibitor in man. Thromb Haemostas 1991; 65: 130-133
10. Brommer EJP: The level of extrinsic Plasminogen Activator (t-PA) during clotting determines the rate of fibrinolysis; inefficiency of activators added afterwards. Thromb. Res. 1984; 34: 109-115
11. Zamarron C, Lijnen H.R., Collen D.: Influence of exogenous and endogenous tissue-type plasminogen activator on the lysiability of clots in a plasma milieu in vitro. Thromb Res 1984; 35: 335-345
12. Tran-Thang C, Kruithof EKO, Bachmann F: The mechanism of in vitro clot lysis induced by vascular Plasminogen Activator. Blood 1984; 63: 1331-1337
13. Gottlob R: Plasminogen and plasma Plasmin Inhibitors in arterial and venous thrombi of various ages. In: JF Davidson, MM Samama, PC Desnoyers (eds): Progress in chemical fibrinolysis and thrombolysis, vol. 1. Raven Press, New York, 1975, pp 23-36
14. Sabovic M, Lijnen HR, Keber D, Collen D: Correlation between progressive adsorption of plasminogen to blood clots and their sensitivity to lysis. Thromb. Haemostas. 1990; 64: 450-454

SAMENVATTING

Diabetes mellitus, oftewel suikerziekte, kan behandeld worden met een dieet, tabletten en/of insuline. Met deze behandelingsvormen kan het bloedglucosegehalte verlaagd worden en kunnen de acute gevolgen van suikerziekte goed bestreden worden. Helaas blijken vele diabetespatiënten op den duur toch aandoeningen te krijgen als gevolg van de ziekte: oogafwijkingen, nieraandoeningen, aantasting van zenuwen en hart-en vaatziekten. Waar de eerstgenoemde afwijkingen door een nauwkeurige instelling van het bloedglucosegehalte vertraagd en misschien wel ten dele voorkomen kunnen worden, lijkt de instelling van het bloedglucosegehalte geen invloed te hebben op het al dan niet verkrijgen van hart- en vaatziekten bij diabetes patienten. De ontwikkeling van hart- en vaatziekten bij diabetes mellitus is het gevolg van (slag)aderverkalking (atherosclerose). Hierbij ontwikkelen zich in de wand van de slagaderen afwijkingen die uiteindelijk kunnen leiden tot dichtslibben van het betreffende bloedvat. Het weefsel dat door dit vat van bloed voorzien wordt, krijgt dan onvoldoende zuurstof en voedingsstoffen, zodat het minder goed functioneert en vaak zelfs afsterft.

Het is niet bekend wat de oorzaak is van de versnelde atherosclerose bij suikerziekte. In het bloed en in de wanden van bloedvaten worden afwijkingen gevonden, die -theoretisch- tot gevolg hebben dat het bloed in de bloedbaan bij personen met suikerziekte meer tot stollen zou neigen. Daardoor zouden bloedvaten en haarvaatjes bij diabetespatiënten sneller verstopt raken, hetgeen leidt tot de bovenbeschreven gevolgen.

Een bloedstolsel bestaat uit fibrine en bloedcellen. Fibrine is een eiwit, dat wordt gevormd onder invloed van stollingsfactoren. Het fibrine fungeert als het ware als het "skelet" van een bloedstolsel. Als het fibrine in een bloedstolsel afgebroken wordt (**FIBRINOLYSE**), zal het bloedstolsel snel uiteenvallen. Hiervan maakt men gebruik bij de moderne behandeling van het hartinfarct, waarbij patiënten vaak kort na opname in het ziekenhuis een fibrinolytische stof krijgen toegediend.

Binnen het lichaam is sprake van doorlopende bloedstolling èn van oplossing van bloedstolsels. Doordat de twee in evenwicht zijn, merken wij er in het dagelijks leven niets van, want de gevormde bloedstolsels worden snel opgeruimd. Als de neiging tot stolling toeneemt, zoals bij diabetes mellitus, kan de balans tussen stolling en fibrinolyse verstoord raken. Er treedt langzamerhand meer stolling op en uiteindelijk zullen vaten eerder verstopt raken, wat leidt tot versneld ontstaan van hart- en vaatziekten.

Het systeem van eiwitten in het bloed dat leidt tot afbraak van fibrine heet het **FIBRINOLYTISCH SYSTEEM.** Een vereenvoudigd schema van dit systeen is weergegeven op bladzijde 13. Het eiwit dat uiteindelijk fibrine afbreekt heet plasmine. Plasmine wordt gevormd uit het onwerkzame plasminogeen. Dit is een voorloperstof die onder invloed van plasminogeen activator pas wordt omgezet in plasmine.

In hoeverre het fibrinolytisch systeem bij diabetes mellitus afwijkt van normaal was tot op heden niet duidelijk. Men heeft zowel versterkte als verzwakte, als ook normale activiteit van het fibrinolytisch systeem beschreven. Er zijn een aantal redenen aan te geven als verklaring voor de verscheidenheid aan uitkomsten: verschillende soorten suikerziekte (jeugddiabetes tegenover ouderdomsdiabetes, oftewel insuline-afhankelijke diabetes mellitus (IADM) tegenover niet-van-insuline-afhankelijke diabetes mellitus (NIADM)), verschillen in de mate van instelling van het bloedglucosegehalte, verschillen in de ontwikkeling van lange termijn complicaties van diabetes en verschillen in gebruikte laboratoriumtechnieken.

Daarnaast is de laatste jaren duidelijk geworden dat de activiteit van het fibrinolytisch systeem voornamelijk bepaald wordt door twee componenten: een stof die fibrinolytische activiteit verlaagt, het plasminogeen activator remmer type 1 (PAI-1), en het weefsel-type plasminogeen activator (t-PA). In rust is er een overmaat PAI-1 aanwezig en is de fibrinolytische activiteit laag, terwijl na stimulatie van het fibrinolytisch systeem er meer t-PA in het bloed komt met als gevolg een toegenomen fibrinolytische activiteit. Het fibrinolytisch syteem kan gestimuleerd worden door stuwing, door infusie met een hormoon, of door .....inspanning !

Van dit laatste hebben wij gebruik gemaakt bij het uitvoeren van de studies vermeld in de hoofdstukken 2 en 3. In **Hoofdstuk 2** beschrijven wij de invloed van bloedglucoseregulatie op het fibrinolytisch syteem bij 8 goed gereguleerde IADM (d.w.z. bloedglucosewaarden over het algemeen genomen bijna normaal) en bij 7 matig tot slecht gereguleerde IADM (d.w.z. bloedglucosewaarden in het algemeen hoog). De bloedglucoseregulatie bij de slecht gereguleerde IADM werd zodanig verbeterd, dat deze gemiddeld gelijk was aan die van de groep, die goed was gereguleerd. Na een periode van vijf maanden werden alle patienten opnieuw onderzocht.

Wij vonden dat het gehalte aan PAI-1 in het bloedplasma in de slecht gereguleerde groep lager was dan dat van de goed gereguleerde groep. Bovendien bleek dat verbetering van de bloedglucoseregulatie in eerstgenoemde groep resulteerde in een stijging van het PAI-1. Het t-PA-gehalte van het bloedplasma was in de slecht gereguleerde groep lager dan in de goed gereguleerde groep en dit veranderde niet na verbetering van de bloedglucoseregulatie. Het t-PA-gehalte na stimulatie door in-

spanning was in de slecht gereguleerde groep lager, zij het niet significant. Ook na verbetering van de regulatie was het gestimuleerde t-PA-gehalte in de slecht gereguleerde groep lager, maar ditmaal was het verschil wel significant.

Deze resultaten wijzen erop dat verbetering van bloedglucoseregulatie bij (langdurig) slecht gereguleerde IADM een ongunstig effect kan hebben op het fibrinolytisch systeem. Door enerzijds een verhoging van het PAI-1-gehalte van het bloedplasma en anderzijds het uitblijven van een toename van t-PA-spiegels wordt de fibrinolytische activiteit -theoretisch- verlaagd. Ervan uitgaande dat een verlaagde fibrinolytische activiteit van het bloedplasma een rol kan spelen bij het ontstaan van versnelde atherosclerose bij diabetes mellitus, kunnen deze resultaten een verklaring geven voor het (tot nog toe) ontbreken van aanwijzingen dat verbeterde bloedglucoseregulatie minder hart- en vaatziekten bij diabetes mellitus tot gevolg heeft.

In **Hoofdstuk** 3 worden 7 insuline-afhankelijke diabetische marathonrenners vergeleken met 8 niet-diabetische marathonrenners, die als "gezonde controlepersonen" dienden. De diabetische patiënten hadden geen van allen lange-termijn complicaties. Wij constateerden dat de bloedspiegels van de plasminogeenactivatoren t-PA en urokinase-type plasminogeen activator (u-PA) bij zowel diabetespatiënten als bij gezonde controlepersonen duidelijk stegen. Wel viel op dat de diabetes patienten tijdens inspanning minder hoge bloedspiegels van t-PA hadden dan de gezonde controlepersonen, gerelateerd aan de geleverde inspanning. Omdat t-PA afkomstig is van de vaatwand, duidt dit op een stoornis in de functie daarvan.

De uiteindelijke activering van het fibrinolytisch systeem bleek bij de diabetespatiënten toch voldoende, omdat een andere plasminogeen activator, het u-PA, tijdens inspanning in hoge mate aanwezig bleek te zijn. De praktische consequentie van dit onderzoek is dat langdurige inspanning bij IADM, door verhoging van fibrinolytische activiteit, kan bijdragen tot bescherming tegen het versneld ontwikkelen van hart-en vaatziekten.

Bij NIADM is veelal de spiegel van het PAI-1 verhoogd. Dit kan leiden tot een verminderde fibrinolytische activiteit van het bloedplasma, wat mogelijk bijdraagt tot het versneld optreden van hart- en vaatziekten bij dit type diabetes mellitus. De bloedplasmaspiegel van het PAI-1 blijkt samen te hangen met de plasma-insuline spiegel, zowel in patiënten met NIADM als in niet-diabetische patiëntengroepen en in gezonde controlepersonen. Er is gesuggereerd dat insuline de productie van PAI-1 door de lever stimuleert. Bij de mens is dit echter nooit aangetoond.

In **Hoofdstuk 4** onderzoeken wij de relatie tussen de spiegel van PAI-1 in het bloedplasma enerzijds, en insulinespiegel en insulinewerking anderzijds. Dit laatste werd met de zogenaamde clamp techniek gemeten. Daarbij wordt insuline geïnfundeerd en wordt het bloedglucosegehalte op een vast niveau vastgezet, in ons geval 5 mmol/l. Uit deze studie blijkt dat er niet zozeer samenhang is tussen het PAI-1 en de insulinespiegel, maar tussen het PAI-1 en insulineresistentie. Als de insuline onvoldoende werkzaam is (zoals bij NIADM) is het PAI-1 in het bloedplasma hoog.
De verklaring voor de samenhang tussen het PAI-1 en de insulinespiegel, zoals deze gevonden is door anderen, ligt hierin dat bij insulineresistentie naast het PAI-1 als een compensatiemechanisme ook de insulinespiegel in het plasma stijgt. De proinsulinespiegels in het plasma bleek evenmin een directe samenhang te vertonen met die van PAI-1.

In **Hoofdstuk 5** rapporteren wij de PAI-1 waarden in het plasma tijdens insulineinfusie in het kader van de clamp studies vermeld in Hoofdstuk 4. Wij zagen dat verhoging van de insulinespiegel gedurende minstens 8 uren tot zelfs extreem hoge waarden geen verhoging gaf van het plasmagehalte van PAI-1. Dit bevestigt de bevindingen uit Hoofdstuk 4 dat insuline en PAI-1 niet oorzakelijk verbonden zijn.

Vooralsnog is onopgehelderd hoe insulineresistentie aanleiding geeft tot verhoging van de PAI-1 spiegel. In elk geval lijkt dit niet via een verhoging van de insulinespiegel te gebeuren.

Hoofdstukken 6 en 7 gaan over de resultaten van een onderzoek naar de factoren die van invloed zijn op de oplosbaarheid van een menselijk bloedstolsel (thrombus). Kwantitatieve gegevens over de samenstelling van thrombi zijn niet voorhanden. Evenmin is het bekend of de bij kunstmatig gevormde plasmastolsels vastgestelde relatie tussen samenstelling en oplosbaarheid van het stolsel toepasbaar is op menselijke thrombi.

In **Hoofdstuk 6** is onderzocht in hoeverre de oplosbaarheid van een thrombus afhangt van de samenstelling. Thrombi, zowel slagaderlijk als aderlijk, bleken beter op te lossen in streptokinase (een fibrine oplossende stof) naarmate ze meer aan-fibrine-gebonden plasminogeen bevatten. Het aan-fibrine-gebonden plasminogeen-gehalte van een thrombus wordt op zijn beurt voor een belangrijk deel bepaald door de hoeveelheid aan-fibrine-gebonden t-PA. Deze gegevens wijzen erop dat de tot dusverre gevonden in vitro gegevens inderdaad vertaald kunnen worden naar de menselijke thrombus.

In **Hoofdstuk 7** wordt bij dezelfde thrombi de histologisch geschatte ouderdom van de thrombus in verband gebracht met de samenstelling en met de oplosbaarheid. In tegenstelling tot wat algemeen aangenomen wordt, vonden wij dat oudere thrombi veelal goed oplossen in streptokinase. Dit bleek samen te hangen met een hoge aan-fibrine-gebonden plasminogeen-gehalte van deze thrombi in vergelijking met verse thrombi. Op grond van deze gegevens verdient het aanbeveling de huidige opinie, dat alleen verse thrombi met streptokinase behandeld dienen te worden, kritisch te bezien.

De studies in dit proefschrift zijn gerapporteerd:

IXth Int. Congress on Fibrinolysis, Amsterdam, 1988: hoofstukken 6 en 7

Fibrinolysis Workshop, Leiden, 1990: hoofdstukken 3 en 5

XIth Int. Congress on Thrombosis, Ljubljana, 1990: hoofdstuk 6

Nederlandse Internisten Dagen, Veldhoven, 1991: hoofdstuk 3

XIIIth Int. Congress on Thrombosis & Haemostasis, Amsterdam, 1991: hoofdstuk 4

27th Congress of the European Association for the Study of Diabetes,
Dublin, 1991: hoofdstuk 2

Hoofdstuk 3 is gedeeltelijk verschenen in Fibrinolysis 1990; 4 (Suppl. 2): 102-104
Hoofdstuk 5 is verschenen in Fibrinolysis 1990; 4 (Suppl. 2): 93-94
Hoofdstuk 6 is ter perse (Thrombosis & Haemostasis)

NAWOORD

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En, Most of All, *see page 5*

CURRICULUM VITAE

De schrijver werd op 9 augustus 1956 geboren te Kaapstad, Zuid-Afrika, waar hij tot zijn negende jaar woonde. Drie lagere en drie middelbare scholen werden bezocht en afgesloten met het behalen van het diploma Atheneum B in 1974 op het Rijnlands Lyceum te Oegstgeest. De studie geneeskunde werd aan de Rijksuniversiteit te Leiden gevolgd, waar kandidaats-, doctoraal- en artsexamen werden afgelegd in 1977, 1979 en 1981. In dat laatste jaar werd eveneens het Visa Qualifying Exam behaald en werd in het Westeinde Ziekenhuis te Den Haag de opleiding tot internist aangevangen (opleiders: Dr. E.J. Buurke en Dr. E. van Leer). Op 1 oktober 1986 volgde inschrijving als internist in het specialistenregister. Vanaf oktober 1986 tot september 1990 was de schrijver werkzaam in het Academisch Ziekenhuis Leiden, aanvankelijk op de afdeling nierziekten (Hoofd: Prof. Dr. L.A. van Es) en vanaf januari 1988 op de afdeling Stofwisselingsziekten en Endocrinologie (Hoofd: Prof. Dr. H.M.J. Krans). Van september 1990 tot oktober 1991 werkte hij als internist/chef de clinique in het Onze Lieve Vrouwe Gasthuis te Amsterdam (Hoofd: Dr. J. Silberbusch) en als klinisch beoordelaar bij het College ter Beoordeling van Geneesmiddelen te Rijswijk (Hoofd: Dr. C. de Visser). Sindsdien is hij werkzaam als internist/chef de clinique op de afdeling nefrologie/interne geneeskunde van het St. Lucas Ziekenhuis te Amsterdam (Hoofd: Dr. H. Schreuder). Hij is tevens sinds 1986 gastmedewerker van het Gaubius Laboratorium van het IVVO-TNO te Leiden.