Relations Between Coagulation And Fibrinolysis

A study on plasminogen activators

Ellen Braat

Stellingen

1. Trombine inactiveert scu-PA *in vivo*.
   dit proefschrift

2. Trombomoduline kan de inactivatie van scu-PA door trombine in plasma zowel stimuleren als voorkomen.
   dit proefschrift

3. Remming van urokinase-gemedieerde fibrinolyse in de gewrichten van patiënten met reumatoide arthritis via inactivatie van scu-PA door trombine kan een bijdrage leveren aan het degeneratieve verloop van deze ziekte.
   dit proefschrift

4. Het toeschrijven van onverklaarde contact activatie-afhankelijke fibrinolyse aan één onbekende plasminogeen activator is onjuist.
   dit proefschrift

5. De huidige kennis dat u-PA van groot belang is voor fibrinolyse in de muis geeft nog geen uitsluitsel over de betrokkenheid van dit enzym bij fibrinolyse in de mens.
   Carmeliet et al., Nature 1994; 368: 419-424 en Bugge et al., Proc Natl Acad Sci USA 1996; 93: 5899-5904

6. De aanwezigheid van een remmer van humaan plasma carboxypeptidase B, oftewel 'thrombin activatable fibrinolysis inhibitor' (TAFI), in de bloedzuiger *Hirudo medicinalis* is een duidelijke aanwijzing dat TAFI een fysiologische rol van betekenis speelt.
   Reverter et al., J Biol Chem 1998; 273: 32927-32933

7. De 'revised model'van de stolling, waarin voor de intrinsieke route via het contact activatie systeem geen plaats meer is, gaat voorbij aan het feit dat in een aantal situaties de stolling *in vivo* wel degelijk geïnitieerd wordt door contact activatie.
   Gailani et al., Science 1991; 253: 909-912

8. Aangezien het roken van sigaren leidt tot een verhoogd risico op cardiovasculaire aandoeningen en kanker van de bovenste luchtwegen, zou de Europese Unie in navolging op het reclameverbod voor sigaretten ook reclame voor sigaren moeten verbieden.
Iribarren et al., N Eng J Med 1999; 340: 1773-1780

9. De bevinding dat het voeden van larven van de monarch vlinder met stuifmeel van transgene maïs leidt tot een verminderde ontwikkeling en een verhoogd sterftecijfer van deze larven, moet beschouwd worden als een waarschuwing dat genetisch gemodificeerd voedsel een gevaar voor de gezondheid kan zijn.
Losey et al., Nature 1999; 399: 214

10. Tegen snurkers die 's nachts meer decibellen produceren dan wettelijk is toegestaan dienen juridische stappen te worden ondernomen.
Wilson et al., Chest 1999; 115: 762-770

11. De geschiedenis van de 20ste eeuw leert dat het instabiele fenotype van de beschaafde *Homo Sapiens* onder onfortuinlijke omstandigheden eenvoudig muteert in dat van een wrede *Homo Stupidus*.

12. Leven is het meervoud van lef.
Loesje

Stellingen behorende bij het proefschrift: *"Relations between coagulation and fibrinolysis. A study on plasminogen activators."*

Leiden, 25 november 1999 Ellen Braat

Relations between coagulation and fibrinolysis

A study on plasminogen activators

Proefschrift
ter verkrijging van de graad van Doctor
aan de Universiteit Leiden,
op gezag van de Rector Magnificus Dr. W.A. Wagenaar,
hoogleraar in de faculteit der Sociale Wetenschappen,
volgens besluit van het College voor Promoties
te verdedigen op 25 november 1999
te klokke 15.15 uur

door

Ellen Anne Martien Braat

geboren te 's-Gravenhage in 1970

Promotiecommissie

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Cover: 'Couple aux têtes pleines de nuages', Salvador Dali

Hoewel de menselijke vindingrijkheid verscheidene uitvindingen kan doen, zal zij nooit uitvindingen bedenken die mooier, eenvoudiger of doelmatiger zijn dan die der Natuur; want aan haar uitvindingen ontbreekt niets, en is niets overbodig.

Leonardo da Vinci

Voor Peter

Contents

CHAPTER 1

General Introduction

THE HAEMOSTATIC BALANCE

In the human body the haemostatic balance between clot formation (coagulation) and clot degradation (fibrinolysis) regulates the fluid state of blood under physiological conditions and the prevention of blood loss after vascular injury. Under physiological circumstances the endothelium, the inner layer of the vessel wall, maintains blood fluidity by inhibiting coagulation and platelet aggregation (1). When the vascular wall ruptures, the endothelium changes into a procoagulant surface and several processes are initiated to prevent blood loss (1). First, the muscular layer of the vessel wall contracts resulting in the narrowing of the vascular lumen, a process called vasoconstriction. Subsequently, a plug of platelets is formed at the injury site by adhesion and aggregation of platelets. Simultaneous activation of the coagulation system results in the generation of a solid fibrin network in and around the platelet plug. These processes are closely interacting. Activated platelets secrete several components that stimulate vasoconstriction and coagulation, they provide a promoting surface for coagulation and they bind fibrin(ogen) via specific receptors. Thrombin, the final product of the coagulation system, activates platelets and generates a fibrin network that reinforces the platelet plug. Once the clot has been formed at the injury site, coagulation ceases to prevent occlusion of the vessel. Wound healing can take place, the fibrin network is degraded via the fibrinolytic system, and the vascular wall can return to its original state.

The haemostatic balance is disturbed when coagulation or fibrinolysis is affected. Disturbances in the coagulation or fibrinolytic system are caused by genetic disorders (2,3), and also by factors like smoking, obesity, diabetes mellitus and alcohol (4-9). A defect in the coagulation system can result in severe bleeding symptoms, as seen in haemophilic patients who have a deficiency in coagulation factor VIII or IX (10). On the other hand, impaired fibrinolysis or excessive coagulation may lead to the development of cardiovascular diseases (11-16). Since cardiovascular diseases are the major cause of death in Western countries (17,18), it is obvious that an efficient haemostatic balance is critical in men.

COAGULATION

In 1964, a model of coagulation was independently proposed by Davie and Ratnoff (19) and by MacFarlane (20), which formed the basis for the current view of coagulation. Components of the coagulation system circulate as inactive pro-enzymes and are activated in an amplifying cascade or waterfall of enzymatic reactions. The coagulation cascade can be initiated by two pathways, namely the extrinsic pathway via tissue factor/factor VII and the intrinsic pathway via the contact activation system (Figure 1). As a result, thrombin is generated and a solid fibrin network is formed.

Coagulation cascade

Extrinsic pathway

Most of the enzymatic reactions in the extrinsic pathway are calcium-dependent and need a phospholipid surface, which is provided by activated platelets (21-23). The zymogens factor VII, IX, X and prothrombin possess carboxyglutamic acid groups in the so-called Gla-domain, which are involved in the binding to phospholipid membranes. Since vitamin K is essential in surface binding via the Gla-domain, these coagulation factors are called vitamin K-dependent proteins. Cofactors V and VIII do not possess a Gla-domain, but can bind directly to platelets via specific receptors (23). Factor V and VIII circulate as precursor forms and need to be activated in order to express their cofactor activity.

Activation of the extrinsic pathway is initiated when tissue factor present in the subendothelium is exposed to the circulation due to damage of the endothelium (Figure 1). Factor VII binds to tissue factor and is then converted into factor VIIa, either by autoactivation or by trace amounts of factor IXa, Xa or thrombin (24). The factor VIIa/tissue factor complex activates factor X either directly or indirectly via activation of factor IX. Factor IXa, forming the tenase complex with its cofactor VIIIa, also activates factor X. Subsequently, factor Xa forms the prothrombinase complex with its cofactor Va. The prothrombinase complex is able to cleave prothrombin (factor II) into thrombin (factor IIa). Thrombin can then exert its procoagulant properties. It cleaves fibrinogen into fibrin, generating a network of fibrin strands, and activates factor XIII. Factor XIIIa in turn stabilizes the fibrin network by crosslinking fibrin strands. In addition, thrombin induces the activation and aggregation of

platelets. These thrombin-mediated effects finally result in the formation of a solid clot composed of fibrin and platelets at the injured site of the vessel wall.

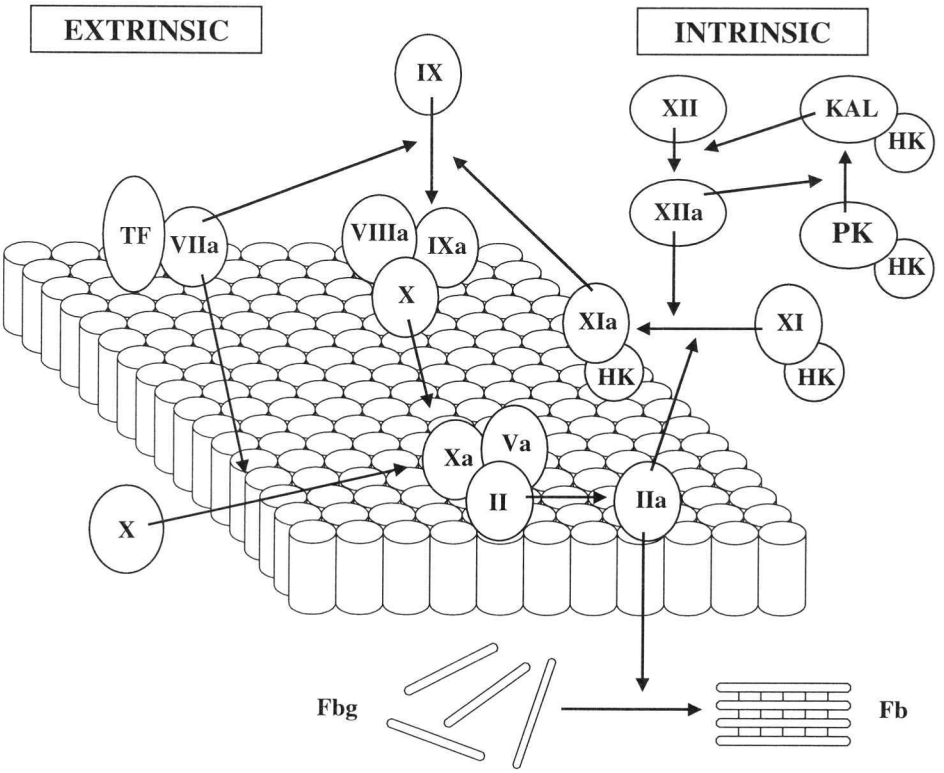

Figure 1. The coagulation cascade. The coagulation cascade can be activated by the extrinsic or the intrinsic pathway. Most of the enzymatic reactions take place on a phospholipid surface, as shown in this model. Initiation of the extrinsic pathway occurs via the activation of factor VII bound to tissue factor, followed by the activation of factor IX and X. The intrinsic pathway is initiated by (auto)activation of surface-bound factor XII, followed by the activation of factor XI , IX and X. At the end of the cascade thrombin is generated and a fibrin network is formed. Abbreviations used are: II, prothrombin; IIa, thrombin; TF, tissue factor; IX/IXa, factor IX(a); V/Va, factor V(a); VIII/VIIIa, factor VIII(a); X/Xa, factor X(a); XI/XIa, factor XI(a); XII/XIIa, factor XII(a); Fb, fibrin; Fbg, fibrinogen; HK, high molecular weight kininogen; KAL, kallikrein; PK, prekallikrein.

Thrombin can amplify its own generation by activating the cofactors V and VIII (25). In addition, a feedback loop via factor XI has recently been described. Thrombin can directly activate factor XI, which in turn activates the tenase complex resulting in additional thrombin formation (26-30).

Intrinsic pathway

The intrinsic pathway is activated by the contact activation system. This system is initiated by activation of factor XII. Factor XII bound to a negatively charged surface via its heavy chain (31) can either be autoactivated or enzymatically be activated by kallikrein into factor XIIa (32,33). A physiological surface for the activation of factor XII has not yet been identified, but phospholipids and glycosaminoglycans may be suitable candidates (34). In addition, binding of factor XII to endothelial cells may also be involved in the (auto)activation of factor XII (35). Factor XIIa can activate prekallikrein and factor XI, which are bound to cell surfaces via the cofactor high molecular weight kininogen (35-37). Kallikrein cleaves factor XII into factor XIIa, forming a reciprocal activating pathway. Via activation of factor IX by factor XIa, the contact activation system triggers the generation of thrombin (Figure 1). The contact activation system is not only involved in coagulation, but also in fibrinolysis, blood pressure regulation, and activation of the complement system.

The physiological importance of the intrinsic pathway in the coagulation cascade is nowadays questioned. Factor XI can be efficiently activated by thrombin that is generated via the extrinsic pathway (26-30,38). In addition, deficiencies of factor XII and prekallikrein are not associated with bleeding tendencies (39,40), while deficiencies of proteins like factor VIII, factor IX and factor XI all lead to bleeding disorders (10). As a result, a revised model of coagulation has been postulated in which there is no distinction between extrinsic and intrinsic pathways (26,41). However, under pathophysiological circumstances the intrinsic pathway seems to play an important role in the activation of the coagulation cascade. Due to activation of the intrinsic pathway by therapeutically generated plasmin, thrombolytic therapy in acute myocardial infarction is hampered by procoagulant effects which frequently lead to reocclusion of the vessel (42-44). Activation of the contact activation system is also a major problem in the clinical use of biomaterials for medical devices like haemodialysis membranes and coronary artery bypass grafts (45-50). Binding of factor XII to biomaterials results in activation of the coagulation cascade, a phenomenon which explains the

thrombogenicity of these devices. Furthermore, levels of factor XIIa have been reported to be associated with an increased risk of coronary artery disease (51,52).

Regulation of coagulation

The coagulation system is regulated by several pathways. Coagulation enzymes are inhibited by various protease inhibitors. Tissue factor pathway inhibitor inhibits the generation of thrombin by forming an inactive complex with factor VIIa/tissue factor/factor Xa (53,54). The activity of thrombin, factor IXa, factor Xa and factor XIa is blocked by antithrombin (55,56). Factor XIIa is inhibited by C1-inhibitor, and kallikrein by C1-inhibitor and α_2-macroglobulin (57-60). Other regulators of the coagulation system are heparin cofactor II, protein C inhibitor and α_1-antitrypsin (61). As well as by regulation by protease inhibitors, the coagulation cascade is also regulated by the protein C pathway. Protein C is activated by thrombin complexed to its cofactor thrombomodulin, a process which will be discussed in more detail later in this chapter. Activated protein C inactivates cofactors Va and VIIIa and in this way prevents the generation of thrombin (62,63). In addition to the pathways described above, there are several other regulatory mechanisms which will not be discussed in this chapter, like the regulation of synthesis and secretion and the clearance of coagulation factors.

FIBRINOLYSIS

The key enzyme in the fibrinolytic system is plasmin. Plasmin circulates as an inactive pro-enzyme called plasminogen and needs to be activated by plasminogen activators before it can degrade the fibrin network. The main physiological plasminogen activators are tissue-type plasminogen activator (t-PA) and urokinase-type plasminogen activator (u-PA). In addition, components of the contact activation system have been reported to directly or indirectly activate plasminogen (Figure 2).

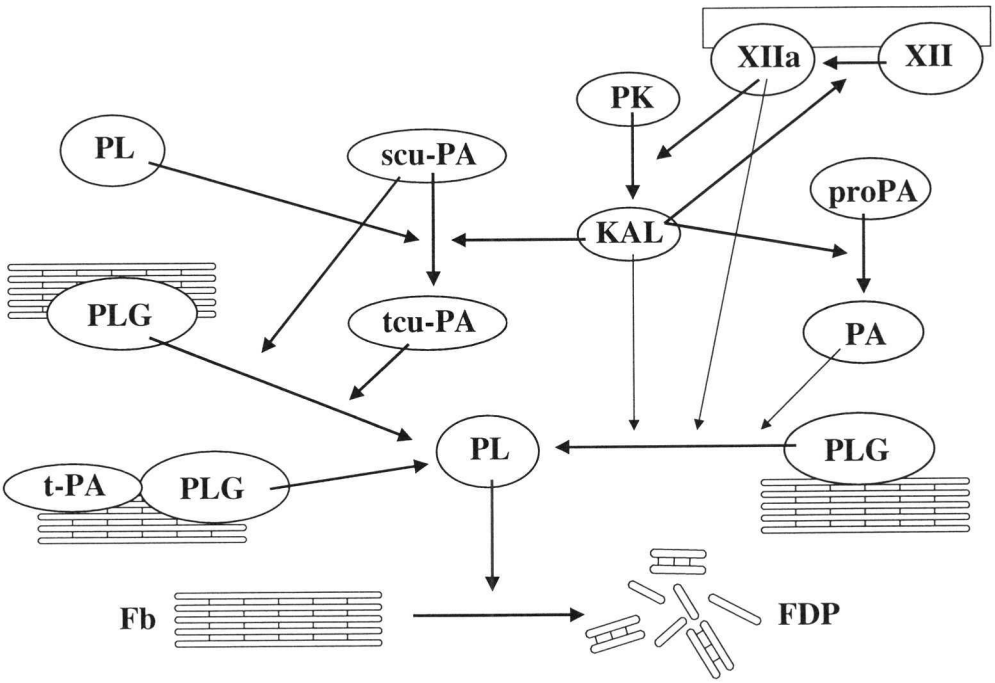

Figure 2. The fibrinolytic system. The key enzyme in fibrinolysis is plasmin, which is able to degrade the fibrin network into fibrin degradation products. The pro-enzyme plasminogen is activated by t-PA, u-PA or other plasminogen activators. Non-fibrin specific plasminogen activators like tcu-PA and components of the contact activation system activate both plasminogen in the fluid phase and fibrin-bound plasminogen. Fibrin-specific plasminogen activators like t-PA and scu-PA prefer to activate fibrin-bound plasminogen. Components of the contact activation system are able to activate plasminogen both indirectly and directly. Factor XIIa activates plasminogen indirectly via the activation of prekallikrein into kallikrein, which in turn cleaves scu-PA into tcu-PA. Another possible indirect pathway may be the activation of an unidentified factor XII-dependent plasminogen proactivator by kallikrein. Factor XIIa, kallikrein and factor XIa (not included in this figure) can also activate plasminogen directly. Abbreviations used are: XII/XIIa, factor XII/XIIa; Fb, fibrin; FDP, fibrin degradation products; KAL, kallikrein; PK, prekallikrein; PL, plasmin; PLG, plasminogen; proPA, factor XII-dependent plasminogen proactivator; PA, factor XII-dependent plasminogen activator; scu-PA, single-chain urokinase-type plasminogen activator; tcu-PA, two-chain urokinase-type plasminogen activator; t-PA, tissue-type plasminogen activator.

Components of the fibrinolytic system

Plasmin(ogen)

Like all the other fibrinolytic enzymes, plasmin is a serine protease. The pro-enzyme plasminogen is activated after cleavage at Arg 561 – Val 562 by t-PA, u-PA or other plasminogen activators. The native form of plasminogen is called Glu-plasminogen. Cleavage of the N-terminal region by plasmin results in a second form called Lys-plasminogen, which is more easily activated into plasmin than Glu-plasminogen (64).

Plasminogen contains five kringle domains and a protease domain (65). Via the kringle domains plasminogen binds to intact fibrin and to C-terminal lysines on partially degraded fibrin (66,67). Fibrin-bound plasminogen is efficiently activated into plasmin and fibrin-bound plasmin is protected from inhibition by its inhibitor α_2-antiplasmin (68,69). In this way, fibrinolysis is localized at the site of a fibrin clot. By degrading the fibrin network into fibrin degradation products, which are rapidly cleared from the circulation, plasmin is able to dissolve a blood clot. Plasmin is also involved in extracellular proteolysis and in this way plays an important role in cell migration, tumour invasion and rheumatoid arthritis (70).

Tissue-type plasminogen activator

Tissue-type plasminogen activator (t-PA) is believed to be the most important plasminogen activator for the degradation of fibrin (71,72). This enzyme occurs both in a single-chain and a two-chain form. The single-chain form of t-PA is not an inactive pro-enzyme like plasminogen, but is almost as active as the two-chain form that is generated after cleavage at Arg 275 – Ile 276 by plasmin or other enzymes (73). t-PA consists of a finger-like domain, an epidermal growth factor-like domain, two kringle domains and a protease domain. In the absence of fibrin, t-PA is a poor plasminogen activator. However, binding of t-PA to fibrin via its finger domain and second kringle domain is associated with a strong plasminogen activator activity of t-PA (74,75). Due to this fibrin specificity, t-PA is considered a clot-specific thrombolytic agent (76-78). Binding to fibrin may also protect t-PA from rapid inhibition by its inhibitor plasminogen activator inhibitor-1 (79).

Urokinase-type plasminogen activator

Urokinase-type plasminogen activator (u-PA) circulates as a single-chain pro-enzyme (scu-PA) (73). Activation into two-chain u-PA (tcu-PA) occurs after cleavage of scu-PA at Lys 158- Ile 159 by plasmin, kallikrein or other activators (80-84). Cleavage of scu-PA at Arg 156 – Phe 157 by thrombin results in an inactive two-chain molecule called thrombin-cleaved tcu-PA (tcu-PA/T) (81). This process will be discussed in more detail later in this chapter. Although the intrinsic plasminogen activator activity of scu-PA seems to be little (85-89), plasminogen activation by scu-PA is substantially promoted by fibrin-derived fragments (90-92). In addition, the enzymatic activity of scu-PA bound to the urokinase receptor is said to be comparable to that of tcu-PA (93-95). u-PA consists of an N-terminal part, containing an epidermal growth factor-like domain and a kringle domain, and a protease domain. The N-terminal part is involved in binding u-PA to the urokinase receptor. In contrast to t-PA, tcu-PA does not bind to fibrin and can induce systemic plasminogen activation. Plasminogen bound to partially degraded fibrin is efficiently activated by scu-PA, suggesting that scu-PA can mediate fibrin-specific fibrinolysis and may be a potential candidate for clot-specific thrombolytic therapy (96-98).

For a long time, the role of u-PA as a plasminogen activator has been focussed on non-fibrinolytic processes like extracellular proteolysis. However, recent animal studies have indicated that u-PA is importantly involved in intravascular fibrinolysis. Fibrin deposition in endotoxin-treated mice was found to correlate with changes in u-PA mRNA but not t-PA mRNA (99). In a study using knock-out mice, spontaneous intravascular fibrin deposition was observed in u-PA-deficient mice rather than t-PA deficient mice (100). In addition, the fibrinolytic potential of u-PA was found to be sufficient for the clearance of fibrin in combined t-PA-deficient and urokinase receptor-deficient mice (101). Furthermore, expression of u-PA by human venous endothelial cells *in vivo* has been described, which is consistent with a physiological role of u-PA in fibrinolysis (102).

Contact activation system

Activation of surface-bound factor XII, either by autoactivation or by kallikrein, is the initial step in contact activation and in the intrinsic pathway of the coagulation cascade. There is substantial evidence that components of the contact activation system are also involved in fibrinolysis (103).

Factor XII structurally resembles plasminogen, t-PA and u-PA (33,104,105). Furthermore, the activation of plasminogen is indirectly triggered by factor XIIa through the cleavage of prekallikrein into kallikrein, which in turn can activate scu-PA into tcu-PA (81,82). There is also evidence that prekallikrein bound to endothelial cells via its cofactor high molecular weight kininogen can be activated independently of factor XIIa (106), which may result in the activation of cell-bound scu-PA (107,108). In addition, the existence of an as yet unidentified factor XII-dependent plasminogen proactivator has been postulated (103,109,110). When the total fibrinolytic activity was measured in a dextran sulphate euglobulin fraction of plasma, it appeared that 35% of this activity could be ascribed to a factor XII-dependent plasminogen proactivator different from the known plasminogen activators (103). The identification of this unknown plasminogen activator is still in progress (110,111). Another profibrinolytic effect of the contact activation system is induced by bradykinin. Bradykinin, released after the cleavage of the cofactor high molecular weight kininogen by kallikrein, stimulates the secretion of t-PA by endothelial cells (112). Besides the indirect activation of plasminogen, factor XIIa and kallikrein, and factor XIa as well, are also known to directly activate plasminogen (34,113-115). The physiological importance of plasminogen activation by these components is still unclear.

Evidence for a role of the contact activation system in fibrinolysis is also provided by clinical studies. Fibrinolytic activity was found to be reduced in factor XII-deficient patients (116). Deficiencies of factor XII and kallikrein have been reported to be associated with thromboembolic tendencies (39,117). Furthermore, a relationship has been described between depressed contact activation-dependent fibrinolysis and cardiovascular diseases (118-121).

Regulation of fibrinolysis

The fibrinolytic system is regulated by several serine protease inhibitors, the so-called serpins (122). The main inhibitor of plasmin is α_2-antiplasmin (68,69,123). The inhibition of plasmin by α_2-antiplasmin is strongly reduced when the lysine-binding sites of plasmin are occupied by binding to fibrin (68,69). The plasminogen activators t-PA and u-PA are primarily inhibited by plasminogen activator inhibitor-1 (PAI-1) (124-126). PAI-2 is a strong inhibitor of tcu-PA, and to a lesser extent of t-PA (126). Another inhibitor of tcu-PA is PAI-3, which is identical to protein C inhibitor (127,128). Interestingly, scu-PA, the precursor form of tcu-PA, has also been reported to interact

with PAI-1 and PAI-2 (129,130). Inhibition of factor XIIa and kallikrein is mediated by C1-inhibitor and α_2-macroglobulin (57-60).

Besides via serpins, fibrinolysis is also regulated by the regulation of synthesis and secretion, and by the clearance of its components. The half-life of t-PA and u-PA in plasma is only 5 minutes due to very rapid and specific receptor-mediated clearance by the liver (131,132).

Recently, another mechanism regulating the fibrinolytic system has been discovered by the isolation of a plasma carboxypeptidase B called thrombin activatable fibrinolysis inhibitor (TAFI). TAFI is able to cleave the C-terminal lysines of partially degraded fibrin. In this way, the binding of plasminogen to fibrin is diminished, resulting in the inhibition of fibrinolysis (133-135).

LINKS BETWEEN COAGULATION AND FIBRINOLYSIS

The coagulation and fibrinolytic system regulate the haemostatic balance and interact closely. Several mechanisms that link the two systems have been described. A selection of these links is described below.

Contact activation system

The contact activation system plays a dual role in the haemostatic balance by both mediating coagulation and fibrinolysis (see above). This system comprises the intrinsic pathway of the coagulation cascade, while its components are also able to directly or indirectly activate plasminogen. In addition, the cofactor kininogen has prominent effects on fibrinolysis and coagulation. Kininogen circulates in a high molecular weight form and a low molecular weight form. Cleavage of kininogen by kallikrein results in the release of bradykinin, a potent vasodilatator and a stimulator of the release of t-PA by endothelial cells. Another profibrinolytic effect of kininogen is that this cofactor assembles prekallikrein on the cell surface, which in turn is activated and can then cleave scu-PA into tcu-PA (107,108). Kininogen also exerts anticoagulant properties by selectively inhibiting thrombin-induced platelet aggregation and secretion (136-139). In addition, enzymes of the contact activation system can interact with PAI-1, which may result in the consumption of PAI-1 (140).

The significance of the role of the contact activation system in coagulation seems to be more prominent in pathological conditions than in physiological conditions. The participation in fibrinolysis via the activation of scu-PA is well established, but the physiological importance of factor XIIa, kallikrein, factor XIa and the unidentified factor XII-dependent plasminogen proactivator as plasminogen activators remains to be elucidated.

Regulation of the synthesis and secretion of fibrinolytic components by thrombin

Thrombin modulates the synthesis and secretion of several components of the fibrinolytic system. Production of t-PA (141-143), u-PA (144-146), PAI-1 (141-143,147,148), PAI-2 (149) and urokinase receptor (150) by various cells is upregulated by thrombin. Thrombin induces the release of PAI-1 by platelets, which contain about 90% of the total amount of PAI-1 present in blood, resulting in resistance to clot lysis (151-153). In addition, thrombin may cleave the urokinase receptor from cells and in this way decrease u-PA binding (154). Via these pathways, thrombin can regulate the fibrinolytic system (154,155).

Interactions between thrombin and PAI-1

In addition to the inhibition of t-PA and u-PA, PAI-1 efficiently inhibits thrombin in the presence of vitronectin or heparin (156-158). The interaction between PAI-1 and thrombin most likely occurs within the vessel wall, where extracellular matrix proteins are available to serve as cofactors for this reaction. The inhibition of thrombin by PAI-1 occurs via a "suicide substrate mechanism" that is regulated by vitronectin and heparin (159,160), meaning that either stable PAI-1/thrombin complexes are formed (160) or that the inhibitor PAI-1 is degraded by the enzyme thrombin followed by recycling of thrombin (161-164). This mechanism can lead to mutual neutralization of PAI-1 and thrombin and may therefore affect both coagulation and fibrinolysis. The same phenomenon has been described for the interaction between PAI-1 and factor Xa (164,165).

Besides the suicide substrate reaction between PAI-1 and thrombin, the interaction between PAI-1 and thrombin in the presence of vitronectin promotes the cellular clearance of thrombin by the low density lipoprotein receptor-related protein (166).

Thrombin/thrombomodulin complex

The role of thrombin in the haemostatic balance is very complex, since thrombin both promotes and inhibits coagulation and fibrinolysis via several pathways. Various effects of thrombin are potentiated by binding to its cofactor thrombomodulin. Thrombomodulin is a membrane protein on endothelial cells and forms a 1:1 complex with thrombin (167), resulting in the acceleration of both the anticoagulant and antifibrinolytic effects of thrombin. The thrombin/thrombomodulin complex is less efficient in the cleavage of fibrinogen into fibrin and in the activation of platelets, while it is more efficiently inhibited by antithrombin than free thrombin (62,168,169). The main effect of the thrombin/thrombomodulin complex is the activation of protein C. The complex also activates TAFI and inactivates scu-PA (Figure 3). Via these mechanisms both coagulation and fibrinolysis are affected.

Activation of protein C

Protein C is a vitamin K-dependent protein that circulates in an inactive precursor form. It is efficiently activated by thrombin complexed to its cofactor thrombomodulin (62,63,170). Activated protein C (APC) forms a complex with its cofactor protein S on the surface of platelets or endothelial cells (63). These complexes prevent additional thrombin generation by proteolytic inactivation of cofactors Va and VIIIa (62) (Figure 3). In this way APC not only inhibits the coagulation cascade (171) but also promotes fibrinolysis by decreasing the activation of the fibrinolysis inhibitor TAFI (172,173).

The importance of protein C in the regulation of coagulation and fibrinolysis is reflected by the fact that disturbances in the protein C pathway result in thrombosis. Inherited deficiencies of protein C or protein S are associated with an increased risk of venous thromboembolism (2). In addition, a point mutation in the gene for factor V has been described that results in the replacement of Arg 506 to Gln (174). This variant of factor V, called factor V^{Leiden}, cannot be cleaved at Arg 506 by APC and is therefore resistant to inactivation by APC. Factor V^{Leiden} is associated with thrombotic tendencies (15,175,176).

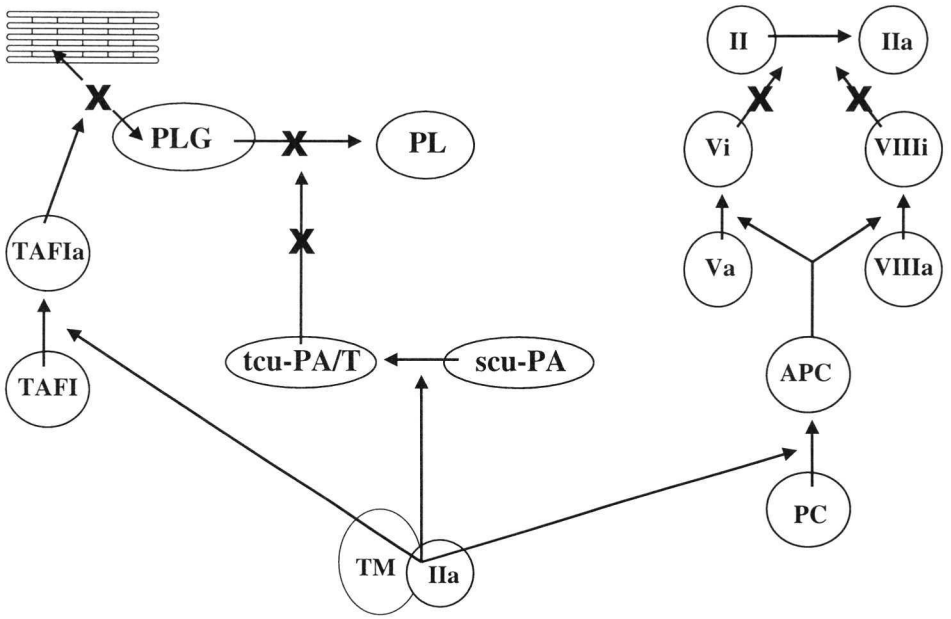

Figure 3. Effects of the thrombin/thrombomodulin complex on coagulation and fibrinolysis. Thrombin forms a complex with its cofactor thrombomodulin. As a result, both the antifibrinolytic and anticoagulant properties of thrombin are potentiated. In this figure some of the effects of the thrombin/thrombomodulin complex are depicted. The activation of TAFI and the inactivation of scu-PA will result in the inhibition of fibrinolysis, and the activation of protein C will prevent further generation of thrombin. Abbreviations used are: APC, activated protein C; II, prothrombin; IIa, thrombin; PC, protein C; PL, plasmin; PLG, plasminogen; scu-PA, single-chain urokinase-type plasminogen activator; TAFI, thrombin activatable fibrinolysis inhibitor; TAFIa, activated thrombin activatable fibrinolysis inhibitor; tcu-PA/T, thrombin-cleaved two-chain urokinase-type plasminogen activator; TM, thrombomodulin; Va/Vi, active/inactive factor V; VIIIa/VIIIi, active/inactive factor VIII.

Activation of TAFI

It has been well recognized that thrombin attenuates fibrinolysis via an antifibrinolytic mediator and that APC promotes fibrinolysis by preventing thrombin generation and activation of this mediator (29,172,177). In 1995, the antifibrinolytic mediator was identified as a thrombin activatable enzyme with carboxypeptidase B-like activity and was therefore named thrombin activatable fibrinolysis inhibitor (TAFI) (133). TAFI circulates as an inactive precursor form and is efficiently activated by the thrombin/thrombomodulin complex (178). Activated TAFI is able to remove C-terminal lysine and arginine residues from partially degraded fibrin, in this way reducing the binding of plasminogen to fibrin (134,135,179). In addition, high amounts of TAFI can inactivate plasmin (179). As a result, both t-PA-mediated and u-PA-mediated fibrinolysis is inhibited even at therapeutic concentrations (135,180-183).

There is no knowledge about the physiological relevance of TAFI. Levels of TAFI in the plasma of healthy individuals were found to correlate with the clot lysis time (184). Data on the disturbance of TAFI levels and its consequences are not available at the moment.

Inactivation of scu-PA

Thrombin cleaves scu-PA two residues prior to the activation site, which results in an inactive form called thrombin-cleaved tcu-PA (tcu-PA/T) (81). In a purified system, the inactivation of scu-PA by thrombin is accelerated by thrombomodulin and also by heparin and heparin-related glycosaminoglycans (185,186). The amidolytic and fibrinolytic activity of tcu-PA/T appears to be little *in vitro* (81,187), and tcu-PA/T is hardly activated by plasmin (188). However, tcu-PA/T has been described as a potent and fibrin-specific thrombolytic agent *in vivo* (189,190). This may be explained by re-activation of tcu-PA/T, since the lysosomal enzyme cathepsin C was found to re-activate tcu-PA/T (191). Another explanation may be that plasminogen bound to fibrin fragment E-2 is efficiently activated by tcu-PA/T (187), a phenomenon comparable to fibrin-specific plasminogen activation by scu-PA. Binding to soluble urokinase receptor protects scu-PA from inactivation by thrombin (192). In the presence of thrombomodulin, however, this protective effect is abolished, suggesting that the urokinase receptor and thrombomodulin play an important role in the regulation of the inactivation of scu-PA by thrombin.

The inactivation of scu-PA by thrombin may affect systemic plasminogen activation in plasma as well as local plasminogen activation on cells. This process is considered as a mechanism to protect a blood clot from early degradation (193). The inactivation of scu-PA by thrombin has been extensively studied in a purified system, but the physiological relevance remains to be established.

Aim of the study

The links between coagulation and fibrinolysis provide essential information for understanding the regulation of the haemostatic balance. This information can be of major importance for therapeutic intervention in both thrombotic and bleeding disorders. The aim of this study was to get more insight into two specific links between coagulation and fibrinolysis, namely the inactivation of scu-PA by thrombin and the contact activation system.

The inactivation of scu-PA into tcu-PA/T by thrombin has been extensively studied in a purified system. This process may have a significant effect on the haemostatic balance and on extracellular proteolysis, and may affect the efficacy of scu-PA as a thrombolytic agent. However, there is no knowledge about the occurrence of tcu-PA/T *in vivo* or the physiological relevance of the inactivation of scu-PA by thrombin. In **Chapter 2**, the development of a sensitive bioimmunoassay for tcu-PA/T in human body fluids is described. Using this assay, the occurrence of tcu-PA/T was examined in the plasma of healthy individuals and sepsis patients and in the synovial fluid of rheumatoid arthritis patients. In **Chapter 3**, the bioimmunoassay was used to study the inactivation of scu-PA by thrombin in plasma, a natural milieu in which physiological inhibitors and substrates of thrombin are present. In addition, the effect of thrombomodulin on this process in plasma was investigated. In **Chapter 4**, a study is described that was performed to examine under what circumstances tcu-PA/T can be generated systemically *in vivo*. The occurrence of tcu-PA/T was assessed in the plasma of subjects with a varying degree of hypercoagulability. Relationships between the level of tcu-PA/T and the levels of parameters reflecting thrombin generation were analysed. A role for the inactivation of scu-PA by thrombin in an extravascular milieu is described in **Chapter 5**. Levels of tcu-PA/T were measured in the synovial fluid of patients with rheumatoid arthritis or osteoarthritis and of healthy individuals in order to provide an explanation for the fibrin accumulation observed in the synovial fluid and tissue of rheumatoid arthritis patients. In **Chapter**

6, cell-mediated inactivation of scu-PA by thrombin was studied using human endothelial cells. In **Chapter 7**, the structural elements of thrombomodulin that are essential for the acceleration of the inactivation of scu-PA by thrombin were identified.

The physiological importance of the contact activation system in fibrinolysis is still unclear. To get more insight into the relevance of this sytem in fibrinolysis, this study focussed on the role of factor XIIa as a direct plasminogen activator and on the unidentified factor XII-dependent plasminogen activator. In **Chapter 8**, the contribution of factor XIIa as a plasminogen activator to contact activation-dependent fibrinolysis in plasma was quantified by examining the fibrinolytic properties of various molecular forms of FXIIa both in a purified system and in a dextran sulphate euglobulin fraction of plasma. **Chapter 9** describes the preliminary results and conclusions of a study on the characterization and the isolation of the factor XII-dependent plasminogen activator.

In **Chapter 10** the results of this study are summarized and general conclusions are made.

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CHAPTER 2

A sensitive bioimmunoassay for thrombin-cleaved two-chain urokinase-type plasminogen activator in human body fluids

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Summary

Thrombin cleaves single-chain urokinase-type plasminogen activator (scu-PA) into a two-chain form (tcu-PA/T), which is virtually inactive in plasminogen activator assays. Little is known about the physiological importance of tcu-PA/T. To examine the occurrence of tcu-PA/T *in vivo*, we developed a sensitive and specific bioimmunoassay (BIA) for the assessment of tcu-PA/T in human body fluids. In this BIA urokinase antigen was immuno-immobilized in microtiter plates and treated with cathepsin C, a specific activator of tcu-PA/T, after which plasminogen activator activity was measured. The occurrence of tcu-PA/T was examined in the plasma of 27 healthy individuals and of 17 sepsis patients, and in the synovial fluid of 16 rheumatoid arthritis patients. In addition, the concentration of urokinase antigen and scu-PA were measured in all three groups. In the plasma of the healthy individuals no measurable amounts of tcu-PA/T could be found (< detection limit of 0.2 ng/ml). In the plasma of almost all sepsis patients tcu-PA/T could be detected (median value 0.4 ng/ml). The amount of tcu-PA/T was 12% of the amount of scu-PA and accounted for about 9% of urokinase antigen. In the synovial fluid of all rheumatoid arthritis patients tcu-PA/T could be measured (median value 5.4 ng/ml) at a concentration which was twofold higher than the concentration found for scu-PA. In this group tcu-PA/T contributed to about 47% of the urokinase antigen. From these data we conclude that inactivation of scu-PA by thrombin can take place *in vivo* under pathological conditions which involve the production of large amounts of thrombin. In this way thrombin may regulate fibrinolysis and extracellular proteolysis. The BIA for tcu-PA/T can be of use for further research on the physiological role of tcu-PA/T.

Introduction

Single-chain urokinase-type plasminogen activator (scu-PA) is the precursor form of two-chain urokinase-type plasminogen activator (tcu-PA), a serine protease composed of two polypeptide chains linked together by a disulphide bond. This zymogen seems to have little amidolytic or plasminogen activator activity (1-6). However, the intrinsic plasminogen activator activity of scu-PA can be substantially promoted by fibrin-derived fragments (7,8). scu-PA can be activated to tcu-PA through cleavage of its Lys 158 - Ile 159 peptide bond by plasmin, kallikrein, factor XIIa,

thermolysin, tryptase (9-13) and several other proteases (for review see (14)). Active tcu-PA in turn converts plasminogen into plasmin, an enzyme which plays a major role in fibrinolysis and extracellular proteolysis.

Besides activation to tcu-PA, cleavage of scu-PA can also lead to inactivation. Thrombin cleaves scu-PA at Arg 156 - Phe 157, two residues before the activation site, which results in an inactive tcu-PA form called thrombin-cleaved tcu-PA (tcu-PA/T) (11). The inactivation of scu-PA by thrombin is accelerated by thrombomodulin, heparin and heparin-related glycosaminoglycans (15,16). *In vitro*, tcu-PA/T appears to have little amidolytic or fibrinolytic activity (11,17). However, it has been reported that tcu-PA/T is a potent and fibrin-specific plasminogen activator *in vivo* (18,19), which implies the involvement of specific factors that are able to re-activate tcu-PA/T. Liu and Gurewich have suggested that the plasminogen activating capacity of tcu-PA/T might be ascribed to fibrin fragment E-2, which can be generated *in vivo* via plasmin formation by endogenous tissue-type plasminogen activator (17).

Although the possible relevance of tcu-PA/T has been established *in vitro* ((20); for review see (21)), its physiological importance is still uncertain. It has been suggested that the inactivation of scu-PA by thrombin may contribute to the maintainance of the integrity of a fresh blood clot by suppressing fibrinolysis (20,21). We were interested to find out if tcu-PA/T could be detected in human plasma and other body fluids under physiological and pathological circumstances. However, until now no method was available for detecting tcu-PA/T. In the present study we developed a sensitive bioimmunoassay (BIA) for the assessment of tcu-PA/T. This assay enabled us to examine the occurrence of tcu-PA/T in the plasma of healthy individuals and sepsis patients and in the synovial fluid of rheumatoid arthritis patients.

Materials and Methods

Materials

Single-chain urokinase-type plasminogen activator (scu-PA) from a transformed human kidney cell line was kindly provided by Dr. F. Hammerschmidt, Sandoz (Vienna, Austria). The amount of scu-PA was determined in a bioimmunoassay using pooled normal plasma, containing 2.2 ng/ml scu-PA, as a standard (22). Bovine serum albumin, bovine spleen cathepsin C, L-cysteine and human thrombin were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Glu-plasminogen and plasmin were purchased from Biofine (Leiden, The Netherlands). Aprotinin (Trasylol^{R})

was obtained from Bayer AG (Leverkusen, Germany) and hirudin was obtained from Pentapharm (Basel, Switzerland). Tissue-type plasminogen activator (t-PA) obtained from a human melanoma cell culture was a gift of Dr. J.H. Verheijen from this laboratory. Pyro-Glu-Gly-Arg-p-nitroanilide (S-2444) was obtained from Chromogenix (Mölndal, Sweden). The plasmin substrate was H-D-Val-Leu-Lys-p-nitroanilide.

Urokinase-depleted plasma was obtained by immunoadsorption with immobilized anti-urokinase IgGs as described before (23). Citrated platelet-poor plasma was obtained from 27 healthy individuals from this institute (mean age 38.1 ± 11.2 years). Plasma obtained from 17 sepsis patients was kindly donated by Dr. J. Philippé of the University Hospital Gent (Belgium). Synovial fluid of 16 rheumatoid arthritis patients was kindly provided by Dr. K. Ronday of the Academic Hospital Leiden (The Netherlands).

Preparation of thrombin-cleaved two-chain urokinase-type plasminogen activator (tcu-PA/T)

In order to obtain tcu-PA/T, scu-PA (1 µg/ml) was treated with thrombin (17 NIH U/ml) for 1 h at 37°C in 50 mM Tris/HCl, pH 7.5, 100 mM NaCl, 0.1% BSA, 0.01% Tween 80 according to De Munk *et al.* (15). After incubation thrombin was inhibited by hirudin (50 ATU/ml). The amidolytic activity of tcu-PA/T was tested against S-2444 (15). The activity of tcu-PA/T was about 5.0% as compared with the activity of scu-PA, after treatment of the two preparations in solution with 50 nM plasmin for 1 h at 37°C, while untreated scu-PA and tcu-PA/T were found to have 2.7% and 1.5% activity of plasmin-treated scu-PA, respectively.

Bioimmunoassay (BIA) for scu-PA and tcu-PA/T

The potential activity of scu-PA was measured by the bioimmunoassay (BIA) for scu-PA as described before (22,24). Potential activity of tcu-PA/T was assessed using a modified form of the BIA for scu-PA as follows. Sample dilutions of 150 µl in 10 mM sodium phosphate buffer, pH 7.4, 150 mM NaCl, 0.01% Tween 80 and 0.02% NaN_3 (buffer A), supplemented with 0.1% BSA and 700 KIU/ml aprotinin, were incubated overnight at room temperature in polyvinylchloride 96-wells plates coated with rabbit polyclonal anti-u-PA antibody. Subsequently, the immobilized material was washed five times with buffer A and activated for 2 hours at room temperature by 150 µl of cathepsin C dilution in 50 mM sodium phosphate buffer, pH 6.0, 100 mM NaCl, 2 mM EDTA, 10 mM L-cysteine, 0.1% BSA and 0.01% Tween 80. After washing five times with buffer A, urokinase activity was determined at 25°C by the addition of 150 µl of 1.2 mM of the plasmin substrate H-D-Val-Leu-Lys-p-nitroanilide together with 0.5 µM Glu-plasminogen in 100 mM Tris/HCl, pH 8.5, 1 mM 6-aminohexanoic acid and 0.1% Tween 80. Measurements were performed at timed intervals at 405 nm with a Titertek Multiscan spectrophotometer and activity was expressed as delta A/h^2 ($\Delta A/h^2$).

Routinely, the amounts of scu-PA and tcu-PA/T in samples were determined in parallel wells through activation by 1 nM plasmin in 50 mM Tris/HCl, pH 7.5, 100 mM NaCl, 0.1% BSA and 0.01% Tween 80 (scu-PA assay) and through activation by 20 nM cathepsin C (tcu-PA/T assay). These concentrations of plasmin and cathepsin C were found to be sufficient for the conversion to active tcu-PA of scu-PA and tcu-PA/T, respectively. After subtraction of background activity obtained without activating enzyme, the concentrations of scu-PA and tcu-PA/T were calculated from the calibration curves obtained by serial dilutions of purified scu-PA and purified tcu-PA/T, respectively.

Measurement of scu-PA and tcu-PA/T in thrombin-clotted plasma

One volume of pooled normal plasma was clotted with one volume thrombin solution of a concentration range (0-100 NIH U/ml) in 26 mM sodium 5,5-diethylbarbiturate buffer/HCl, pH 7.35, 26 mM sodium acetate, 108 mM NaCl for 1 h at 37°C. The plasma clot was squeezed and the concentration of scu-PA and tcu-PA/T in the supernatant was determined in the two BIAs essentially as described above. To inhibit thrombin activity during sample incubation in the BIA, equal amounts (units/ml) of hirudin were added to the wells.

Assessment of scu-PA, tcu-PA/T and urokinase antigen (u-PA:Ag) in human plasma and synovial fluid

The amount of scu-PA and tcu-PA/T was measured in 25 µl plasma of healthy individuals and sepsis patients, and in 12.5 µl synovial fluid of rheumatoid arthritis patients using the two BIAs described above, while urokinase antigen was quantitated by the urokinase ELISA as described before (25).

To test possible generation of tcu-PA/T by thrombin during sample incubation in the BIA, 10 ATU/ml hirudin was added during incubation of randomly selected samples.

Statistics

For values of scu-PA, tcu-PA/T and u-PA:Ag in the plasma of healthy individuals and sepsis patients and in the synovial fluid of rheumatoid arthritis patients, as well as for the different ratios, median and interquartile ranges were calculated. Differences between groups were tested for significance with the Mann-Whitney test. Correlations between parameters were tested using Spearman rank correlation coefficient.

Results

Validation of the assay

Activation of immobilized scu-PA and tcu-PA/T

As shown in Figure 1A, immobilized purified scu-PA was activated by plasmin in a concentration-dependent way. Immobilized tcu-PA/T was about 50-fold less effectively activated by plasmin, as compared with scu-PA. At a concentration of 1 nM plasmin discrimination between scu-PA and tcu-PA/T was maximal. Therefore, this concentration was used routinely in the BIA for the assessment of scu-PA. Cathepsin C activated immobilized tcu-PA/T in a concentration-dependent way (Figure 1B). Activity of immobilized scu-PA treated with cathepsin C was not higher than the activity of scu-PA treated with buffer. A concentration of 20 nM (4.2 µg/ml) cathepsin C was selected to be used routinely in the BIA for the assessment of tcu-PA/T. The optimal concentrations of plasmin and cathepsin C proved to be independent of the scu-PA and tcu-PA/T concentration (tested between 50 and 200 pg/well, data not shown).

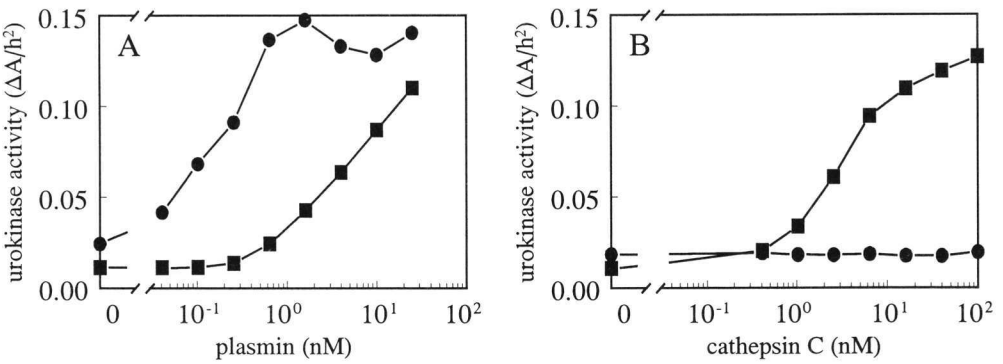

Figure 1. Urokinase activity of 200 pg immobilized scu-PA (•) or tcu-PA/T (■) after activation for 2 h by increasing concentrations of plasmin (A) or cathepsin C (B).

Calibration

The calibration curves of scu-PA activated by plasmin (1 nM) and of tcu-PA/T activated by cathepsin C (20 nM) are shown in Figure 2. As expected, scu-PA and tcu-PA/T could only be detected after activation by plasmin and cathepsin C, respectively. Plasmin induced low activity at high concentrations of tcu-PA/T.

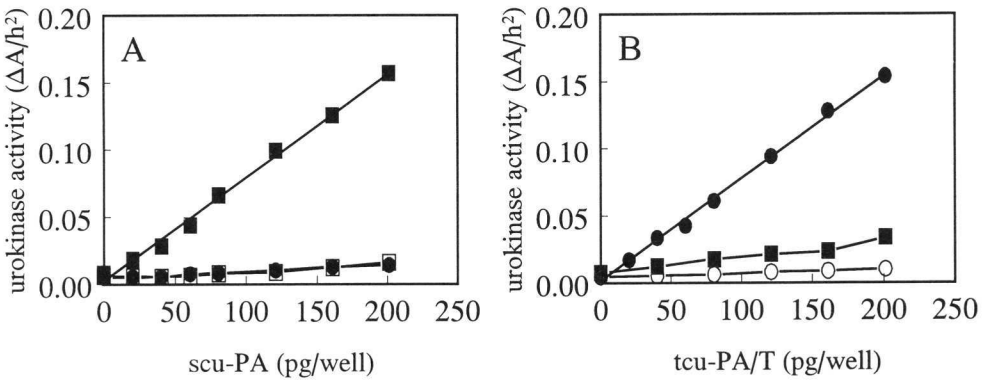

Figure 2. Urokinase activity of increasing amounts of immobilized scu-PA (A) or tcu-PA/T (B) after activation for 2 h by 1 nM plasmin (■), 20 nM cathepsin C (●) or blank (□ Tris/HCl pH 7.5, ○ phosphate buffer pH 6.0).

Background and detection limit of the assay for tcu-PA/T

The response of 25 µl u-PA immunodepleted plasma was equal to that of buffer and amounted to about 0.006 $\Delta A/h^2$, both with and without cathepsin C. The detection limit of the assay was expressed as the dose which provided a response 2 SD away from the activity of u-PA immunodepleted plasma, and was calculated to be 0.2 ng/ml tcu-PA/T corresponding to 5 pg/well.

Specificity

In human plasma serine proteases structurally related to urokinase may occur, such as t-PA and plasminogen. These enzymes could interfere in the assay. Activity of concentration ranges of t-PA (highest concentration 100 ng/ml) and Glu-plasminogen (highest concentration 150 µg/ml) added to buffer or pooled normal plasma were tested in triplicate in the BIA for tcu-PA/T. No activity higher than the background was measured.

Reproducibility

The reproducibility of the measurements of tcu-PA/T, expressed as the coefficient of variation (SD/mean x 100%), was estimated by determination of the activity of 25 µl pooled normal plasma supplemented with 50, 100 and 150 pg tcu-PA/T. The samples were analyzed six times in a single assay and an intra-assay variability of 4.3% was found. The same samples were analyzed four times in four consecutive assays and an inter-assay variability of 10.5% was found.

Efficiency in buffer and plasma

To examine whether the assay was equally efficient in buffer and in plasma, 50, 100 and 150 pg scu-PA or tcu-PA/T were added to 25 µl pooled normal plasma and immobilized in the two BIAs. Recovery of added scu-PA and tcu-PA/T activity in plasma was determined after activation by plasmin (1 nM) and cathepsin C (20 nM), respectively, using the calibration curves shown in Figure 2. As demonstrated in Table 1, recovery of scu-PA activity by plasmin and of tcu-PA/T activity by cathepsin C was about 100%, indicating that the two assays were equally efficient in buffer and in plasma. Recovery of scu-PA by cathepsin C and of tcu-PA/T by plasmin was very low, which is in agreement with Figures 1 and 2.

Table 1. Recovery of scu-PA and tcu-PA/T in the BIA.

	pg added	recovery (%) by plasmin	recovery (%) by cathepsin C
scu-PA	50	100 ± 23	3 ± 1
	100	98 ± 28	4 ± 1
	150	116 ± 17	6 ± 1
tcu-PA/T	50	7 ± 9	117 ± 5
	100	0 ± 5	126 ± 4
	150	15 ± 3	126 ± 7

Different amounts of scu-PA and tcu-PA/T were added to 25 µl pooled normal plasma and immobilized in the BIA. After activation by plasmin (1 nM) and cathepsin C (20 nM), recovery (mean ± SD, n=4) was determined using the calibration curves shown in Figure 2. For calculation of recovery by plasmin the calibration curve of scu-PA activated by plasmin was used, while for calculation of recovery by cathepsin C the calibration curve of tcu-PA/T activated by cathepsin C was used.

tcu-PA/T in thrombin-clotted plasma

To examine whether tcu-PA/T could be generated in plasma and detected in the BIA, pooled normal plasma was clotted by a concentration range of thrombin. As can be seen in Figure 3, tcu-PA/T was formed when plasma was clotted by 10 NIH U/ml or more thrombin, reaching the level of the original scu-PA concentration in plasma at 100 NIH U/ml thrombin. Simultaneously, the concentration of scu-PA decreased with increasing amounts of thrombin higher than 10 NIH U/ml. The sum of the concentrations of scu-PA and tcu-PA/T at 100 NIH U/ml thrombin slightly exceeded the original scu-PA concentration, which is most likely due to experimental errors.

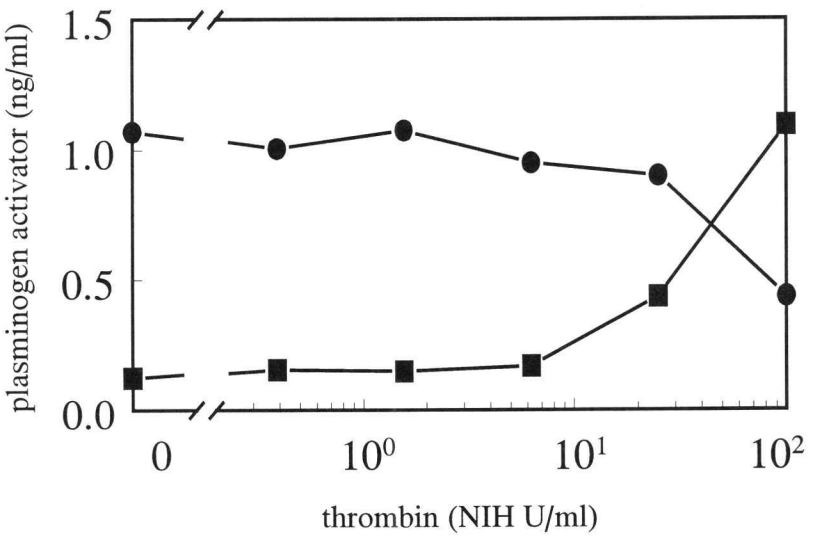

Figure 3. Concentration of scu-PA (•) or tcu-PA/T (■) in twofold diluted clotted plasma derived from pooled normal plasma after clotting by increasing concentrations of thrombin for 1 h at 37°C.

Application of the assay

The values of u-PA:Ag, scu-PA and tcu-PA/T measured in the plasma of healthy individuals and sepsis patients, and in the synovial fluid of rheumatoid arthritis patients are represented in Figure 4.

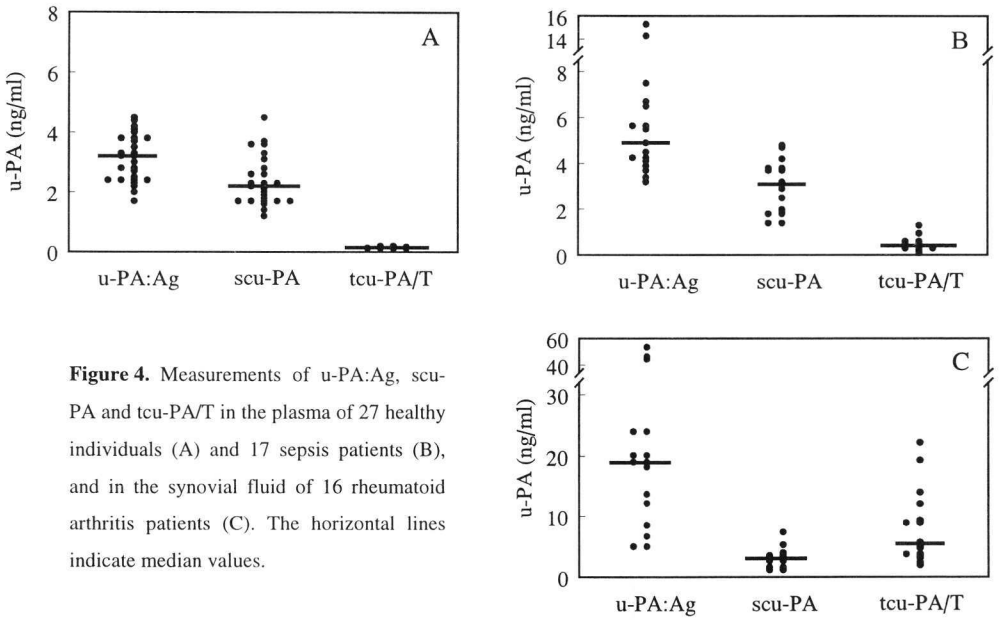

Figure 4. Measurements of u-PA:Ag, scu-PA and tcu-PA/T in the plasma of 27 healthy individuals (A) and 17 sepsis patients (B), and in the synovial fluid of 16 rheumatoid arthritis patients (C). The horizontal lines indicate median values.

In the plasma of 27 healthy individuals the u-PA:Ag concentration amounted to 3.2 (2.4 - 3.8) ng/ml (median and interquartile ranges) and the scu-PA concentration to 2.2 (1.7 - 2.8) ng/ml. The concentration of tcu-PA/T was below the detection limit of 0.2 ng/ml.

In the plasma of 17 sepsis patients the u-PA:Ag concentration was found to be 4.9 (4.1 - 6.5) ng/ml, while the concentration of scu-PA was 3.1 (1.9 - 3.8) ng/ml. In the plasma of 15 sepsis patients tcu-PA/T could be detected. The concentration of tcu-PA/T was found to be 0.4 (0.3 - 0.5) ng/ml. In the group of sepsis patients u-PA:Ag, scu-PA and tcu-PA/T were significantly higher than in the group of healthy individuals ($p < 0.0001$, $p = 0.0706$, $p < 0.0001$, respectively).

In the synovial fluid of 16 rheumatoid arthritis patients the u-PA:Ag concentration was 18.9 (10.4 - 23.8) ng/ml and the scu-PA concentration 3.0 (1.6 - 3.8) ng/ml. In all cases tcu-PA/T could be detected. The concentration of tcu-PA/T amounted to 5.4 (3.6 - 10.7) ng/ml. The assessment of tcu-PA/T in human body fluids was not affected by the addition of excess hirudin during sample incubation in the BIA.

In Table 2 the ratios between tcu-PA/T and scu-PA, between scu-PA and u-PA:Ag and between tcu-PA/T and u-PA:Ag are represented for all three groups.

Table 2. Ratios (%) between tcu-PA/T and scu-PA, between scu-PA and u-PA:Ag and between tcu-PA/T and u-PA:Ag (median and interquartile ranges) in the plasma of healthy individuals and sepsis patients, and in the synovial fluid of rheumatoid arthritis patients.

	plasma		synovial fluid
parameter	healthy individuals n = 27	sepsis patients n = 17	rheumatoid arthritis patients n = 16
tcu-PA/T as % of scu-PA	7[#] (5 - 9)	12[**] (11-18)	207 (134 - 365)
scu-PA as % of u-PA:Ag	76 (62 - 87)	57 (47 - 86)	19 (9 - 32)
tcu-PA/T as % of u-PA:Ag	5[#] (4 - 6)	9[*] (6 - 10)	47 (38 - 55)

Comparisons are made between healthy individuals and sepsis patients. [*] $p < 0.01$; [**] $p < 0.0001$. [#] The ratios between tcu-PA/T and scu-PA and between tcu-PA/T and u-PA:Ag in the plasma of healthy individuals are only indications, since tcu-PA/T was measured below the detection limit.

In the plasma of healthy individuals scu-PA accounted for about 76% of u-PA antigen. Since tcu-PA/T in this group was below the detection limit it could not be determined definitely whether tcu-PA/T also contributed to u-PA antigen. In the plasma of sepsis patients scu-PA accounted for about 57% of u-PA antigen and tcu-PA/T for about 9%. The ratio between tcu-PA/T and scu-PA and between tcu-PA/T and u-PA:Ag were significantly higher in the group of sepsis patients, as compared with healthy individuals ($p < 0.0001$, $p < 0.01$, respectively, Table 2).

In the synovial fluid of rheumatoid arthritis patients the concentration of tcu-PA/T was about twofold higher than the scu-PA concentration. scu-PA accounted for about 19% of u-PA antigen, while tcu-PA/T accounted for about 47%.

Significant correlations were found between the concentration of u-PA:Ag and scu-PA ($r = 0.57$, $p < 0.01$) in the plasma of healthy individuals as well as between scu-PA and tcu-PA/T ($r = 0.58$, $p < 0.05$) and between u-PA:Ag and tcu-PA/T ($r = 0.67$, $p < 0.01$) in the synovial fluid of rheumatoid arthritis patients.

Discussion

The present study describes a bioimmunoassay for the assessment of tcu-PA/T using a modified form of the BIA for scu-PA (22,24).

Bioimmunoassays are specific and sensitive assays which have been developed for the assessment of levels of active enzymes in biological samples (26,27). The BIA described for scu-PA is based on the potent capacity of plasmin to activate scu-PA, thus making it possible to discriminate between active tcu-PA and inactive scu-PA (22). For the assessment of tcu-PA/T in a bioimmunoassay a specific activator of tcu-PA/T is needed. It is known that tcu-PA/T can be activated by plasmin, thermolysin, trypsin and cathepsin C (12,28,29). For the assessment of tcu-PA/T in samples containing both tcu-PA/T and scu-PA, plasmin, thermolysin and trypsin are far less suitable candidates, when compared with cathepsin C. Plasmin and trypsin are very weak activators of tcu-PA/T and strong activators of scu-PA, and thus are not useful for detecting tcu-PA/T specifically in a bioimmunoassay. Thermolysin, a bacterial protease, activates scu-PA and tcu-PA/T to the same extent. As a result, the use of thermolysin in an assay does not allow for the discrimination between scu-PA and tcu-PA/T (30). Cathepsin C does not affect scu-PA but is an efficient activator of tcu-PA/T (29). In this way cathepsin C can serve as a specific indicator for tcu-PA/T in a BIA which can distinguish tcu-PA/T from other urokinase forms. Therefore, cathepsin C was used in the BIA for the assessment of tcu-PA/T. The findings of this study indicate that cathepsin C is indeed suitable for detecting tcu-PA/T specifically.

Optimal activation of tcu-PA/T in the BIA by cathepsin C occurred at a concentration higher than the concentration found for optimal activation of tcu-PA/T in solution (29). This is probably the result of decreased accessibility of tcu-PA/T for cathepsin C due to both the immuno-immobilization of tcu-PA/T and the considerable dimensions of cathepsin C (210 kDa). Immuno-

immobilized tcu-PA/T was more efficiently activated by plasmin than tcu-PA/T in solution (29). The finding that, in the BIA for scu-PA, tcu-PA/T is about 50-fold less effectively activated by plasmin compared to scu-PA is in agreement with the results of Lijnen *et al.* (28).

The BIA for tcu-PA/T is precise and sensitive, as demonstrated by the intra-assay (4%) and inter-assay (11%) variability, and the detection limit of 0.2 ng tcu-PA/T per ml plasma, respectively. In the BIA, cathepsin C did not activate scu-PA. Other structurally urokinase-related serine proteases, such as t-PA and plasminogen, did not interfere with the assay. This indicates that the BIA is highly specific for tcu-PA/T. Recovery experiments showed that the BIA for tcu-PA/T is equally efficient in buffer and in plasma. In addition, the generation of tcu-PA/T in pooled normal plasma through clotting by thrombin could also be demonstrated in the assay. These findings imply that tcu-PA/T in human plasma samples can be easily assessed using the BIA. Furthermore, the detection limit of the BIA for tcu-PA/T (0.2 ng/ml) makes the assay potentially appropriate for the determination of physiological or pathological amounts of tcu-PA/T. These conclusions are confirmed by the results of the assessment of tcu-PA/T in plasma and synovial fluid performed in this study.

In the plasma of 27 healthy individuals no tcu-PA/T above the detection limit could be measured. This finding may seem to contradict the indications of ongoing thrombin generation in normal plasma. The occurrence of thrombin-antithrombin III-complexes (31-34) and prothrombin fragment F_{1+2} (31,34,35) in the plasma of healthy individuals has been described in several studies. However, the concentration of physiologically circulating thrombin may not be sufficient to induce measurable amounts of tcu-PA/T in spite of the presence of thrombomodulin on the endothelial cells. Thrombomodulin not only accelerates the inactivation of scu-PA by thrombin (15,16), but also abolishes the protective effect of u-PA binding to the urokinase receptor against inactivation by thrombin (36).

In the plasma of almost all sepsis patients measurable amounts of tcu-PA/T were found (median value 0.4 ng/ml). The occurrence of tcu-PA/T is probably the result of an increased thrombin concentration in the plasma of these patients. It is known that endotoxins in septicemia induce a variety of pathological reactions, including the generation of high amounts of thrombin (37,38). The finding that the concentrations of u-PA antigen and scu-PA in the plasma of the sepsis patients were higher than in the plasma of the healthy individuals is in agreement with other studies (39,40).

In the synovial fluid of all the 16 rheumatoid arthritis patients examined in this study tcu-PA/T could be detected (median value 5.4 ng/ml). The concentration of tcu-PA/T was about twofold

higher compared to the concentration of scu-PA (median value 3.0 ng/ml). Fibrin deposits found in the joints of rheumatoid arthritis patients indicate that high amounts of thrombin are generated extravascularly (41,42). Furthermore, thrombomodulin has been observed on synovial surfaces (43,44) which may facilitate the inactivation of scu-PA by thrombin. This can explain the occurrence of such high concentrations of tcu-PA/T. The positive correlations found between u-PA antigen and tcu-PA/T and between scu-PA and tcu-PA/T concentrations imply an ongoing accumulation of scu-PA in the synovium of rheumatoid arthritis patients, of which a significant part is inactivated by thrombin.

In this study the assessment of tcu-PA/T was not affected by possible *in vitro* generation of tcu-PA/T by thrombin present in the sample during incubation in the BIA. However, to avoid *in vitro* generation the addition of about 0.5 ATU/ml hirudin during sample incubation in the BIA is to be recommended when tcu-PA/T is determined in material containing high amounts of thrombin.

Several clinical studies describe how the concentration of u-PA antigen measured in various body fluids could only partly be explained by the presence of tcu-PA and scu-PA (40,45,46). This study shows that in pathological circumstances tcu-PA/T can account for a significant part of u-PA antigen. However, a considerable amount of u-PA antigen still remains unexplained. Possible components which may account for this unidentified part of u-PA antigen may be tcu-PA/inhibitor complexes, scu-PA/inhibitor complexes, degraded urokinase or scu-PA inactivated by other enzymes such as elastase (47-49).

It may be hypothesized from the results of this study that generation of tcu-PA/T can occur in inflammatory processes. This hypothesis is favoured by the fact that thrombin generation is increased under these conditions, as indicated by the findings of fibrin deposition. It may be speculated that in this way thrombin can play a role in regulating the fibrinolytic system and extracellular proteolysis by inactivating scu-PA.

In conclusion, the BIA for tcu-PA/T developed in this study provides evidence for the occurrence of tcu-PA/T *in vivo* under pathological circumstances. For clinical studies the BIA for tcu-PA/T can be of use in identifying considerable amounts of u-PA antigen in human body fluids which cannot be explained by tcu-PA and scu-PA. In addition, tcu-PA/T might have a prognostic value for thrombotic and inflammatory diseases. Further research needs to be done to evaluate the importance of tcu-PA/T *in vivo*.

Acknowledgements

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CHAPTER 3

The inactivation of single-chain urokinase-type plasminogen activator by thrombin in a plasma milieu: effect of thrombomodulin

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Summary

Thrombin cleaves single-chain urokinase-type plasminogen activator (scu-PA) into a virtually inactive two-chain form (tcu-PA/T), a process which may contribute to the maintainance of a fresh blood clot. We have examined the inactivation of scu-PA by thrombin in a plasma milieu to get more insight in the physiological relevance of this phenomenon. Citrated pooled normal plasma was treated with thrombin in the absence and presence of thrombomodulin (TM). After an incubation period of 30 min the concentrations of scu-PA and tcu-PA/T were measured using specific bioimmunoassays. The inactivation of scu-PA in citrated plasma was found to be stimulated 4-fold by TM. Kinetic experiments showed that the inactivation of scu-PA by thrombin in the absence and presence of TM occurred rapidly and declined within one minute due to rapid inhibition by antithrombin III (ATIII) and other possible inhibitors. Calcium had no direct effect on the inactivation of scu-PA by exogenously added thrombin in the absence and presence of TM. However, recalcification of plasma induced significant inactivation of scu-PA in plasma due to endogenous thrombin generation through the contact activation system. This calcium-induced inactivation of scu-PA was completely abolished in the presence of TM, most likely due to activation of protein C by the complex formed between TM and endogenously generated thrombin. TM thus appeared to play a dual role by both stimulating the inactivation of scu-PA by thrombin and inhibiting calcium-induced inactivation of scu-PA in plasma. In the plasma from a patient heterozygous for protein C deficiency, TM could not prevent calcium-induced generation of tcu-PA/T, while the stimulating effect of TM predominated instead. This result implied that disturbance of the protein C pathway may lead to the inactivation of substantial amounts of scu-PA in plasma under (patho)physiological circumstances and may provide an additional explanation for the association found between thromboembolism and deficiencies in the protein C pathway. This study shows that the amount of scu-PA that is inactivated in plasma depends mainly on the generation of thrombin and on TM. We conclude that the inhibition of scu-PA-induced fibrinolysis thus appears to be regulated by activation of the coagulation system, so providing a link between coagulation and fibrinolysis.

Introduction

Single-chain urokinase-type plasminogen activator (scu-PA) is the precursor form of two-chain urokinase-type plasminogen activator (tcu-PA), a serine protease composed of two polypeptide chains linked together by a disulphide bond. Active tcu-PA plays a role in fibrinolysis and extracellular proteolysis by converting plasminogen into plasmin. Plasmin is able to dissolve a blood clot via degradation of the fibrin network and to degrade the extracellular matrix (1). scu-PA can be activated through cleavage of its Lys 158 - Ile 159 peptide bond. The main activator of scu-PA is plasmin, although several other activators are known (2-7). Besides activation to tcu-PA, cleavage of scu-PA can also lead to inactivation. Thrombin cleaves scu-PA at Arg 156 - Phe 157, two residues before the activation site, which results in an inactive tcu-PA form called thrombin-cleaved tcu-PA (tcu-PA/T) (4). *In vitro*, tcu-PA/T appears to have little amidolytic or fibrinolytic activity (4,8). However, it has been reported that tcu-PA/T is a potent and fibrin-specific plasminogen activator *in vivo*, which implies the involvement of specific factors that are able to re-activate tcu-PA/T (9,10). Recently, we developed a sensitive bioimmunoassay (BIA) for tcu-PA/T (11). Using this BIA, we were able to demonstrate the occurrence of tcu-PA/T in human body fluids under pathological conditions which involve the production of large amounts of thrombin, such as sepsis and rheumatoid arthritis. Although tcu-PA/T can be demonstrated *in vivo*, the physiological role of the inactivation of scu-PA is still uncertain. It has been suggested that the inactivation of scu-PA by thrombin may contribute to the maintainance of the integrity of a fresh blood clot by suppressing fibrinolysis (12,13).

The mechanism of the inactivation of scu-PA by thrombin has been described in detail in a purified system. It was found that the inactivation of scu-PA by thrombin is strongly accelerated by thrombomodulin and to a lesser extent by heparin and heparin-related glycosaminoglycans (14,15). Binding of scu-PA to the urokinase receptor protects scu-PA from inactivation by thrombin (16). However, this protective effect is abolished in the presence of thrombomodulin. Thrombomodulin is a membrane protein on endothelial cells, which forms a 1:1 complex with thrombin (for review see (17)). It stimulates both the anticoagulant and antifibrinolytic properties of thrombin by accelerating the activation of protein C and the activation of a thrombin-activatable fibrinolysis inhibitor (TAFI), respectively (18-21). The effect of thrombomodulin on the inactivation of scu-PA by thrombin in a natural plasma environment is still unknown.

To get more insight into the physiological relevance of this phenomenon, we describe the inactivation of scu-PA by thrombin in plasma, a natural milieu in which physiological inhibitors and substrates of thrombin are present. For this purpose, we investigated the inactivation of scu-PA by thrombin in citrated and recalcified plasma, and the effect of thrombomodulin on this process. We demonstrated that the inactivation of scu-PA by thrombin in plasma is regulated by activation of the coagulation system mainly involving thrombin, thrombomodulin and protein C.

Materials and Methods

Materials

Single-chain urokinase-type plasminogen activator (scu-PA) from a transformed human kidney cell line was kindly provided by Dr. F. Hammerschmidt, Sandoz (Vienna, Austria). The amount of scu-PA was determined in a bioimmunoassay using pooled normal plasma, containing 2.2 ng/ml scu-PA, as a standard (22). Bovine serum albumin and human thrombin (specific activity of 3.4 NIH U/μg, as indicated by the manufacturer, molecular mass 37 kD) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Rabbit lung thrombomodulin (specific activity of 1 U/μg, as indicated by the manufacturer, molecular mass 75 kD) was obtained from American Diagnostica (Greenwich, CT, USA). Hirudin was purchased from Pentapharm (Basel, Switzerland). Polypropylene tubes were obtained from Greiner (Alphen a/d Rijn, The Netherlands).

Citrated platelet-poor normal plasma was obtained from healthy individuals from this institute, factor II-deficient plasma from Biopool (Umea, Sweden) and factor XI- and factor XII-deficient plasma from George King Biomedical (Overland Park, KS, USA). Antithrombin III deficient plasma was a kind gift from Mr. C. Kortmann (Kordia bv, Leiden, The Netherlands). Plasma from a patient heterozygous for protein C deficiency was kindly donated by Prof. R.M. Bertina (Leiden University Medical Centre, Leiden, The Netherlands).

Experiments in plasma

In order to study the effect of a wide concentration range of thrombin and thrombomodulin on the inactivation of scu-PA in plasma, the incubation volume was restricted to 40 μl. For this reason, experiments were performed in normal or deficient plasma supplemented with exogenous scu-PA to a final scu-PA concentration of 11 ng/ml or 0.2 nM (5x physiological concentration). In this way, inactivation of scu-PA in plasma could still be assessed in our assays despite the minimal amount of plasma used. In all experiments plasma was not diluted more than 30%. Plasma incubations were performed in polypropylene tubes.

Inactivation of scu-PA by thrombin in buffer and in plasma in the presence and absence of thrombomodulin

The effect of thrombomodulin (TM) on the inactivation of scu-PA by thrombin was measured in a purified system and in a plasma milieu. In a purified system, scu-PA (final concentration of 0.2 nM) was incubated in 40 µl final volume for 30 minutes at 37°C with a concentration range of thrombin (0-200 nM) or a concentration range of thrombin (0-8 nM) preincubated with TM (1:1 complex) for 5 minutes at 37°C in 0.05 M Tris/HCl, pH 7.5, 0.1 M NaCl, 0.1% BSA, 0.01% Tween 80. After the incubation period of 30 minutes hirudin (U/ml) was added in a twofold excess to thrombin to inhibit thrombin activity. The concentrations of scu-PA and tcu-PA/T were assessed in 5 µl samples using a bioimmunoassay (BIA) for scu-PA and a BIA for tcu-PA/T (see below) (11,22,23).

Citrated pooled normal plasma supplemented with exogenous scu-PA (final concentration of 0.2 nM) was incubated in 40 µl final volume for 30 minutes at 37°C with a concentration range of thrombin (0-320 nM) or a concentration range of thrombin (0-100 nM) 1:1 complexed with TM. After incubation, any plasma clot present was squeezed using a plastic spatula and a twofold excess of hirudin (U/ml) was added to the plasma or plasma-derived serum to inhibit thrombin activity. The concentrations of scu-PA and tcu-PA/T were assessed in 5 µl plasma or plasma-derived serum using the BIA for scu-PA and the BIA for tcu-PA/T.

To examine the effect of antithrombin III (ATIII) on the inactivation of scu-PA in plasma, ATIII-deficient plasma was incubated with a concentration range of thrombin with or without TM as described above for normal plasma.

The time course of the inactivation of scu-PA was measured during incubation (0-30 minutes) at 37°C in plasma using 320 nM thrombin or 80 nM thrombin/TM complex. After incubation hirudin was added and the concentrations of scu-PA and tcu-PA/T were assessed as described above. A similar experiment was performed in ATIII-deficient plasma using 160 nM thrombin or 20 nM thrombin/TM complex.

Inactivation of scu-PA in recalcified plasma

The effect of calcium on the inactivation of scu-PA by thrombin was studied in recalcified plasma by adding calcium to citrated plasma. To examine whether inactivation of scu-PA by exogenously added thrombin or thrombin/TM complex was affected by calcium, plasma was incubated with 160 nM thrombin or 40 nM thrombin/TM complex for 30 minutes at 37°C in the presence of a concentration range of calcium (0-20 mM, resulting in a free calcium concentration of 0-2 mM). After incubation hirudin was added and the concentrations of scu-PA and tcu-PA/T were assessed in the BIAs.

It was studied in normal and deficient plasmas whether plasma recalcification could induce the inactivation of scu-PA. Citrated normal plasma was recalcified with a concentration range of calcium (0-20 mM) for 30 minutes at 37°C and clot formation was observed. After incubation 2 U/ml hirudin was added and the concentrations of scu-PA and tcu-PA/T were assessed in the BIAs. Inactivation of scu-PA was also measured in normal plasma, factor II-deficient, factor XI-deficient and factor XII-deficient plasma during recalcification with 20 mM calcium for 0 to 60 minutes at 37°C.

After incubation 2 U/ml hirudin was added to inhibit thrombin activity and the concentrations of scu-PA and tcu-PA/T were assessed in the BIAs.

Effect of TM on calcium-induced inactivation of scu-PA in plasma

Normal plasma (30 µl) was incubated in 40 µl final volume with a concentration range of TM (0-40 nM) in the presence of 20 mM calcium for 30 minutes at 37°C. After incubation 2 U/ml hirudin was added and the concentrations of scu-PA and tcu-PA/T were assessed in the BIAs.

The effect of TM was studied further in the plasma from a patient heterozygous for protein C deficiency. 30 µl plasma was incubated in 40 µl final volume with 20 mM calcium in the absence and presence of 20 nM TM for 30 minutes at 37°C. Normal plasma was used as a control. After incubation 2 U/ml hirudin was added and the concentrations of scu-PA and tcu-PA/T were assessed in the BIAs.

Bioimmunoassay (BIA) for scu-PA and tcu-PA/T

The inactivation of scu-PA was measured using a BIA for scu-PA and a BIA for tcu-PA/T as described before (11,22,23). Briefly, 5 µl samples in duplicate were incubated in 96-well plates coated with rabbit polyclonal anti-u-PA antibody. After washing, the immobilized material was activated in parallel wells with 1 nM plasmin (BIA for scu-PA) or 20 nM cathepsin C (BIA for tcu-PA/T). Urokinase activity was determined using Glu-plasminogen and the plasmin substrate H-D-Val-Leu-Lys-p-nitroanilide. The concentrations of scu-PA and tcu-PA/T were calculated from calibration curves obtained by serial dilutions of purified scu-PA and purified tcu-PA/T, respectively.

Statistics

Student's t-test was used for statistical analysis and $p<0.05$ was considered as statistically significant.

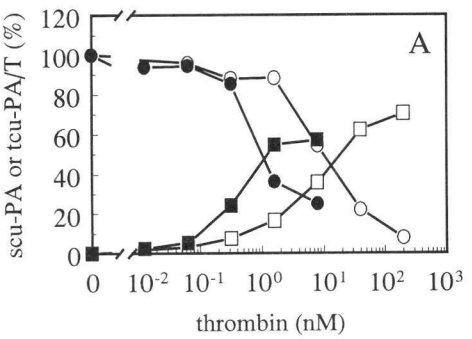
Results

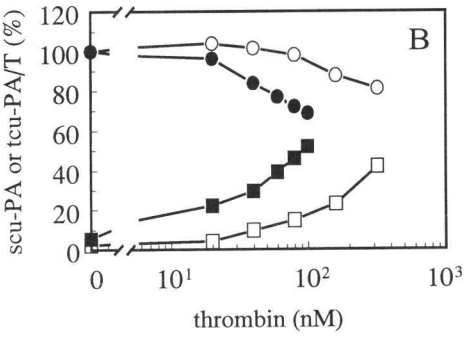
Effect of TM on the inactivation of scu-PA by thrombin in buffer and in plasma

To compare the effect of TM on the inactivation of scu-PA by thrombin in a purified system and in a plasma milieu, scu-PA was incubated for 30 min in buffer and in plasma with a concentration range of thrombin in the absence and presence of TM. In buffer, generation of tcu-PA/T was found at a concentration of 0.08 nM thrombin or more (Figure 1A). The inactivation of scu-PA by thrombin was

found to be stimulated about 20-fold by thrombomodulin, i.e. in the presence of TM 20-fold less thrombin was needed to reach the same extent of inactivation as compared with free thrombin, which is in line with earlier results (14).

In plasma, more thrombin was needed for the inactivation of scu-PA as compared with inactivation in buffer (Figure 1B). Generation of tcu-PA/T was found when plasma was clotted with 40 nM thrombin or more. The inactivation of scu-PA in plasma was stimulated 4 to 5-fold by thrombomodulin, which was less than compared with stimulation by TM in buffer.





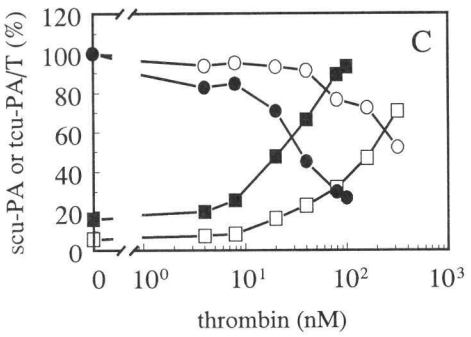


Figure 1. Concentration of scu-PA (○,●) or tcu-PA/T (□,■) in buffer (A), pooled normal plasma (B) and ATIII-deficient plasma (C) incubated for 30 minutes at 37°C with increasing concentrations of thrombin in the absence (open symbols) or presence (closed symbols) of thrombomodulin. The concentration of scu-PA and tcu-PA/T was expressed as a percentage of the scu-PA concentration in buffer or plasma measured after incubation in the absence of thrombin and TM.

In ATIII-deficient plasma, about twofold less thrombin was needed to reach the same extent of scu-PA inactivation as compared with normal plasma (Figure 1C). In addition, inactivation of scu-PA was stimulated about 8-fold by TM, which is twofold stronger as compared with stimulation in normal plasma. These findings suggested a role of ATIII in the inactivation of scu-PA in plasma by rapid inhibition of thrombin or the thrombin/TM complex, and by decreasing the stimulating effect of TM.

To investigate the time course of inactivation of scu-PA and the role of ATIII in this process, the generation of tcu-PA/T was studied during incubation of normal or ATIII-deficient plasma with thrombin in the absence and presence of TM. As shown in Figure 2, inactivation of scu-PA by free thrombin in normal plasma was found to occur rapidly and declined within one minute most likely due to rapid neutralization of thrombin by its inhibitors. Similar results were found for inactivation of scu-PA by the thrombin/TM complex. In ATIII-deficient plasma, inactivation of scu-PA was found to be prolonged to about 5 minutes, demonstrating that ATIII indeed rapidly inhibits inactivation of scu-PA by thrombin or the thrombin/TM complex (data not shown).

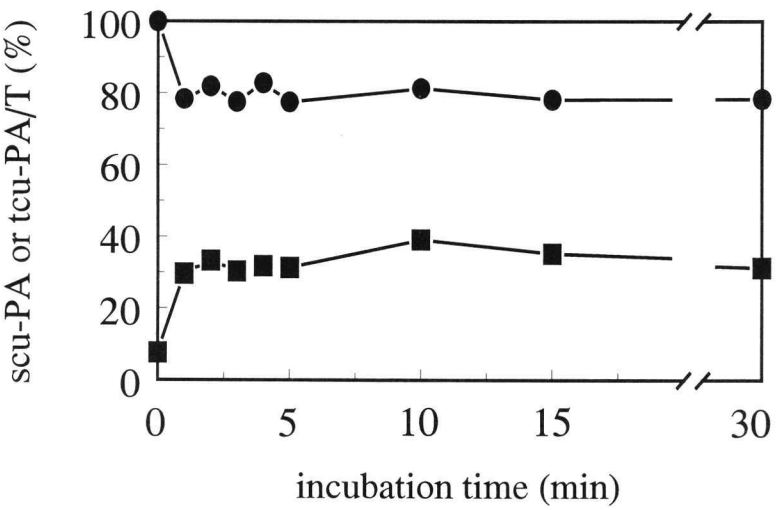


Figure 2. Concentration of scu-PA (•) and tcu-PA/T (■) in pooled normal plasma during incubation in separate tubes with 320 nM thrombin at 37°C. For t=0, a twofold excess of hirudin was added in advance to plasma before the addition of thrombin. The concentration of scu-PA and tcu-PA/T was expressed as a percentage of the scu-PA concentration in plasma at t=0.

Effect of calcium on the inactivation of scu-PA in plasma by thrombin or the thrombin/TM complex

To examine whether the inactivation of scu-PA by exogenously added thrombin or thrombin/TM complex was affected by calcium, plasma was incubated with thrombin with or without TM in the presence of a concentration range of calcium for 30 minutes at 37°C. As shown in Figure 3, calcium induced a twofold increase in the generation of tcu-PA/T by free thrombin (160 nM) in a concentration-dependent way. However, in the presence of TM no effect of calcium was found. When plasma was incubated with thrombin in the presence of a concentration range of calcium for 5 minutes, the period in which inactivation of scu-PA in plasma by thrombin was found to take place, no stimulating effect of calcium was found (data not shown).

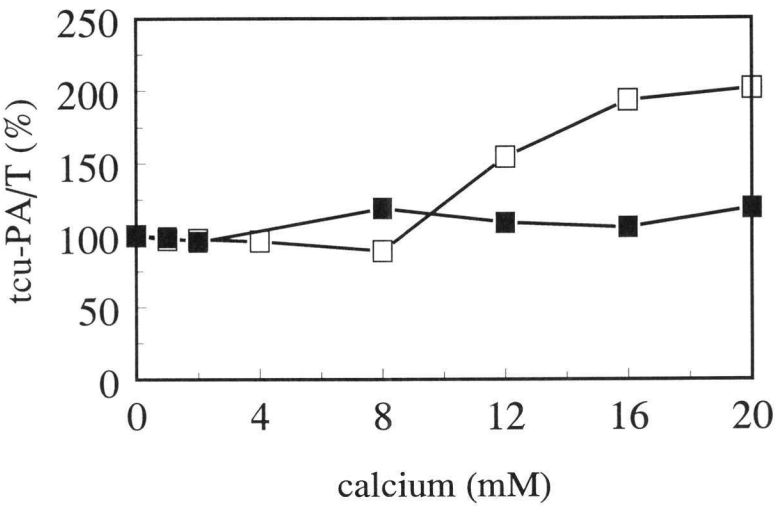


Figure 3. Effect of calcium on the generation of tcu-PA/T in pooled normal plasma incubated for 30 minutes at 37°C with 160 nM thrombin (□) or 40 nM thrombin/TM complex (■). The concentration of tcu-PA/T was expressed as a percentage of the tcu-PA/T concentration induced by thrombin (0.07 nM tcu-PA/T) and thrombin + TM (0.08 nM tcu-PA/T) in the absence of calcium.

These findings suggested that calcium had no direct effect on the inactivation of scu-PA by thrombin or the thrombin/TM complex. However, calcium appeared to increase the inactivation of scu-PA in plasma most likely due to endogenous thrombin generation, a process that was found to be inhibited by the thrombin/TM complex. Therefore, the induction of inactivation of scu-PA by recalcification of plasma was studied further.

Effect of plasma recalcification on the inactivation of scu-PA

Recalcification of plasma resulted in the inactivation of scu-PA, a process dependent on the calcium concentration (Figure 4). It was observed that calcium induced clotting of plasma in a concentration-dependent way as found for the inactivation of scu-PA. These findings suggested that calcium-induced endogenous thrombin generation resulted in the generation of tcu-PA/T.

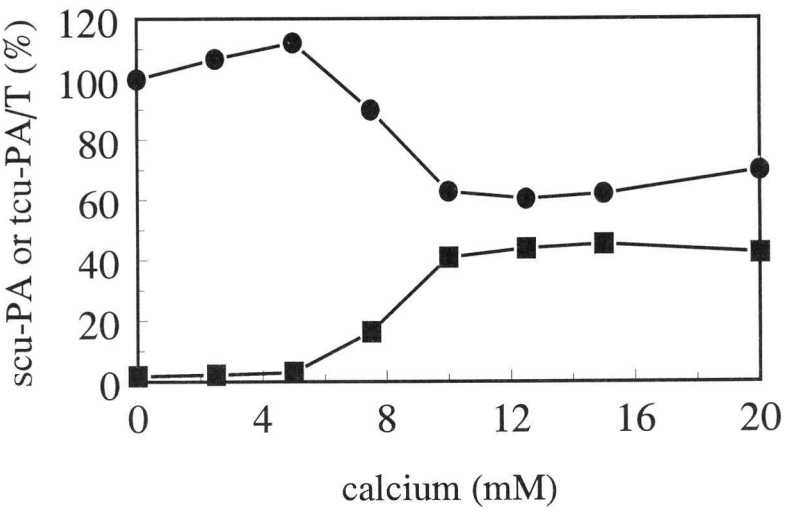


Figure 4. Concentration of scu-PA (●) and tcu-PA/T (■) in pooled normal plasma recalcified with a concentration range of calcium for 30 minutes at 37°C. The concentration of scu-PA and tcu-PA/T was expressed as a percentage of the scu-PA concentration in plasma measured after incubation in the absence of calcium.

To examine by which pathway tcu-PA/T was generated endogenously, normal plasma, factor II-deficient, factor XI-deficient and factor XII-deficient plasma was incubated with 20 mM calcium (resulting in 2 mM free calcium) and generation of tcu-PA/T was measured during 60 minutes. As shown in Figure 5, tcu-PA/T was generated during recalcification of normal plasma after a lag-phase of 10 minutes, reaching a plateau after 30 minutes, which coincided with the time-course of clot formation. In recalcified factor II-deficient, factor XI-deficient and factor XII-deficient plasma no generation of tcu-PA/T was found while no clot formation could be observed. These findings implied that calcium could mediate endogenous thrombin generation through the contact activation system, resulting in significant inactivation of scu-PA.

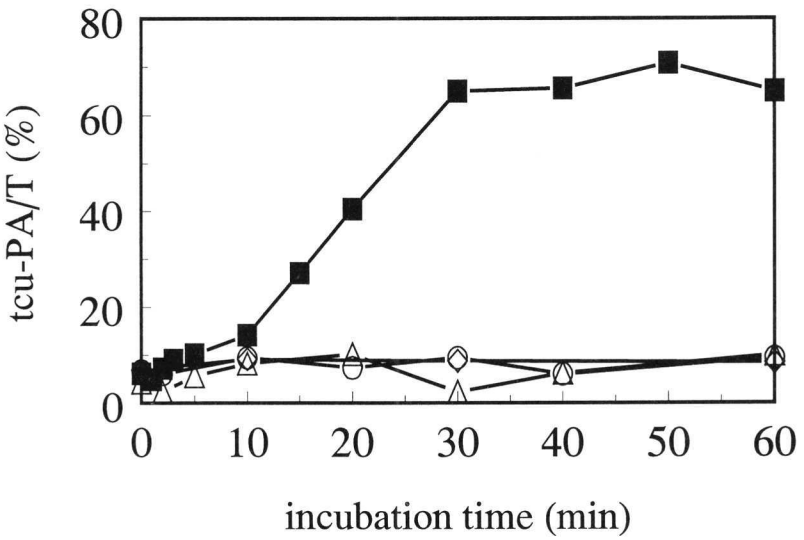


Figure 5. Generation of tcu-PA/T during recalcification of plasma in separate tubes with 20 mM calcium at 37°C in pooled normal plasma (■), factor II-deficient plasma (◊), factor XI-deficient plasma (Δ) and factor XII-deficient plasma (○). For t=0, 2 U/ml hirudin was added in advance to plasma before the addition of calcium. The concentration of tcu-PA/T was expressed as a percentage of the scu-PA concentration in plasma at t=0.

Effect of TM on calcium-induced inactivation of scu-PA in plasma

When TM was added during the recalcification of plasma, it was found that calcium-induced clot formation and generation of tcu-PA/T was completely inhibited by TM in a concentration-dependent way, reaching a baseline level of about 10% tcu-PA/T (representing the detection limit of the BIA) (Figure 6). This finding suggested that TM could form a complex with endogenously generated thrombin and in this way convert protein C to activated protein C (APC). Activation of protein C then would result in the inhibition of thrombin generation and as a result in the inhibition of inactivation of scu-PA.

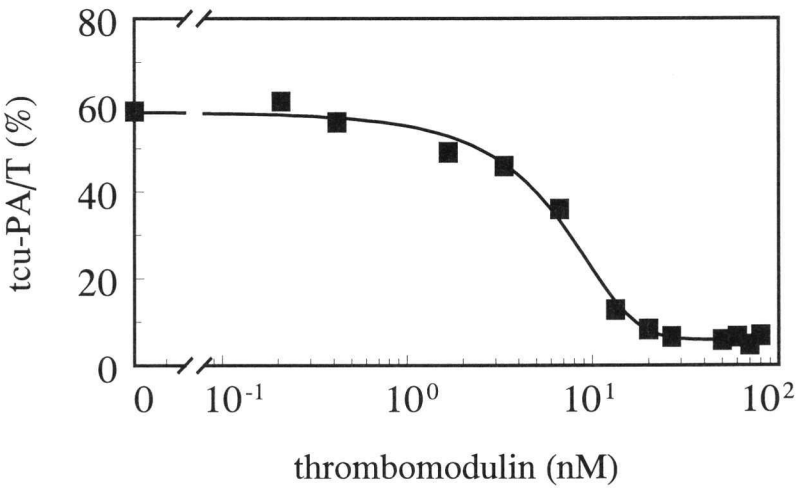


Figure 6. Effect of TM on the generation of tcu-PA/T in pooled normal plasma recalcified with 20 mM calcium for 30 minutes at 37°C. The concentration of tcu-PA/T was expressed as a percentage of the scu-PA concentration in plasma measured after incubation in the absence of calcium and TM.

This hypothesis was confirmed by experiments performed in the plasma from a patient heterozygous for protein C deficiency. In the absence of TM, the extent of inactivation of scu-PA was similar in both normal plasma and the protein C deficient plasma. In normal plasma, calcium-induced generation of tcu-PA/T was significantly decreased from 60% in the absence of TM to about 12% in the presence of

TM (Figure 7). However, in the protein C deficient plasma TM could not inhibit calcium-induced inactivation of scu-PA. Instead, TM induced a statistically significant increase in the generation of tcu-PA/T, most likely due to the stimulating effect of TM described above.

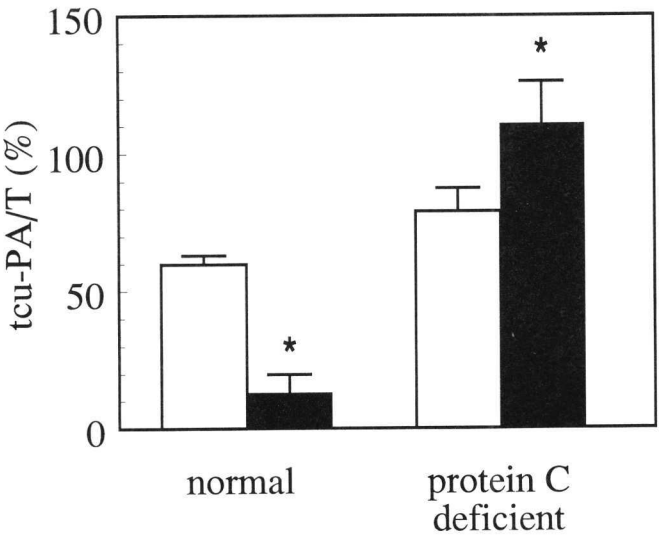


Figure 7. Concentration of tcu-PA/T in pooled normal plasma and in the plasma from a patient heterozygous for protein C deficiency recalcified with 20 mM calcium for 30 minutes at 37°C in the absence (open bars) and presence (solid bars) of 20 nM TM. The concentration of tcu-PA/T was expressed as a percentage (mean ± SD, n=3) of the scu-PA concentration in plasma measured after incubation in the absence of calcium and TM. * $p<0.001$.

Discussion

We have examined the inactivation of scu-PA by thrombin in plasma, a natural milieu in which the physiological inhibitors and substrates for thrombin are present that may influence this process. We found that in plasma far more thrombin was needed to generate tcu-PA/T as compared with a purified system. The inactivation of scu-PA occurred rapidly and declined within one minute. This result suggests that in plasma more thrombin is needed for the inactivation of scu-PA due to rapid neutralization of thrombin by its inhibitors. Indeed, in ATIII-deficient plasma in which the main thrombin inhibitor is absent less thrombin was needed to induce inactivation of scu-PA. However, there was still a significant difference in the competence of thrombin as compared with the inactivation of scu-PA in a purified system. Besides ATIII other inhibitors could be present in plasma

which may inhibit thrombin activity rapidly, such as heparin cofactor II, protease nexin I and α_2-macroglobulin (24). In plasma, about 150-300 nM of free thrombin can potentially be generated (25). In this study, inactivation of scu-PA by exogenous thrombin in citrated plasma was found at a concentration of 40 nM thrombin or more. This result implies that inactivation of scu-PA can indeed occur *in vivo* during activation of the coagulation system.

The inactivation of scu-PA in citrated plasma was stimulated 4-fold by thrombomodulin, i.e. in the presence of TM 4-fold less thrombin was needed to reach the same extent of inactivation as compared with free thrombin. In ATIII-deficient plasma, stimulation by thrombomodulin was increased twofold as compared with normal plasma. This finding implies that the thrombin/TM complex is more rapidly inhibited by ATIII than free thrombin, which is in agreement with other studies (26,27). In our system, we were not able to measure inactivation of scu-PA below one minute of incubation and therefore we could not demonstrate a clear difference in neutralization rate of free thrombin or thrombin complexed to thrombomodulin in normal plasma. Since stimulation by TM in ATIII-deficient plasma (8-fold) was still less than stimulation found in a purified system (20-fold), other inhibitors may be involved that inhibit the thrombin/TM complex faster than free thrombin.

Complexing of thrombin to thrombomodulin is known to result in stimulation of the antifibrinolytic behaviour of thrombin. An antifibrinolytic property induced by thrombomodulin is the stimulation of the activation of TAFI by thrombin (20). Activated TAFI acts as a carboxypeptidase and most likely removes C-terminal lysines from partially degraded fibrin (21,28). In this way, t-PA-mediated fibrinolysis is inhibited (20,21,28). Through stimulation of thrombin-mediated inactivation of scu-PA in plasma, thrombomodulin appears to promote the antifibrinolytic behaviour of thrombin via an alternative pathway. As demonstrated by experiments performed in knock-out animal models, urokinase appears to play a significant role in fibrinolysis and thrombolysis (29). By modulating the fibrinolytic activity through stimulation of both the activation of TAFI and the inactivation of scu-PA, the formation and maintainance of a blood clot *in vivo* may be governed by the thrombin/TM complex.

We could not find any direct effect of calcium on the activity of thrombin or the thrombin/TM complex towards scu-PA. In a purified system, it has been described that calcium slightly inhibits the inactivation of scu-PA by thrombin or the thrombin/TM complex (14). To our surprise, recalcification of plasma induced the generation of a significant amount of tcu-PA/T, an effect comparable to

generation by more than 160 nM thrombin exogenously added to citrated plasma. This finding confirms that inactivation of scu-PA may occur *in vivo* during activation of the coagulation system. The fact that no calcium-induced inactivation of scu-PA could be found in factor XI- and factor XII-deficient plasma indicates that in our system calcium mediates endogenous thrombin generation via the contact activation system, a process which may be amplified by direct activation of factor XI by endogenously generated thrombin (30). In our experimental set-up, no phospholipids were added to the platelet-poor plasma. It is known that phospholipids catalyze the enzymatic cascades in the coagulation system, thus the effect of recalcification on the inactivation of scu-PA may be stronger in the presence of an excess of phospholipids.

In the presence of thrombomodulin, no calcium-induced inactivation of scu-PA in plasma could be found, because the complex formed between thrombomodulin and endogenously generated thrombin prevented, most likely through the activation of protein C, further endogenous thrombin generation and in this way the inactivation of scu-PA. APC inactivates cofactors Va and VIIIa and thereby inhibits prothrombin activation (18,19). It is known that APC is profibrinolytic by attenuating thrombin generation and in this way inhibiting the activation of TAFI (31-33). This study shows another possible profibrinolytic property of APC, namely preventing the inactivation of scu-PA by the inhibition of thrombin generation.

As shown by this study, thrombomodulin plays a dual role in the inactivation of scu-PA in plasma. On the one hand it stimulates the inactivation of scu-PA by thrombin, while on the other hand thrombomodulin prevents inactivation of scu-PA in recalcified plasma by inhibiting endogenous thrombin generation. Physiologically, the inactivation of scu-PA thus seems to be regulated by a delicate balance between several coagulation components mainly involving thrombin, thrombomodulin and protein C. In the static microenvironment of a blood clot especially or in the microcirculation where the local concentration of endothelium-associated thrombomodulin is very high (up to 500 nM), this balance will determine whether scu-PA is inactivated or not (19). In this study, 13 nM thrombomodulin was found to be sufficient to inhibit calcium-induced inactivation of scu-PA. Therefore, it may be speculated that under physiological circumstances the balance favours the inhibition of inactivation of scu-PA, which may be an explanation for the fact that no tcu-PA/T could be found in the plasma of healthy individuals (11). However, disturbance of the balance may

lead to the inactivation of scu-PA, as shown in this study. We have demonstrated that in the plasma from a patient heterozygous for protein C deficiency thrombomodulin did not inhibit calcium-induced generation of tcu-PA/T, but instead stimulated the inactivation process. It is known that disturbance of the protein C pathway results in the resistance of the blood clot to fibrinolysis, as shown for factor Va^{Leiden} (34). Extensive inactivation of scu-PA due to a disturbed protein C pathway may contribute to the resistance of a blood clot to fibrinolysis. This finding may be an additional explanation for the association found between thromboembolism and deficiencies in the protein C pathway (35-38).

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APPENDIX TO CHAPTER 3

The inactivation of single-chain urokinase-type plasminogen activator by thrombin may provide an additional explanation for the antifibrinolytic effect of factor XI

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Dear Sir,

We have read with great interest the recent review article by Bouma *et al.* (1) on factor XI and the protection of the fibrin clot against lysis. In this article, the authors state that thrombin activatable fibrinolysis inhibitor (TAFI) is responsible for the factor XI-dependent downregulation of the activation of the fibrinolytic system *in vitro*. In addition, it is implied that the inactivation of single-chain urokinase-type plasminogen activator (scu-PA) by thrombin does not play a significant role in this process, since the inactivation of scu-PA requires only low amounts of thrombin that can easily be produced by the tissue factor pathway (1). Although we agree with Bouma *et al.* that TAFI is a potentially important component, we believe that the contribution of the inactivation of scu-PA by thrombin to factor XI-dependent inhibition of fibrinolysis is underestimated. In a purified system, low amounts of thrombin are indeed sufficient to inactivate scu-PA (2,3). However, in a recent paper we showed that in plasma, a natural milieu in which physiological inhibitors and substrates of thrombin are present, higher levels of thrombin are needed for this process (4). Inactivation of scu-PA in plasma was only found when 40 nM thrombin or more was used. Furthermore, recalcification of plasma without the addition of exogenous thrombin induced significant inactivation of scu-PA, comparable to the effect of more than 160 nM exogenously added thrombin, due to endogenous thrombin generation. The calcium-induced inactivation of scu-PA did not occur in factor XII- or factor XI-deficient plasma, indicating that this process was mediated by the contact activation system and may be amplified by feedback activation of factor XI by thrombin (4). These findings not only show that in plasma high concentrations of thrombin are necessary for the inactivation of scu-PA, comparable to the amounts of thrombin needed for both the activation of TAFI (5) and the antifibrinolytic effect of factor XI (6), but also that the inactivation of scu-PA may depend on thrombin that is additionally generated via factor XI, providing a link between coagulation and fibrinolysis.

In the studies on the antifibrinolytic properties of factor XI, clot lysis experiments were performed in 50% plasma to which about 50 ng/ml tissue-type plasminogen activator (t-PA) was added (6,7). Taking the plasma concentration of scu-PA of 2.2 ng/ml into consideration, one can expect that scu-PA-induced fibrinolysis will be completely overruled by t-PA-induced fibrinolysis

in this experimental set-up. As a result, any effect of factor XI-mediated inactivation of scu-PA by thrombin on scu-PA-induced fibrinolysis will not be detectable.

In conclusion, the inactivation of scu-PA by thrombin may provide an additional explanation for factor XI-dependent downregulation of fibrinolysis under (patho)physiological conditions. It is intriguing that the action of thrombin on both TAFI (8,9) and scu-PA (4,10) is accelerated by the endothelial cell cofactor thrombomodulin. The antifibrinolytic effect of the inactivation of scu-PA by thrombin needs further research.

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CHAPTER 4

Inactivation of single-chain urokinase-type plasminogen activator by thrombin in human subjects

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Summary

Thrombin cleaves single-chain urokinase-type plasminogen activator (scu-PA) into a virtually inactive two-chain form (tcu-PA/T), a process which may protect a blood clot from early fibrinolysis. Is is not known under what circumstances tcu-PA/T can be generated *in vivo*. We have studied the occurrence of tcu-PA/T in human subjects with a varying degree of hypercoagulability. tcu-PA/T was assessed in the plasma of patients with disseminated intravascular coagulation (DIC), endotoxin-treated volunteers, patients with unstable angina pectoris, and patients selected for hip replacement. Relationships between tcu-PA/T and several markers reflecting thrombin generation were examined. tcu-PA/T was only observed in the plasma of DIC patients and was associated with all thrombin markers and with scu-PA and urokinase antigen. Prothrombin fragment 1+2 and urokinase antigen were independent predictors of tcu-PA/T. The fact that tcu-PA/T could not be detected in the other three groups was explained by a lower extent of thrombin generation, greater inhibition of thrombin by antithrombin, or less available urokinase antigen in these groups. The contribution of scu-PA to total urokinase antigen was decreased in the DIC patients due to inactivation by thrombin, which may be an additional explanation for the inadequate fibrinolysis observed in these patients. These findings show that scu-PA can be inactivated in the circulation under severe pathophysiological circumstances and that the process of inactivation not only depends on the generation of thrombin but also on the control of thrombin activity by its inhibitor antithrombin.

Introduction

Single-chain urokinase-type plasminogen activator (scu-PA) is the precursor form of two-chain urokinase-type plasminogen activator (tcu-PA), a serine protease composed of two polypeptide chains linked together by a disulphide bond. Active tcu-PA, generated after the cleavage of scu-PA by plasmin or other activators, plays a role in fibrinolysis and extracellular proteolysis by converting plasminogen into plasmin (1). Cleavage of scu-PA can also result in inactivation. Thrombin cleaves scu-PA at Arg 156 - Phe 157, two residues prior to the activation site, in this way generating an inactivated tcu-PA form called thrombin-cleaved tcu-PA (tcu-PA/T) (2). *In vitro*, tcu-PA/T appears

to have little amidolytic and fibrinolytic activity (2,3). However, in a rabbit jugular vein thrombosis model tcu-PA/T was found to be a potent and fibrin-specific thrombolytic agent, suggesting the involvement of specific factors that can re-activate tcu-PA/T (4,5). Indeed, tcu-PA/T is re-activated by dipeptidyl peptidase I (cathepsin C), as well as by platelets (6,7). An alternative explanation for the observed thrombolytic potency of tcu-PA/T may be the promoting effect of fibrin fragment E-2 on plasminogen activation by tcu-PA/T (8).

The inactivation of scu-PA has been postulated as a mechanism for protecting a fresh blood clot from early fibrinolysis (3,9). This process does indeed take place *in vivo*, as we have demonstrated the presence of tcu-PA/T in human body fluids under pathological conditions such as sepsis and rheumatoid arthritis, which involve the production of large amounts of thrombin (10). Recently, we described the inactivation of scu-PA by thrombin in a plasma milieu (11). It was observed that the inactivation is accelerated by thrombomodulin, a phenomenon also demonstrated in a purified system, and that the inhibition of scu-PA-induced fibrinolysis through inactivation by thrombin is mainly regulated by the activation of the coagulation system (11-13).

The mechanism of the inactivation of scu-PA by thrombin has been described in detail both in a purified system and in a plasma milieu (11-14). However, it is not known under what conditions tcu-PA/T is generated in the circulation *in vivo*. It can be speculated that the inactivation of scu-PA depends on the production of thrombin, although there is no direct evidence. To study this hypothesis, the occurrence of tcu-PA/T was assessed in the plasma of subjects with a varying degree of hypercoagulability, namely patients with disseminated intravascular coagulation, endotoxin-treated volunteers, patients with unstable angina pectoris and patients selected for hip replacement. Relationships between the level of tcu-PA/T and the levels of parameters reflecting thrombin generation were analysed in order to gain more insight into the occurrence of pathophysiological inactivation of scu-PA by thrombin.

Materials and Methods

Study groups

All studies were approved by local research ethics committees.

DIC patients

Fourteen patients with sepsis (as evidenced by positive blood cultures) and DIC were included in this study. DIC was diagnosed if the following criteria were met: 1) platelet count < $100x10^9$/l or a drop in platelet count of at least $40x10^9$/l in the previous 24 h, 2) prolongation of activated partial thromboplastin time and prothrombin time of at least 3 sec, 3) antithrombin (AT) levels < 0.75 U/ml and 4) fibrin degradation products > 40 µg/ml. Exclusion criteria consisted of the lack of the informed consent of the patient or his/her relatives, and the concurrent use of anticoagulant agents at a therapeutic dose less than 24 h before inclusion.

Blood from the patients was obtained from an antecubital vein using 21-gauge butterfly needles and collected in plastic tubes containing 3.2% (w/v) sodium citrate in a ratio of 9 volumes of blood : 1 volume of sodium citrate. Blood samples were immediately placed in melting ice and centrifuged at 4°C for 20 min at 1600 x g. Plasma samples were stored in aliquots at -70°C until assayed.

endotoxin-treated volunteers

Three healthy male volunteers with a normal medical history and physical and laboratory examination were included in this study. Written informed consent was obtained from each subject. The subjects were treated with endotoxin i.v. as part of a study on the effect of recombinant interleukin-10 on endotoxin-induced effects on coagulation and fibrinolysis, as described by Pajkrt *et al.* (15), and blood was collected at selected time-points (0 - 12 h). Maximum thrombin generation was found 4 h after the administration of endotoxin.

unstable angina pectoris patients

A group of 15 unstable angina pectoris patients was taken from the Acute Phase Reactions and Ischaemic Syndromes (APRAIS) Study (16). In this group, 11 patients were randomly selected and 4 patients were selected on the basis of high prothrombin fragment 1+2 (F1+2) and thrombin-antithrombin complexes (TAT) levels.

hip patients

A group of 29 patients was randomly selected from the ECAT DVT Study, a collaborative study of preoperative haemostatic tests for predicting deep venous thrombosis after elective hip replacement (17). The patients from the ECAT DVT Study have increased plasma levels of thrombin markers F1+2 and TAT as compared to the reference ranges (17), implying ongoing thrombin generation in these patients due to preoperative underlying conditions.

Assays

In all groups plasma levels of F1+2 and TAT were measured using Enzygnost F1+2 and TAT kits (Behringwerke AG, Marburg, Germany; reference ranges 0.4 to 1.1 nM and 1.0 to 4.1 ng/ml, respectively). AT activity was measured by an amidolytic assay as described by Peters *et al.* (reference range 80 to 120%) (18) in the plasma of the DIC patients and the endotoxin-treated volunteers, and by a spectrophotometric assay (Coamate Antithrombin, Chromogenix AB, Mölndal, Sweden; reference range 80 to 120%) in the plasma of the unstable angina pectoris patients and the hip patients. Soluble fibrin was measured in the plasma of the DIC patients and the endotoxin-treated volunteers using the Fibrinostika Soluble Fibrin Microelisa (Organon Teknika, Boxtel, The Netherlands; reference range 26 to 59 ng/ml) (19) and fibrin(ogen) degradation products (FDP) were assessed using a latex agglutination test (Thrombo Wellcotest, Murex Biotechnology Ltd, Temple Hill, England; reference range less than 10 μg/ml). Plasma levels of scu-PA (reference range 2.0 to 2.6 ng/ml) and tcu-PA/T (reference range less than 0.2 ng/ml) were measured in all groups using sensitive bioimmunoassays (10,20,21). Urokinase antigen was measured in all groups using a urokinase ELISA (reference range 2.1 to 4.9 ng/ml) (22). As an additional measure for the inactivation of scu-PA, the ratio between scu-PA and urokinase antigen was calculated by expressing scu-PA as a percentage of urokinase antigen.

Statistical analysis

Since the variables were not normally distributed in all groups, median and interquartile ranges were determined and non-parametric statistical methods were used. Correlations between tcu-PA/T and other parameters were tested using Spearman rank correlation method. To determine a set of independent variables that best predicted the level of tcu-PA/T, multiple linear regression analysis (backward method) was performed. To evaluate whether there were significant differences of levels of single variables between the different groups, Fisher's least significant differences procedure was used: first a Kruskal Wallis test was performed and if this test indicated a significant difference between groups, then two groups at a time were compared using a Mann-Whitney test to determine which differences between groups were significant. A value of $p < 0.05$ was considered statistically significant.

Results

Thrombin-cleaved urokinase (tcu-PA/T) was only detected in the plasma of the DIC patients (Figure 1). In the plasma of one DIC patient the level of tcu-PA/T was below the detection limit of the assay (0.2 ng/ml), but in all other patients levels of up to 5.6 ng/ml were observed. In the other groups, tcu-PA/T values above the detection limit of the assay could not be found.

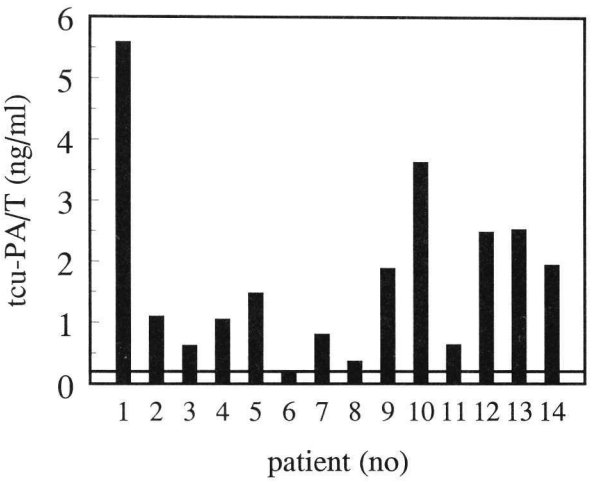

Figure 1. Levels of tcu-PA/T in the plasma of 14 DIC patients. The horizontal line represents the detection limit of the bioimmunoassay for tcu-PA/T (0.2 ng/ml) (10). In one patient (no 6) the plasma level of tcu-PA/T was below the detection limit.

Table 1. Levels of urokinase and coagulation parameters in plasma of patients with DIC, and correlations between tcu-PA/T and other parameters.

parameter	median (interquartile range)	correlation with tcu-PA/T*
tcu-PA/T (ng/ml)	1.3 (0.6 - 2.5)	-
scu-PA (ng/ml)	4.4 (2.6 - 7.0)	0.802***
urokinase antigen (ng/ml)	11.0 (5.8 - 17.5)	0.802***
F1+2 (nM)	3.8 (2.4 - 5.0)	0.833***
TAT (ng/ml)	27.4 (22.3 - 38.3)	0.739***
AT (%)	50.5 (33.5 - 66.3)	-0.678***
FDP (μg/ml)	120 (80 - 160)	0.627**
soluble fibrin (ng/ml)	1220 (465 - 1760)	0.389

*Correlations between tcu-PA/T and other parameters were tested using Spearman rank correlation method. ** $p < 0.05$; *** $p < 0.01$.

In the DIC patients significant correlations between tcu-PA/T and scu-PA, urokinase antigen, F1+2, TAT, AT and FDP, but not soluble fibrin, were found (Table 1). Multiple regression analysis, with tcu-PA/T as the dependent variable and all other parameters as independent variables, demonstrated that the model that best predicts the level of tcu-PA/T includes urokinase antigen ($\beta = 0.59$, $p <$

0.001) and F1+2 ($\beta = 0.51$, $p < 0.001$). This finding indicated that tcu-PA/T is likely to increase as a result of increasing levels of urokinase antigen and F1+2. tcu-PA/T explained about 13.5% (median value) of total urokinase antigen, and scu-PA about 41.8%.

To examine why tcu-PA/T was only observed in the plasma of the DIC patients, a Kruskal Wallis test was used to evaluate whether there were significant differences of levels of single variables between the four groups. In Figure 2, the individual levels of F1+2, TAT, AT, urokinase antigen, scu-PA and the ratio between scu-PA and urokinase antigen are presented for the four groups. For the endotoxin-treated volunteers, measurements were selected at the time-point of maximal thrombin generation, namely t = 4 h. Plasma levels of FDP and soluble fibrin were not known for the unstable angina pectoris patients and the hip patients, and therefore could not be included in this statistical analysis.

Significant differences between groups were found for all variables tested, namely F1+2 ($p < 0.01$), TAT ($p < 0.001$), AT ($p < 0.001$), urokinase antigen ($p < 0.001$), scu-PA ($p < 0.001$) and the ratio between scu-PA and urokinase antigen ($p < 0.001$).

Next, two groups at a time were compared in order to determine which differences between groups were significant, using a Mann-Whitney test. In comparison with the unstable angina pectoris patients and the hip patients, levels of F1+2, TAT, urokinase antigen and scu-PA were significantly higher in the plasma of the DIC patients (Figure 2). Urokinase antigen and scu-PA levels were also significantly higher than those of the endotoxin-treated volunteers. F1+2 and TAT levels did not differ between the DIC patients and the endotoxin-treated volunteers. AT was significantly lower in the plasma of the DIC patients in comparison with the other three groups. The ratio between scu-PA and urokinase antigen was also significantly lower in the plasma of the DIC patients compared with the other groups.

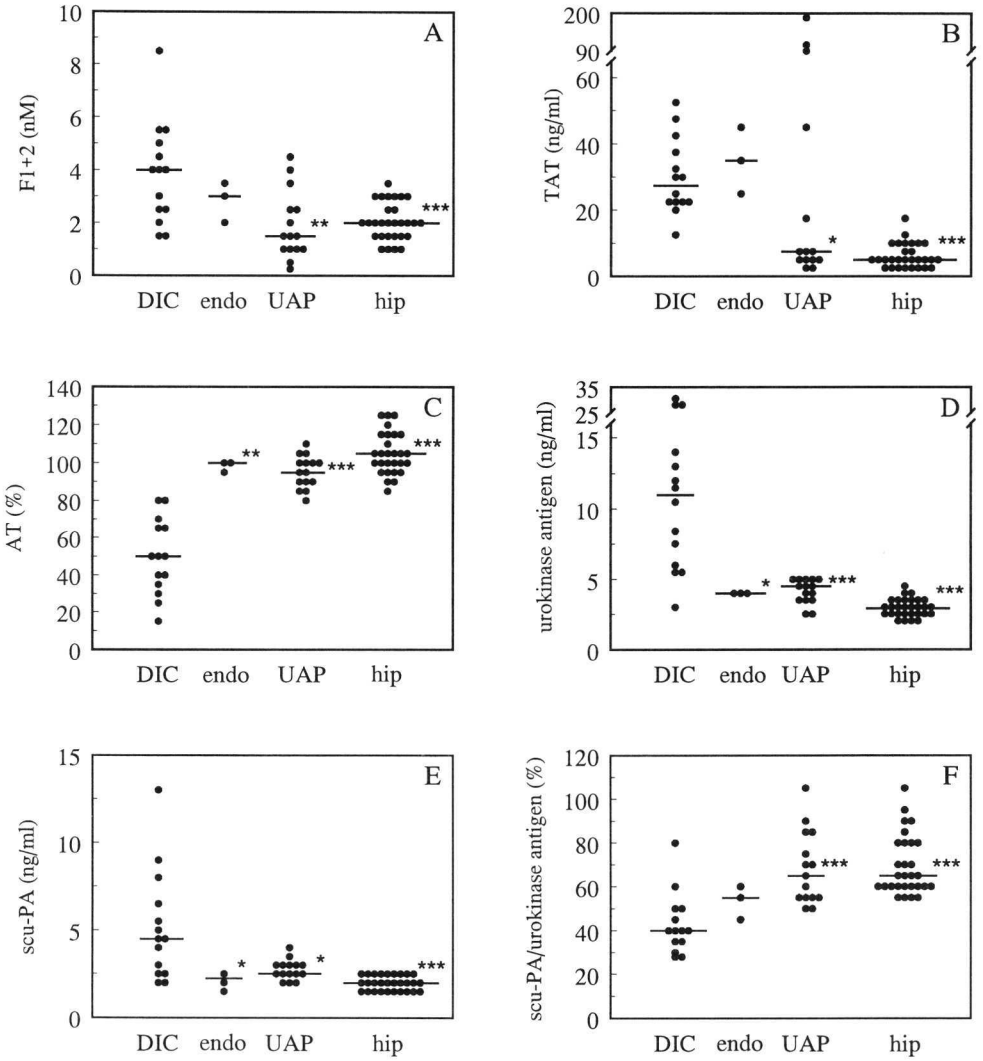

Figure 2. Levels of F1+2 (A), TAT (B), AT (C), urokinase antigen (D) and scu-PA (E) in the plasma of 14 DIC patients (DIC), 3 endotoxin-treated volunteers (endo), 15 unstable angina pectoris patients (UAP) and 29 hip patients (hip). The ratio between scu-PA and urokinase antigen was calculated by expressing scu-PA as a percentage of urokinase antigen (F). For the endotoxin-treated volunteers measurements at t = 4 h were selected. The horizontal lines represent median values. Comparisons are made between the DIC patients and the other groups. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ (Mann-Whitney test).

No significant differences were found between the levels of the parameters in the endotoxin-treated volunteers and the unstable angina pectoris patients. Compared with the hip patients, levels of TAT ($p < 0.01$) and urokinase antigen ($p < 0.05$) were significantly higher in the endotoxin-treated volunteers, while levels of urokinase antigen ($p < 0.001$) and scu-PA ($p < 0.001$) were significantly higher in the unstable angina pectoris patients. Levels of AT were significantly lower in the unstable angina pectoris patients compared with the hip patients ($p < 0.05$), but were still within the reference range (80 - 120%).

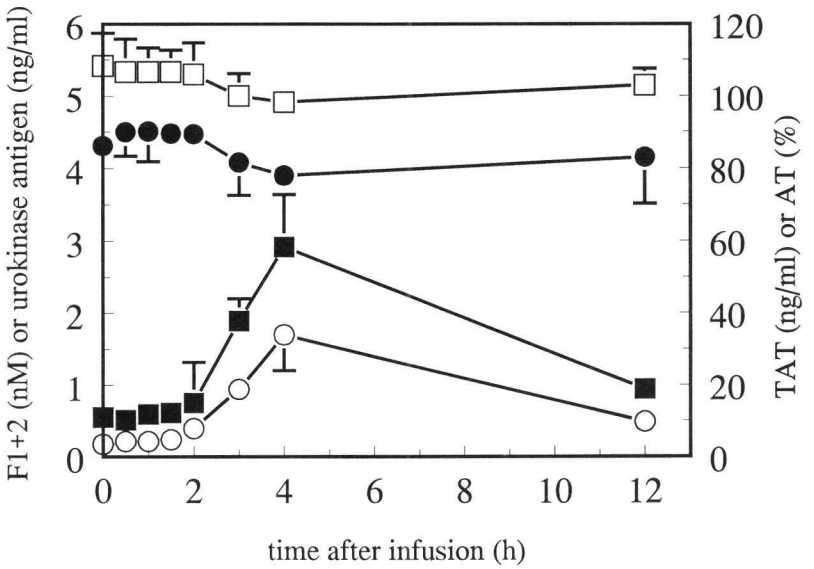

Figure 3. Mean levels (± SD) of F1+2 (■), TAT (○), AT (□) and urokinase antigen (●) in the plasma of 3 healthy volunteers measured over a period time after the infusion of endotoxin.

When the time-course of thrombin generation in the endotoxin-treated volunteers was studied, it appeared that thrombin was generated after 2 h, reaching a maximum after 4 h (Figure 3). The maximum amount of thrombin that was generated in the endotoxin-treated volunteers, as reflected by F1+2 and TAT, was comparable to the levels of thrombin generation seen in the DIC patients. In

contrast to the DIC patients who showed very low plasma levels of AT, the levels of AT in the endotoxin-treated volunteers remained within the reference range suggesting that all thrombin generated was immediately inhibited by AT (Figure 3). Levels of urokinase antigen (Figure 3), scu-PA and soluble fibrin were all within the reference ranges and did not change during time. No significant generation of FDP was observed, while tcu-PA/T could not be detected.

Discussion

The inactivation of scu-PA by thrombin in plasma is regulated by activation of the coagulation system and thus may depend mainly on the generation of thrombin (11). The concentration of physiologically circulating thrombin seems to be insufficient to induce this process, since tcu-PA/T could not be found in the plasma of healthy individuals (10). It can be hypothesized that the generation of tcu-PA/T is likely to occur under pathophysiological conditions involving the production of large amounts of thrombin. Indeed, significant levels of tcu-PA/T were observed in the plasma of the DIC patients, accounting for about 13% of urokinase antigen. DIC is associated with various clinical disorders and is manifested by severe symptoms (23). Systemic generation of thrombin in these patients leads to deposition of fibrin in the microvasculature which can result in multiple organ failure (24,25). In this study, plasma levels of tcu-PA/T in the DIC patients were positively associated with F1+2 and TAT, which are markers of thrombin generation, supporting a relationship between the inactivation of scu-PA and the generation of thrombin *in vivo*. Furthermore, it was observed that F1+2, together with urokinase antigen, is an independent predictor of tcu-PA/T.

Unstable angina pectoris is associated with increased levels of thrombin generation (26,27), while during the infusion of endotoxins in healthy subjects activation of the coagulation pathway is induced (15,28). Nevertheless, in the plasma of these groups or in the plasma of the patients selected for hip replacement, detectable levels of tcu-PA/T could not be observed. In the unstable angina pectoris patients and the hip patients the median plasma levels of F1+2 and TAT were significantly lower compared with the DIC patients. It may be concluded that, although a lower level of tcu-PA/T could be expected given the lower urokinase antigen level, the amount of thrombin generated in these persons was not sufficient to generate levels of tcu-PA/T above the detection limit of the assay

(0.2 ng/ml). In contrast, in the plasma of the endotoxin-treated volunteers median levels of F1+2 and TAT at maximum thrombin generation did not differ from the plasma levels found in the DIC patients, suggesting that tcu-PA/T would be generated in these subjects. Since tcu-PA/T accounted for about 13% of urokinase antigen in the plasma of the DIC patients and since plasma levels of urokinase antigen in the endotoxin-treated volunteers were about 4 ng/ml, detectable tcu-PA/T levels would be expected in the plasma of these subjects. However, no tcu-PA/T could be measured suggesting that the actual thrombin activity was less compared with the DIC patients, in spite of comparable thrombin generation in the endotoxin-treated subjects. This hypothesis is supported by the finding that soluble fibrin, which can also be considered as a product of thrombin activity (29), remained within the reference range (not shown). These results suggest a difference in pathogenesis between the DIC patients and the endotoxin-treated volunteers. In the endotoxin-treated volunteers, the thrombin generation is present for only a short period of time, whereas patients with DIC may have ongoing and sustained generation of thrombin for days resulting in the consumption of AT, the main inhibitor of thrombin (30). Consequently, thrombin is efficiently inhibited during short-lasting endotoxin-induced thrombin generation but is less efficiently eliminated during DIC. Plasma levels of AT were indeed substantially lower in the DIC patients in comparison with the other three groups, which in this study showed levels within the reference range. Due to this severe decrease of AT, the activity of thrombin generated will be less well controlled, favouring the generation of tcu-PA/T during DIC. This hypothesis is supported by our recent finding that less thrombin is needed to induce the inactivation of scu-PA in AT-deficient plasma (11). In the endotoxin-treated volunteers, the unstable angina pectoris patients and the hip patients, the plasma levels of AT were within the reference range, suggesting that although thrombin is generated in these persons, it is efficiently inhibited and in this way prevented from inactivating scu-PA. In the literature, soluble fibrin has been described in the plasma of unstable angina pectoris patients (31), suggesting that in these patients the thrombin activity generated may be sufficient to cleave fibrinogen but not to inactivate scu-PA. The absence of soluble fibrin in the plasma of the endotoxin-treated volunteers may be ascribed to the fact that in these subjects thrombin generation is present for only a short period, while during unstable angina pectoris thrombin generation is an ongoing process. Besides depending on thrombin, the generation of tcu-PA/T also appeared to depend on the amount of

circulating urokinase antigen, which can be explained by the fact that scu-PA is a substrate for thrombin. Both urokinase antigen and scu-PA levels were increased in the plasma of the DIC patients compared to the reference ranges, which is in agreement with other studies (10,32,33). The high plasma levels of urokinase antigen and scu-PA may be explained by both a decreased metabolism of urokinase due to organ failure and by an increased production of urokinase. In the DIC-patients mild renal and hepatic failure was observed, which may affect the clearance of urokinase. In addition, it is known that thrombin, which is abundantly generated during DIC, stimulates the production of urokinase by human renal endothelial cells (34). The contribution of scu-PA to the total amount of urokinase was lowered in comparison with the other groups, suggesting a decrease of scu-PA-dependent fibrinolysis in DIC due to the inactivation of scu-PA by thrombin. In DIC patients an imbalance between fibrin formation and fibrin degradation is thought to play a significant role in the pathogenesis of (micro)thrombosis. Impaired fibrin degradation is ascribed to an insufficient function of the fibrinolytic system, presumably due to high levels of plasminogen activator inhibitor-1 (23,35). The present mechanism may provide an additional explanation for inadequate fibrinolysis in DIC and may contribute to the pathogenesis of pathological fibrin deposition and multiple organ dysfunction (23,35).

From these results it can be concluded that the inactivation of scu-PA in the circulation occurs under severe pathophysiological conditions and depends on a balance between several components of the coagulation system. Both the generation of thrombin and the control of thrombin activity by inhibitors like AT seem to be of major importance. In addition, this study indicates that tcu-PA/T may serve as an interesting marker for the diagnosis of DIC.

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CHAPTER 5

Urokinase-mediated fibrinolysis in synovial fluid of rheumatoid arthritis patients may be affected by thrombin-mediated inactivation of single-chain urokinase-type plasminogen activator

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Summary

Excessive fibrin deposition within the inflamed joints of rheumatoid arthritis (RA) patients suggests that local fibrinolysis is inefficient, which appears to be in contrast to the observed increased levels of urokinase-type plasminogen activator (u-PA). Thrombin-mediated inactivation of single-chain u-PA (scu-PA) into an inactive form called thrombin-cleaved two-chain u-PA (tcu-PA/T) may provide a possible explanation for this contradiction. The aim of this study was to assess the occurrence of tcu-PA/T in the synovial fluid of patients with RA and with osteoarthritis (OA), and in the synovial fluid of controls in order to find support for thrombin-mediated inactivation of scu-PA in RA. Levels of scu-PA and tcu-PA/T were measured in the synovial fluid of 20 RA patients, 9 OA patients and 14 controls using sensitive bioimmunoassays. Total urokinase antigen was quantified by a urokinase ELISA. tcu-PA/T was found in the synovial fluid of all RA and OA patients. Only in 7 out of 14 control samples, levels of tcu-PA/T could be measured above the detection limit of the assay (0.2 ng/ml). The concentrations of tcu-PA/T, scu-PA and u-PA:Ag were significantly higher in the synovial fluid of the RA and OA patients as compared with the controls, while the RA patients had significantly higher levels of tcu-PA/T and u-PA:Ag than the OA patients. In RA, tcu-PA/T appeared to account for more than 40% of total urokinase antigen, while the contribution of tcu-PA/T to total urokinase antigen was only minor in OA and the controls (9.0 and 6.6%, respectively). From this study it is concluded that a significant part of the high total urokinase antigen in the synovial fluid of RA patients can be attributed to tcu-PA/T, implying that a large amount of scu-PA is not available for fibrinolysis due to its inactivation by thrombin. Thus, thrombin may promote the inflammation process in RA by inhibiting the fibrinolytic system and preventing the removal of fibrin.

Introduction

Rheumatoid arthritis (RA) is a systemic inflammatory disease characterized by cartilage and bone destruction. Intraarticular fibrin deposition is a prominent finding in the disease and may be of major importance for the perpetuation of RA due to proinflammatory effects of fibrin (1).

Intraarticular fibrin formation is induced by thrombin generated extravascularly by coagulation proteins that are either leaked from the circulation or produced by synovial macrophages (1). Several studies have demonstrated that the excessive fibrin formation is counteracted by increased levels of fibrinolytic components, predominantly that of urokinase-type plasminogen activator (u-PA) (2-5). Recently, Busso *et al.* demonstrated exacerbation of arthritis in u-PA-deficient mice, showing increased amounts of fibrin within the inflamed joints (6). Depletion of fibrinogen decreased the sustained joint inflammation in u-PA-deficient mice, and all observations were comparable to what was found in plasminogen-deficient mice. These findings strongly suggested that u-PA may play a beneficial role in arthritis by mediating the removal of fibrin via the activation of plasminogen. However, excessive deposition of fibrin within the inflamed joints of RA patients suggests that local fibrinolysis is inefficient, which appears to be in contrast to the previously observed increased levels of u-PA in synovial fluid (SF) and synovial tissue of RA patients (2-5). This contradiction may be explained by the presence of large amounts of thrombin in SF of RA patients. Thrombin is able to cleave single-chain u-PA (scu-PA) into an inactive form called thrombin-cleaved two-chain u-PA (tcu-PA/T) (7). Since thrombin is abundantly generated in SF of RA patients, we hypothesized that besides activation of scu-PA into two-chain u-PA (tcu-PA), inactivation of scu-PA by thrombin into tcu-PA/T could take place. This may provide a possible explanation for the inefficient intraarticular fibrinolysis in RA patients. To find support for thrombin-mediated inactivation of scu-PA, we investigated the occurrence of tcu-PA/T in SF of patients with RA and with osteoarthritis (OA), and in SF of controls.

Materials and Methods

Specimens of SF were obtained from 20 patients with RA and from 9 patients with advanced OA, who required joint surgery for severe disease, and post mortem from 14 individuals without any sign of joint disease, who were regarded as controls. All RA patients fulfilled the established criteria for RA and were operated at the Orthopaedic Department of the Leiden University Medical Centre, The Netherlands (8). The OA patients corresponded to grade 3-4 in the Kellgren classification system and were operated at the Department of Orthopaedics of Rijnland Hospital, Leiderdorp, The Netherlands (9). Some of the RA patients were part of a previous study (10).

Levels of tcu-PA, scu-PA and tcu-PA/T were measured using sensitive bioimmunoassays (10-12). Total urokinase antigen (u-PA:Ag) was quantitated by the urokinase ELISA as described before (13). In addition, scu-PA and tcu-PA/T were expressed as a percentage of total urokinase antigen.

Median and interquartile ranges were calculated for all values. Differences between groups were tested for significance with the Mann-Whitney test.

Results

The values of tcu-PA/T, scu-PA and u-PA:Ag measured in SF of the controls, OA patients and RA patients are represented in Figure 1. Active tcu-PA was not detectable in SF of the controls nor the OA patients, and only in very low amounts (< 0.4 ng/ml) in some of the RA patients.

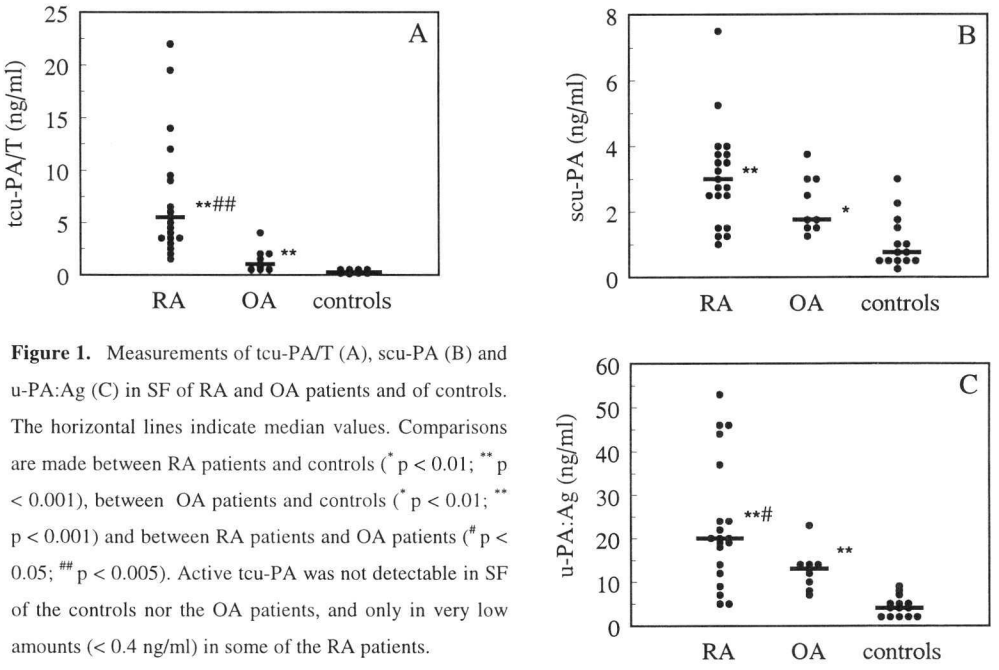

Figure 1. Measurements of tcu-PA/T (A), scu-PA (B) and u-PA:Ag (C) in SF of RA and OA patients and of controls. The horizontal lines indicate median values. Comparisons are made between RA patients and controls (* $p < 0.01$; ** $p < 0.001$), between OA patients and controls (* $p < 0.01$; ** $p < 0.001$) and between RA patients and OA patients ($^{\#}$ $p < 0.05$; $^{\#\#}$ $p < 0.005$). Active tcu-PA was not detectable in SF of the controls nor the OA patients, and only in very low amounts (< 0.4 ng/ml) in some of the RA patients.

tcu-PA/T was found in SF of all RA and OA patients (Figure 1A). However, only in 7 out of 14 control SF samples, levels of tcu-PA/T could be measured above the detection limit of the assay (0.2 ng/ml). The concentrations of tcu-PA/T, scu-PA and u-PA:Ag were significantly higher in SF of the RA patients (median values of 5.4, 2.9 and 19.9 ng/ml, respectively) and OA patients (1.1, 1.9 and 12.6 ng/ml, respectively) as compared with the controls (0.2, 0.8, and 3.8 ng/ml, respectively), while the RA patients had significantly higher levels of tcu-PA/T and u-PA:Ag than the OA patients (Figure 1).

The contribution of tcu-PA/T to total urokinase antigen was significantly higher in SF of the RA patients compared with the OA patients and the controls (Table 1). In RA, tcu-PA/T appeared to account for more than 40% of total urokinase antigen, while the contribution of tcu-PA/T to total urokinase antigen was only minor in OA and the controls (9.0 and 6.6%, respectively).

Table 1. Ratios (%) between tcu-PA/T and u-PA:Ag and between scu-PA and u-PA:Ag (median and interquartile ranges) in the synovial fluid of RA and OA patients and of controls.

parameter	RA	OA	controls
	n = 20	n = 9	n = 14
tcu-PA/T / u-PA:Ag	42.4$^{**\#\#}$	9.0	6.6
(%)	(13.6 - 51.4)	(6.3 - 17.1)	(4.6 - 8.9)
scu-PA / u-PA:Ag	14.1	18.8	22.9
(%)	(8.4 - 29.5)	(12.2 - 24.5)	(17.2 - 33.4)

Comparisons are made between RA patients and controls (** $p < 0.001$) and between RA patients and OA patients ($^{\#\#}$ $p < 0.005$). No significant differences were found between the OA patients and the controls.

Discussion

The results of this study show that a significant part of the increased total urokinase antigen in SF of RA patients can be attributed to tcu-PA/T. This implies that a large amount of scu-PA is not available for fibrinolysis due to its inactivation by thrombin. Thrombin may therefore promote the inflammation process in RA by inhibiting the fibrinolytic system and preventing the removal of fibrin.

In the OA patients, the contribution of tcu-PA/T to total urokinase antigen was far lower than in the RA patients. This discrepancy between OA and RA can be explained by a higher expression of coagulation proteins in RA and thus a higher extent of thrombin formation (1). Furthermore, elevated SF levels of soluble thrombomodulin in RA patients, which is known to accelerate the inactivation of scu-PA by thrombin, may contribute to the high levels of tcu-PA/T (14-16). Finally, it has been suggested that a significant part of antithrombin, the main inhibitor of thrombin, is inactivated in SF of RA patients (17). Altogether these data suggest that the conditions for the inactivation of scu-PA by thrombin are highly favourable in RA. Although tcu-PA/T accounts for about 42% of total urokinase antigen, a significant part of total urokinase antigen in SF of RA patients remains unexplained. The unexplained part of total urokinase antigen may be ascribed to other inactive forms of u-PA like u-PA/PAI-1 complexes or degraded u-PA (2,3), which may also contribute to impaired intraarticular fibrinolysis in RA. Brommer *et al.* have described that levels of tissue-type plasminogen activator (t-PA) were depressed in SF of RA patients compared to levels in plasma (2,3). Removal of intraarticular fibrin in RA may therefore not only be hampered by the inactivation of scu-PA by thrombin, but also by decreased t-PA-mediated plasminogen activation. In SF of OA patients, the contribution of tcu-PA/T to urokinase antigen was only minor, implying that fibrinolysis may be more efficient than in RA patients. In SF of healthy individuals tcu-PA/T is negligible, as has been observed earlier in normal plasma (10).

Inactivation of scu-PA by thrombin provides an explanation for the excessive amounts of fibrin being present in the inflamed joints of RA patients despite increased expression of u-PA in the synovial tissue and fluid of these patients. Thrombin might decrease u-PA-mediated fibrinolysis and could therefore be of major importance for sustaining inflammation in RA (6).

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CHAPTER 6

The inactivation of single-chain urokinase-type plasminogen activator by thrombin on cultured human endothelial cells

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submitted

Summary

Single-chain urokinase-type plasminogen activator (scu-PA) is cleaved by thrombin, resulting in an inactive molecule called thrombin-cleaved two-chain urokinase-type plasminogen activator (tcu-PA/T). It has been reported that binding to soluble urokinase receptor protects scu-PA from inactivation by thrombin, but there is no knowledge about cell-mediated inactivation of scu-PA. We have studied whether scu-PA bound to cultured human umbilical vein endothelial cells (HUVEC) could be inactivated by thrombin. High molecular weight scu-PA was bound to HUVEC and incubated with increasing amounts of thrombin for 30 min at 37°C. Cell-bound urokinase-type plasminogen activator (u-PA) was released and levels of scu-PA, tcu-PA/T and active two-chain u-PA were measured using sensitive bioimmunoassays. Cell-bound high molecular weight scu-PA was efficiently inactivated by thrombin. 50% inactivation of scu-PA occurred at about 0.2 nM thrombin. Only low binding of low molecular weight scu-PA to HUVEC was observed. In the presence of monoclonal anti-urokinase receptor IgG, at least 50% of the binding of scu-PA to HUVEC was inhibited. The relative amount of tcu-PA/T that was generated by thrombin was not affected by the monoclonal antibody. These results indicated that scu-PA bound to HUVEC via the urokinase receptor can be inactivated by thrombin. The efficient inactivation of cell-bound scu-PA suggests that a cofactor for thrombin may be involved, like thrombomodulin or glycosaminoglycans. It is concluded that scu-PA bound to the urokinase receptor on a cell surface can be inactivated by thrombin, which may have profound effects on u-PA-mediated local fibrinolysis and extracellular proteolysis during processes in which thrombin is also involved.

Introduction

Urokinase-type plasminogen activator (u-PA) plays an important role in extracellular proteolysis and fibrinolysis by converting plasminogen into plasmin (1). This serine protease circulates as a single-chain precursor form (scu-PA), which can be activated into two-chain u-PA (tcu-PA) after cleavage at Lys 158 – Ile 159 by plasmin or other activators (1). Cleavage of scu-PA can also result in inactivation. Thrombin cleaves scu-PA at Arg 156 - Phe 157, two residues prior to the activation site, in this way generating an inactive tcu-PA form called thrombin-cleaved tcu-PA (tcu-PA/T) (2).

The inactivation of scu-PA has been postulated as a mechanism for protecting a fresh blood clot from early fibrinolysis (3,4). This process does indeed take place *in vivo*, as we have demonstrated the presence of tcu-PA/T in human body fluids under pathological conditions which involve the production of large amounts of thrombin (5,6).

The inactivation of scu-PA by thrombin is accelerated by thrombomodulin, both in a purified system and in a plasma milieu (7-9). Thrombomodulin is a membrane protein on endothelials cells which forms a 1:1 complex with thrombin, in this way promoting both the anticoagulant and antifibrinolytic properties of thrombin (for review see (10,11)). Wilhelm *et al.* have shown that binding of scu-PA to soluble urokinase receptor protects scu-PA from inactivation by thrombin (12). However, this protective effect is abolished in the presence of soluble thrombomodulin (12). These data imply that the urokinase receptor and thrombomodulin may play a significant role in regulating the inactivation of scu-PA by thrombin. Binding of scu-PA to the urokinase receptor promotes the activation of plasminogen either by augmentation of the intrinsic activity of scu-PA or via reciprocal activation of scu-PA and plasminogen (13-16). Inactivation of receptor-bound scu-PA by thrombin or the thrombin/thrombomodulin complex thus may affect processes that are mediated by plasminogen activation via receptor-bound u-PA, such as local fibrinolysis, cell migration, tumor invasion, tissue remodelling and angiogenesis (17,18). However, there is no knowledge about thrombin-mediated inactivation of scu-PA on a cell surface. In the present study, we examined the inactivation of scu-PA bound to cultured human endothelial cells by thrombin.

Materials and Methods

Materials

Penicillin/streptomycin, L-glutamine and medium M199 were obtained from Biowitthaker (Verviers, Belgium). Heat-inactivated newborn calf serum was obtained from Gibco/BRL (Paisley, UK). Human serum was prepared from pooled fresh blood of 10-20 healthy donors obtained from a local bloodbank. A crude preparation of endothelial cell growth factor was prepared from bovine brain as described by Maciag *et al.* (19). Heparin was obtained from Leo Pharmaceutical Products (Weesp, The Netherlands). Gelatin was obtained from Merck (Darmstadt, Germany) and tissue culture plastics from Costar (Cambridge, MA, USA).

High molecular weight (HMW) single-chain urokinase-type plasminogen activator (scu-PA) from a transformed human kidney cell line was a gift from Dr. F. Hammerschmidt (Sandoz, Vienna, Austria) (20). Low molecular weight (LMW) scu-PA purified from human embryonic kidney cell cultures was a gift from Dr. J. Henkin (Abbott Laboratories, Abbott Park, Ill, USA) (7). Human thrombin (specific activity of 2.9 NIH U/μg, as indicated by the manufacturer) and bovine serum albumin (BSA) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Hirudin was obtained from Pentapharm (supplied by Kordia, Leiden, The Netherlands). Monoclonal anti-human urokinase receptor IgG (Moab H-2) was a gift from Dr. Weidle (Boehringer Mannheim, Penzburg, Germany) (18,21). An inhibitory monoclonal IgG directed against epidermal growth factor-like domain 5 of human thrombomodulin was obtained from American Diagnostica (Greenwich, CT, USA). Monoclonal anti-human mannose receptor IgG (15-2) was a gift from M. Barrett-Bergshoeff of this institute (22).

Cell culture

Human umbilical vein endothelial cells (HUVEC) were isolated, cultured and characterized as described previously (23,24). Cells were cultured in a 12-wells gelatin-coated (1%) culture plate until confluence at 5% CO_2/95% air in medium M199 supplemented with 2 mM L-glutamine, 20 mM HEPES (pH 7.3), 10% heat-inactivated human serum, 10% heat-inactivated newborn calf serum, 150 μg/ml crude endothelial cell growth factor, 5 U/ml heparin, 100 IU/ml penicillin and 100 μg/ml streptomycin at 37°C. Confluent cells were used from passage 2.

Inactivation of HMW scu-PA or LMW scu-PA by thrombin on HUVEC

Confluent HUVEC cultured in 3.8 cm^2 wells of a 12-wells culture plate were incubated for 2 h at 37°C with serum-free medium. Prior to the incubation with scu-PA, endogenous ligand bound to the urokinase receptor or other binding sites was removed by washing the cells with 50 mM glycine/HCl, pH 3.0, 100 mM NaCl for 3 min at 37°C, followed by immediate neutralization with M199 containing 1% BSA (buffer A). Cells were then incubated with 1 μg/ml HMW scu-PA or LMW scu-PA in buffer A for 30 min at 37°C in a final volume of 400 μl. Unbound scu-PA was removed and increasing amounts of thrombin (0 - 10 nM) in buffer A were added for 30 min at 37°C in a final volume of 400 μl. After incubation thrombin was removed and cells were washed once with buffer A supplemented with 1 ATU/ml hirudin to inhibit thrombin. Cell-bound u-PA was released by adding 405 μl 50 mM glycine/HCl, pH 3.0, 100 mM NaCl, 0.1% BSA for 20 min at 37°C, and supernatants were collected. The supernatants were neutralized by adding 135 μl 150 mM Tris, 0.4% BSA, 0.04% Tween 80 containing 4 ATU/ml hirudin and centrifuged. Levels of tcu-PA, scu-PA and tcu-PA/T were assessed in 25 μl supernatant using bioimmunoassays for tcu-PA, scu-PA and tcu-PA/T (see below) (5,25,26). Total u-PA antigen was determined in 20 μl supernatant using a urokinase ELISA (27). As a control, the experiment with HMW scu-PA and LMW scu-PA was also performed in blank wells without HUVEC.

Effect of monoclonal anti-urokinase receptor IgG (Moab H-2) on the inactivation of HMW scu-PA by thrombin on HUVEC

HUVEC were incubated with 1 µg/ml HMW scu-PA in the absence and presence of 2.5 and 10 µg/ml monoclonal anti-urokinase receptor IgG (Moab H-2) in buffer A for 30 min at 37°C, as described above. Unbound scu-PA and Moab H-2 were removed and bound scu-PA was inactivated by 0.1 nM thrombin in buffer A for 30 min at 37°C. Cell-bound urokinase was released and levels of tcu-PA, scu-PA and tcu-PA/T were assessed in 25 µl supernatant using the bioimmunoassays, as described above.

Effect of monoclonal anti-thrombomodulin IgG on the inactivation of HMW scu-PA by thrombin on HUVEC

HUVEC were incubated with 1 µg/ml HMW scu-PA in buffer A for 30 min at 37°C, as described above. Unbound scu-PA was removed and bound scu-PA was inactivated by 0.1 and 1 nM thrombin in buffer A in the absence and presence of 2.5 and 25 µg/ml monoclonal anti-thrombomodulin IgG for 30 min at 37°C. As a control antibody, 25 µg/ml monoclonal anti-mannose receptor IgG was used. Cell-bound urokinase was released and levels of tcu-PA, scu-PA and tcu-PA/T were assessed in 25 µl supernatant using the bioimmunoassays, as described above.

Bioimmunoassay (BIA) for tcu-PA, scu-PA and tcu-PA/T

The inactivation of scu-PA on HUVEC was measured using a BIA for tcu-PA and scu-PA, and a BIA for tcu-PA/T as described before (5,25,26). In these assays, the (potential) activity of the various u-PA forms is measured. Briefly, 25 µl supernatant in duplicate was incubated in 96-well plates coated with rabbit polyclonal anti-u-PA antibody. After washing, the immobilized material was activated in parallel wells with buffer (BIA for tcu-PA), 1 nM plasmin (BIA for scu-PA) or 20 nM cathepsin C (BIA for tcu-PA/T). Urokinase activity was determined using Glu-plasminogen and the plasmin substrate H-D-Val-Leu-Lys-p-nitroanilide. The levels of tcu-PA and scu-PA were calculated from a calibration curve obtained by serial dilutions of purified scu-PA, and the level of tcu-PA/T was calculated from a calibration curve obtained by serial dilutions of purified tcu-PA/T.

Statistics

Correlations between levels of u-PA measured in the BIA and levels of u-PA measured in the ELISA were tested using the Pearson correlation coefficient method.

Results

The total amount of cell-bound HMW u-PA was calculated as the sum of the levels of tcu-PA, scu-PA and tcu-PA/T as measured in the BIAs. The recovery of total cell-bound HMW u-PA was found to decrease with increasing amounts of thrombin, while no effect was observed on the binding of HMW u-PA to blank wells (Table 1). The same phenomenon was found when total u-PA antigen was measured by ELISA (data not shown). In addition, a positive correlation was observed between the levels of the total amount of u-PA recovered in the BIAs and the levels of total u-PA antigen recovered in the ELISA ($r = 0.903$, $p < 0.001$), indicating a simultaneous decrease of both u-PA activity and u-PA antigen (data not shown). The effect of thrombin on the recovery of cell-bound u-PA may be explained by thrombin-mediated release of u-PA bound to HUVEC, resulting in a decreased amount of cell-bound u-PA. To correct for this phenomenon, levels of tcu-PA, scu-PA and tcu-PA/T were expressed as a percentage of the total amount of u-PA bound to HUVEC per thrombin concentration (Figure 1A).

Table 1. Effect of thrombin on the recovery of cell-bound u-PA in the BIAs.

thrombin (nM)	total HMW u-PA bound to HUVEC (pg)	total HMW u-PA bound to blank wells (pg)	total LMW u-PA bound to HUVEC (pg)	total LMW u-PA bound to blank wells (pg)
0	4357 ± 791	628	241 ± 31	112
0.1	3747 ± 824	619	249 ± 35	113
1	2940 ± 623	586	251 ± 27	112
10	2391 ± 763	611	238 ± 30	110

HMW scu-PA or LMW scu-PA (1 μg/ml) were bound to HUVEC for 30 min at 37°C in four (n=4) and three experiments (n=3), respectively. As a control, HMW scu-PA (n=2) and LMW scu-PA (n=1) were also bound to blank wells. Cells were subsequently incubated with increasing amounts of thrombin for 30 min at 37°C. Cell-bound u-PA was released and levels of tcu-PA, scu-PA and tcu-PA/T in the supernatants (total volume of 540 μl) were determined using bioimmunoassays (see Materials and Methods). The total amount of bound u-PA (mean ± SD, n=4 for HMW scu-PA bound to HUVEC; mean, n=2 for HMW scu-PA bound to blank wells; mean ± SD, n=3 for LMW scu-PA bound to HUVEC; n=1 for LMW scu-PA bound to blank wells) was expressed as the sum of the amounts of tcu-PA, scu-PA and tcu-PA/T.

Both the absolute levels of scu-PA (data not shown) and the levels of scu-PA expressed as a percentage of total cell-bound u-PA were found to decrease with increasing amounts of thrombin (Figure 1A). Simultaneously, tcu-PA/T was generated which was reflected both by an increase of absolute levels of tcu-PA/T (data not shown) and an increase of tcu-PA/T expressed as a percentage of total cell-bound u-PA (Figure 1A). About 0.2 nM thrombin was needed to induce 50% inactivation of scu-PA. The levels of tcu-PA were very low and were not affected by thrombin, suggesting that scu-PA was hardly activated during the incubation.

About 10% binding of HMW u-PA to the blank wells was found as compared to binding to HUVEC in the absence of thrombin (Table 1). scu-PA bound to the blank wells was also found to be inactivated by thrombin, but to a lesser extent than scu-PA bound to HUVEC (Figure 1B). In the blank wells, 50% inactivation was not even reached at the highest concentration of thrombin used (10 nM).

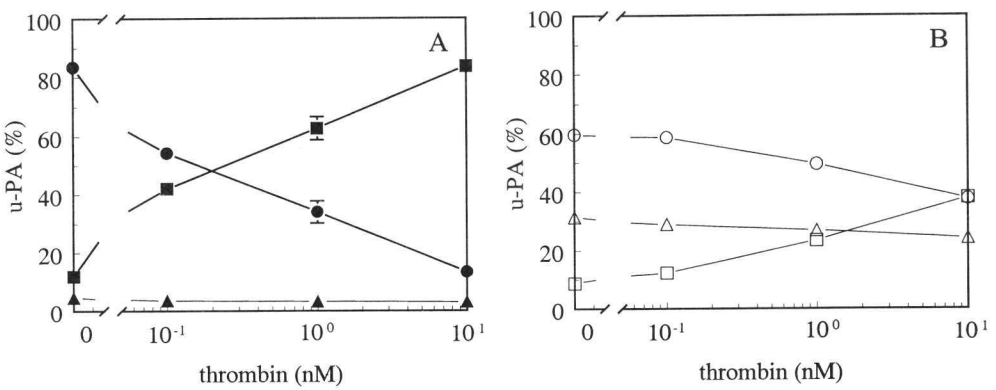

Figure 1. HMW scu-PA (1 μg/ml) was bound to HUVEC (A) in four experiments (n=4) or to blank wells (B) in two experiments (n=2) for 30 min at 37°C and subsequently incubated with increasing amounts of thrombin for 30 min at 37°C. Levels of cell-bound tcu-PA (▲), scu-PA (●) and tcu-PA/T (■) were measured in bioimmunoassays (see Materials and Methods) and were expressed as a percentage (mean ± SD, n=4) of the total amount of cell-bound u-PA per thrombin concentration (see Table 1). Levels of tcu-PA (Δ), scu-PA (○) and tcu-PA/T (□) bound to the blank wells were expressed as a percentage (mean, n=2) of the total amount of bound u-PA per thrombin concentration (see Table 1).

In the absence of thrombin, about 5% binding of LMW scu-PA to HUVEC was found as compared to HMW scu-PA (Table 1). Thrombin did not affect the recovery of bound LMW u-PA in the BIAs. The binding of LMW scu-PA to HUVEC was about twofold higher than the binding of LMW scu-PA to blank wells (Table 1).

These findings implied that in this experimental set-up about 90% of the binding of HMW scu-PA to HUVEC was specific and mediated via the N-terminal part of scu-PA. In addition, scu-PA bound to HUVEC via cellular binding sites was efficiently inactivated by thrombin.

To examine specifically whether scu-PA bound to the urokinase receptor could be inactivated by thrombin, we studied in a second experiment the effect of monoclonal anti-urokinase receptor IgG (Moab H-2) on the inactivation of HMW scu-PA by thrombin. The binding of HMW scu-PA to HUVEC was inhibited by Moab H-2 in a concentration-dependent manner (Figure 2A). At 10 µg/ml monoclonal antibody, 50% inhibition of scu-PA binding was found. This finding indicated that in this experimental set-up at least 50% of HMW scu-PA is bound to HUVEC via the urokinase receptor. Since cell-bound HMW scu-PA could be completely inactivated by 10 nM thrombin (Figure 1A), the urokinase receptor must be at least partially involved in this process. The involvement of the urokinase receptor in the inactivation of cell-bound scu-PA by thrombin was confirmed by the finding that the relative amount of generated tcu-PA/T was not affected by Moab H-2 (Figure 2B). If only scu-PA bound to HUVEC via other binding sites than the urokinase receptor had been inactivated by thrombin, then an increase in the relative amount of generated tcu-PA/T could have been expected in the presence of Moab H-2.

Monoclonal anti-thrombomodulin IgG and the control antibody monoclonal anti-mannose receptor IgG did not affect the total amount of HMW u-PA that was recovered in the BIA and had no significant effect on the inactivation of scu-PA by thrombin at the concentrations used (data not shown).

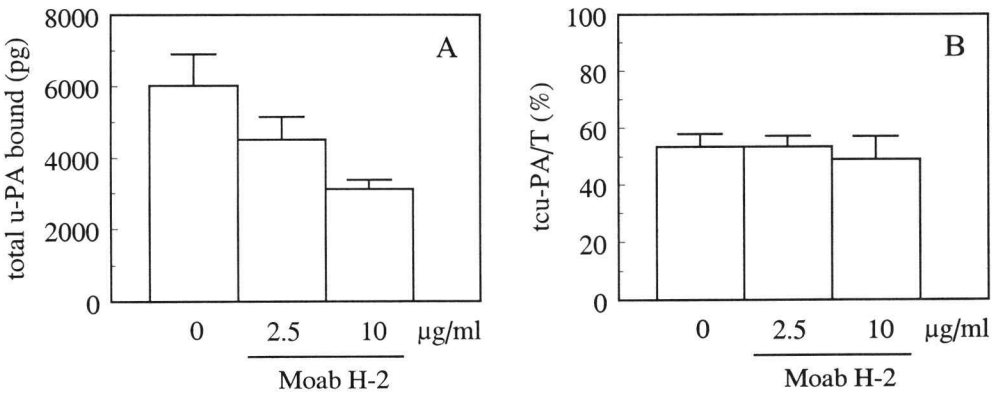

Figure 2. Effect of monoclonal anti-urokinase receptor IgG (Moab H-2) on the binding of HMW scu-PA to HUVEC and on the inactivation of scu-PA by thrombin on HUVEC. HMW scu-PA (1 µg/ml) was bound to HUVEC in the absence (0 µg/ml) and presence of 2.5 µg/ml or 10 µg/ml Moab H-2 for 30 min at 37°C in three experiments (n=3). Subsequently, cell-bound scu-PA was incubated with 0.1 nM thrombin for 30 min at 37°C. Levels of cell-bound tcu-PA, scu-PA and tcu-PA/T were measured in bioimmunoassays (see Materials and Methods). The total amount of bound u-PA (mean ± SD, n=3) was expressed as the sum of the amounts of tcu-PA, scu-PA and tcu-PA/T (A) and generated tcu-PA/T was expressed as a percentage of the total amount of bound u-PA (B).

Discussion

In this study, thrombin-mediated inactivation of scu-PA bound to HUVEC was examined. The results demonstrated that binding of scu-PA to HUVEC does not protect scu-PA from inhibition by thrombin. Cell-bound scu-PA was almost completely inactivated into tcu-PA/T by 10 nM thrombin. In this experimental set-up, at least 50% of the binding of scu-PA to HUVEC appeared to be mediated by the urokinase receptor. The involvement of other binding sites besides the urokinase receptor, like protease nexins and glycosaminoglycans cannot be excluded (28-31). From our results it is concluded that binding of scu-PA to HUVEC via the urokinase receptor offers no protection against inactivation, as has been claimed for the binding of scu-PA to soluble urokinase receptor by Wilhelm *et al.* (12). An explanation may be that due to the binding of thrombin to an endothelial cofactor, the protective effect of the urokinase receptor is abolished. In the study of Wilhelm *et al.*,

thrombin bound to its cofactor thrombomodulin was capable of inactivating urokinase receptor-bound scu-PA (12).

Cell-bound scu-PA was very efficiently inactivated by thrombin. About 0.2 nM thrombin was needed to induce 50% inactivation of scu-PA, which is far less than observed in buffer or in a plasma milieu (7,9). In the absence of thrombomodulin, 50% inactivation of scu-PA in buffer is induced by 5-15 nM thrombin, while more than 300 nM is needed in a plasma milieu because of the presence of thrombin inhibitors (7,9). In the presence of thrombomodulin, about 0.2-1 nM thrombin is needed to induce 50% inactivation in buffer, and about 80-90 nM is needed in a plasma milieu (7,9). These data suggest that the inactivation of cell-bound scu-PA by thrombin may be potentiated by a cofactor. Thrombomodulin is an endothelial cofactor for thrombin and potentiates the activation of protein C and thrombin activatable fibrinolysis inhibitor by thrombin at the endothelial cell surface (11,32-34). In this study, the inactivation of cell-bound scu-PA by thrombin was not affected by 25 µg/ml (170 nM) monoclonal anti-thrombomodulin IgG, suggesting that the role of thrombomodulin is of minor importance. However, the amount of monoclonal antibody used in this study may not have been sufficient to prevent the binding of thrombin to thrombomodulin. Molinari *et al.* have demonstrated that endogenous thrombomodulin enhances the inactivation of scu-PA by thrombin in a perfused rabbit heart model (8). In addition to thrombomodulin, other components on the endothelial surface may serve as a cofactor for thrombin. It is known that heparin and related glycosaminoglycans like heparin sulphate and chondroitin sulphate potentiate the inactivation of scu-PA by thrombin (8,35). Heparin-like molecules are present on endothelial cells and are able to bind both thrombin and u-PA (30,31,36-38). Further research is needed to examine the role of thrombomodulin or other cofactors like glycosaminoglycans in the inactivation of cell-bound scu-PA by thrombin.

A strong and rapid decreasing effect of thrombin on the recovery of cell-bound u-PA was observed. This observation may be explained by the release of the urokinase receptor from HUVEC by thrombin (39). Cleavage of the ligand-binding domains of the urokinase receptor by tcu-PA as described by Hoyer-Hansen *et al.* (40) is not likely, since no activation of scu-PA into active tcu-PA was observed.

The inactivation of cell-bound scu-PA seems not to be restricted to a specific cell type. In a preliminary experiment, we found that scu-PA bound to cultured human smooth muscle cells is also

inactivated by thrombin (unpublished observations). Recently, it was demonstrated that scu-PA associated to human platelets via an unidentified urokinase binding protein is inactivated by thrombin into tcu-PA/T. This reaction is followed by re-activation of tcu-PA/T due to thrombin-mediated release of a lysosomal enzyme by platelets (41). This phenomenon may be of importance for the regulation of fibrinolysis. Besides in fibrinolysis, thrombin-mediated inactivation of cell-bound scu-PA may have profound effects on processes in which both u-PA and thrombin are involved, like angiogenesis, atherosclerosis and restenosis.

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CHAPTER 7

Identification of the epidermal growth factor-like domains of thrombomodulin essential for the acceleration of the inactivation of single-chain urokinase-type plasminogen activator by thrombin

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Summary

Single-chain urokinase-type plasminogen activator (scu-PA) can be cleaved by thrombin into a virtually inactive form called thrombin-cleaved two-chain urokinase-type plasminogen activator (tcu-PA/T), a process accelerated by thrombomodulin. This cofactor of thrombin contains six epidermal growth factor (EGF)-like domains, which are involved in thrombin binding and cofactor activity. In this study, we identified the EGF-like domains of thrombomodulin required for the acceleration of the inactivation of scu-PA by thrombin using various forms of thrombomodulin (TM). scu-PA was treated with thrombin in the absence and presence of full length rabbit TM (containing EGF1-6), recombinant TM comprising all of the extracellular domains including EGF1-6 (TM_{LEO}) and recombinant TM comprising EGF4-6 plus the interconnecting region between EGF3 and EGF4 ($TM_{E}i4$-6) for 45 min at 37°C. Generated tcu-PA/T was re-activated by cathepsin C and activity was measured using the chromogenic substrate S-2444. Rabbit TM accelerated the inactivation of scu-PA about 35-fold, while both recombinant forms accelerated it about 3-fold. Subsequently, $TM_{E}5$-6 was prepared by cyanogen bromide digestion of $TM_{E}i4$-6. After digestion $TM_{E}i4$-6 bound thrombin but did not accelerate the activation of protein C. In contrast, the inactivation of scu-PA by thrombin was accelerated to the same extent as that induced by TM_{LEO} and $TM_{E}i4$-6. In addition, $TM_{E}5$-6 isolated from the digested material by SDS-PAGE accelerated the inactivation of scu-PA, but did not accelerate the activation of protein C. This study demonstrates that only EGF-like domains 5 and 6 are essential for the acceleration of the inactivation of scu-PA by thrombin, which differs from the domains that are critical for activation of protein C (EGF-like domains i4-6) and thrombin activatable fibrinolysis inhibitor (EGF-like domains 3-6).

Introduction

Single-chain urokinase-type plasminogen activator (scu-PA) is the precursor form of two-chain urokinase-type plasminogen activator (tcu-PA), which plays a role in fibrinolysis and extracellular proteolysis by converting plasminogen into plasmin (1). Activation of scu-PA into tcu-PA occurs

after cleavage at Lys 158 - Ile 159 by plasmin or other activators. Cleavage of scu-PA at another site can result in inactivation. Thrombin cleaves scu-PA at Arg 156 - Phe 157, two residues prior to the activation site, in this way generating an inactivated tcu-PA form called thrombin-cleaved tcu-PA (tcu-PA/T) (2). *In vitro*, tcu-PA/T appears to have little amidolytic and fibrinolytic activity (2,3). However, in a rabbit jugular vein thrombosis model tcu-PA/T was found to be a potent and fibrin-specific thrombolytic agent (4,5), which may be explained by re-activation of tcu-PA/T by dipeptidyl peptidase I (cathepsin C) and by platelets (6,7), or by the promoting effect of fibrin fragment E-2 on plasminogen activation by tcu-PA/T (8). The inactivation of scu-PA has been postulated as a mechanism for protecting a fresh blood clot from early fibrinolysis (3,9). The inactivation does indeed take place *in vivo*, as we have demonstrated the presence of tcu-PA/T in human body fluids under pathological conditions, which involve the production of large amounts of thrombin (10).

Thrombomodulin accelerates the inactivation of scu-PA by thrombin, both in a purified system and in a plasma milieu (11-13). Binding of scu-PA to the urokinase receptor protects scu-PA from inactivation by thrombin (14). However, this protective effect is abolished in the presence of thrombomodulin. These findings suggest that thrombomodulin plays an important role in the inactivation of scu-PA by thrombin. Thrombomodulin is an endothelial cell membrane protein, which can form a 1:1 complex with thrombin (15). It promotes both the anticoagulant and antifibrinolytic properties of thrombin by accelerating the activation of protein C and the activation of a thrombin activatable fibrinolysis inhibitor (TAFI), respectively (16-19). Thrombomodulin contains six epidermal growth factor (EGF)-like domains. EGF-like domains are involved in the binding of thrombin and the cofactor activity (17). It has been demonstrated that the EGF-like domains of thrombomodulin essential for the activation of protein C and TAFI are distinct. EGF-like domains 4-6 plus the connecting region between EGF-like domain 3 and EGF-like domain 4 are critical for the activation of protein C (20-22), while for the activation of TAFI EGF-like domains 3-6 are needed (23-25). Thrombomodulin containing all six EGF-like domains can accelerate the inactivation of scu-PA by thrombin (11,13), but the structural elements required for this acceleration have not been identified precisely. De Munk *et al.* have demonstrated that the presence of chondroitin sulfate on thrombomodulin is of major importance for the acceleration of the

inactivation of scu-PA by thrombin (26). However, chondroitin sulfate itself was hardly capable to accelerate the inactivation of scu-PA by thrombin (26), suggesting that chondroitin sulfate should be linked to the thrombomodulin polypeptide chain and that other domains of thrombomodulin may be involved. In the present study, we have localized the EGF-like domains that are essential for the acceleration of the inactivation of scu-PA by thrombin using various forms of thrombomodulin, demonstrating that the critical domains differ from the domains required for activation of protein C and TAFI.

Materials and Methods

Materials

Single-chain urokinase-type plasminogen activator (scu-PA, molecular mass 54 kD) from a transformed human kidney cell line was kindly provided by Dr. F. Hammerschmidt (Sandoz, Vienna, Austria). Human thrombin (specific activity of 2.9 NIH U/μg, as indicated by the manufacturer, molecular mass 37 kD), bovine spleen cathepsin C, L-cysteine, cystatin and bovine serum albumin were obtained from Sigma Chemical Co. (St. Louis, MO, USA). In Figure 1, a schematic representation of the various forms of thrombomodulin used in this study is shown. Rabbit lung thrombomodulin (full-length thrombomodulin containing the extracellular domains which consist of the lectin domain, EGF1-6, and the O-link domain with chondroitin sulfate, a transmembrane domain and a cytoplasmatic tail, residues 1-557, molecular mass 75 kD, specific activity of 1 U/μg, as indicated by the manufacturer) was obtained from American Diagnostica (Greenwich, CT, USA). Two recombinant forms of thrombomodulin, TM_{LEO} (the extracellular domains of human thrombomodulin which consist of the lectin domain, EGF1-6, and the O-link domain, residues 4-497 without chondroitin sulfate, molecular mass 52 kD) and $TM_{E}i4\text{-}6$ (EGF4-6 plus the connecting region between EGF3 and 4, residues 345-462, molecular mass 13 kD) were produced in CHO cells and purified as described previously (27,28). There was no chondroitin sulfate present on TM_{LEO} since the plasmid used for transfection of the CHO cells encoded a mutation (S474A) that prevents addition of chondroitin sulfate (27). Purity of the preparations was verified by SDS-PAGE analysis in which both proteins migrated as a single band with their predicted molecular weight. Monoclonal antibodies directed against EGF5 (Moab 531) and against EGF2 (Moab 43B) of human thrombomodulin were prepared as described previously (22). Hirudin was obtained from Pentapharm (supplied by Kordia, Leiden, The Netherlands). Human protein C (molecular mass 62 kD) was obtained from Enzyme Research Laboratories (supplied by Kordia). The chromogenic substrates pyroGlu-Gly-Arg-p-nitroanilide (S-2444), H-D-Phe-Pip-Arg-p-nitroanilide (S-2238) and pyroGlu-Pro-Arg-p-nitroanilide (S-2366) were obtained from Chromogenix AB (Mölndal, Sweden). Cyanogen bromide was obtained from Fluka Chemika (Buchs, Switzerland).

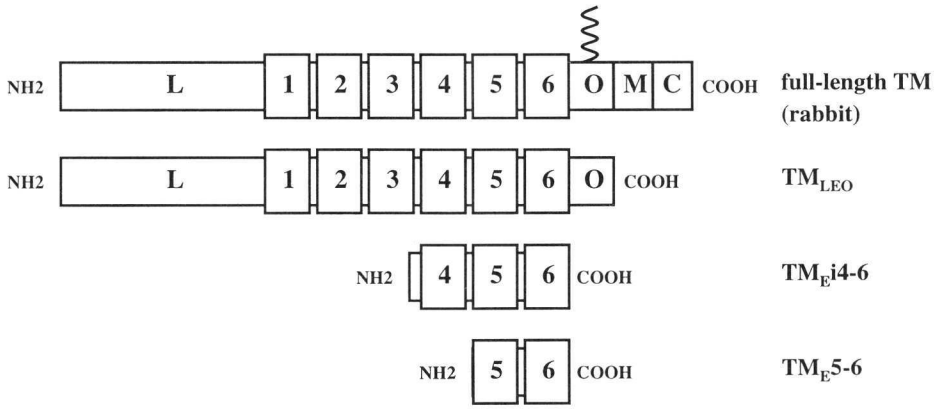

Figure 1. Schematic representation of the types of TM used in this study. Full-length TM consists of all of the domains in wild type TM including the lectin domain (L), six EGF-like domains (1-6), an O-link domain (O), a transmembrane domain (M) and a cytoplasmatic tail (C). In these studies rabbit TM was used as a representative full-length TM, and it is modified with chondroitin sulfate in the O-link domain (coiled line). Recombinant TM_{LEO} contains all of the extracellular domains without the chondroitin sulfate modification. Recombinant TM_Ei4-6 contains EGF-like domains 4-6 plus the connecting region between EGF-like domain 3 and 4. TM_E5-6, prepared from TM_Ei4-6 by CNBr digestion, contains EGF-like domains 5-6.

Determination of the effects of thrombomodulin on the inactivation of scu-PA by thrombin

scu-PA (final concentration of 15 nM) was incubated in 50 µl final volume for 45 min at 37°C with increasing amounts of thrombin (0-10 nM for rabbit TM and 0-100 nM for recombinant TM) in the absence and presence of 15 nM rabbit TM or 150 nM recombinant TM in 50 mM Tris/HCl, pH 7.5, 100 mM NaCl, 3 mM $CaCl_2$, 0.1% (w/v) BSA, 0.01% (v/v) Tween 80. As a control for complete inactivation of scu-PA into tcu-PA/T by thrombin, scu-PA was treated with 17 NIH U/ml (160 nM) thrombin for 45 min at 37°C (10). After the incubation period of 45 min, 1 µl hirudin was added in a twofold excess to thrombin to inhibit thrombin activity. Subsequently, generated tcu-PA/T was re-activated during 90 min at 37°C by the addition of 100 µl cathepsin C (final concentration of 20 nM) in 50 mM sodium phosphate buffer, pH 6.0, 100 mM NaCl, 2 mM EDTA, 10 mM L-cysteine, 0.1% BSA, 0.01% Tween 80. After the incubation, cathepsin C was inhibited by the addition of 20 µl cystatin (final concentration of 20 nM). Amidolytic activity of re-activated tcu-PA/T was measured by adding 75 µl aliquot in duplicate to 175 µl S-2444 (final concentration of 0.3 mM) in 50 mM

Tris/HCl, pH 8.8, 38 mM NaCl, 0.01% Tween 80. Measurements were performed at timed intervals at 405 nm with a Titertek Multiskan spectrophotometer and activity was calculated as ΔA405/h. The activity of tcu-PA/T was expressed as a percentage of the activity of the control for complete inactivation of scu-PA (see above). In order to quantify acceleration by the various TM forms, the concentration of thrombin needed to generate 50% tcu-PA/T was determined by fitting the data to a sigmoidal curve.

To examine the effects of Moab 531 (anti-EGF5) and Moab 431B (anti-EGF2) on the activity of TM_{LEO} and TM_Ei4-6, scu-PA (final concentration of 15 nM) was inactivated by 10 nM thrombin and 30 nM TM_{LEO} or TM_Ei4-6 in the absence and presence of 30 μg/ml Moab 531 or Moab 431B for 45 min at 37°C. The generation of tcu-PA/T was determined as described above.

Preparation of TM_E5-6 by cyanogen bromide digestion of TM_Ei4-6

TM_E5-6 was prepared by cyanogen bromide (CNBr) digestion of TM_Ei4-6 (29). A volume of 150 μl TM_Ei4-6 (0.7 mg/ml in phosphate-buffered saline) was digested with 350 μl cyanogen bromide (20 mg/ml in 100% formic acid) for 24 h at room temperature (RT). After lyophilization, the digest was dissolved in 150 μl distilled water. The CNBr-digest was analysed on a 15% SDS-polyacrylamide gel under reducing conditions according to the method of Laemmli (30). Proteins were visualized using silver staining (31).

Thrombin binding assay

To examine whether TM_Ei4-6 after CNBr-digestion was still able to bind thrombin, a competitive solid-phase assay was performed essentially as described by Jandrot-Perrus *et al.* (32). Briefly, wells of a 96-wells polystyrene plate were coated overnight at 4°C with 100 μl TM_{LEO} (0.25 μg/ml) in 50 mM carbonate buffer, pH 9.5. The remaining binding sites were blocked by incubation with 1% BSA in 50 mM HEPES, pH 7.5, 100 mM NaCl for 2 h at RT. Subsequently, the wells were incubated with 150 μl thrombin (final concentration of 15 nM) in the presence of increasing amounts of TM_Ei4-6 before and after CNBr-digestion (0-400 nM) in 10 mM Tris/HCl, pH 7.5, 100 mM NaCl, 2.5 mM $CaCl_2$, 0.05% Tween 20 for 1 h at RT. After washing the wells with 50 mM HEPES, pH 7.5, 100 mM NaCl, 0.05% Tween 20, thrombin activity was assayed using 150 μl 0.1 mM S-2238 in 50 mM Tris/HCl, pH 8.0, 200 mM NaCl, 0.1% PEG 6000 at 25°C. Measurements were performed at timed intervals at 405 nm with a Titertek Multiskan spectrophotometer and activity was calculated as ΔA405/h. The concentrations of TM_Ei4-6 before and after CNBr-digestion needed to induce 50% inhibition of thrombin binding were determined.

Protein C activation assay

The functional activity of TM_Ei4-6 after CNBr-digestion was tested in a two-stage protein C activation assay, essentially as described by Tsiang *et al.* (33). In the first stage, 0.5 μM protein C (final concentration) was incubated with 1 nM thrombin in the presence of increasing amounts of TM_Ei4-6 before and after CNBr-digestion (0-400 nM) in 50 mM

HEPES, pH 7.4, 150 mM NaCl, 5 mM $CaCl_2$, 0.01% Tween 80 for 30 min at 37°C, in a final volume of 30 µl. After the incubation period of 30 min, 20 µl hirudin (final concentration of 10 ATU/ml) was added to inhibit thrombin. In the second stage, 100 µl S-2366 (final concentration of 0.2 mM) was added to determine activated protein C (APC) activity at 37°C. Measurements were performed at timed intervals at 405 nm with a Titertek Multiskan spectrophotometer and activity was calculated as ΔA405/h.

Effect of TM_E5-6 on the inactivation of scu-PA by thrombin

Inactivation of scu-PA by TM_Ei4-6 after CNBr-digestion

scu-PA (final concentration of 15 nM) was incubated with increasing amounts of thrombin in the presence of 150 nM TM_Ei4-6 after CNBr-digestion for 45 min at 37°C. The generation of tcu-PA/T was determined as described above. In order to quantify the acceleration, the concentration of thrombin needed to generate 50% tcu-PA/T was determined as described above for TM_{LEO} and TM_Ei4-6.

Isolation of TM_E5-6 from TM_Ei4-6 after CNBr-digestion by extraction of SDS-PAGE slices

Following CNBr-digestion, 10.5 µg TM_Ei4-6 was run on a 15% SDS-polyacrylamide gel under non-reducing conditions. Slices of 3 mm (16 slices in total) were cut into small pieces using a razor and proteins were eluted in 60 µl 50 mM Tris/HCl, pH 7.5, 100 mM NaCl, 3 mM $CaCl_2$, 0.1% BSA, 0.01% Tween 80 overnight at 4°C. In a parallel lane, proteins from the digested material were visualized by silver staining. The eluates of the slices were tested for acceleration of the inactivation of scu-PA by thrombin (2 µl eluate) and for acceleration of the activation of protein C by thrombin (1 µl eluate), as described above.

Results

Effect of various forms of thrombomodulin on the inactivation of scu-PA by thrombin

All tested forms of thrombomodulin accelerated the inactivation of scu-PA by thrombin (Figure 2). Acceleration by rabbit thrombomodulin was about 35-fold, meaning that about 35-fold less thrombin was needed to induce 50% inactivation. This increment is an order of magnitude larger than the acceleration by recombinant TM_{LEO} and TM_Ei4-6, which both showed an accelerating effect of about 3-fold (Table 1).

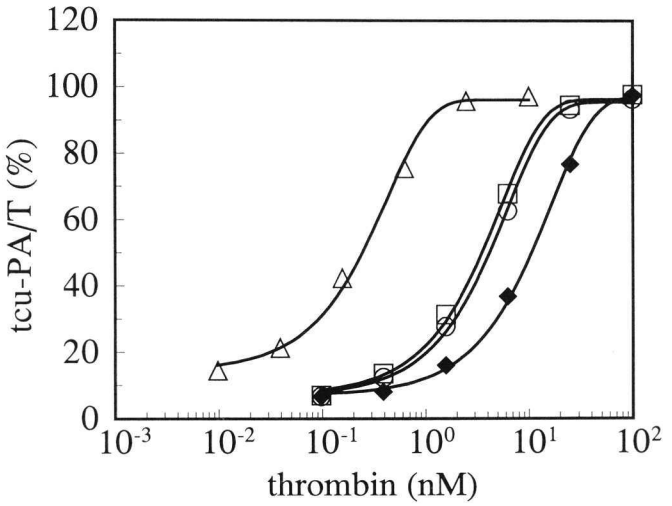

Figure 2. Effect of various forms of TM on the inactivation of scu-PA by thrombin. scu-PA (15 nM) was incubated with increasing amounts of thrombin in the absence (♦) and presence of 15 nM rabbit TM (Δ), 150 nM recombinant TM_{LEO} (□) or 150 nM $TM_{E}i4\text{-}6$ (○) for 45 min at 37°C. After the incubation period of 45 min, the generated amount of tcu-PA/T was measured. As a control for complete inactivation, scu-PA was incubated with 17 NIH U/ml (160 nM) thrombin for 45 min at 37°C (10). tcu-PA/T was expressed as a percentage of this control. This figure represents the mean data of three to four experiments. The acceleration induced by the various TM forms was quantified by determination of the concentration of thrombin needed to reach 50% inactivation of scu-PA (Table 1).

The acceleration induced by TM_{LEO} and by $TM_{E}i4\text{-}6$ could be completely blocked by the antibody directed against EGF-like domain 5 (Figure 3). In contrast, the antibody directed against EGF-like domain 2 had no effect on the acceleration. These results suggested that EGF-like domains 4-6 plus the connecting region between EGF-like domain 3 and 4 contain the structural elements of recombinant full length TM required for the acceleration of the inactivation of scu-PA by thrombin and that EGF-like domain 5, the binding domain for thrombin, is essential.

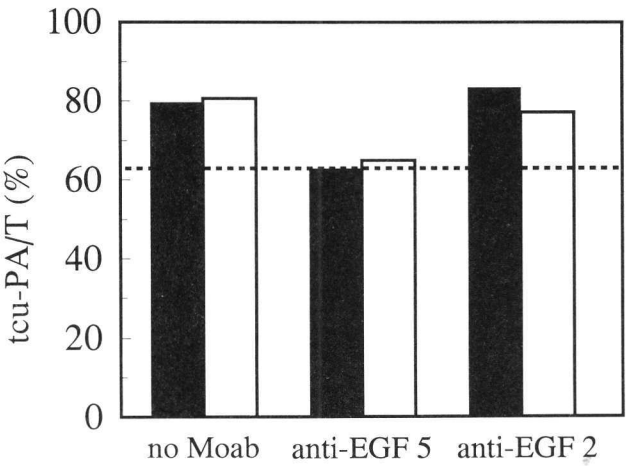

Figure 3. Effect of an antibody directed against EGF-like domain 5 and an antibody directed against EGF-like domain 2 on the accelerating effect of TM_{LEO} and $TM_{E}i4\text{-}6$ on the inactivation of scu-PA by thrombin. scu-PA was inactivated by 10 nM thrombin in the presence of 30 nM TM_{LEO} (solid bars) or 30 nM $TM_{E}i4\text{-}6$ (open bars) with and without 30 μg/ml Moab 531 (anti-EGF5) or Moab 431B (anti-EGF2) for 45 min at 37°C. After the incubation period of 45 min, the generated amount of tcu-PA/T was measured. As a control for complete inactivation, scu-PA was incubated with 17 NIH U/ml (160 nM) thrombin for 45 min at 37°C. tcu-PA/T was expressed as a percentage of this control. The dotted line represents the percentage of tcu-PA/T generated by 10 nM thrombin in the absence of TM.

Effect of $TMi_{E}4\text{-}6$ after CNBr-digestion on the inactivation of scu-PA by thrombin

To identify the essential EGF-like domains of thrombomodulin more precisely, we tested whether EGF-like domain 4 was critical for the acceleration of the inactivation of scu-PA by thrombin as it is for activation of protein C and TAFI. EGF-like domains 5-6 ($TM_{E}5\text{-}6$) were prepared by CNBr-digestion of $TM_{E}i4\text{-}6$ (29). Since there is only one methionine residue present in $TM_{E}i4\text{-}6$ at position 388, cleavage by CNBr will lead to two fragments consisting of $TM_{E}5\text{-}6$ and EGF-like domain 4 plus the interconnecting region between EGF-like domains 3 and 4. In Figure 4 the CNBr-digestion pattern of $TM_{E}i4\text{-}6$ is shown. Before CNBr-digestion, $TM_{E}i4\text{-}6$ migrates as one single band. After CNBr-digestion, the major part of the digest migrates as a band of a lower molecular

mass, showing that most of TM_Ei4-6 is digested into TM_E5-6. A minor part of TM_Ei4-6 is still present after CNBr-digestion, suggesting that the CNBr-digestion was not complete. EGF-like domain 4 could not be detected after CNBr-digestion due to its very low molecular mass, probably because it is washed out of the gel during the staining procedure.

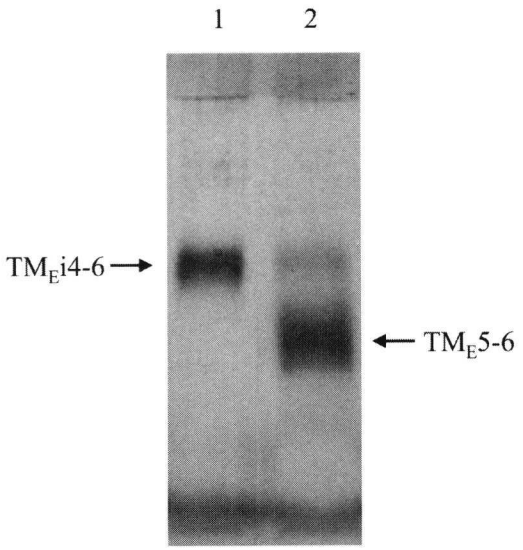

Figure 4. CNBr-digestion pattern of TM_Ei4-6. TM_Ei4-6 before and after CNBr-digestion was run on a 15% SDS-polyacrylamide gel under reducing conditions. Lane 1 shows TM_Ei4-6 before CNBr-digestion and lane 2 TM_Ei4-6 after CNBr-digestion. The arrows indicate the positions of TM_Ei4-6 and TM_E5-6. EGF-like domain 4 could not be visualized due to its very low molecular mass.

About 50 nM of TM_Ei4-6 before CNBr-digestion was needed to reach 50% inhibition of thrombin binding in the competitive thrombin binding assay, while after CNBr-digestion about 180 nM was needed (data not shown). This result demonstrated that TM_Ei4-6 after CNBr-digestion was still capable of complexing to thrombin.

After CNBr-digestion, TM_Ei4-6 did not accelerate the activation of protein C (Figure 5), while TM_Ei4-6 showed strong acceleration of the activation of protein C, in agreement with the literature (20-22).

Altogether, these data indicated that TM_Ei4-6 had been digested into TM_E5-6 and EGF-like domain 4, resulting in the loss of cofactor activity for protein C activation while retaining the ability to bind thrombin.

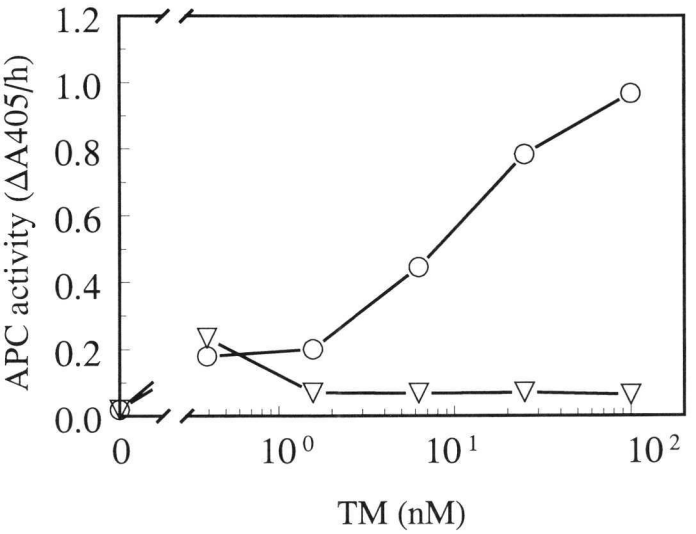

Figure 5. Effect of TM_Ei4-6 before and after CNBr-digestion on the activation of protein C by thrombin. Protein C (0.5 μM) was activated by 1 nM thrombin in the presence of increasing amounts of TM_Ei4-6 before (○) and after (▽) CNBr-digestion for 30 min at 37°C. Activated protein C (APC) activity was measured using S-2366.

We then tested whether the complex of thrombin and the fragments of TM_Ei4-6 generated by CNBr-digestion could accelerate the inactivation of scu-PA by thrombin. scu-PA was inactivated by increasing amounts of thrombin in the presence of a fixed amount of TM_Ei4-6 after CNBr-digestion, in a similar experiment to that performed for the other recombinant forms of TM shown in Figure 2. Inactivation of scu-PA in the presence of TM_Ei4-6 after CNBr-digestion was similar to its inactivation in the presence of TM_{LEO} and TM_Ei4-6 before CNBr-digestion. No significant differences in either the thrombin concentration needed to induce 50% inactivation of scu-PA or the acceleration factor were found between TM_{LEO} and TM_Ei4-6 before and after CNBr-digestion (Table 1). These results strongly suggested that EGF-like domain 4 is not essential for the acceleration of the inactivation of scu-PA by thrombin, while TM_E5-6 is essential.

Table 1 Acceleration of the inactivation of scu-PA by thrombin by various forms of TM.

TM	50% inactivation (nM)	acceleration factor
-	12.3 ± 3.6	
rabbit TM	0.3 ± 0.1**	35.5 ± 6.2
TM_{LEO}	3.7 ± 0.6**	3.3 ± 0.5 ^{NS}
$TM_{E}i4\text{-}6$	4.4 ± 0.5**	2.8 ± 0.6 ^{NS}
$TM_{E}i4\text{-}6$ after CNBr-digestion	5.5 ± 1.3*	2.2 ± 0.4 ^{NS}

scu-PA was inactivated for 45 min at 37°C by increasing amounts of thrombin in the absence and presence of rabbit TM or recombinant TM_{LEO}, $TM_{E}i4\text{-}6$ or $TM_{E}i4\text{-}6$ after CNBr-digestion (see Materials and Methods). After the incubation period of 45 min, the generated amount of tcu-PA/T was measured. The concentrations of thrombin needed to induce 50% inactivation were determined (mean ± SD, n=3-4). Comparisons are made between thrombin in the absence of TM and thrombin in the presence of TM using Student's t test (** $p < 0.01$, * $p < 0.05$). The acceleration factors of the various forms of TM were calculated by dividing the 50%-inactivation value of thrombin in the absence of TM by the 50%-inactivation value of thrombin in the presence of TM. Differences between the acceleration factors of the recombinant TM forms were tested using Student's t test (^{NS} = not significant).

Effect of EGF-like domains 5-6 on the inactivation of scu-PA by thrombin

To confirm the hypothesis that $TM_{E}5\text{-}6$ is essential for the acceleration of the inactivation of scu-PA by thrombin, $TM_{E}5\text{-}6$ was isolated from $TM_{E}i4\text{-}6$ after CNBr-digestion by SDS-PAGE, slicing of the gel and extraction of slices. The material eluted from individual slices was then tested for acceleration of the activation of protein C and the inactivation of scu-PA by thrombin. It was found that material eluted from slices 10-11 accelerated the inactivation of scu-PA, but had no effect on the activation of protein C (Figure 6). When a parallel lane of $TM_{E}i4\text{-}6$ digested with CNBr was silver stained, two bands were visualized. One band found in slices 7-8 comigrated with undigested $TM_{E}i4\text{-}6$, the other band corresponds to slices 10-11 and consists of $TM_{E}5\text{-}6$. Thus the fact that material from slices 10 and 11, which is $TM_{E}5\text{-}6$, can accelerate the inactivation of scu-PA by thrombin confirms the hypothesis that EGF-like domains 5 and 6 are sufficient.

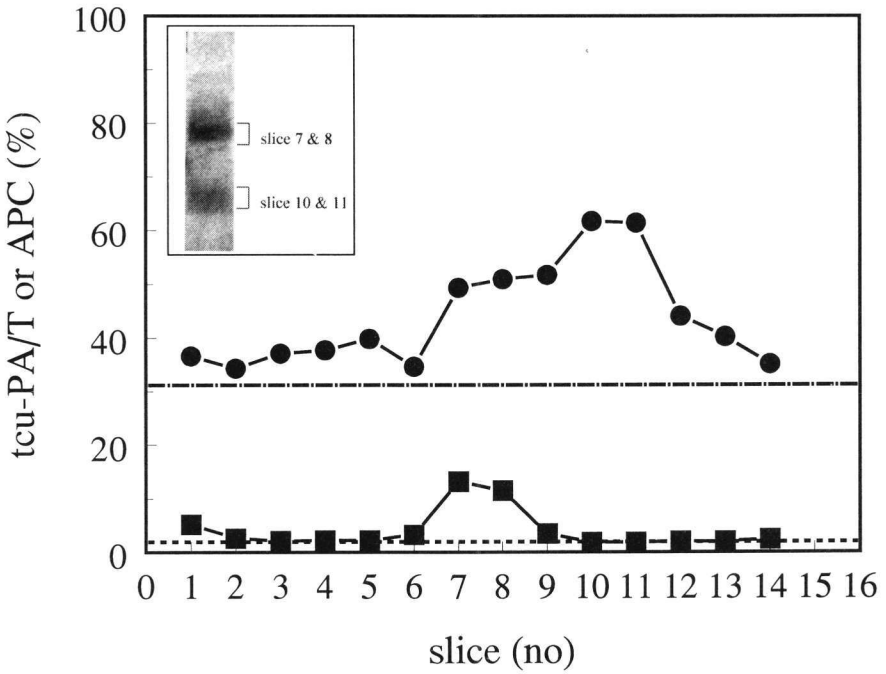

Figure 6. Effect of TM_E5-6, eluted after SDS-PAGE, on the inactivation of scu-PA by thrombin and on the activation of protein C by thrombin. TM_Ei4-6 after CNBr-digestion was run on a 15% SDS-polyacrylamide gel under non-reducing conditions. The gel was cut into 16 slices of 3 mm and proteins were eluted overnight in 60 µl buffer at 4°C. The inactivation of scu-PA by thrombin was assessed by incubating scu-PA with 25 nM thrombin in the presence of 2 µl of eluate of consecutive slices for 10 min at 37°C, and was measured as the generated amount of tcu-PA/T (●). As a control for complete inactivation, scu-PA was incubated with 17 NIH U/ml (160 nM) thrombin during 45 min at 37°C. tcu-PA/T was expressed as a percentage of this control. The dashed line (— · —) represents the generation of tcu-PA/T by 25 nM thrombin in the absence of eluate. The activation of protein C by thrombin was assessed by incubating protein C with 1 nM thrombin in the presence of 1 µl of eluate of consecutive slices for 30 min at 37°C, and activated protein C (APC) activity (■) was measured using S-2366. As a control for complete activation, protein C was incubated with 1 nM thrombin in the presence of 100 nM TM_Ei4-6 before CNBr-digestion during 30 min at 37°C. APC was expressed as a percentage of this control. The dotted line (- - -) represents the generation of APC by 1 nM thrombin in the absence of eluate.

The *insert* shows a parallel lane of TM_Ei4-6 after CNBr-digestion stained with silver staining. Intact TM_Ei4-6 and putative complexes between EGF-like domain 4 and TM_E5-6 were eluted from slices 7 and 8, while TM_E5-6 was eluted from slices 10 and 11.

It is interesting to note that on SDS-PAGE under non-reducing conditions a larger part of TM_Ei4-6 after CNBr-digestion was found at the position of undigested TM_Ei4-6 (slices 7-8) as compared to SDS-PAGE under reducing conditions (Figure 4). This material, which was found to be able to accelerate the activation of protein C as well as the inactivation of scu-PA (Figure 6), consisted of a small amount of intact TM_Ei4-6 and most likely of EGF-like domain 4 complexed to TM_E5-6.

Discussion

The inactivation of scu-PA by thrombin is accelerated by thrombomodulin, but the structural elements required for this acceleration are not known. In this study, we have identified the essential EGF-like domains of thrombomodulin by comparing various forms of thrombomodulin (Figure 1).

Both recombinant TM_{LEO} containing all six EGF-like domains and TM_Ei4-6 containing EGF-like domains 4-6 accelerated the inactivation of scu-PA about 3-fold. Surprisingly, isolated TM_E5-6 containing EGF-like domains 5-6 was also found to accelerate the inactivation of scu-PA. This finding was in contrast with the activation of protein C, which was accelerated by TM_Ei4-6 but not by isolated TM_E5-6, in agreement with earlier reports (20-22). These data indicate that EGF-like domains 5 and 6 are required but also sufficient for the enhancement in the rate of the inactivation of scu-PA by thrombin. EGF-like domains 5 and 6 have previously been shown to bind to the anion exosite I of thrombin, and in this way inducing a conformational change that alters the specificity of thrombin (29,34).

Rabbit TM, containing all six EGF-like domains, appeared to accelerate the inactivation of scu-PA by thrombin about 35-fold, which is in agreement with the previous study of De Munk *et al.* (11). The acceleration by the recombinant TM forms was about 3-fold. This difference is explained by the absence of chondroitin sulfate (CS) on the recombinant TM forms. It has been demonstrated that the absence of CS on TM strongly diminishes TM-induced acceleration of the inactivation of scu-PA by thrombin (26). CS on TM provides an additional binding site for thrombin, besides EGF-like domains 5 and 6, resulting in a decrease of the Kd for thrombin binding (35-37). CS mediates the anticoagulant properties of the thrombin/TM complex by potentiating the inhibitory effects of TM on fibrinogen and factor V cleavage by thrombin (38) and by facilitating the inhibition of

thrombin by antithrombin (38,39). In addition, TM-induced acceleration of the activation of protein C by thrombin can be enhanced by CS (36,37), but CS does not have a prominent role in this specific cofactor activity of TM (35,38,39). In contrast, CS seems to be of substantial importance for the accelerating effect of thrombomodulin on the inactivation of scu-PA.

The findings of this study demonstrate that binding of thrombin to EGF-like domains 5 and 6 of thrombomodulin is sufficient for the acceleration of the inactivation of scu-PA and that no additional EGF-like domains are required. The domains of thrombomodulin essential for the inactivation of scu-PA differ from the domains essential for the activation of protein C (EGF-like domains 4-6 plus the connecting region between EGF-like domain 3 and EGF-like domain 4 (20-22)) and TAFI (EGF-like domains 3-6 (23-25)). Protein C and TAFI both need additional domain(s) beside EGF-like domains 5 and 6 in order to be efficiently activated by the thrombin/thrombomodulin complex, suggesting a direct interaction with thrombomodulin in regions that are different from the thrombin-binding domains (20,25,40). The mechanism of the accelerating effect of thrombomodulin on the inactivation of scu-PA by thrombin is not only distinguished from that of the accelerating effect on the activation of protein C and TAFI by which structural domains of thrombomodulin are needed, but also by a difference in the dependence on calcium. The inactivation of scu-PA by thrombin and the thrombin/thrombomodulin complex is calcium-independent (11), while the activation of both protein C and TAFI is calcium-dependent (24,37,41).

Two simple models are possible to explain how thrombomodulin enhances the inactivation of scu-PA by thrombin. In the first model, EGF-like domains 5 and 6 of thrombomodulin bind to the anion exosite I of thrombin, allosterically altering its substrate specificity, and there are no interactions between thrombomodulin and scu-PA. In the second model, in addition to the interactions in the first model there are also interactions between thrombomodulin and scu-PA. Currently available data do not allow us to distinguish between these models.

Although the mechanism of the accelerating effect of thrombomodulin differ between scu-PA and TAFI, the inactivation of scu-PA and the activation of TAFI by the thrombin/thrombomodulin complex will result in similar antifibrinolytic effects. Fibrinolysis will be inhibited due to the inactivation of scu-PA and the removal of C-terminal lysines necessary for the binding of plasminogen to partially degraded fibrin by activated TAFI (18,19,42) and in this way blood clots

might be protected from degradation. In contrast, the activation of protein C by the thrombin/thrombomodulin complex will result both in an anticoagulant and a profibrinolytic effect. Thrombin generation is attenuated due to the anticoagulant effects of APC via the inactivation of cofactors Va and VIIIa by APC. This results in a paradoxal decrease in the activation of TAFI and the inactivation of scu-PA by thrombin (13,43-45). Measurements *in vivo* will be necessary to determine the relative magnitude of these potential mechanisms.

In conclusion, the conformational change of thrombin induced by binding to EGF-like domains 5 and 6 of thrombomodulin appears to be sufficient for the acceleration of the inactivation of scu-PA, while additional EGF-like domains are required for the activation of protein C (EGF-like domains i4-6) and TAFI (EGF-like domains 3-6).

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CHAPTER 8

Fibrinolytic properties of activated FXII

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Summary

Activated factor XII (FXIIa), the initiator of the contact activation system, has been shown to activate plasminogen in a purified system. However, the quantitative role of FXIIa as a plasminogen activator in contact activation-dependent fibrinolysis in plasma is still unclear. In this study, the plasminogen activator activity (PAA) of FXIIa was examined both in a purified system and in a dextran sulphate euglobulin fraction of plasma by measuring fibrinolysis in a fibrin microtiter plate assay. FXIIa was found to have low PAA in a purified system. Dextran sulphate potentiated the PAA of FXIIa about sixfold, but had no effect on the PAA of smaller fragments of FXIIa, missing the binding domain for negatively charged surfaces. The addition of low amounts of factor XII (FXII) to FXII-deficient plasma induced a strong increase of contact activation-dependent PAA, as measured in a dextran sulphate euglobulin fraction, which may be ascribed to FXII-dependent activation of plasminogen activators like prekallikrein. When more FXII was added PAA continued to increase but to a lesser extent. In normal plasma, the addition of FXII also resulted in an increase of contact activation-dependent PAA. These findings suggested a significant contribution of FXIIa as a direct plasminogen activator. Indeed, at least 20% of contact activation-dependent PAA could be extracted from a dextran sulphate euglobulin fraction prepared from normal plasma by immunodepletion of FXIIa and therefore be ascribed to direct PAA of FXIIa. PAA of endogenous FXIIa immunoadsorped from plasma could only be detected in the presence of dextran sulphate. From these results it is concluded that FXIIa can contribute significantly to fibrinolysis as a plasminogen activator in the presence of a potentiating surface.

Introduction

Factor XII (FXII), or Hageman factor, is involved in the contact activation system together with prekallikrein and the cofactor high molecular weight kininogen (1). It is a single-chain glycoprotein with a molecular mass of 80 kD and circulates in plasma as a zymogen at a concentration of about 30 µg/ml (2,3). Activation of FXII, the initial step in contact activation, occurs after the binding of FXII to a negatively charged surface. Surface-bound FXII can either be autoactivated or activated

enzymatically by kallikrein into two-chain FXIIa (FXIIa) (2,4). Subsequently, FXIIa can be cleaved into smaller FXII fragments, of which β-FXIIa is a well-known active product (2,5,6).

There is evidence that the physiological role of FXII may be related to fibrinolysis (7). FXII structurally resembles the fibrinolytic proteins plasminogen, tissue-type plasminogen activator (t-PA) and urokinase-type plasminogen activator (u-PA) (2,8). FXIIa indirectly triggers fibrinolysis by activating prekallikrein, which in turn cleaves single-chain u-PA into active two-chain u-PA (9,10), and following activation of prekallikrein, the kallikrein generated can liberate bradykinin from high molecular weight kininogen. Both kallikrein and bradykinin liberate t-PA *in vivo* (3). In addition, an as yet unidentified FXII-dependent plasminogen proactivator in euglobulin fractions of contact-activated plasma has been described (7,11,12). Furthermore, studies in purified systems have demonstrated that FXII can also activate plasminogen directly, a process potentiated by negatively charged surfaces and zinc ions (13-16). Although the specific activity of FXII was found to be orders of magnitude less compared with the specific activity of u-PA (14), based on the plasma concentrations of both proteins FXII might be equally potent in activating plasminogen *in vivo* as u-PA.

The role of FXII in fibrinolysis *in vivo* is still unclear. Deficiencies of FXII have been reported to be associated with thromboembolic tendencies (17,18), and in addition a relationship has been described between depressed FXII-dependent fibrinolysis, i.e. contact activation-dependent fibrinolysis, and cardiovascular diseases (19-22). Studies with knock-out mice have demonstrated that the general predisposition to thrombosis is comparable between plasminogen-deficient and combined t-PA-deficient and u-PA-deficient mice (23-25). However, some notable differences were also observed such as the development of thrombotic lesions in various tissues in the plasminogen-deficient mice, which were absent or only seen in old age in the combined t-PA-deficient and u-PA-deficient mice (24). These differences may be related to the presence of alternative plasminogen activators *in* vivo, like FXII.

The interest in the role of FXII in fibrinolysis and its potential involvement in the thrombotic process is growing. However, although plasminogen activation by FXII has been extensively studied in purified systems, the quantitative role of FXII as a plasminogen activator in the physiological process of fibrinolysis has not yet been established. Therefore, we studied the fibrinolytic properties

of various molecular forms of FXIIa both in a purified system and in a dextran sulphate euglobulin fraction of plasma. In this way, we were able to quantify the contribution of FXIIa as a plasminogen activator to contact activation-dependent fibrinolysis, as measured in a dextran sulphate euglobulin fraction, providing more insight into the physiological role of FXII.

Part of this work has been presented at the XIVth International Congress on Fibrinolysis and Thrombolysis, Ljubljana, Slovenia (26).

Materials and Methods

Materials

Human FXII was obtained from Enzyme Research Laboratories (supplied by Kordia, Leiden, The Netherlands). Bovine trypsin was obtained from Boehringer Mannheim (Mannheim, Germany). Human kallikrein (specific activity of 4.9 U/mg protein as indicated by the manufacturer) and soybean trypsin inhibitor (SBTI) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The chromogenic substrate H-D-Pro-Phe-Arg-p-nitroanilide (S-2302) was obtained from Chromogenix AB (Mölndal, Sweden). Urinary two-chain u-PA (Ukidan) was obtained from Serono (Aubonne, Switzerland). Dextran sulphate (DXS, molecular mass 500 kD), CNBr-activated Sepharose 4B and Sephadex G-25 coarse were purchased from Pharmacia (Uppsala, Sweden). Bovine thrombin was obtained from Leo Pharmaceutical Products (Ballerup, Denmark). Plasminogen-rich bovine fibrinogen was prepared according to Brakman (27). Goat anti-human FXII antiserum and polyclonal peroxidase-conjugated rabbit anti-goat IgG were obtained from Nordic (Tilburg, The Netherlands). Goat anti-human FXII IgGs were isolated from the antiserum using sodium sulphate precipitation according to Kekwick, as described by Heide and Schwick (28). Normal goat serum was a kind gift of Dr. S. Emeis from this institute. Nitrocellulose sheets were obtained from Schleicher and Schuell (Dassel, Germany). BM blue Peroxidase substrate was obtained from Boehringer Mannheim. Citrated platelet-poor pooled normal plasma was obtained from healthy individuals from this institute. Plasma of a patient deficient in FXII (< 1% activity) was obtained from George King Biomedical (Overland Park, Kansas, USA). Polyclonal rabbit anti-human u-PA IgGs isolated from antisera raised against a urinary u-PA preparation were obtained and affinity purified on Protein A-Sepharose as described previously (29). Polyclonal rabbit anti-human t-PA IgGs were kindly provided by Mr. A. de Bart from this Institute.

Effect of DXS on plasminogen activator activity of FXIIa

FXIIa was prepared by autoactivation of a high concentration of FXII (0.8 mg/ml) during 90 min at 37°C in the presence of 5 µg/ml DXS (4), which resulted in the conversion of more than 90% FXII into FXIIa as judged on SDS-PAGE under reducing conditions. FXIIa was then diluted in 50 mM Tris/HCl, pH 7.4, 100 mM NaCl, 0.1% Tween 80 to a final

concentration of 40 µg/ml in the fibrin microtiter plate assay, containing a low concentration of DXS (0.25 µg/ml). In this way, the effect of DXS on plasminogen activation by FXIIa could be studied.

The effect of DXS on plasminogen activator activity (PAA) of FXIIa was measured in a fibrin microtiter plate assay, essentially as described by Sidelmann *et al.* (30). Briefly, 200 µl of a solution containing 0.1% (w/v) plasminogen-rich bovine fibrinogen in 50 mM sodium barbital/HCl, pH 7.75, 20 mM $CaCl_2$, 0.1% Tween 80 was mixed with 20 µl 1 NIH U/ml bovine thrombin in 150 mM NaCl, 0.1% Tween 80 in a 96-well microtiter plate. The plate was incubated for at least 4 h at 25°C to obtain a stable fibrin gel. FXIIa (40 µg/ml, containing 0.25 µg/ml DXS) was placed on top of the fibrin gel and a concentration range of DXS (0 - 100 µg/ml) was added in a final volume of 30 µl. Subsequently, 10 µl flufenamate (final concentration of 2 mM) was added and the plate was incubated at 25°C. Flufenamate was used in experiments studying plasminogen activation by FXIIa in dextran sulphate euglobulin fractions of plasma in order to inhibit plasma inhibitors like C1-inhibitor (31). In order to have similar experimental conditions both in a purified system and in a dextran sulphate euglobulin fraction of plasma, flufenamate was also used in experiments studying plasminogen activation by FXIIa in buffer. Measurements were performed at timed intervals at 405 nm with a Titertek Multiscan spectrophotometer and lysis was expressed as the decrease in absorbance between t = 0 and a time-point selected from the linear part of the lysis versus time curve (between t = 18 h and t = 42 h). PAA was calculated from the calibration curve obtained by serial dilutions of urinary two-chain u-PA, and was expressed as International Units (IU) per ml.

Plasminogen activator activity during activation of FXII

FXII (40 µg/ml) was incubated at 37°C with either 0.1 ng/ml trypsin, or 5 µg/ml DXS, or 0.1 U/ml kallikrein in the presence of 5 µg/ml DXS. PAA was measured during incubation (0 - 8 h) by assaying 30 µl sample in the fibrin microtiter plate assay, as described above.

Amidolytic activity of FXIIa was measured by adding 1 µl sample to 120 µl SBTI (final concentration of 250 µg/ml) in 50 mM Tris/HCl, pH 8.0, 0.1% Tween 80. Subsequently, 25 µl S-2302 (final concentration of 0.1 mM) was added and absorbance was measured at 405 nm during incubation at 37°C (4). FXIIa activity was expressed as ΔA/h.

Activation of FXII was also studied using Western blotting. Samples were run on 10% SDS-polyacrylamide gels under non-reducing conditions according to the method of Laemmli (32). After electrophoresis, proteins were immunoblotted on nitrocellulose sheets for 4 h at 1 mA/cm^2 in 25 mM Tris/HCl, pH 8.6, 190 mM glycine, 0.1% SDS, 20% methanol. The sheets were incubated overnight at 4°C in 10 mM Tris/HCl, pH 7.4, 150 mM NaCl, 1% BSA, 0.05% Tween 20. The sheets were then incubated with 1000x diluted goat anti-human FXII antiserum in 10 mM Tris/HCl, pH 7.4, 150 mM NaCl, 0.05% Tween 20 for 2 h at room temperature and subsequently with 5000x diluted polyclonal peroxidase-conjugated rabbit anti-goat IgG. Proteins were visualized with BM blue Peroxidase substrate.

Plasminogen activator activity of a dextran sulphate euglobulin fraction of plasma

Contact activation-dependent fibrinolysis, also called FXII-dependent fibrinolysis, is routinely measured in a dextran sulphate euglobulin fraction (DEF) of plasma using the fibrin plate method (31,33), and is defined as the residual PAA after inhibition of t-PA and u-PA activity by antibodies. Whether the fibrin microtiter plate was a suitable assay to measure contact activation-dependent fibrinolysis, was tested. A DEF was prepared from human normal plasma according to Kluft *et al.* (31), and dissolved in 50 mM Tris/HCl, pH 7.4, 100 mM NaCl, 0.1% Tween 80 to the original plasma volume. 30 µl of the DEF was assayed in the fibrin microtiter plate assay in the presence of 10 µl flufenamate (final concentration of 2 mM) supplemented with either no antibody, 10 µg/ml anti-t-PA, 50 µg/ml anti-u-PA, or both antibodies together (final concentrations). These antibody concentrations were sufficient to completely block t-PA and u-PA activity.

Contact activation-dependent plasminogen activator activity in a DEF of FXII-deficient plasma supplemented with FXII

A concentration range of FXII was added to normal plasma (0 - 80 µg/ml) and FXII-deficient plasma (0 - 160 µg/ml) and subsequently a DEF was prepared. 30 µl of the DEF was assayed in the fibrin microtiter plate assay, as described above. Besides flufenamate, 10 µg/ml anti-t-PA and 50 µg/ml anti-u-PA antibodies were added to inhibit t-PA and u-PA activity.

FXII-immunodepletion of a dextran sulphate euglobulin fraction

A DEF was prepared from 1 ml normal plasma and dissolved in 50 mM Tris/HCl, pH 7.4, 1 M NaCl, 0.1% Tween 80 to 2x the original plasma volume. IgGs isolated from goat anti-human FXII antiserum were coupled to CNBr-activated Sepharose 4B according to the prescription of the manufacturer (5 mg IgG per ml Sepharose 4B). 1.8 ml of the DEF was run on a goat anti-human FXII IgG Sepharose column (0.5 x 1.5 cm) in 50 mM Tris/HCl, pH 7.4, 1 M NaCl, 0.1% Tween 80. The bound material was eluted with 50 mM glycine/HCl, pH 2.2, 1 M NaCl, 0.1% Tween 80 and elution fractions were neutralized with 1 M Tris/HCl, pH 9.0.

Prior to the assessment of contact activation-dependent PAA, the fractions were desalted by exchanging the buffer into 50 mM Tris/HCl, pH 7.4, 100 mM NaCl, 0.1% Tween 80 using Sephadex G-25 coarse. Contact activation-dependent PAA was assessed in 30 µl of the fractions, as described above, and was measured in the presence and absence of 5 µg/ml DXS. Besides 2 mM flufenamate, 10 µg/ml anti-t-PA and 50 µg/ml anti-u-PA antibodies were added to inhibit t-PA and u-PA activity. FXIIa activity on S-2302 was measured in 5 µl of the fractions in the presence of SBTI and 2.5 µl of the fractions were subjected to SDS-PAGE and Western blotting, as described above. As a control for aspecific binding of the antiserum or the conjugate, Western blots were incubated with 1000x diluted normal goat serum instead of the goat anti-human FXII antiserum.

Results

Purified system

FXIIa, generated by autoactivation, was found to have low PAA in the fibrin microtiter plate assay. PAA was potentiated at least 6-fold by DXS, a negatively charged compound, and this potentiating effect exhibited a bell-shaped profile (Figure 1). Since more than 90% of FXII had been converted to FXIIa during the autoactivation, this increase of PAA induced by DXS could mainly be ascribed to a direct effect of DXS on PAA of FXIIa.

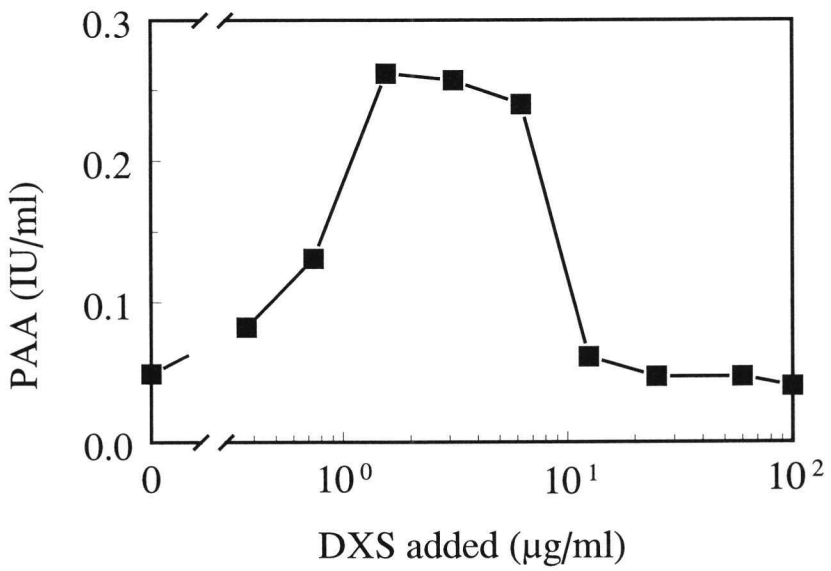

Figure 1. Effect of dextran sulphate (DXS) on plasminogen activator activity (PAA) of 40 µg/ml FXIIa, measured in a fibrin microtiter plate assay. Increasing concentrations of DXS (0 - 100 µg/ml) were added to FXIIa, already containing 0.25 µg/ml DXS.

When FXII was incubated with trypsin in the absence of DXS, activation of FXII occurred within one hour as found in the amidolytic assay (Figure 2A). Simultaneously, PAA was generated and reached a low activity plateau of about 0.06 IU/ml after one hour. Both FXIIa amidolytic activity and PAA remained stable during further incubation. As shown on Western blot, 80 kD FXII was

completely cleaved into smaller FXII fragments, including a 40 kD fragment and β-FXIIa (a doublet of 30 and 28.5 kD), within 8 hours of incubation with trypsin (Figure 2B). These findings suggested that, in the absence of a negatively charged surface, all fragments generated during activation of FXII have comparable amidolytic activity and PAA.

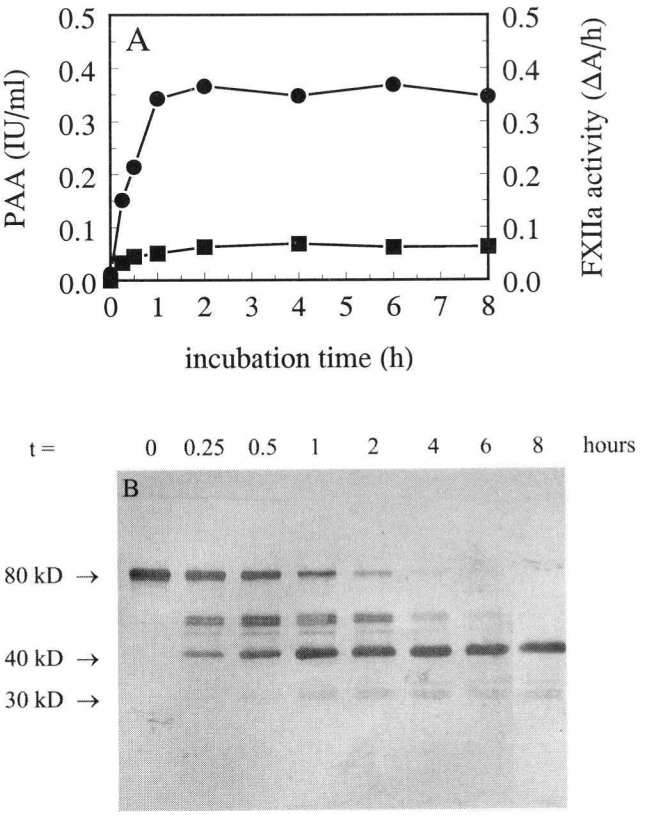

Figure 2. PAA (■) and FXIIa amidolytic activity (●) during activation of 40 μg/ml FXII by 0.1 ng/ml trypsin at 37°C (A). Cleavage of FXII at several time-points was studied by non-reducing SDS-PAGE and Western blot (B). FXII(a) is positioned at 80 kD, smaller FXII fragments between 80 and 40 kD, and a doublet of β-FXIIa at 30 and 28.5 kD. The time-point t = 0 represents (activity of) untreated FXII. Trypsin (0.1 ng/ml) showed no activity both in the fibrin microtiter plate assay and in the amidolytic assay.

Autoactivation of FXII in the presence of DXS occurred within 15 min, coinciding with the fast generation of PAA (Figure 3A). PAA reached a plateau of about 0.35 IU/ml, which is six-fold higher than that found after activation of FXII by trypsin. This finding was most likely due to the potentiating effect of DXS as described in Figure 1. PAA showed a trend to decrease during further incubation for 16 h (data not shown).

Activation of FXII by kallikrein in the presence of DXS also occurred rapidly and initially PAA

reached a maximum similar to the activity found after autoactivation (Figure 3B). However, while FXIIa amidolytic activity remained stable during further incubation, PAA in contrast was found to decrease immediately. After 6 h, a plateau was reached comparable to the PAA found after trypsin activation in the absence of DXS, suggesting that DXS did not potentiate PAA anymore. Very slow activation of FXII by kallikrein was observed in the absence of DXS (data not shown).

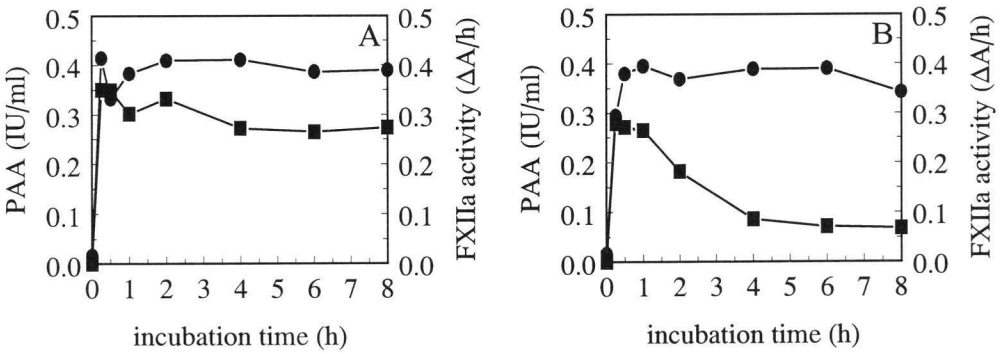

Figure 3. PAA (■) and FXIIa amidolytic activity (●) during autoactivation of 40 µg/ml FXII in the presence of 5 µg/ml DXS (A), and during activation by 0.1 U/ml kallikrein in the presence of 5 µg/ml DXS (B) at 37°C. The time-point t = 0 represents activity of untreated FXII. Kallikrein (0.1 U/ml) showed no activity both in the fibrin microtiter plate assay and in the amidolytic assay.

On Western blot, it was demonstrated that during autoactivation FXIIa was very slowly cleaved into smaller activation products (Figure 4A). After 8 h of incubation 80 kD FXIIa was still the most prominent activation product. In contrast, during activation by kallikrein in the presence of DXS a significant part of 80 kD FXIIa was already cleaved into smaller FXII fragments at the first time-point t = 0.25 h (Figure 4B). Complete cleavage of 80 kD FXIIa was observed after 4 h. Both the pattern and the time-course of the digestion induced by autoactivation and by kallikrein in the presence of DXS were distinct, which is in agreement with the literature (5). Since all FXII fragments appeared to be equally potent in inducing fibrinolysis in the absence of DXS (Figure 2AB), these findings suggested that DXS only potentiated PAA of 80 kD FXIIa in the fibrin microtiter plate assay and not of the smaller FXII fragments missing the binding domain for DXS,

that are predominantly generated during kallikrein activation.

The concentrations of trypsin and kallikrein used were not sufficient to induce fibrinolysis, thus PAA could completely be ascribed to FXIIa.

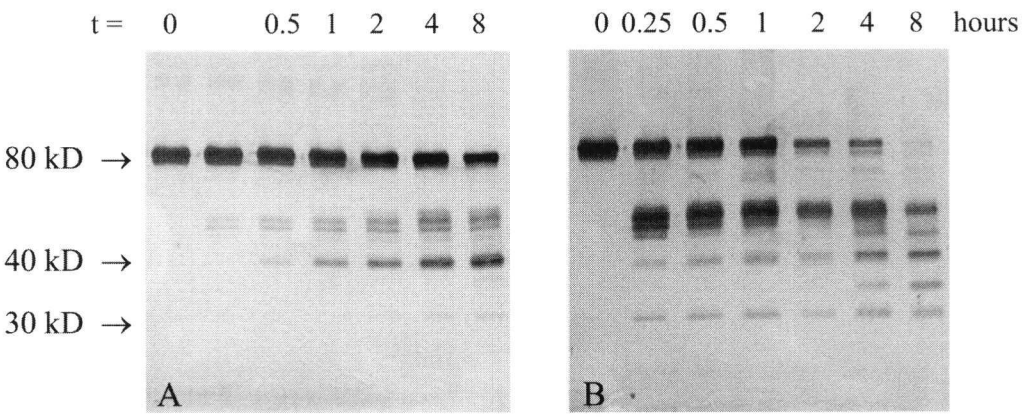

Figure 4. Cleavage of FXII studied on Western blot during autoactivation of 40 µg/ml FXII in the presence of 5 µg/ml DXS (A), and during activation by 0.1 U/ml kallikrein in the presence of DXS (B) at 37°C. FXII(a) is positioned at 80 kD, smaller FXII fragments between 80 and 40 kD, and a doublet of β-FXIIa at 30 and 28.5 kD. The time-point t = 0 represents untreated FXII.

Dextran sulphate euglobulin fractions of plasma

Routinely, contact activation-dependent fibrinolysis is measured in a dextran sulphate euglobulin fraction (DEF) of plasma (31,33). Since DXS was found to potentiate PAA of purified FXIIa, FXII itself may be an important candidate for contact activation-dependent fibrinolysis in the DEF, a milieu in which DXS is present. Therefore, the contribution of FXII as a plasminogen activator to contact activation-dependent fibrinolysis in the DEF was studied.

Assessment of contact activation-dependent fibrinolysis in the fibrin microtiter plate assay

Contact activation-dependent fibrinolysis was assessed in a DEF in the fibrin microtiter plate assay, instead of the classical fibrin plate assay (31,33) (Figure 5). In the absence of antibodies against t-

PA and u-PA, PAA in the DEF was measured at a level of 0.45 ± 0.01 IU/ml (mean ± standard error of the mean, n=24). In the presence of anti-t-PA antibodies, about 92% of the PAA (0.41 ± 0.01 IU/ml) was still present compared with PAA measured in the absence of antibodies. Anti-u-PA antibodies reduced PAA to 62% (0.28 ± 0.01 IU/ml), while a combination of anti-t-PA and anti-u-PA antibodies reduced PAA to 61% (0.28 ± 0.02 IU/ml), which reflects contact activation-dependent PAA. From these data, it was concluded that in this experimental set-up t-PA barely contributed to PAA and that u-PA accounted for about 40% of the activity. The residual 60% of PAA was considered as contact activation-dependent fibrinolysis, as described under "Materials and Methods".

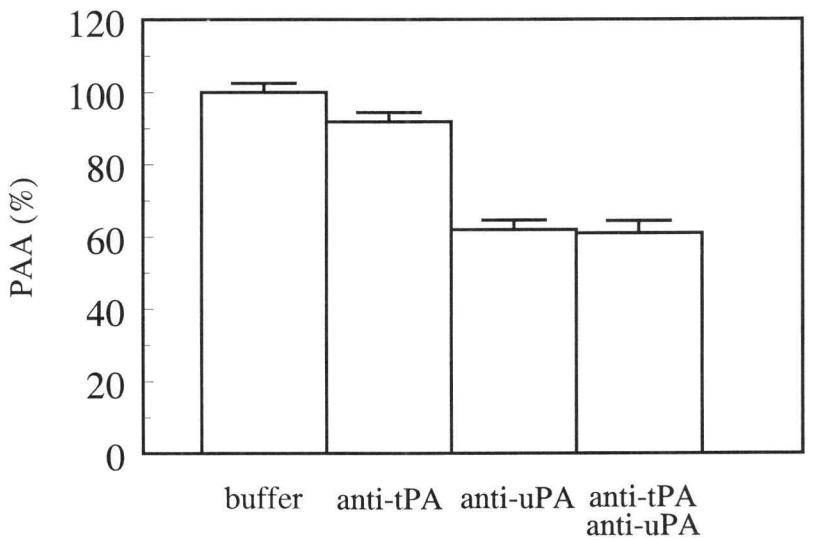

Figure 5. Plasminogen activator activity (PAA) of a DEF prepared from normal plasma, measured in a fibrin microtiter plate assay in the absence and presence of inhibiting anti-t-PA and anti-u-PA antibodies. PAA was expressed as a percentage (mean ± standard error of the mean, n=24) of the activity measured in the absence of antibodies (0.45 ± 0.01 IU/ml).

Effect of addition of FXII to normal plasma and FXII-deficient plasma on contact activation-dependent PAA

Contact activation-dependent PAA in a DEF prepared from a FXII-deficient plasma, as measured in the presence of anti-t-PA and anti-u-PA antibodies, was about 20% compared with contact activation-dependent PAA in a DEF of normal plasma (Figure 6). A possible explanation for this activity could be that low amounts of FXII present in the deficient plasma were (auto)activated into FXIIa in the DEF, which could result in partial activation of other plasminogen activators like prekallikrein, factor XI and possibly an unidentified FXII-dependent plasminogen proactivator. The addition of increasing amounts of FXII to the FXII-deficient plasma resulted initially in a strong increase of contact activation-dependent PAA, most likely due to further activation of the other plasminogen proactivators. When higher concentrations of FXII were added, PAA continued to increase but to a lesser extent, suggesting direct plasminogen activation by FXIIa. In normal plasma, the addition of FXII resulted in a linear increase of contact activation-dependent PAA, providing additional evidence that FXIIa can activate plasminogen directly.

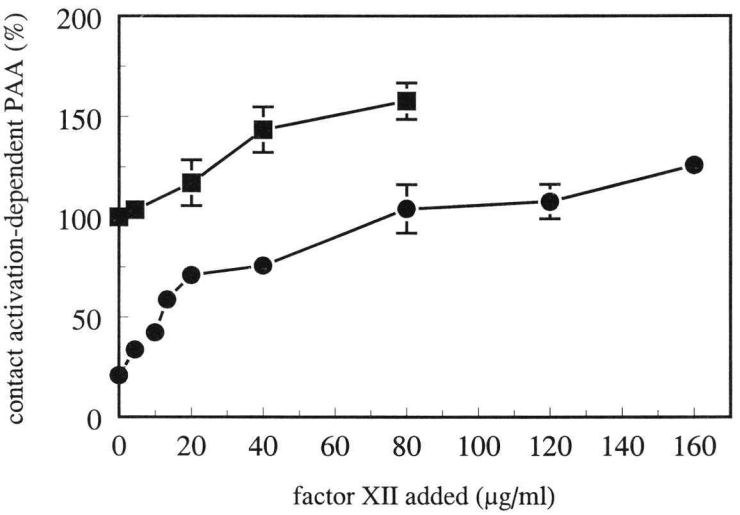

Figure 6. Effect of the addition of FXII to normal plasma (■) and to a FXII-deficient plasma (●) on contact activation-dependent PAA. FXII was first added to the plasma and subsequently a DEF was prepared. Contact activation-dependent PAA, assessed in the presence of anti-t-PA and anti-u-PA antibodies, was expressed as a percentage (mean ± standard error of the mean, $n \geq 3$) of the activity measured in the DEF of normal plasma without FXII added.

FXII-immunodepletion of a DEF

When a DEF of normal plasma was immunodepleted of FXII(a) using a goat anti-human FXII IgG Sepharose column, it appeared that the main part of FXII bound to the column, as demonstrated by FXIIa amidolytic activity (Figure 7A) and Western blotting (Figure 7B-C). On reducing SDS-PAGE and Western blot a substantial amount of the bound 80 kD FXII appeared to be cleaved and thus had been (auto)activated during the preparation of the DEF (data not shown). In the elution fractions containing FXIIa, contact activation-dependent PAA could be detected, as measured in the presence of anti-t-PA and anti-u-PA antibodies, but only when DXS was present (Figure 7A). This finding showed that DXS also potentiated the PAA of endogenous FXIIa generated during dextran sulphate euglobulin fractionation of plasma, as was found for purified FXIIa. PAA accounted for 20% of the original contact activation-dependent activity in the DEF, suggesting that FXIIa is a significant plasminogen activator as measured in this experimental set-up. Since a small amount of FXII(a) was still present in the flow-through (Figure 7B), the contribution of FXII(a) may be even higher. The major part of the high molecular weight proteins observed in the flow-through could be ascribed to aspecific binding of the antiserum or the conjugate, since these high molecular weight proteins were also found on a control Western blot using normal goat serum (data not shown). These data suggest that the major part of the high molecular weight proteins are not related to FXII. Taken the molecular weight of these proteins into consideration, it is unlikely that they can be ascribed to kallikrein or FXIa. The presence of the high molecular weight proteins may be explained by complexes between DXS and plasma proteins.

In the flow-through fractions 80% of the original contact activation-dependent PAA was recovered, which was unaffected by the addition of DXS. This activity may be ascribed to other plasminogen activators of the contact activation system activated by FXIIa during the preparation of the DEF, like prekallikrein, factor XI and possibly an unidentified FXII-dependent plasminogen proactivator, that did not bind to the column.

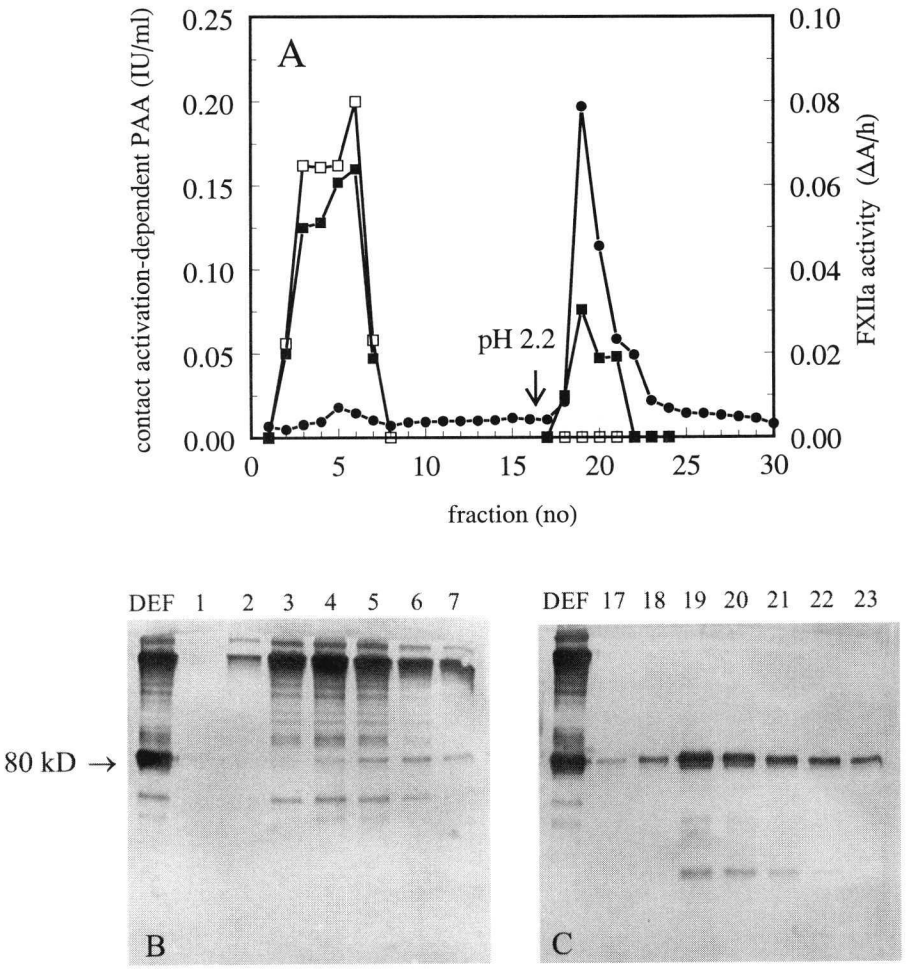

Figure 7. FXII-immunodepletion of a DEF by goat anti-human FXII IgG Sepharose. Fraction volumes of 750 µl were collected, whilst elution of FXIIa commenced from fraction 17. Contact activation-dependent PAA (□,■), assessed in the presence of anti-t-PA and anti-u-PA antibodies, and FXIIa amidolytic activity (●), assessed in the presence of SBTI, were measured in the flow-through and elution fractions (A). Contact activation-dependent PAA was assayed either in the absence (□) and in the presence (■) of 5 µg/ml DXS. Western blotting of FXII was performed in the DEF and the flow-through fractions 1-7 (B) and elution fractions 17-23 (C). FXIIa is positioned at 80 kD. The major part of the high molecular weight proteins in the flow-through could be ascribed to aspecific binding of the antiserum or the conjugate, since these high molecular weight proteins were also found on a control Western blot using normal goat serum.

Discussion

The role of FXII as a plasminogen activator in fibrinolysis is still unknown. In this study the plasminogen activator activity (PAA) of FXII has been examined in a fibrin microtiter plate assay in order to quantify its contribution to fibrinolysis, as measured in a dextran sulphate euglobulin fraction.

Goldsmith *et al.* first reported that FXIIa is able to activate plasminogen in a purified system, in the presence of the negatively charged compound kaolin (13). Recently, Ravon *et al.* described a potentiating effect of DXS on plasminogen activation by FXIIa in a chromogenic assay system (16). In the present study, a negatively charged surface appeared to enhance FXII-induced fibrinolysis as well, since DXS potentiated the PAA of FXIIa in a fibrin microtiter plate assay about 6-fold. A bell-shaped profile was found for this potentiating effect, which may correspond to a template model (34). This model implies the formation of a ternary complex between DXS, plasminogen and FXII, in this way facilitating the activation of plasminogen. From these results it can be concluded that DXS not only acts as an activating surface for FXII, but also enhances the fibrinolytic properties of FXIIa.

The PAA of smaller fragments of FXIIa, such as β-FXIIa, was not potentiated by DXS, which can be explained by the fact that these fragments lack the binding domains for negatively charged surfaces located in the heavy chain of FXII (35). These results suggest that surface-bound FXIIa may efficiently activate plasminogen *in vivo*. Although physiological potentiating surfaces for FXIIa are still unknown, negatively charged components like phospholipids and glycosaminoglycans may be suitable candidates (15).

In the literature, the role of FXII in fibrinolysis is mainly ascribed to the activation of kallikrein-mediated activation of single-chain u-PA (36-38), the indirect release of t-PA via bradykinin generation from high molecular weight kininogen (3), and the activation of an unidentified FXII-dependent plasminogen proactivator (7,11). A direct contribution of FXII as a plasminogen activator to fibrinolysis in plasma, as measured in a dextran sulphate euglobulin fraction (DEF), has been considered negligible. For optimal analysis of contact activation-dependent fibrinolytic activity in plasma, a DEF of plasma is routinely used (11,31). Kluft *et al.* have analyzed the total fibrinolytic activity in a DEF using the fibrin plate method and have ascribed a minor part of the activity to t-

PA, 50% to u-PA and 50% to contact activation-dependent fibrinolysis (7). In addition, contact activation-dependent fibrinolysis was subdivided into 30% of the sum of kallikrein, FXII and factor XI activity (15% of total activity) and 70% of the activity of an unknown FXII-dependent plasminogen proactivator (35% of total activity) (7). The low contribution of t-PA to the total fibrinolytic activity in a DEF may be explained by the fact that citrated plasma is used to prepare a DEF. Since t-PA is rapidly inhibited by plasminogen activator inhibitor 1, blood should be collected into acidic anticoagulant in order to efficiently recover t-PA activity (39). In our fibrin microtiter plate assay, similar contributions were found and 60% of the total activity in a DEF could be ascribed to contact activation-dependent fibrinolysis. On the basis of the observations by Kluft *et al.*, the contribution of FXIIa as a plasminogen activator to contact activation-dependent fibrinolysis in the DEF would only be a few percent (7). However, in this study it was shown that at least 20% of contact activation-dependent fibrinolysis (12% of total fibrinolytic activity) could be extracted from the DEF by immunodepletion of FXII by an anti-FXII Sepharose column and therefore be ascribed to PAA of FXIIa. The fibrinolytic activity eluted from the column could only be detected in the presence of DXS. This finding demonstrates that DXS also potentiates the fibrinolytic properties of endogenous FXIIa in plasma, as measured in a DEF.

These results indicate that the role of FXIIa as a plasminogen activator in plasma, as measured in a DEF, is far more significant than previously assumed. The discrepancy between this study and the results of Kluft *et al.* may be explained by the methods used to establish the contribution of FXIIa to contact activation-dependent fibrinolysis. Kluft *et al.* have quantified this contribution for instance by adding β-FXIIa to a DEF prepared from normal plasma, which resulted in a minor increase in activity (33). As shown in the present study, DXS present in the DEF will not potentiate the PAA of β-FXIIa and thus little activity can be expected. Furthermore, the addition of β-FXIIa at 30% of the plasma level of FXII to a DEF prepared from FXII-deficient plasma was found to be sufficient to restore activity (although normal levels were not reached), suggesting only an indirect contribution by FXII (33). In contrast, in the present study FXII was first added to FXII-deficient plasma before a DEF was prepared, in this way reflecting the generation of contact activation-dependent fibrinolysis in a DEF prepared of normal plasma. Fibrinolytic activity was found to increase rapidly with low amounts of added FXII. This activity may be ascribed to FXII-dependent activation of prekallikrein and factor XI, and possibly of the unidentified plasminogen proactivator mentioned above (7,11,40).

When higher concentrations of FXII were added, contact activation-dependent PAA continued to increase but to a lesser extent due to direct plasminogen activation by FXIIa. This observation suggested that FXII not only induced fibrinolysis in plasma by activating other plasminogen activators, but also directly induced fibrinolysis by activating plasminogen. This finding was confirmed by the fact that the addition of FXII to normal plasma before the DEF-preparation also resulted in a linear increase of fibrinolysis. The addition of 30 μg/ml FXII, the physiological concentration of FXII, to normal plasma resulted in an increase of contact activation-dependent PAA of about 25%, which is in agreement with a contribution of at least 20% found after immunodepletion of a DEF.

In the present study, FXIIa does not account for all contact activation-dependent fibrinolysis in plasma, as measured in a DEF. Although this study does not exclude the existence of an unidentified FXII-dependent plasminogen proactivator, its contribution seems to be less than previously proposed. Other candidates for the residual activity are kallikrein, which already has been shown to be more active than FXII (7), and FXIa (14). The contribution of kallikrein, FXIa or the unidentified FXII-dependent plasminogen activator to contact activation-dependent fibrinolysis needs to be further quantified.

The data described in this study provide new insight into the mechanism of plasminogen activation by FXIIa and demonstrate that its fibrinolytic capacity can be much higher than previously shown for β-FXIIa (14). Potentiation of the PAA of FXIIa appears to be important, but factors that may enhance the PAA of FXIIa *in vivo* are not known at the moment. Since it has recently been demonstrated that FXII binds to endothelial cells, the involvement of cells may not be excluded (41).

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CHAPTER 9

A report of a study on the isolation and characterization of a factor XII-dependent plasminogen activator in plasma

- preliminary results and conclusions –

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Summary

The contact activation system is known to be involved in fibrinolysis. Factor XIIa, kallikrein and factor XIa may promote fibrinolysis by direct or indirect activation of plasminogen. In addition, the presence of an unidentified factor XII-dependent plasminogen proactivator in plasma has been postulated. This factor XII-dependent plasminogen activator accounts for about 35% of the total fibrinolytic activity as measured in a dextran sulphate euglobulin fraction of plasma. There is little knowledge about the identity of the factor XII-dependent plasminogen proactivator. In the present study, we tried to isolate this plasminogen activator and to characterize its identity and its properties as a plasminogen activator. Contact activation-dependent plasminogen activator activity (PAA), defined as the residual PAA after inhibition of tissue-type plasminogen activator and urokinase-type plasminogen activator activity by antibodies, was isolated from fractionated and dextran sulphate-activated plasma by gelfiltration and anion exchange chromatography. Several components were found to contribute to contact activation-dependent PAA. Besides known plasminogen activators like kallikrein, 80 kD factor XIIa and β-factor XIIa, a 140 kD protein with urokinase-type plasminogen activator-related antigenic determinants, most likely corresponding to a previously described 110 kD protein, and a 170 kD protein were observed to be associated with contact activation-dependent PAA. The PAA of the 170 kD protein was strongly potentiated by dextran sulphate. The identity of the 140 kD and 170 kD protein remains to be elucidated.

It can be concluded that the unidentified part of contact activation-dependent PAA in plasma cannot be ascribed to a single factor XII-dependent plasminogen activator, and that several enzymes and cofactors may be involved.

Introduction

The fibrinolytic system is involved in the maintainance of the hemostatic balance. The key enzyme in this process is plasmin. This serine protease is able to degrade the fibrin network, in this way dissolving a blood clot. Plasmin circulates as an inactive precursor form called plasminogen, which is activated by plasminogen activators. The main plasminogen activators are tissue-type plasminogen activator (t-PA) and urokinase-type plasminogen activator (u-PA) (1).

Besides t-PA and u-PA, components of the contact activation system are also known to be involved in the activation of plasminogen (2). The contact activation system consists of factor XII, prekallikrein, factor XI and the cofactor high molecular weight kininogen. The conversion of factor XII into active factor XII (FXIIa) is the initial step in the activation of this system. Factor XII bound to a negatively charged surface can either be autoactivated or enzymatically be activated by kallikrein, followed by the cleavage of FXIIa into smaller factor XII fragments like β-FXIIa (3-7). Subsequently, prekallikrein and factor XI are activated by FXIIa into kallikrein and factor XIa (FXIa), respectively. FXIIa indirectly triggers the activation of plasminogen by activating prekallikrein into kallikrein, which in turn can activate single-chain u-PA into active two-chain u-PA (8,9). FXIIa, kallikrein and FXIa are also able to activate plasminogen directly (10-13). In addition, the existence of an as yet unidentified factor XII-dependent plasminogen pro-activator has been postulated (2,14). This factor XII-dependent plasminogen proactivator was found in a dextran sulphate euglobulin fraction (DEF) prepared of citrated plasma. Kluft *et al.* have described that the total fibrinolytic activity measured in a DEF could be divided in 50% of u-PA-dependent fibrinolysis and 50% of contact activation-dependent fibrinolysis (14). The contribution of t-PA to the total fibrinolytic activity was minor most likely due to rapid inhibition of t-PA by plasminogen activator inhibitor-1 in citrated plasma (15). Factor XIIa, kallikrein and factor XIa accounted for about 30% of contact-activation dependent fibrinolysis (15% of total fibrinolytic activity). The remaining 70% of contact activation-dependent fibrinolysis (35% of total fibrinolytic activity) was ascribed to an unidentified factor XII-dependent plasminogen proactivator (2,14). This unknown plasminogen proactivator could be activated by the contact activation system and its activity was absent in plasma deficient in factor XII or prekallikrein (14).

Various studies have tried to identify the factor XII-dependent plasminogen proactivator. Binnema *et al.* have described that this component is an inactive 110 kD single-chain polypeptide that is cleaved upon activation by the contact activation system (16). The 110 kD polypeptide appeared to comprise some u-PA-related antigenic determinants, but its fibrinolytic activity could not be inhibited by anti-u-PA antibodies and its isoelectric point of 4.8 was much lower than that of u-PA (16,17). From these results, it was concluded that the factor XII-dependent plasminogen proactivator shares some homology with u-PA. Recently, another study has described a 30 kD

protein that is involved in plasminogen activation in a DEF prepared of plasma depleted both of t-PA and u-PA (18). Activation of this protein was also mediated by the contact activation system.

The factor XII-dependent plasminogen proactivator has still not been identified. In this study, we have tried to isolate this plasminogen activator and to characterize its identity and its properties as a plasminogen activator.

Materials and Methods

Materials

Dextran sulphate (DXS, molecular mass 500 kD), Protein A-Sepharose 4B, Sephacryl S-300 High Resolution (S-300 HR), MonoQ HR 5/5, and Sephadex G-25 coarse were purchased from Pharmacia (Uppsala, Sweden). Ammonium sulphate was obtained from Serva (Heidelberg, Germany). Flufenamic acid (flufenamate) was obtained from Aldrich-Europe (Beerse, Belgium). Human kallikrein (specific activity of 4.9 U/mg protein as indicated by the manufacturer) and soybean trypsin inhibitor (SBTI) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The chromogenic substrate Bz-Pro-Phe-Arg-pNA.AcOH (Chromozym PK) was obtained from Pentapharm (Basel, Switzerland) and the chromogenic substrate H-D-Pro-Phe-Arg-p-nitroanilide (S-2302) from Chromogenix AB (Mölndal, Sweden). Urinary two-chain u-PA (Ukidan) was obtained from Serono (Aubonne, Switzerland). Bovine thrombin was obtained from Leo Pharmaceutical Products (Ballerup, Denmark). Plasminogen-rich bovine fibrinogen was prepared according to Brakman (19).

Citrated platelet-poor pooled normal plasma was obtained from healthy individuals. Polyclonal rabbit anti-human u-PA IgGs isolated from antisera raised against a urinary u-PA preparation were obtained and affinopurified on Protein A-Sepharose as described previously (20). Polyclonal rabbit anti-human t-PA IgGs were kindly provided by Mr. A. de Bart from this institute. Goat anti-human factor XII antiserum, goat anti-human kallikrein antiserum, goat anti-human high molecular weight kininogen antiserum, polyclonal rabbit anti-goat IgG peroxidase and polyclonal goat anti-rabbit IgG peroxidase were obtained from Nordic (Tilburg, The Netherlands). Goat anti-human FXI IgG was obtained from Affinity Biochemicals Inc. (supplied by Kordia, Leiden, The Netherlands). Goat anti-human factor XII IgGs and goat anti-human kallikrein IgGs were isolated from the antisera using sodium sulphate precipitation according to Kekwick, as described by Heide and Schwick (21). Nitrocellulose sheets were obtained from Schleicher and Schuell (Dassel, Germany). BM blue Peroxidase substrate was obtained from Boehringer Mannheim.

Methods

Fractionation of plasma

Iso-electric precipitation of plasma followed by fractionation in the presence of dextran sulphate (DXS) was performed as described by Kluft *et al.* (14). Briefly, a volume of 100 ml plasma was mixed with 650 ml aqua dest and titrated to pH 5.9 with 1 M acetic acid. After stirring for 45 min, the plasma was centrifuged and 3 mg DXS was added to the supernatant. The supernatant was diluted with aqua dest to a final volume of 1000 ml and stirred for 45 min. After centrifugation, the pellet was resuspended in the appropriate fractionation buffer (Table 1) and ammonium sulphate fractionation was performed as described by Kluft *et al.* (14). To the resuspended pellet 3.8 M ammonium sulphate (100% solution) was added to a final concentration of 30% ammonium sulphate. The solution was stirred for 30 min and centrifuged. To the supernatant 3.8 M ammonium sulphate was added to a final concentration of 50% ammonium sulphate, followed by stirring for 30 min. After centrifugation the pellet was resuspended in the appropriate fractionation buffer (Table 1). The resuspended pellet was run on a Protein A-Sepharose 4B column (12x1.7 cm) in fractionation buffer in order to remove IgGs (16). The flow through fractions were pooled and proteins were precipitated by 50% ammonium sulphate, as described above. The pellet was resuspended in the appropriate gelfiltration buffer (Table 1). All procedures were performed at 4°C.

Gelfiltration

Fractionated plasma was run on a Sephacryl S-300 HR gelfiltration column (104 x 2.3 cm) at 4°C under various conditions, as described in Table 1. Fractions were collected and contact activation-dependent plasminogen activator activity (PAA), kallikrein activity and FXIIa activity was measured, as described below. Prior to the assessment of contact activation-dependent PAA, fractions containing 1 M NaCl or 1.6 M KSCN were desalted by exchanging the buffer into 50 mM Tris/HCl, pH 7.4, 100 mM NaCl, 0.1% Tween 80 using Sephadex G-25 coarse. In addition, samples were run on SDS-PAGE and analyzed by Western blot or by fibrin zymography, as described below.

Table 1. Conditions used for three different gelfiltration runs of fractionated plasma on a Sephacryl S-300 HR column .

run	fractionation	gelfiltration	protein on column	
(no)	buffer	buffer	mg	ml
1	PBS[1]	phosphate/NaCl[3]	23.2	6.7
2	Tris/NaCl[2]	Tris/NaCl[2]	49.9	4.9
3	Tris/NaCl[2]	Tris/KSCN[4]	47.4	7.4

The fractionation buffer consisted of [1]10 mM phosphate buffer, pH 7.4, 140 mM NaCl or [2]50 mM Tris/HCl, pH 7.4, 100 mM NaCl. The gelfiltration buffer consisted of [2]50 mM Tris/HCl, pH 7.4, 100 mM NaCl, [3]10 mM sodium phosphate buffer, pH 7.4, 1 M NaCl or [4]50 mM Tris/HCl, pH 7.4, 1.6 M KSCN.

Anion exchange chromatography

A pool was prepared of fractions containing contact activation-dependent PAA of the Sephacryl S-300 HR run in 1 M NaCl (run 1, Table 1). The pool was applied to a MonoQ HR 5/5 column (1 ml) in 20 mM Tris/HCl, pH 8.0 using an FPLC system. Proteins were eluted from the column using a stepwise gradient of 0 to 2 M NaCl and fractions were collected. Fractions were desalted by exchanging the buffer into 50 mM Tris/HCl, pH 7.4, 100 mM NaCl, 0.1% Tween 80, prior to the assessment of contact activation-dependent PAA.

Assays

Contact activation-dependent plasminogen activator activity

Contact activation-dependent plasminogen activator activity (PAA) was defined as the residual PAA after inhibition of t-PA and u-PA activity by antibodies. Contact activation-dependent PAA was measured in a fibrin microtiter plate assay, essentially as described by Sidelmann *et al.* (22). Briefly, 200 µl of a solution containing 0.1% (w/v) plasminogen-rich bovine fibrinogen in 50 mM sodium barbital/HCl, pH 7.75, 20 mM $CaCl_2$, 0.1% Tween 80 was mixed with 20 µl 1 NIH U/ml bovine thrombin in 150 mM NaCl, 0.1% Tween 80 in a 96-well microtiter plate. The plate was incubated for at least 4 h at 25°C to obtain a stable fibrin gel. On top of the fibrin gel 10 µl flufenamate (final concentration of 2 mM) supplemented with 10 µg/ml anti-t-PA and 50 µg/ml anti-u-PA (final concentrations) was placed. These antibody concentrations were sufficient to completely block t-PA and u-PA activity. Flufenamate was used in order to inhibit plasma inhibitors like C1-inhibitor (23). Subsequently, 30 µl (desalted) sample was added and the fibrin microtiter plate was incubated at 25°C. Measurements were performed at timed intervals at 405 nm with a Titertek Multiscan spectrophotometer and lysis was expressed as the decrease in absorbance between t=0 and a time-point selected from the linear part of the lysis versus time curve (between t=18 h and t=42 h). Contact activation-dependent PAA was calculated from the calibration curve obtained by serial dilutions of urinary two-chain u-PA (Ukidan), and was expressed as International Units (IU) per ml.

In some experiments, contact activation-dependent PAA was determined in the presence of goat anti-human kallikrein IgG, goat anti-human factor XII IgG or goat anti-human factor XI IgG. In addition, the effect of DXS on contact activation-dependent PAA was studied by analyzing the PAA in the absence or presence of 5 µg/ml DXS.

Kallikrein activity

Amidolytic activity of kallikrein was measured by adding 1 µl sample to 210 µl Tris-imidazole buffer (μ = 0.15), pH 7.9, containing 0.1% polyethyleneglycol 6000 and 0.1% Polybrene in microtiter plates (14). Subsequently, 40 µl 1 mM Chromozym PK was added and absorbance was measured at 405 nm during incubation at 37°C. Activity was expressed as ΔA405/h. Kallikrein activity in the samples was calculated from the calibration curve obtained by serial dilutions of purified human kallikrein, and was expressed as U/ml.

FXIIa activity

Amidolytic activity of FXIIa was measured by adding 5 µl sample to 120 µl SBTI (final concentration of 250 µg/ml) in 50 mM Tris/HCl, pH 8.0, 0.1% Tween 80 in microtiter plates. Subsequently, 25 µl S-2302 (final concentration of 0.1 mM) was added and absorbance was measured at 405 nm during incubation at 37°C (5). FXIIa activity was expressed as ΔA405/h.

SDS-PAGE and Western blotting

Samples were run on SDS-polyacrylamide gels under non-reducing conditions according to the method of Laemmli (24). After electrophoresis, proteins were immunoblotted on nitrocellulose sheets for 4 h at 1 mA/cm^2 in 25 mM Tris/HCl, pH 8.6, 190 mM glycine, 0.1% SDS, 20% methanol. The sheets were incubated overnight at 4°C in 10 mM Tris/HCl, pH 7.4, 150 mM NaCl, 1% BSA, 0.05% Tween 20. The sheets were then incubated with either 1 µg/ml rabbit anti-human u-PA IgG, 1000x diluted goat anti-human kallikrein antiserum, 1000x diluted goat anti-human factor XII antiserum, 1 µg/ml goat anti-human factor XI IgG or 1000x diluted goat anti-human high molecular weight kininogen in 10 mM Tris/HCl, pH 7.4, 150 mM NaCl, 0.05% Tween 20 for 2 h at RT. Subsequently, sheets treated with rabbit IgG were incubated with 10,000x diluted polyclonal goat anti-rabbit IgG peroxidase. Sheets treated with goat antiserum or goat IgG were incubated with 10,000x diluted polyclonal rabbit anti-goat IgG peroxidase. Proteins were visualized with BM blue Peroxidase substrate.

SDS-PAGE and fibrin zymography

Samples were run on SDS-polyacrylamide gels under non-reducing conditions. After electrophoresis, gels were washed for 2 h at 37°C with 2.5% Triton X-100 and subsequently transferred to plasminogen-rich fibrin-agarose underlays to measure the fibrinolytic activity of the proteins (25).

Isolation of contact activation-dependent PAA by extraction of SDS-PAGE slices

Samples were run on a 7.5% SDS-polyacrylamide gel under non-reducing conditions. After electrophoresis, slices were cut using a razor and proteins were eluted for 3 h in 50 mM Tris/HCl, pH 7.4, 100 mM NaCl, 0.1% Tween 80 at room temperature.

Results

In general, the fibrinolytic activity that was observed during the isolation procedures in the fibrin microtiter plate or on fibrin zymography was plasminogen-dependent, since no activity was found when plasminogen-depleted fibrinogen was used in the assays.

Fractionation of plasma

Contact activation-dependent PAA originated during the fractionation of plasma in the presence of DXS. About 10–25% of the contact activation-dependent PAA was recovered after the ammonium sulphate precipitations (data not shown), suggesting that a significant amount of this fibrinolytic activity was lost during these procedures. After Protein A-Sepharose chromatography contact activation-dependent PAA was completely recovered.

Gelfiltrations

The recovery of contact activation-dependent PAA after gelfiltration varied between 17 and 86%, depending on the conditions used (Table 2). Since the recovery of total protein was close to 100% for all runs (Table 2), this variation could probably not be explained by a loss of protein.

Table 2. Recovery of contact activation-dependent PAA and of protein after gelfiltration of fractionated plasma on a Sephacryl S-300 HR column under various conditions (Table 1), expressed as a percentage of the amount of total contact activation-dependent PAA or protein that was applied to the column, respectively.

run (no)	recovery contact activation-dependent PAA (%)	recovery protein (%)
1	18	100
2	52	96
3	86	90

Gelfiltration in 1 M NaCl

After gelfiltration of fractionated plasma on the Sephacryl S-300 HR column in 1 M NaCl (run 1, Figure 1), only 17.5% of contact activation-dependent PAA could be recovered. Contact activation-dependent PAA was found to elute as one peak around an apparent molecular weight (M_r) of 250-300 kD (fractions 20-30) and co-eluted with kallikrein activity. In these fractions little FXIIa activity could be measured, and the peak of FXIIa activity was found to elute at a much lower molecular weight (M_r 36 kD). On Western blot, a band of about 140 kD immunoreactive with rabbit anti-human u-PA IgG was detected in the fractions containing contact activation-dependent PAA

(Figure 1, *insert*). This band most likely corresponded to the 110 kD protein with u-PA-related antigenic determinants described by Binnema *et al.* (16,17).

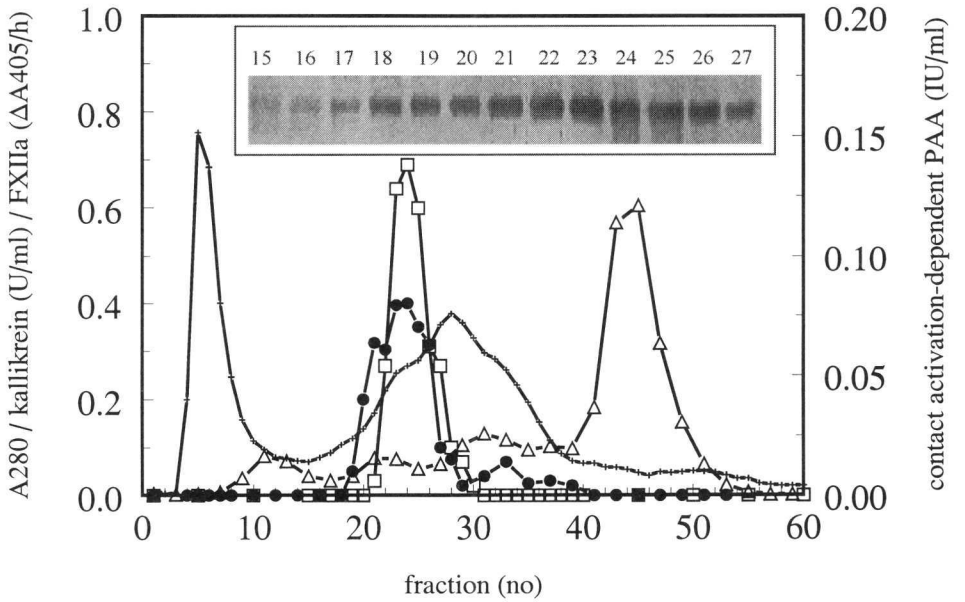

Figure 1. Gelfiltration of fractionated plasma (6.7 ml) on a Sephacryl S-300 HR column in 10 mM sodium phosphate buffer, pH 7.4, 1 M NaCl (run 1, Table 1). Collection of fractions was started after 130 ml and per fraction 2.5 ml was collected. In the fractions A280 (+), kallikrein activity (□), contact activation-dependent PAA (•) and FXIIa activity (Δ) was measured. The *insert* shows a 140 kD band immunoreactive with rabbit anti-human u-PA IgG on Western blot in the fractions containing contact activation-dependent PAA.

To examine whether kallikrein could be separated from the contact activation-dependent PAA, as has been described by Kluft *et al.*(14), fractions of the contact activation dependent-PAA-peak were pooled and applied to an anion exchanger column. Contact activation-dependent PAA was again found to co-elute with kallikrein activity (Figure 2), suggesting that kallikrein accounted for the activity. However, on fibrin zymography a major lysis zone was observed around 170 kD in the contact activation-dependent PAA peak (Figure 2, *insert*), and no lysis was found at the expected position of kallikrein (88 kD). Neither contact activation-dependent PAA in the fibrin microtiter plate nor fibrinolytic activity on fibrin zymography could be inhibited by 75 µg/ml goat anti-human

kallikrein IgG, suggesting that the presence of another plasminogen activator cannot be excluded. The 140 kD band immunoreactive with rabbit anti-human u-PA IgG eluted at a higher salt concentration around fraction 24 and 25 (data not shown), which contained only low contact activation-dependent PAA. In these fractions, lysis around 135 kD was observed on fibrin zymography (Figure 2, *insert*). These findings indicated that the 140 kD band immunoreactive with rabbit anti-human u-PA IgG is not associated with the major contact activation-dependent PAA peak during anion exchange chromatography.

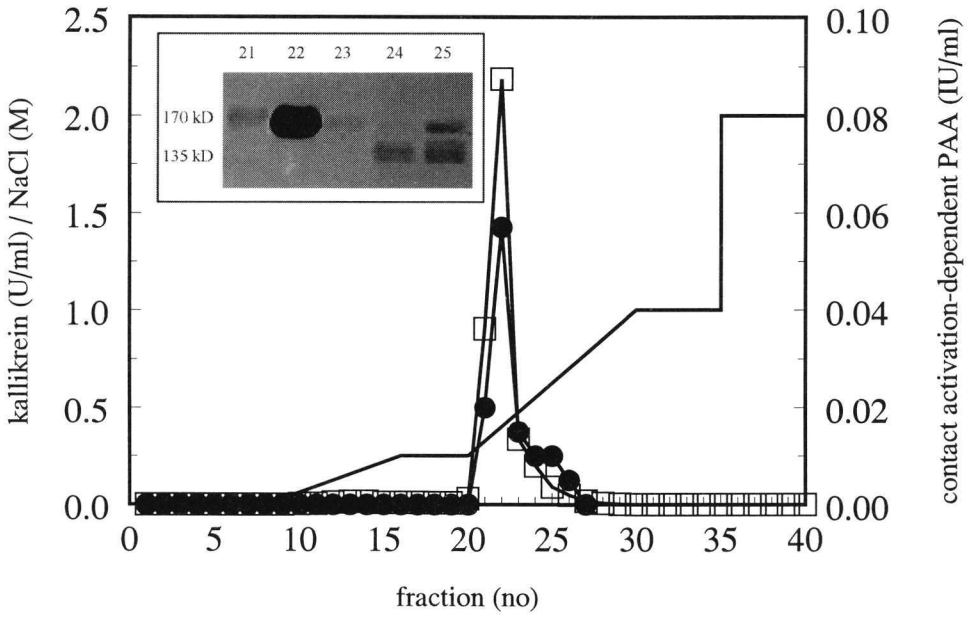

Figure 2. Anion exchange chromatography of a pool of Sephacryl S-300 HR fractions containing contact activation-dependent PAA (Figure 1). Fractions 21-28 of the contact activation-dependent PAA peak in Figure 1 were pooled and 5.7 ml of the pool was run on a MonoQ HR 5/5 column. Proteins were eluted from the column using a stepwise gradient from 0 to 2 M NaCl. Per fraction 1 ml was collected. In the fractions kallikrein activity (□) and contact activation-dependent PAA (●) was measured. The solid line represents the NaCl-gradient. The *insert* shows fibrinolytic activity of fractions 21-25 on fibrin zymography after 36 h incubation at 37°C.

Gelfiltration in 0.1 M NaCl

In the study of Binnema *et al.*, gelfiltration was performed in PBS in order to isolate the factor XII-dependent plasminogen activator (16). To examine whether the ionic strength would affect the recovery of contact activation-dependent PAA after gelfiltration, a run on the Sephacryl S-300 HR column was performed at low ionic strength (run 2, Tables 1 and 2). Two additional peaks of contact activation-dependent PAA were found besides the peak that co-eluted with kallikrein (M_r 345 kD, fractions 17-25, recovery 22.0%) (Figure 3). The first peak was found in the void volume of the column (M_r > 1000 kD, fractions 5-10, recovery 16.4%). In this peak, low kallikrein and FXIIa activity was found, suggesting that these enzymes are not involved in this activity. The 140 kD band immunoreactive with rabbit anti-human u-PA IgG on Western blot was detected both in the contact activation-dependent PAA peak in the void volume as well as in the peak that co-eluted with kallikrein (data not shown). The contact activation-dependent PAA peak in the void volume may be ascribed to a complex of components that were separated during gelfiltration in 1 M NaCl.

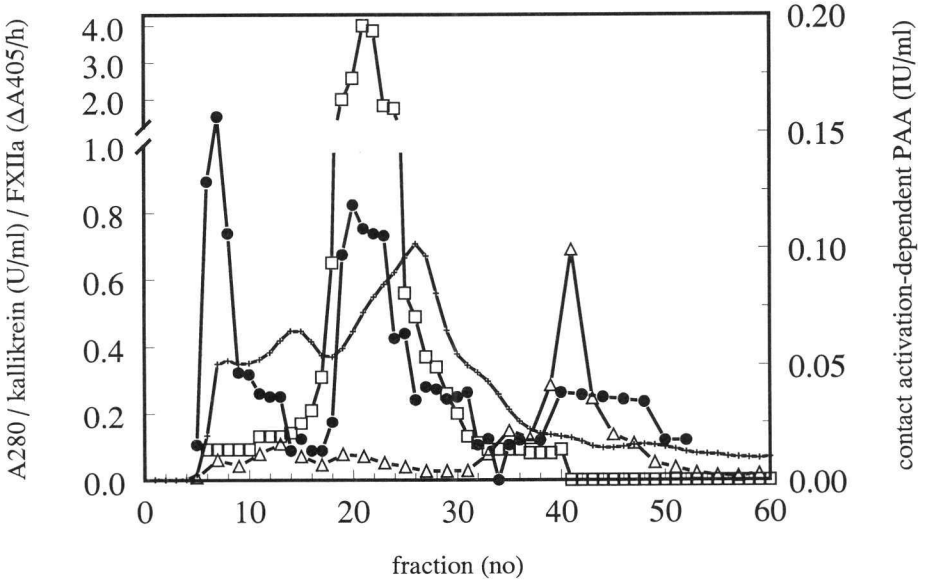

Figure 3. Gelfiltration of fractionated plasma (4.9 ml) on a Sephacryl S-300 HR column in 50 mM Tris/HCl, pH 7.4, 0.1 M NaCl (run 2, Table 1). Collection of fractions was started after 130 ml and per fraction 2.5 ml was collected. In the fractions A280 (+), kallikrein activity (□), contact activation-dependent PAA (•) and FXIIa activity (Δ) was measured.

The second additional peak co-eluted more or less with the FXIIa activity peak (M_r 40 kD, fractions 35-50, recovery 13.3%). On Western blot a doublet with a molecular weight of 30 and 28.5 kD was found (Figure 4), which most likely can be ascribed to β-FXIIa, the smallest active factor XII fragment (3,4,6). To examine whether β-FXIIa was responsible for the contact activation-dependent PAA in the FXIIa activity peak, contact activation-dependent PAA in these fractions was analyzed in the presence of goat anti-human factor XII IgG. Contact activation-dependent PAA could be completely inhibited by anti-human factor XII IgG (data not shown), indicating that β-FXIIa indeed accounted for this fibrinolytic activity.

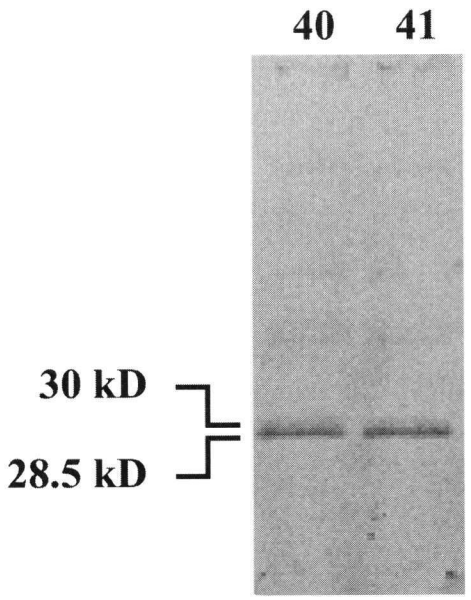

Figure 4. Western blot analysis of fraction 40 and 41 of the Sephacryl S-300 HR gelfiltration run in 0.1 M NaCl (Figure 3) using goat anti-human factor XII antiserum. A doublet of β-FXIIa was found at 30 and 28.5 kD.

Effect of dextran sulphate on contact activation-dependent PAA

The recovery of contact activation-dependent PAA after gelfiltration on the Sephacryl S-300 HR column was poor in both runs (Table 1), suggesting that activity was lost during gelfiltration. An explanation may be that separation of components during gelfiltration resulted in a decrease of PAA. Recently, we demonstrated that plasminogen activation by 80 kD FXIIa is potentiated by

dextran sulphate (DXS) (26). Since a substantial amount of DXS was added to plasma during the fractionation procedure, it was hypothesized that before gelfiltration DXS potentiated contact activation-dependent PAA. During gelfiltration DXS may have been separated from 80 kD FXIIa or other possible plasminogen activators, resulting in a loss of contact activation-dependent PAA. To test this hypothesis, the effect of DXS on contact activation-dependent PAA was studied.

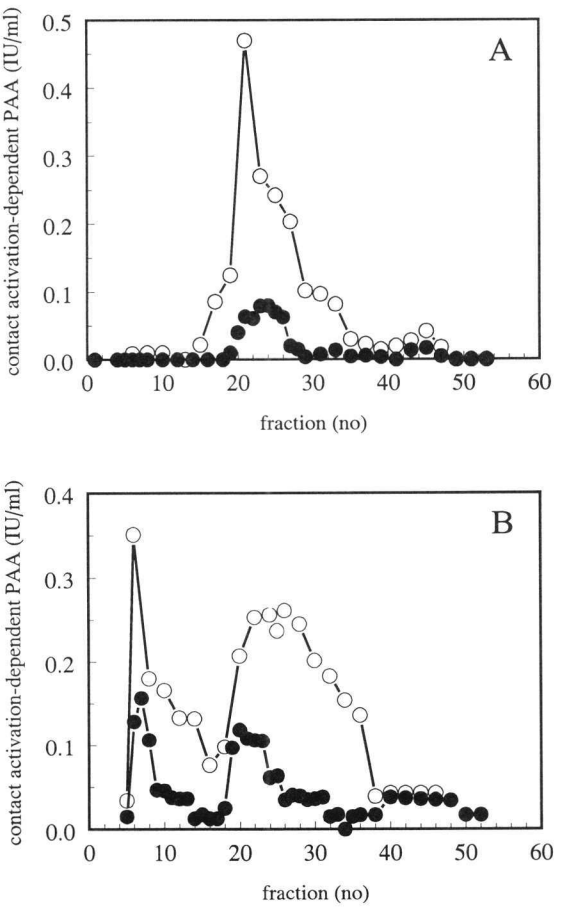

Figure 5. Effect of DXS on contact activation-dependent PAA in fractions of the Sephacryl S-300 HR gelfiltration runs in 1 M NaCl (A) and in 0.1 M NaCl (B) (see Figures 1 and 3, respectively). Contact activation-dependent PAA was measured in the absence (•) and presence (○) of 5 μg/ml DXS.

DXS potentiated contact activation-dependent PAA in the fractions of both gelfiltration runs (Figure 5). In the run in 1 M NaCl (Figure 5A), potentiation of contact activation-dependent PAA was found in the peak that co-eluted with kallikrein (fractions 20-30), but hardly in the fractions containing β-FXIIa (fractions 40-50). In the run in 0.1 M NaCl (Figure 5B), potentiation of contact activation-dependent PAA was found in fractions 5-40, including the void peak and the peak that co-eluted with kallikrein, but also not in the fractions containing β-FXIIa (Figure 5B). The fact that PAA of β-FXIIA was not potentiated by DXS is in agreement with our earlier observations (26). In all contact activation-dependent PAA containing fractions that were potentiated by DXS, 80 kD FXIIa was found on Western blot (data not shown). Since 80 kD FXIIa was present in all contact activation-dependent PAA containing fractions that were potentiated by DXS and since PAA of 80 kD FXIIa is known to be potentiated by DXS (26), this plasminogen activator may contribute to a substantial amount of the DXS-potentiated contact activation-dependent PAA.

From these results it was concluded that the low recovery of contact activation-dependent PAA after gelfiltration may be ascribed to separation of at least one plasminogen activator from its cofactor DXS. The possible role of FXIIa in contact activation-dependent PAA was studied in more detail by performing another gelfiltration run in 1.6 M KSCN. Complexes of proteins are expected to dissociate in 1.6 M KSCN, which should result in a sharp elution profile of 80 kD FXIIa.

Gelfiltration in 1.6 M KSCN

After gelfiltration of fractionated plasma on a Sephacryl S-300 HR column in 1.6 M KSCN, three peaks of contact activation-dependent PAA were found (Figure 6). One peak was found right after the void volume of the column (fractions 5-15, recovery 5.9%). In this peak no kallikrein activity and only low FXIIa activity was present, while the 140 kD band immunoreactive with rabbit anti-human u-PA IgG was observed. The second peak was located at the same position as the kallikrein activity peak (fractions 25-35, recovery 62%). This peak contained low FXIIa activity and only low amounts of the 140 kD band immunoreactive with rabbit anti-human u-PA IgG were observed. The third peak co-eluted with the FXIIa activity peak (fractions 35-50, recovery 17.9%). Contact activation-dependent PAA in these fractions was completely inhibited by goat anti-human factor XII IgG, while on Western blot a doublet of β-FXIIa was found (data not shown), confirming the results obtained in run 2.

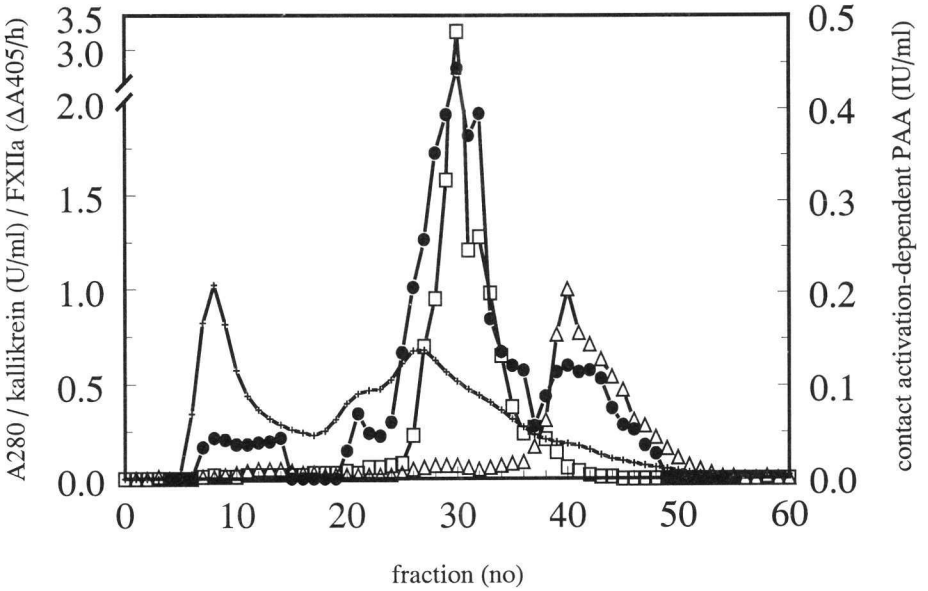

Figure 6. Gelfiltration of fractionated plasma (7.4 ml) on a Sephacryl S-300 HR column in 50 mM Tris/HCl, pH 7.4, 1.6 M KSCN (run 3, Table 1). Collection of fractions was started after 125 ml and per fraction 2.5 ml was collected. In the fractions A280 (+), kallikrein activity (□), contact activation-dependent PAA (•) and FXIIa activity (Δ) was measured.

Potentiation of contact activation-dependent PAA by DXS was found in fractions 5-25, but not in the β-FXIIa peak (Figure 7). On Western blot, sharp elution of 80 kD FXIIa was found in fractions 25-35 (data not shown), which also contained kallikrein activity. Surprisingly, contact activation-dependent PAA in these fractions was not potentiated by DXS. This was explained by the fact that during the incubation period used in the fibrin microtiter plate assay, 80 kD FXIIa appeared to be completely cleaved into smaller factor XII fragments in the presence of DXS (data not shown). Cleavage of 80 kD FXIIa into smaller factor XII fragments was most likely caused by kallikrein present in these fractions and PAA of these smaller fragments is not potentiated by DXS (26). These results indicated that neither kallikrein nor 80 kD FXIIa was responsible for the observed DXS-potentiated contact activation-dependent PAA in fractions 5-25.

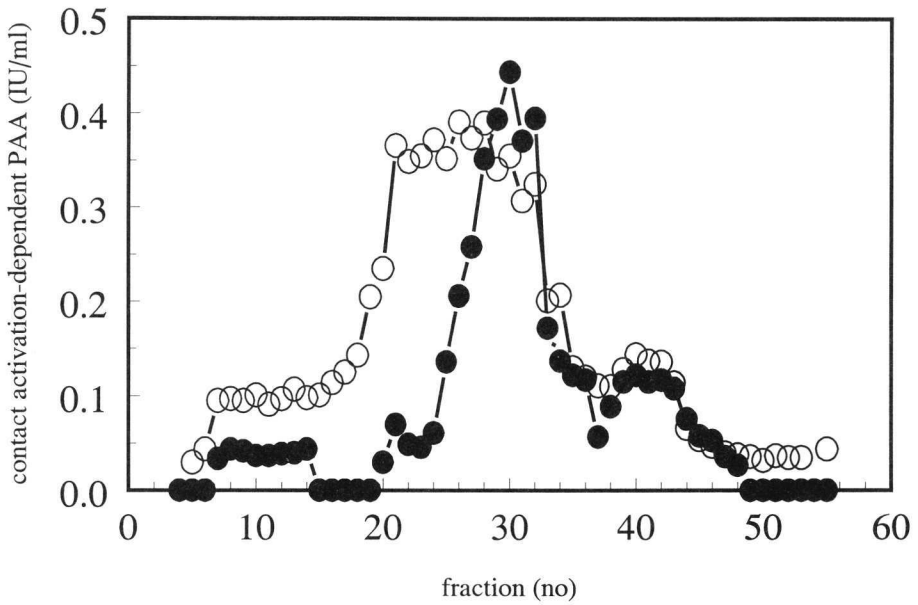

Figure 7. Effect of DXS on contact activation-dependent PAA in fractions of the Sephacryl S-300 HR gelfiltration runs in 1.6 M KSCN (see Figure 6). Contact activation-dependent PAA was measured in the absence (•) and presence (○) of 5 μg/ml DXS.

Characterization of DXS-potentiated contact activation-dependent PAA

To identify the plasminogen activator responsible for the DXS-potentiated contact activation-dependent PAA, pools were made of gelfiltration fractions of the run in 1.6 M KSCN and analysed on fibrin zymography (Figure 8). In the fractions of the void volume peak (pool 8-12), a high molecular weight band > 210 kD was found. In pool 20-24 and pool 25-29, lysis was observed around 170 kD, as well as a weaker lysis zone around 135 kD. In pool 25-29, lysis by kallikrein (88 kD) and by FXIIa (80 kD) was only observed after a longer incubation period (data not shown). In pool 39-43, lysis was found around 30 kD, corresponding to β-FXIIa.

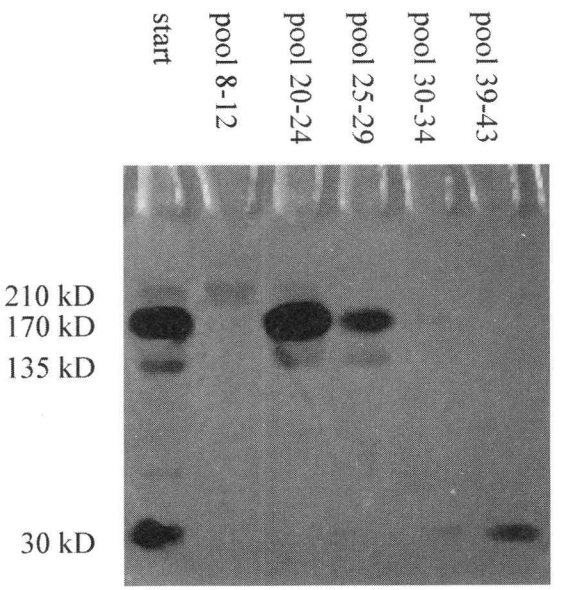

Figure 8. Fibrin zymography of pools of fractions of the Sephacryl S-300 HR gelfiltration run in 1.6 M KSCN (Figure 6) after overnight incubation at 37°C. The sample applied to the Sephacryl S-300 HR column was also included ('start').

It is known that enzyme/inhibitor complexes after SDS-PAGE can be active on fibrin zymography. To test whether the 170 kD lysis zone on fibrin zymography observed in pool 20-24 could be explained by an enzyme/inhibitor complex, proteins were isolated by extraction of SDS-PAGE slices and tested for DXS-potentiated contact activation-dependent PAA. Slices containing the 170 kD lysis zone also appeared to express DXS-potentiated contact activation-dependent PAA on the fibrin microtiter plate (data not shown), indicating that the involvement of an enzyme/inhibitor complex may not be likely. Contact activation-dependent PAA in pool 20-24 in the absence or presence of DXS could not be inhibited by anti-FXII or anti-FXI antibodies (Figure 9).

On Western blot, no proteins immunoreactive with goat anti-human factor XII antiserum, goat anti-human kallikrein antiserum or rabbit anti-human u-PA IgG were observed in pool 20-24. In contrast, immunoreactivity was observed with goat anti-human high molecular weight kininogen and with goat anti-human factor XI IgG (data not shown).

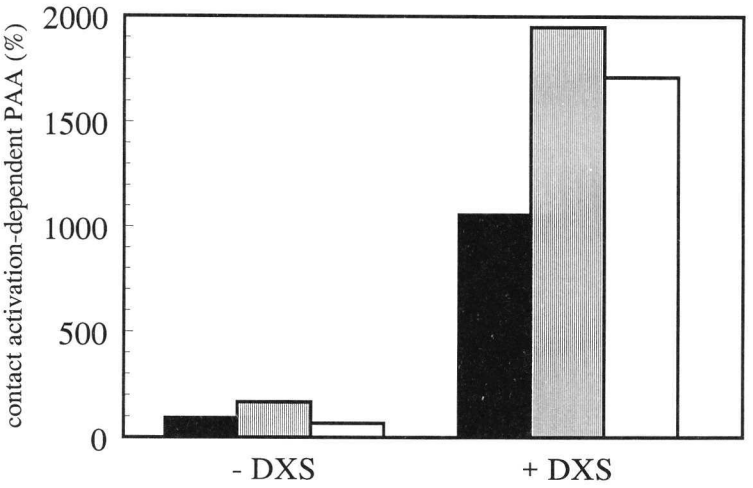

Figure 9. Effect of 500 µg/ml goat anti-human FXII IgG (hatched bars) or 250 µg/ml goat anti-human FXI IgG (open bars) on contact activation-dependent PAA in pool 20-24 of the S-300 HR gelfiltration run in 1.6 M KSCN (Figure 6) in the absence and presence of 5 µg/ml DXS. Contact activation-dependent PAA was also measured in the absence of antibody (solid bars) and was expressed as a percentage of contact activation-dependent PAA measured in the absence of antibody and DXS.

Discussion

In this study, we have tried to isolate and characterize an unidentified factor XII-dependent plasminogen proactivator that has been described in plasma (2,14). During the isolation procedures, various components related to contact activation-dependent PAA were observed and contact activation-dependent PAA was divided into several peaks after gelfiltration, depending on the conditions used.

In all gelfiltration runs performed, kallikrein was found to co-elute with a peak of contact activation-dependent PAA. Further analysis using anion exchange chromatography of the contact activation-dependent PAA peak recovered after the gelfiltration run in 1 M NaCl showed that contact activation-dependent PAA remained associated with kallikrein activity (Figure 2), suggesting that kallikrein is the responsible plasminogen activator. However, the presence of

another plasminogen activator cannot be excluded since on fibrin zymography lysis was found at 170 kD and not at the expected position for kallikrein (88 kD). In addition, contact activation-dependent PAA could not be inhibited by goat anti-human kallikrein IgG, although this may be ascribed to the high concentration of kallikrein in plasma (35-50 µg/ml). Kluft *et al.* have also described that kallikrein co-elutes with a contact activation-dependent PAA peak after gelfiltration (14). This activity could be divided in activity by kallikrein and activity by an unknown plasminogen activator distinct from kallikrein, pointing to the presence of another component.

The plasminogen activator responsible for the contact activation-dependent PAA peak around M_r 30 kD observed during the runs in 0.1 M NaCl and 1.6 M KSCN was identified as β-FXIIa. Sidelmann *et al.* have described a 30 kD protein involved in plasminogen activation in a DEF of plasma (18). This 30 kD plasminogen activator was absent in FXII-deficient plasma, but no further identification was performed. From the results of our study it can be concluded that β-FXIIa is the most likely candidate for this 30 kD plasminogen activator.

In the void volume of the column, a contact activation-dependent PAA peak was found when gelfiltration was performed in 0.1 M NaCl, but not in 1 M NaCl. This is in agreement with the studies of Kluft *et al.* and Binnema *et al.* (14,16). The void peak was also found when gelfiltration was performed in 1.6 M KSCN. No significant kallikrein- or FXIIa-related activity or antigen could be detected, indicating that these plasminogen activators do not contribute to the activity in this peak. In the void peak, a 140 kD band immunoreactive with rabbit anti-human u-PA IgG was observed, most likely corresponding to the 110 kD protein with u-PA-related antigenic determinants described by Binnema *et al.* (16,17). Since elution of this component was found in the void volume, the 140 kD protein was most likely complexed to other proteins. In contrast, after gelfiltration in 1 M NaCl, no contact activation-dependent PAA peak and no 140 kD protein was found in the void volume, suggesting dissociation of complexes. These observations are in agreement with Binnema *et al.* (16). Instead, the 140 kD protein was found to co-elute at a lower M_r with kallikrein activity and contact activation-dependent PAA. However, the 140 kD protein was separated from the main contact activation-dependent PAA peak during anion exchange chromatography, implying that this component is of minor importance as a plasminogen activator. Although the 140 kD protein seems to be associated with contact activation-dependent PAA to some extent, its role is not clear. The 140

kD protein itself might exert little PAA, and complexing to an unidentified cofactor may potentiate its PAA.

Another component involved in contact activation-dependent PAA was discovered when the effect of DXS was studied. DXS appeared to potentiate contact activation-dependent PAA that could not be ascribed to 80 kD FXIIa or kallikrein, suggesting the presence of (an)other plasminogen activator(s) that need(s) DXS as a cofactor. The effect of DXS was more prominent in the fractions of the gelfiltration run in 1 M NaCl than in fractions of the gelfiltration run in 0.1 M NaCl, supporting the hypothesis that complexes between a plasminogen activator and its cofactor were dissociated at high ionic strength. Further analysis of DXS-potentiated contact activation-dependent PAA demonstrated the presence of a 170 kD band on fibrin zymography, which was also able to activate plasminogen in the fibrin microtiter plate in the presence of DXS. This band may be similar to the band observed in the contact activation-dependent PAA peak after anion exchange chromatography (Figure 2, *insert*). The 170 kD protein may therefore contribute to the contact-activation dependent PAA that co-eluted with kallikrein. During the fractionation of plasma, the 170 kD band on fibrin zymography originated after the treatment of plasma with DXS (data not shown), suggesting that this protein is also a factor XII-dependent plasminogen proactivator.

The nature of the 170 kD protein remains to be elucidated. Plasminogen activators like t-PA (70 kD), u-PA (55 kD), FXIIa (80 kD) and kallikrein (88 kD) most likely can be excluded based on their molecular weight and the results of this study. In the fractions containing the 170 kD protein, immunoreactivity was observed with anti-factor XI antibody and anti-high molecular weight kininogen, but the contact activation-dependent PAA could not be inhibited by anti-factor XI antibody. Factor XIa (140-160 kD) has been described to activate plasminogen (10,12), and its role in the results of this study needs further research, as well as the role of high molecular weight kininogen. The physiological importance of factor XI as a plasminogen activator will be minor, since deficiencies of factor XI are associated with bleeding disorders and not with thrombotic tendencies as found for factor XII and prekallikrein deficiencies (27-29).

Complexes between proteins may provide another explanation for the 170 kD protein. Active complexes between contact activation system enzymes and inhibitors have been demonstrated on fibrin zymography. Tsuda *et al.* (30) have described complexes of FXIIa with C1-inhibitor (210 kD) and with α_2-antiplasmin (145 kD) in dextran sulphate-activated plasma, while Hauert *et al.* (31)

have identified a complex between kallikrein and C1-inhibitor (190 kD) in a DEF of normal plasma. These complexes may correspond to the lysis bands observed in this study (Figure 7). Enzym/inhibitor complexes are not expected to be active in the fibrin microtiter plate, as was found for the 170 kD protein after elution of SDS-PAGE slices, but hypothetically DXS may potentiate PAA of these complexes. In addition, Sidelmann *et al.* have described complexes between plasminogen activators and DXS (18). Such complexes may be active in the fibrin microtiter plate and be potentiated by the addition of extra DXS.

After gelfiltration in 1 M NaCl only the contact activation-dependent PAA peak that co-eluted with kallikrein was found, while in 0.1 M NaCl the two other peaks were detected as well. An explanation may be that complexes of proteins remain intact at low ionic strength. This may result in contact activation-dependent PAA of a complex of proteins (void peak) and in further cleavage of 80 kD FXIIa into β-FXIIa (β-FXIIa peak). It was expected that in 1.6 M KSCN, a strong dissociating milieu, complexes of proteins would not remain intact. However, the recovery of contact activation-dependent PAA was high and three peaks of contact activation-dependent PAA were found similar to the peaks found in the run in 0.1 M NaCl, a phenomenon which cannot be explained at the moment.

It is concluded from this study that contact activation-dependent PAA as measured in a DEF can be ascribed to several plasminogen activators. Isolation of these components is very complicated. During the procedures, plasminogen activators can be dissociated from cofactors resulting in a loss of contact activation-dependent PAA and thus in low recoveries of contact activation-dependent PAA after plasma fractionation and gelfiltration. Due to enzymatic effects during the isolation, properties of plasminogen activators can change. For instance, the cleavage of 80 kD FXIIa into β-FXIIa results in a loss of DXS-potentiated contact activation-dependent PAA. On fibrin zymography, it is difficult to discriminate between active enzymes and active complexes between enzymes and inhibitors. In addition, identification of the plasminogen activators is hampered by the fact that due to their high plasma concentrations factor XII (30-40 μg/ml), prekallikrein (35-50 μg/ml) and factor XI (2-7 μg/ml) can hardly be inhibited by antibodies. In this way, the involvement of these components cannot easily be established or excluded.

In conclusion, unidentified contact activation-dependent plasminogen activation in plasma cannot simply be ascribed to the existence of a single factor XII-dependent plasminogen activator.

Extensive research will be needed to identify the components that contribute to contact activation-dependent fibrinolysis.

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CHAPTER 10

Summary and General Conclusions

Summary

The haemostatic balance between coagulation and fibrinolysis regulates blood fluidity under physiological conditions and prevents blood loss when the vessel wall is injured. Several mechanisms that link coagulation and fibrinolysis are known. In this thesis, two specific links are investigated, namely the inactivation of scu-PA by thrombin and the contact activation system.

Inactivation of scu-PA by thrombin

Recent experiments using knock-out mice models have demonstrated that u-PA plays an important role in fibrinolysis (1,2). By the inactivation of scu-PA, thrombin may therefore regulate fibrinolysis. In order to analyse the occurrence of the inactivation of scu-PA by thrombin *in vivo*, a sensitive and specific bioimmunoassay (BIA) was developed in **Chapter 2** for the assessment of tcu-PA/T, the molecule that originates from the inactivation of scu-PA by thrombin (3). In this assay, urokinase antigen from human body fluids was immuno-immobilized in microtiter plates and subsequently treated with cathepsin C. This lysosomal enzyme cleaves the two N-terminal residues of the B-chain of tcu-PA/T, resulting in the re-activation of tcu-PA/T (4). Cathepsin C does not affect the activity of scu-PA or tcu-PA and in this way tcu-PA/T can be discriminated from these two u-PA forms. The BIA was used to examine the occurrence of tcu-PA/T in the plasma of healthy individuals and of sepsis patients, and in the synovial fluid of rheumatoid arthritis (RA) patients. In the plasma of the healthy individuals, no tcu-PA/T could be detected above the detection limit of the assay (0.2 ng/ml), suggesting that the concentration of physiologically circulating thrombin is not sufficient to induce the inactivation of scu-PA (5-7). In the plasma of almost all sepsis patients tcu-PA/T could be detected, most likely due to endotoxin-mediated thrombin generation in these patients (8). Substantial amounts of tcu-PA/T were found in the synovial fluid of the RA patients and tcu-PA/T accounted for about 47% of total urokinase antigen. These high levels may be explained by extravascular thrombin generation and by the presence of thrombomodulin on synovial cells, which may accelerate the inactivation (9-11). Thus, the inactivation of scu-PA by thrombin can take place *in vivo* under pathological conditions that involve the production of large amounts of thrombin.

The inactivation of scu-PA by thrombin is accelerated by the cofactor thrombomodulin in a purified system (12). **Chapter 3** consists of an investigation on whether thrombomodulin can also

potentiate this process in a plasma milieu. Citrated pooled plasma was treated with thrombin in the absence and presence of thrombomodulin. After the incubation, levels of tcu-PA/T were measured in the BIA. Due to the presence of antithrombin and other thrombin inhibitors, far more thrombin was needed to induce the inactivation of scu-PA in a plasma milieu than in a purified system. Thrombomodulin accelerated the inactivation of scu-PA by thrombin in plasma about fourfold, which was less than the acceleration found in a purified system (12). This difference could also partially be ascribed to the fast inhibition of the thrombin/thrombomodulin complex by antithrombin, suggesting that this inhibitor plays a significant role in the regulation of the inactivation of scu-PA by thrombin in plasma. Recalcification of plasma in the absence of thrombin and thrombomodulin resulted in strong inactivation of scu-PA by endogenously generated thrombin. In factor XII- and factor XI-deficient plasma, no calcium-induced inactivation of scu-PA was observed, indicating that the inactivation was mediated by endogenous thrombin that was generated via activation of the intrinsic pathway of coagulation. In the presence of thrombomodulin, calcium-induced inactivation of scu-PA was completely abolished. It was hypothesized that thrombomodulin formed a complex with endogenously generated thrombin and prevented further thrombin generation by the activation of the protein C pathway. Indeed, in the plasma of a patient heterozygous for protein C deficiency, thrombomodulin did not inhibit the calcium-induced inactivation of scu-PA and potentiated this process instead. Thrombomodulin thus seems to play a dual role in the inactivation of scu-PA by thrombin in plasma. It can either potentiate the activity of thrombin towards scu-PA or inhibit the inactivation of scu-PA during thrombin generation via activation of the protein C pathway. The net effect of thrombomodulin *in vivo* is unclear and will depend on a balance between several coagulation components, especially in the microcirculation where the local concentration of endothelial thrombomodulin is very high. Disturbance of this balance, for instance due to resistance to APC, can shift the effect towards the potentiation of the inactivation of scu-PA. The inactivation of scu-PA by thrombin may therefore contribute to the thrombotic tendency observed in patients with APC resistance (13-15).

It has been reported that fibrinolysis is downregulated by additional thrombin that is generated via feedback activation of factor XI by thrombin (16). The activation of TAFI has been postulated as a potential mechanism for this phenomenon (17,18). The findings described in **Chapter 3** showed that in plasma, high concentrations of thrombin are necessary for the inactivation of scu-PA, comparable to the amounts needed for the antifibrinolytic effect of factor XI (16), and that the

inactivation of scu-PA may depend on thrombin that is additionally generated via factor XI. The inactivation of scu-PA may therefore provide an additional explanation for the factor XI-dependent downregulation of fibrinolysis (**Appendix to Chapter 3**).

The occurrence of the inactivation of scu-PA by thrombin *in vivo* was studied in more detail in **Chapter 4**. Levels of tcu-PA/T were assessed in the plasma of patients with a varying degree of hypercoagulability. The four groups studied consisted of patients with disseminated intravascular coagulation (DIC), endotoxin-treated volunteers, patients with unstable angina pectoris, and patients selected for hip replacement. tcu-PA/T was only observed in the plasma of the DIC-patients and was associated with levels of F1+2, TAT, antithrombin, urokinase antigen and scu-PA, indicating that inactivation of scu-PA in the circulation depends on thrombin generation and on the amount of u-PA available. In addition, F1+2 and urokinase antigen were independent predictors of tcu-PA/T. In DIC, inadequate fibrinolysis is observed which is ascribed to an increased production of PAI-1 (19,20). The decreased fibrinolytic potential results in fibrin deposition, followed by multiple organ failure (19,20). The inactivation of scu-PA by thrombin may also contribute to a decreased fibrinolytic potential. In the other groups tested, no tcu-PA/T was observed above the detection limit due to lower levels of thrombin generation, higher levels of antithrombin or lower levels of urokinase antigen. The inactivation of scu-PA *in vivo* thus not only seems to depend on the generation of thrombin but also on the control of thrombin activity by inhibitors like antithrombin. The regulatory role of antithrombin was also observed in the study performed in **Chapter 3**. Decreased levels of antithrombin may therefore promote the inactivation of scu-PA by thrombin, which may contribute to the thrombotic tendency associated with antithrombin deficiency (21,22).

In **Chapter 5**, the inactivation of scu-PA by thrombin was studied in an extravascular milieu. Levels of tcu-PA/T were measured in the synovial fluid (SF) of patients with RA and osteoarthritis (OA) and of controls. It was found that more than 40% of total urokinase antigen in the SF of the RA patients could be ascribed to tcu-PA/T, confirming the results of **Chapter 2**. In contrast, in the SF of the OA patients and of the controls the contribution of tcu-PA/T to total urokinase antigen was only minor. Busso *et al.* recently demonstrated the exacerbation of arthritis in u-PA-deficient mice due to a decrease of fibrin removal, suggesting that u-PA plays a beneficial role in arthritis by mediating fibrinolysis (23). Elevated levels of u-PA have been reported in the synovial fluid and tissue of RA patients (24,25). However, the increased production of u-PA cannot prevent the excessive articular deposition of fibrin in RA (9). This discrepancy can at least partially be

explained by the fact that a large amount of scu-PA is inactivated by thrombin in RA-affected joints and can therefore not contribute to fibrinolysis. In this way, fibrinolysis is hampered and inflammation in RA is sustained.

Binding of u-PA to the urokinase receptor plays a role in extracellular proteolysis as well as in fibrinolysis at the cell surface (2,26). Therefore, inactivation of urokinase receptor-bound scu-PA by thrombin may affect these processes. In **Chapter 6**, it was found that thrombin was able to inactivate scu-PA that was bound to HUVEC. The major part of the binding of scu-PA to HUVEC was mediated via the urokinase receptor, indicating that binding of scu-PA to the urokinase receptor offers no protection against inactivation, as has been claimed for the binding of scu-PA to soluble urokinase receptor by Wilhelm *et al.* (27). The results point to the presence of cofactors like thrombomodulin or glycosaminoglycans, as Wilhelm *et al.* demonstrated that the protective effect of soluble urokinase receptor is abolished in the presence of thrombomodulin. In addition, the inactivation of cell-bound scu-PA by thrombin was very efficient (**Chapter 6**), which also points to the presence of a cofactor. Further research is needed to examine the possible involvement of thrombomodulin or other cofactors in the inactivation of cell-bound scu-PA by thrombin.

Thrombomodulin contains six epidermal growth factor (EGF)-like domains, which are involved in the binding of thrombin and the cofactor activity (28). It has been demonstrated that the EGF-like domains of thrombomodulin essential for the activation of protein C and TAFI by thrombin are distinct. EGF-like domains 4-6 plus the connecting domain between EGF-like domain 3 and 4 are critical for the activation of protein C (29-31), while for the activation of TAFI EGF-like domains 3-6 are needed (32-34). It is not known which EGF-like domains are involved in the acceleration of the inactivation of scu-PA by thrombin. In a study by De Munk *et al.*, it was demonstrated that the chondroitin sulphate on thrombomodulin plays an important role in the acceleration of the inactivation of scu-PA by thrombin (35). This glycosaminoglycan provides an additional binding site for thrombin, besides the primary binding site located in EGF-like domains 5 and 6. The EGF-like domains required for the chondroitin sulphate-independent acceleration of the inactivation of scu-PA by thrombin were identified in **Chapter 7** using various forms of thrombomodulin. Thrombomodulin containing chondroitin sulphate stimulated the inactivation of scu-PA about 35-fold, while recombinant thrombomodulin lacking this glycosaminoglycan showed about 3-fold stimulation, confirming the results of the study of De Munk *et al.* (35). It was found that EGF-like domains 5 and 6 were sufficient for the enhancement in the rate of chondroitin sulphate-

independent inactivation of scu-PA by thrombin. EGF-like domains 5 and 6 bind to the anion exosite I of thrombin, in this way inducing a conformational change that alters the specificity of thrombin (36,37). The mechanism of the accelerating effect of thrombomodulin on the inactivation of scu-PA by thrombin thus appears to differ from that of the accelerating effect on the activation of protein C and TAFI, which both need an additional domain or domains in addition to the EGF-like domains 5 and 6 in order to be efficiently activated by the thrombin/thrombomodulin complex. It has been reported that protein C and TAFI can compete for the thrombin/thrombomodulin complex despite the differences in structural domains of thrombomodulin that are required for the activation of these components (34). Whether scu-PA competes with protein C and TAFI for the thrombin/thrombomodulin complex remains to be studied.

Contact activation system

This thesis focussed on the role of the contact activation system in fibrinolysis. In **Chapter 8**, the fibrinolytic properties of activated factor XII (FXIIa) were studied. FXIIa exerted little plasminogen activator activity (PAA) as measured in a fibrin microtiter plate. The PAA of FXIIa was strongly potentiated by the negatively charged surface dextran sulphate (DXS), confirming and extending the results of a recent study by Ravon *et al.* (38). In contrast, the PAA of smaller factor XII fragments missing the binding domain for negatively charged surfaces, like β-FXIIa, was not potentiated by DXS. A dextran sulphate euglobulin fraction (DEF) of plasma is routinely used for the analysis of contact activation-dependent fibrinolysis in plasma (39,40). A study was then made on whether FXIIa contributes as a direct plasminogen activator to contact activation-dependent fibrinolysis as measured in a DEF, a milieu in which DXS is present. At least 20% of contact activation-dependent PAA could be extracted from a DEF prepared from normal plasma by immunodepletion of factor XII(a) and could therefore be ascribed to direct plasminogen activation by FXIIa. The contribution of FXIIa as a direct plasminogen activator as measured in a DEF is thus far more significant than previously assumed (41). The PAA of endogenous FXIIa immunodepleted from plasma could only be detected in the presence of dextran sulphate. These findings suggest that FXIIa may be a significant plasminogen activator *in vivo* in the presence of a potentiating surface.

The total fibrinolytic activity measured in a DEF can be divided into 50% of u-PA-dependent activity and 50% of contact activation-dependent activity (40,41). The contribution of t-PA to the total fibrinolytic activity in a DEF is minor, because t-PA is rapidly inhibited by plasminogen

activator inhibitor 1 in citrated plasma. About 70% of contact activation-dependent fibrinolysis is ascribed to an unidentified factor XII-dependent plasminogen proactivator. In **Chapter 9**, an attempt was made to isolate and characterize this unknown plasminogen activator. Contact activation-dependent PAA, defined as the residual PAA after the inhibition of t-PA and u-PA activity by antibodies, was isolated from normal plasma by fractionation, gelfiltration and anion exchange chromatography. Several components appeared to contribute to contact activation-dependent PAA. Known plasminogen activators found here were kallikrein, FXIIa and β-FXIIa. β-FXIIa may be a likely candidate for the 30 kD factor XII-dependent plasminogen activator that was recently described by Sidelmann *et al.* (42). In addition, two unknown proteins associated with contact activation-dependent PAA were observed. The first protein was a 140 kD protein with u-PA-related antigenic determinants, which most likely corresponds to the 110 kD protein described by Binnema *et al.* (43,44). The PAA of the second protein of about 170 kD was strongly potentiated by DXS. This 170 kD protein could not be explained by known plasminogen activators such as t-PA, u-PA, kallikrein and FXIIa. The identity of both proteins remains to be elucidated.

In **Chapter 8** and **9**, the contact activation system was found to promote fibrinolysis. This system was, however, also found to inhibit fibrinolysis under certain conditions, since activation of the intrinsic pathway by recalcification of plasma resulted in the inactivation of scu-PA by endogenously generated thrombin (**Chapter 3**).

General Conclusions

The inactivation of scu-PA by thrombin can take place systemically under pathological conditions. This process not only depends on the presence of large amounts of thrombin, but is also mediated by a balance between several coagulation components. Control of thrombin activity by antithrombin and control of thrombin generation by the protein C pathway is of major importance for the regulation of the inactivation of scu-PA. Disturbances in these two pathways may result in favourable conditions for the inactivation of scu-PA, which may contribute to the thrombotic tendency observed in patients with antithrombin deficiency, protein C deficiency or APC resistance (13-15,21,22). Plasma levels of tcu-PA/T in patients with these specific disorders have not yet been studied.

The effectiveness of scu-PA in local fibrinolysis, a process which is not detectable in the circulation, may also be affected by the inactivation by thrombin. In the micro-environment of a blood clot, high concentrations of thrombin are generated which may be sufficient to inactivate scu-PA that is present in and around the blood clot. Inactivation of scu-PA by thrombin at the cell surface may affect local fibrinolysis as well. Thrombin efficiently inactivates scu-PA bound to human endothelial cells via the urokinase receptor. In addition, scu-PA bound to human platelets has also been reported to be inactivated by thrombin (45). About 20% of endogenous scu-PA is claimed to be associated to platelets in blood (46), thus the inactivation of scu-PA by thrombin at the cell surface may have significant effects on local fibrinolysis. The inactivation of cell-bound scu-PA may also hamper extracellular proteolysis in processes in which both u-PA and thrombin are involved, like atherosclerosis and restenosis. Further research is needed to elucidate the (patho)physiological effects of the inactivation of scu-PA by thrombin on fibrinolysis and extracellular proteolysis.

The role of thrombomodulin in the inactivation of scu-PA by thrombin is dual. On the one hand, thrombomodulin potentiates this process which will contribute to the antifibrinolytic effects of the thrombin/thrombomodulin complex. On the other hand, thrombomodulin can prevent the inactivation of scu-PA by inhibiting additional thrombin generation via the protein C pathway, in this way promoting fibrinolysis. A similar role for thrombomodulin has been described for the activation of thrombin activatable fibrinolysis inhibitor (TAFI) by thrombin (47,48). Mutations in the thrombomodulin gene are associated with thrombosis (49-51), and in animal models deficiency of thrombomodulin results in fibrin deposition while local overexpression of thrombomodulin can prevent arterial thrombosis (52,53). On the basis of these observations, thrombomodulin may primarily have an anticoagulant/profibrinolytic effect under physiological conditions and thus prevent the inactivation of scu-PA by thrombin. Studies *in vitro* and *in vivo* will be necessary to determine the net effect of thrombomodulin on the inactivation of scu-PA by thrombin under (patho)physiological conditions. Furthermore, as yet no study has been conducted on whether soluble fragments of thrombomodulin in the circulation may contribute to the inactivation of scu-PA by thrombin. These fragments have been reported in plasma and levels are increased in several diseases, including DIC (54,55). Since soluble thrombomodulin fragments are functionally active *in vivo*, they may enhance the inactivation of scu-PA by thrombin under pathological conditions (56).

Contact activation-dependent fibrinolysis as measured in a DEF can be ascribed to several

enzymes, including FXIIa. For plasminogen activation by FXIIa, the presence of a potentiating surface seems to be essential. Until now, studies on the effect of potentiating surfaces on plasminogen activation by FXIIa have been performed using artificial materials like dextran sulphate and kaolin (38,57,58). It remains to be elucidated which physiological compounds may serve as a potentiating surface for FXIIa-mediated plasminogen activation. Phospholipids and glycosaminoglycans may be suitable candidates, as well as cellular receptors for factor XII (58,59). The residual contact activation-dependent fibrinolytic activity as measured in a DEF, which has been ascribed to a factor XII-dependent plasminogen proactivator, cannot simply be explained by a single unknown plasminogen activator. At least two components seem to be involved, besides the known plasminogen activators of the contact activation system. The identity of these proteins needs to be clarified. Interactions between plasminogen activators and possible cofactors seem to play an important role in contact activation-dependent fibrinolysis. This phenomenon will hamper the isolation and characterization of the separate plasminogen activators. Extensive research will be needed to unmask all the components that contribute to contact activation-dependent fibrinolysis.

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Samenvatting

In het lichaam is een mechanisme aanwezig dat er zorg voor draagt dat bloed onder normale omstandigheden vloeibaar gehouden wordt en dat bloedverlies voorkomen wordt bij beschadiging van de vaatwand. Dit mechanisme wordt hemostase genoemd. Hemostase wordt gereguleerd door een balans tussen de stolling (het stollen van bloed) en de fibrinolyse (het oplossen van bloedstolsels). Het belangrijkste enzym in de stolling is trombine, dat gegenereerd wordt via een cascade van enzymatische reacties. Trombine zorgt voor het vormen van een stolsel bestaande uit fibrine draden en bloedplaatjes, waarmee het gat in de vaatwand wordt gedicht. Het stolsel dient vervolgens afgebroken te worden zodat de vaatwand zich kan herstellen en terug kan keren in de oorspronkelijke staat. Het fibrine netwerk wordt afgebroken door het fibrinolytische enzym plasmine. Plasmine circuleert als een inactieve pro-vorm, plasminogeen genaamd, en dient geactiveerd te worden door zogenaamde plasminogeen activatoren. De bekendste plasminogeen activatoren zijn weefsel-type plasminogeen activator (t-PA) en urokinase-type plasminogeen activator (u-PA). Daarnaast zijn er aanwijzingen dat componenten uit het contact activatie systeem een rol spelen bij de fibrinolyse. Het contact activatie systeem bestaat uit factor XII, prekallikreine, factor XI en de cofactor kininogeen. Naast de fibrinolyse is dit systeem ook betrokken bij de stolling, de regulatie van de bloeddruk en het immuunsysteem.

Er bestaan verschillende relaties tussen de stolling en de fibrinolyse. In dit proefschrift is nader onderzoek verricht naar twee specifieke relaties, namelijk de inactivatie van de pro-vorm van u-PA door trombine, en de rol van het contact activatie systeem in de fibrinolyse.

De plasminogeen activator u-PA circuleert in plasma als een inactieve éénketenige pro-vorm (scu-PA). Deze pro-vorm kan door specifieke enzymen geknipt worden, wat leidt tot activatie van u-PA. Wanneer scu-PA echter geknipt wordt door het stollingsenzym trombine, ontstaat een inactief molekuul genaamd trombine-geknipt u-PA (tcu-PA/T). Dit molekuul is niet in staat om plasminogeen te activeren, waardoor er geen fibrinolyse kan plaatsvinden. In **Hoofdstuk 2** is een biologische immunoassay opgezet om tcu-PA/T te kunnen meten in plasma en gewrichtsvloeistof. In plasma van gezonde personen kon geen tcu-PA/T worden aangetoond, wat suggereert dat er onder normale omstandigheden geen of nauwelijks inactivatie van scu-PA door trombine plaatsvindt. In plasma van patiënten met een ernstige bacteriële infectie (sepsis) werd wel tcu-PA/T aangetroffen, wat verklaard kan worden door het feit dat tijdens sepsis de stolling sterk geactiveerd

wordt. In gewrichtsvloeistof van patiënten met reumatoïde artritis (RA), een ziekteproces waarbij in hoge mate trombine in de gewrichten wordt gevormd, kon eveneens veel tcu-PA/T aangetoond worden. Uit deze resultaten werd geconcludeerd dat de inactivatie van scu-PA door trombine *in vivo* kan voorkomen in situaties waarbij grote hoeveelheden trombine gegenereerd worden.

De inactivatie van scu-PA door trombine is tot op heden alleen in een gezuiverd systeem bestudeerd, waarbij gevonden is dat dit proces gestimuleerd wordt door de cofactor trombomoduline. Trombomoduline is een membraaneiwit op endotheelcellen, de cellen die de binnenkant van de vaatwand bekleden. Deze cofactor bindt trombine en moduleert op deze wijze de activiteit van trombine. In **Hoofdstuk 3** is de inactivatie van scu-PA door trombine bestudeerd in een natuurlijke plasma omgeving en is bekeken of trombomoduline in dit milieu ook in staat is de inactivatie van scu-PA te stimuleren. In plasma bleken er hogere concentraties trombine nodig te zijn voor de inactivatie van scu-PA in vergelijking met een gezuiverd systeem, wat toegeschreven kon worden aan de aanwezigheid van trombine remmers zoals antitrombine. De inactivatie van scu-PA in plasma werd door trombomoduline gestimuleerd, maar in mindere mate dan in een gezuiverd systeem. Dit kon eveneens verklaard worden door de aanwezigheid van remmers in plasma. Daarnaast werd gevonden dat toevoeging van calcium aan citraat plasma resulteerde in inactivatie van scu-PA door endogeen gegenereerd trombine. Het contact activatie systeem, dat de intrinsieke route van de stolling vormt, bleek essentieel voor deze trombine generatie. Trombomoduline remde de calcium-geïnduceerde inactivatie van scu-PA volledig door een complex te vormen met endogeen gegenereerd trombine, resulterend in de activatie van proteine C. Proteine C is in staat om trombine generatie te remmen en voorkomt zo de inactivatie van scu-PA. Uit deze studie blijkt dat trombomoduline de inactivatie van scu-PA door trombine in plasma zowel kan stimuleren als voorkomen. In de circulatie kan trombine via een intrinsieke route (het contact activatie systeem) en een extrinsieke route (tissue factor route) gegenereerd worden. Daarnaast bestaat er een positieve terugkoppelingsroute, waarbij trombine factor XI activeert. Actief factor XI kan vervolgens factor IX activeren, wat uiteindelijk resulteert in extra trombine generatie. Er is beschreven dat de fibrinolyse geremd kan worden via deze factor XI-afhankelijke route van trombine generatie. Aangezien er in plasma grote hoeveelheden trombine nodig blijken te zijn voor de inactivatie van scu-PA, zou trombine generatie via factor XI een belangrijke rol kunnen spelen bij dit proces. Factor XI-gemedieerde inactivatie van scu-PA zou daarom een bijdrage kunnen leveren aan factor XI-afhankelijke remming van de fibrinolyse (**Appendix Hoofdstuk 3**).

In **Hoofdstuk 4** is de inactivatie van scu-PA door trombine *in vivo* nader bestudeerd door tcu-PA/T te bepalen in plasma van personen met verschillende mate van verhoogde activiteit van de stolling. Het bleek dat tcu-PA/T alleen meetbaar was in plasma van patiënten met diffuse intravasale stolling (DIC), en niet in plasma van vrijwilligers behandeld met endotoxine, van patiënten met onstabiele angina pectoris of van patiënten die een heupoperatie moesten ondergaan. DIC is een zeer ernstig en vaak fataal ziekteproces waarbij zowel de stolling en de fibrinolyse geactiveerd wordt. De fibrinolyse is tijdens DIC echter niet afdoende om een evenwichtige balans met de stolling te kunnen vormen, wat mede verklaard zou kunnen worden door de inactivatie van scu-PA door trombine. Het feit dat tcu-PA/T niet aantoonbaar was in het plasma van de andere personen kon toegeschreven worden aan een lager trombine gehalte, een lager u-PA gehalte en een hoger antitrombine gehalte, de belangrijkste remmer van trombine, in het plasma van deze personen. Naast trombine generatie lijkt het onder controle houden van trombine activiteit door remmers als antitrombine van groot belang te zijn voor de regulatie van de inactivatie van scu-PA door trombine in plasma.

In **Hoofdstuk 5** is tcu-PA/T bepaald in gewrichtsvloeistof van patiënten met RA of met artrose (OA), en in gewrichtsvloeistof van gezonde personen. In RA bleek tcu-PA/T meer dan 40% uit te maken van het totale u-PA gehalte in de gewrichtsvloeistof. Deze bijdrage was in OA veel lager en in de gezonde personen zelfs verwaarloosbaar. In RA vindt extravasculaire generatie van trombine plaats in de gewrichten. Hierdoor kan scu-PA geïnactiveerd worden. Dit zou er toe kunnen bijdragen dat er, ondanks sterk verhoogde gehaltes van u-PA, aanzienlijke fibrine afzettingen worden gevonden in de gewrichten van patiënten met RA. Deze fibrine afzettingen leveren een belangrijke bijdrage aan de progressie van RA.

In **Hoofdstuk 6** is de inactivatie van scu-PA gebonden aan humane endotheelcellen bestudeerd. Trombine bleek celgebonden scu-PA zeer efficiënt te kunnen inactiveren, wat suggereert dat een cellulaire cofactor als trombomoduline bij dit proces betrokken zou kunnen zijn. De binding van scu-PA aan de cellen werd in belangrijke mate gemedieerd door de urokinase receptor. Activatie van plasminogeen door u-PA dat gebonden is aan cellen via de urokinase receptor speelt een grote rol bij lokale fibrinolyse en bij de afbraak van extracellulaire matrix tijdens bijvoorbeeld celmigratie, tumor invasie en wondheling. Inactivatie van celgebonden scu-PA door trombine zou daarom een groot effect op deze processen kunnen hebben.

Trombomoduline bevat zes groeifactordomeinen, welke betrokken zijn bij trombine binding en de cofactor activiteit van trombomoduline. In **Hoofdstuk 7** zijn de groeifactordomeinen geïdentificeerd welke essentieel zijn voor de stimulatie van de inactivatie van scu-PA door trombine. Groeifactordomeinen 5 en 6 bleken voldoende te zijn voor de stimulatie. Deze domeinen bevatten de bindingsplaats voor trombine. In dit opzicht verschilt scu-PA sterk van proteine C en TAFI, twee andere substraten van het trombine/trombomoduline complex. Voor de activatie van proteine C (groeifactordomeinen 4-6 plus het gedeelte tussen groeifactor domein 3 en groeifactor domein 4) en TAFI (groeifactordomeinen 3-6) zijn naast de trombine-bindende groeifactordomeinen ook andere groeifactordomeinen nodig.

De rol van het contact activatie systeem in de fibrinolyse is nog steeds onduidelijk. Het is bekend dat actief factor XII (FXIIa) in staat is om direct plasminogeen te activeren. In **Hoofdstuk 8** zijn de eigenschappen van FXIIa als plasminogeen activator bestudeerd met behulp van een fibrine microtiter plaat. In deze assay wordt fibrinolyse gemeten als maat voor plasminogeen activatie. Plasminogeen activatie door gezuiverd FXIIa bleek gestimuleerd te worden door dextraan sulfaat, een negatief geladen stof. Factor XII circuleert in een inactieve pro-vorm. Tijdens de activatie van factor XII wordt eerst FXIIa gevormd, waarna FXIIa vervolgens in kleinere actieve fragmenten wordt geknipt. Dextraan sulfaat stimuleerde plasminogeen activatie door de kleinere fragmenten niet, wat verklaard kan worden door het feit dat deze fragmenten het bindingsdomein voor negatief geladen oppervlakken missen. Routinematig wordt de totale fibrinolytische activiteit in plasma gemeten in een dextraan sulfaat euglobuline fractie (DEF) van plasma. Hierbij wordt het contact activatie systeem volledig geactiveerd. Dit systeem is verantwoordelijk voor ongeveer 50% van de totale fibrinolytische activiteit. FXIIa bleek een bijdrage van tenminste 20% te leveren aan de totale contact activatie-afhankelijke fibrinolyse in een DEF, hoogstwaarschijnlijk door de aanwezigheid van dextraan sulfaat.

De totale fibrinolytische activiteit in een DEF bereid van citraat plasma kan onderverdeeld worden in 50% u-PA-afhankelijke fibrinolyse en 50% contact activatie-afhankelijke fibrinolyse. De bijdrage van t-PA is minimaal door onmiddellijke complexering van t-PA met de remmer PAI-1 in citraat plasma. Ongeveer 70% van contact activatie-afhankelijke fibrinolyse is tot nu toe onverklaard gebleven en wordt toegeschreven aan een nog onbekende factor XII-afhankelijke plasminogeen activator. In **Hoofdstuk 9** is getracht deze onbekende plasminogeen activator te

isoleren en te karakteriseren. Er werden meerdere componenten gevonden die betrokken waren bij contact activatie-afhankelijke fibrinolyse. Naast bekende plasminogeen activatoren als kallikreine, actief factor XIIa en β-factor XIIa werden twee onbekende componenten aangetroffen, namelijk een eiwit met een molekuulgewicht van 140 kD en een eiwit van 170 kD. Het 140 kD eiwit vertoonde immunoreactiviteit met anti-u-PA antilichamen en komt hoogstwaarschijnlijk overeen met een eerder beschreven eiwit van 110 kD. Plasminogeen activatie door het 170 kD eiwit werd sterk gestimuleerd door dextraan sulfaat. De identiteit van deze componenten dient nog opgehelderd te worden.

In **Hoofdstuk 10** worden de resultaten van de verschillende studies besproken. Uit het onderzoek kan geconcludeerd worden dat de inactivatie van scu-PA door trombine *in vivo* onder bepaalde omstandigheden kan plaatsvinden. Dit proces wordt gereguleerd door verscheidene componenten uit de stolling. Verder is gebleken dat de rol van actief factor XII als fysiologische plasminogeen activator groter is dan eerder werd aangenomen. Daarnaast lijkt de contact activatie-afhankelijke fibrinolyse die toegeschreven werd aan één onbekende plasminogeen activator door meerdere componenten verklaard te kunnen worden.

Abbreviations

APC	activated protein C
AT/ATIII	antithrombin/antithrombin III
BIA	bioimmunoassay
BSA	bovine serum albumin
DEF	dextran sulphate euglobulin fraction
HUVEC	human umbilical vein endothelial cells
DIC	disseminated intravascular coagulation
DXS	dextran sulphate
EGF	epidermal growth factor
F1+2	prothrombin fragment 1+2
FXIIa	activated factor XII
Moab	monoclonal antibody
OA	osteoarthritis
PAA	plasminogen activator activity
RA	rheumatoid arthritis
scu-PA	single-chain urokinase-type plasminogen activator
SF	synovial fluid
TAFI	thrombin activatable fibrinolysis inhibitor
TAT	thrombin-antithrombin complexes
tcu-PA	two-chain urokinase-type plasminogen activator
tcu-PA/T	thrombin-cleaved two-chain urokinase-type plasminogen activator
TM	thrombomodulin
$TM_Ei4\text{-}6$	thrombomodulin comprising EGF-like domains 4-6 and the interconnecting region between EGF-like domain 3 and EGF-like domain 4
$TM_E5\text{-}6$	thrombomodulin comprising EGF-like domains 5-6
TM_{LEO}	thrombomodulin comprising EGF-like domains 1-6
t-PA	tissue-type plasminogen activator
UAP	unstable angina pectoris
u-PA	urokinase-type plasminogen activator
u-PA:Ag	urokinase antigen

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ELLEN

Curriculum Vitae

Ellen Anne Martien Braat werd op 30 juni 1970 geboren te Scheveningen. In 1988 behaalde zij haar VWO diploma aan het Christelijk Gymnasium Sorghvliet te Den Haag. In hetzelfde jaar begon zij aan de studie Biomedische Wetenschappen (destijds Gezondheidswetenschappen genaamd) aan de Universiteit Leiden en in augustus 1989 behaalde zij de propaedeuse. In de doctoraalfase volgde zij in totaal een zestal stages op diverse vakgebieden, waaronder bij de MRC Immunochemistry Unit, Department of Biochemistry, University of Oxford in Engeland in het kader van het Erasmus Uitwisselingsproject. Haar afstudeerstage getiteld 'Influence of macrophage mannose receptor expression on the t-PA concentration and t-PA activity in the microenvironment of the macrophage' voerde ze uit bij TNO Preventie en Gezondheid, Gaubius Laboratorium te Leiden onder begeleiding van Dr. D.C. Rijken en Drs. F. Noorman. In juni 1994 werd de studie cum laude afgerond.

Van augustus 1994 tot mei 1999 was zij werkzaam als AIO bij TNO Preventie en Gezondheid, Gaubius Laboratorium te Leiden onder begeleiding van Dr. D.C. Rijken en Prof. Dr. P. Brakman. De resultaten van het promotie-onderzoek, gesubsidieerd door de Leiden-Leuven Research Stichting, staan beschreven in dit proefschrift. Sinds mei 1999 is zij werkzaam als Research Associate bij Pharming Technologies B.V. te Leiden.