CLINICAL STUDIES ON FIBRINOLYSIS INHIBITORS SYNTHESIZED BY THE LIVER

FIBRINOLYSEREMMERS GESYNTHETISEERD DOOR DE LEVER - KLINISCHE STUDIES -

PROEFSCHRIFT

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CHAPTER 1

INTRODUCTION

INTRODUCTION

The formation of a fibrin clot after tissue injury prevents bleeding and initiates tissue repair by providing a solid matrix for new tissue growth. During and after the process of tissue repair, the clot is degraded and the normal anatomy restored as far as possible. The process of dissolution of fibrin clots is called fibrinolysis (1). The haemostatic mechanism is regarded as a dynamic balance between coagulation and fibrinolysis (2).
In recent years there has been a dramatic increase in knowledge on fibrinolysis due to the discovery of several fibrinolysis proteins and the techniques to study these proteins in health and disease. The development of plasminogen activators for clinical use in the treatment of acute myocardial infarction has additionally caused a marked upsurge in interest and research in fibrinolysis (3).

Fibrinolysis and disease

The clinical relevance of the fibrinolytic system is examplified by the various congenital disorders of fibrinolysis (4,5). For instance patients with a congenital homozygous deficiency of α_2-antiplasmin have a life-long haemorrhagic diathesis, sometimes resulting in severe disability (6,7,8). In addition to the congenital disorders, acquired disorders of fibrinolysis in various disease states, such as liver disease (9), acute promyelocytic leukemia (10), coronary artery disease (11), disseminated intravascular coagulation (12), nephrotic syndrome (13) and diabetes mellitus have also been reported (14).
In patients with congenital or acquired disorders of fibrinolysis, the fibrinolytic system does not function normally and this can lead to either bleeding, due to increased fibrinolysis or to thrombo-embolic complications, due to decreased fibrinolysis (9,10,11,15).
The liver plays a major role in the systemic regulation of fibrinolysis, since it is involved in both the synthesis and the clearance of most proteins of the fibrinolytic system (16). A decrease of liver function in patients with liver disease will therefore lead to multiple changes in fibrinolysis. This can contribute to the bleeding problems observed in these patients (17).

Objectives of the study

In this thesis studies on congenital and acquired disorders of the fibrinolytic system, with emphasis on the inhibitors of the fibrinolytic system, are described. This was initiated to obtain more information on the fibrinolytic system, and to obtain insight into the mechanism of enhancement of fibrinolysis in patients with liver disease.

In chapter 2, a concise review on the fibrinolytic system, on the role of the liver in the regulation of the fibrinolytic system and an overview on the literature on fibrinolysis in liver disease are given. In the other chapters biochemical and clinical studies on fibrinolysis inhibitors are reported. In the last two chapters we report investigations on the fibrinolytic system in patients with liver cirrhosis.

The aims of the studies were:

1. To evaluate the clinical outcome in some patients with a congenital heterozygous deficiency of
   a) α_2-antiplasmin, the major inhibitor of plasmin (chapter 3) or of
   b) plasminogen, the zymogen of plasmin in the circulation (chapter 4).

2. To study, in patients with acute myocardial infarction, the consumption of the plasmin inhibitors in relation to systemic effects during thrombolytic therapy (chapter 5).

3. To obtain more insight into the behaviour of the two molecular forms of α_2-antiplasmin and to evaluate the mechanism of the conversion of the active plasminogen binding form into the less active non-plasminogen binding form of α_2-antiplasmin (chapter 6).

4. To study the fibrinolytic system in patients with liver cirrhosis, with special attention to the balance between pro- and antifibrinolytic factors at several levels of the fibrinolytic system (chapter 7 & 8).

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CHAPTER 2

FIBRINOLYSIS AND THE LIVER

A REVIEW ON FIBRINOLYSIS, ON THE ROLE OF THE LIVER IN THE REGULATION OF FIBRINOLYSIS AND ON FIBRINOLYSIS IN LIVER DISEASE

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2.1 INTRODUCTION

Liver disease is frequently complicated by clinically significant bleeding (1,2,3). The incidence of bleeding has been estimated to be 32 % in patients with cirrhosis of the liver and is sometimes life-threatening (4). The bleeding may be due to local abnormalities, for instance bleeding from varices or ulcers, but may be also due to changes in haemostasis (3). Changes in the haemostatic mechanism have been reported in 75-85 % of the patients with liver disease (5,6). The coagulopathy in patients with liver disease may be caused by changes in coagulation factors, in platelets or vessel wall function or in fibrinolysis, and seems to be multifactorial in most patients (1,2). We have concentrated on the effects of liver disease on the fibrinolytic system and this introduction will give an overview on fibrinolysis in liver diseases. A concise survey of the haemostatic mechanism, with emphasis on the fibrinolytic system will be presented; the role of the liver in the regulation of fibrinolysis will be discussed and, finally, the literature on fibrinolysis in liver disease and the management of disorders of fibrinolysis in liver disease will be reviewed.

2.2 HAEMOSTASIS

The haemostatic mechanism is a dynamic balance between coagulation and fibrinolysis (7). The haemostatic mechanism ensures that, after tissue injury, a clot is formed, which prevents blood from leaking out of vessels. In addition, this mechanism also cares for the dissolution of these clots after tissue repair.

2.2.1. VESSEL WALL, PLATELETS AND COAGULATION

As soon as the vessel wall is injured, platelets attach to the subendothelial collagen (platelet adhesion). This process is mainly mediated by coagulation factor VIII:von Willebrand and fibronectin. During adhesion of platelets, several factors are secreted by the platelets, which induce platelet aggregation. In

addition thromboxane A_2 is formed, which induces vasoconstriction and platelet aggregation. During this process of platelet adhesion and aggregation, the coagulation cascade is activated. Coagulation can be initiated by several types of factors, which are all related to vessel wall injury. The coagulation system consists of the extrinsic and the intrinsic cascade, which have several interrelations. The extrinsic clotting pathway is triggered by tissue factor, which is exposed by the injured cells (figure 1). The intrinsic pathway is started by coagulation factor XII, which is triggered by negatively charged surfaces. Also high molecular weight kininogen and kallikrein are involved. Triggering of each of the coagulation pathways results finally in formation of soluble fibrin monomers, which polymerise and are converted to insoluble fibrin polymers by activated factor XIII (figure 1) (for recent reviews see 8,9).

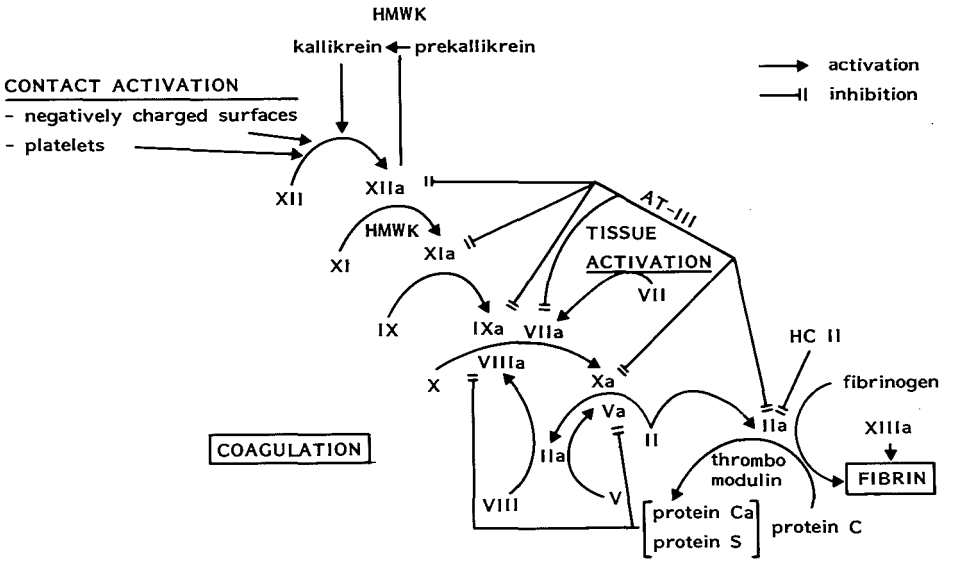

Figure 1: Schematic presentation of the coagulation cascade. Extensive description is given in the text. AT-III= antithrombin III; HC-II= heparin co-factor II; HMWK= high molecular weight kininogen.

2.2.2 FIBRINOLYSIS

Fibrin, the end product of the activated coagulation system has only a temporary function. During tissue repair, fibrin is degraded. The process of fibrin breakdown to soluble fibrin degradation products is called fibrinolysis (10). Various plasma proteins are involved in the regulation of fibrinolysis. The interaction between fibrin and some of the proteins of the fibrinolytic system is responsible for the fibrin specificity of this proteolytic mechanism (11). Disorders of the fibrinolytic mechanism can lead to either a haemorrhagic diathesis, in enhanced fibrinolysis, or a thrombotic diathesis, in reduced fibrinolysis (12).

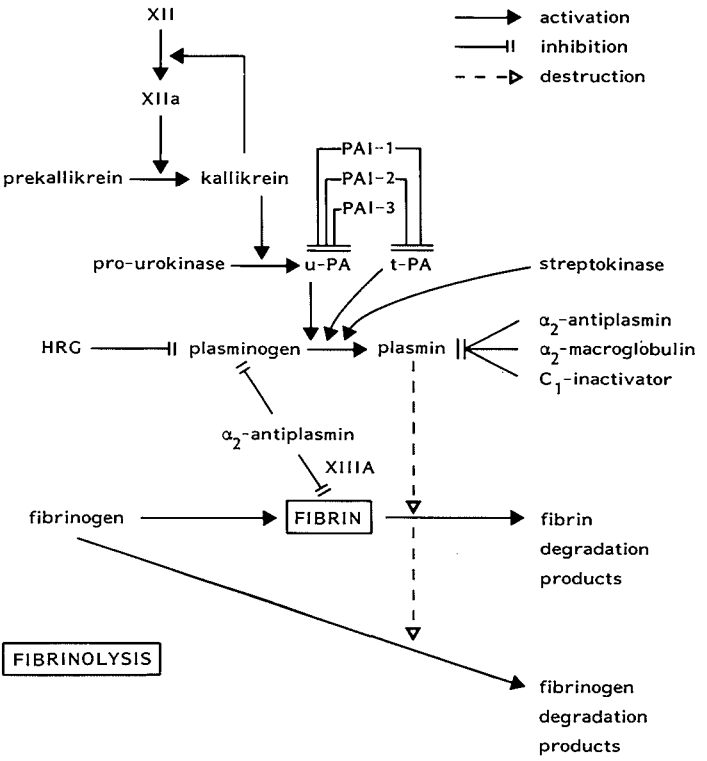

Figure 2: Schematic presentation of the fibrinolytic system. An extensive description is given in the text. HRG= histidine-rich glycoprotein; PAI= plasminogen activator inhibitor; t-PA= tissue-type plasminogen activator; u-PA= urokinase-type plasminogen activator.

As is schematically shown in figure 2, fibrin is proteolytically degraded to fibrin degradation products by plasmin, the key enzyme of fibrinolysis. Plasmin is formed by activation of plasminogen by plasminogen activators, such as tissue-type plasminogen activator and urokinase-type plasminogen activator. Fibrinolysis is controlled by fibrinolysis inhibitors: plasminogen activator inhibitors act at the level of plasminogen activators, histidine-rich glycoprotein regulates the amount of "free" plasminogen in the circulation and α_2-antiplasmin is the main inhibitor of plasmin. α_2-Macroglobulin and C_1-inactivator are less potent inhibitors of plasmin.

2.2.2.1 Plasminogen

Plasminogen, a glycoprotein with a molecular mass of 92,000, is synthesized by the liver and occurs in plasma at a concentration of 1.5 μM (140 mg/l) (13). Plasminogen is incorporated in the clot during clot formation and can thus be activated where plasmin activity is required. Plasminogen is activated to plasmin by plasminogen activators. The native plasminogen molecule (Glu-plasminogen) is a single chain. Lys-plasminogen is formed by plasmin cleavage of the first 76 amino acids from Glu-plasminogen (14). Lys-plasminogen has a higher affinity for fibrin and is also more susceptible to activation to plasmin by activators (15). Activation of plasminogen to plasmin is accomplished by cleavage of the peptide bond between Arg-561 and Val-562 by plasminogen activators. Plasmin consists of a heavy chain with five kringle structures and a light chain (13). The kringle structures and the active site region harbour lysine binding sites (14). Lysine binding sites are involved in the binding, amongst others, to fibrin, α_2-antiplasmin, histidine-rich glycoprotein and thrombospondin (16). The light chain contains the active site of plasmin. Both chains remain bound to each other by a disulfide bridge. The heavy chain binds to fibrin, while fibrin is degraded by the light chain (17).

A deficiency of plasminogen, either due to a decreased synthesis of a normal molecule or due to synthesis of an abnormal dysfunctional molecule, is associated with deep venous thrombosis (18,19).

2.2.2.2 Plasminogen activators

Two separate groups of plasminogen activators can be distinguished: endogenous and exogenous plasminogen activators. The endogenous activators are tissue-type plasminogen activator, pro-urokinase (single chain urokinase-type plasminogen activator, scu-PA), urokinase (two-chain urokinase-type plasminogen activator; tcu-PA) and a factor XII-dependent activator. Exogenous plasminogen activators are streptokinase and acylated plasminogen streptokinase activator complex (APSAC). Some endogenous activators (t-PA and u-PA) and mutants of these plasminogen activators are at present produced in large amounts by recombinant DNA techniques and used experimentally for thrombolysis.

a) Tissue-type plasminogen activator (t-PA)

Tissue-type plasminogen activator (t-PA) is mainly synthesized by endothelial cells and has a plasma concentration of about 5 µg/l (20). The activation of plasminogen by t-PA is markedly accelerated in the presence of fibrin, up to 200-400 times faster (21). This explains the fibrin specificity of t-PA. The amount of t-PA incorporated in the clot is dependent upon the plasma concentration (22). The rate of fibrinolysis in turn is dependent upon the t-PA concentration in the clot. Local triggers of t-PA secretion from endothelial cells seem therefore to be of major importance.

T-PA concentration both in the circulation and locally can be increased by exercise, venous stasis, infusion of Desmopressin (DDAVP), anoxia, acidosis and several clotting factors, such as thrombin, bradykinin and activated protein C (23,24,25). The activity of t-PA in the circulation is controlled by inhibitors (plasminogen activator inhibitors 1,2 and 3) (26).

T-PA has a short half-life in the circulation of about four minutes. It is rapidly cleared by the liver (27,28). An increase of t-PA activity in the circulation may be associated with a bleeding tendency (29), whereas a deficient t-PA response to venous stasis has been found in patients with deep venous thrombosis (30). It has also been suggested that decreased t-PA levels may have predictive value for the recurrence of myocardial infarction (31).

b) Urokinase-type plasminogen activator (u-PA)

Urokinase-type plasminogen activator (u-PA) circulates in plasma as

a pro-enzyme (pro-urokinase or scu-PA) and can be activated to u-PA by plasmin and also by kallikrein (32). Pro-urokinase is a plasminogen activator, with a fibrin specific mechanism, which is different from t-PA. U-PA has been demonstrated in urine and plasma and is secreted by several cell types including fibroblasts, epithelial cells, pneumocytes, endothelial cells and kidney cells (33,34,35,35a). The plasma concentration of u-PA is virtually zero, of pro-urokinase is 2.3 ± 0.3 μg/l and the sum of u-PA, pro-urokinase and u-PA-inhibitor complexes is 3.5 ± 1.5 μg/l (36).

c) Plasminogen activator from the XII dependent pathway

A third plasminogen activator (F XII-dependent plasminogen activator) has been postulated in plasma. Its identity and properties are not fully clarified (37).

d) Exogenous plasminogen activators

Among the exogenous plasminogen activators, streptokinase is most widely used (38). Streptokinase is a protein derived from β-haemolytic Streptococci and has a molecular mass of 47,000 (39). The mechanism by which streptokinase activates plasminogen is different from that of the above mentioned activators. Streptokinase forms a 1:1 complex with plasminogen, which results in a molecular rearrangement in the plasminogen molecule. The plasminogen molecule in the complex can activate plasminogen. The streptokinase-plasminogen complex has affinity for fibrin and, once bound to fibrin, it can also activate plasminogen at the fibrin surface (40).

Other plasminogen activators include acylated plasminogen streptokinase activator complex (APSAC), recombinant tissue-type plasminogen activator and recombinant pro-urokinase. These plasminogen activators are being used in the treatment of acute myocardial infarction, deep venous thrombosis and pulmonary embolism (41).

2.2.2.3 Inhibitors of fibrinolysis

a) α_2-antiplasmin

α_2-antiplasmin (α_2-AP) is a glycoprotein with a molecular mass of 67,000 and a concentration in plasma of 1.0μM (67 mg/l) (42,43,44). It is synthesized by the liver with a synthesis rate of 2.1

mg/kg/day and has a half life in plasma in vivo of about 60 hours (45). α_2-AP occurs in plasma in two molecular forms: a very active plasminogen-binding form (PB-α_2-AP), which is synthesized by the liver and a (less active) non-plasminogen-binding form (NPB-α_2-AP), formed in the circulation by conversion from PB-α_2-AP (46). The ratio of PB-α_2-AP to NPB-α_2-AP in plasma is about 2.1 (46). α_2-AP belongs to the serpin superfamily and is the major inhibitor of plasmin. It acts by forming a 1:1 stoichiometric complex with plasmin (PAP-complex) (47).

PB-α_2-AP not only inactivates plasmin, it also has the ability to bind to plasminogen and can be cross-linked to fibrin by factor XIIIA (48,49). The NPB form can only slowly inhibit plasmin (50). The three aforementioned properties of PB-α_2-AP make it the most important inhibitor of plasmin in circulation. A decrease in α_2-AP may result in enhanced fibrinolysis and a haemorrhagic diathesis. Patients with a congenital homozygous α_2-AP deficiency have been reported to suffer from a life-long, haemophilia-like, haemorrhagic diathesis (51,52,53). Heterozygous deficient patients are reported to have mild or no bleeding problems (for a review see 54). Acquired α_2-AP deficiency has been reported in several disease states most of which are associated with a severe bleeding tendency. These deficiencies may be due to a decreased synthesis such as in liver insufficiency (55) and asparaginase therapy (56), or to an increased consumption, for instance in case of disseminated intravascular coagulation, promyelocytic leukemia (57), amyloidosis (58) and thrombolytic therapy (59). An increase of α_2-AP may theoretically result in a decreased fibrinolysis and therefore predispose to thrombosis (60,61). However only few patients with elevated α_2-AP levels and venous thrombosis have been described so far. In one patient, who was followed over a longer period, the α_2-AP levels normalised spontaneously over a period of one year (61).

b) Histidine-rich glycoprotein

Histidine-rich glycoprotein (HRG) is a glycoprotein with a molecular mass of 80,000, which occurs in plasma at a concentration of 1.8 μM (150 mg/l). It is thought to be synthesized mainly in the liver (62,63). HRG may play a regulatory role in the fibrinolytic mechanism by interacting with the lysine binding sites of

plasminogen (64). By complex formation with plasminogen, HRG reduces the amount of "free" plasminogen in circulation. Plasminogen bound to HRG is not available for binding to fibrin and subsequent activation to plasmin at the fibrin surface. HRG is therefore considered to be an inhibitor of fibrinolysis. The physiological role of HRG has not yet been established (review 65). A familial increase of HRG has been found in some families with thrombophilia (66,67). A decrease of HRG has been reported in several disease states, including liver disease, sepsis, disseminated intravascular coagulation and systemic lupus erythematodes (62,68), and also during pregnancy and in women taking oral contraceptives (69).

c) α_2-Macroglobulin

α_2-Macroglobulin (α_2-M) is a protease inhibitor with a variety of actions. It is a glycoprotein with a molecular mass of 725,000 and a plasma concentration of 3.0 μM (2.2 g/l)(70). It is synthesized by the liver, macrophages and fibroblasts (71). Its role in the inhibition of fibrinolysis is due to inhibition of plasmin and u-PA and t-PA (72). α_2-M only inhibits plasmin after α_2-AP is consumed, for instance during thrombolytic therapy, when the fibrinolytic system is extensively activated (73,74). Plasmin is covalently bound to give an α2-M-plasmin complex, which is recognised by the cells of the reticuloendothelial system and removed from the circulation (73). Increased levels of α_2-M are found in pregnancy and in women taking oral contraceptives (75), in renal disease, rheumatoid arthritis and several other diseases (for review see 76). A congenital deficiency of α_2-macroglobulin has been described occasionally and is not associated with a clinically relevant disorder of haemostasis or changes in coagulation tests (77).

d) C_1-Inactivator

C_1-Inactivator (C_1-INA) is a protease inhibitor, which belongs to the serpin superfamily. It has a molecular mass of 105,000 (78). It is mainly synthesized in the liver and has a plasma concentration of 1.5μM (150 μg/l). It has multiple functions in the regulation of haemostasis. C_1-INA can inhibit components of the complement system and the coagulation factors XIa and XIIa, and is the predominant inhibitor of plasma kallikrein (79). C_1-INA

inhibits plasmin, kallikrein and t-PA (79,80). Little is known however about its role in plasmin inhibition in vivo. A deficiency of C_1-inactivator is associated with hereditary angioedema, but not with clinically evident changes in haemostasis (81).

e) Plasminogen activator inhibitor-1 (PAI-1)

PAI-1 is the most important inhibitor of plasminogen activators in blood (82,83,84). PAI-1 is a glycoprotein with a molecular mass of 52,000. The PAI activity levels in plasma of healthy individuals show a large interindividual variation ranging from 0.0 to about 1.3 nmol/L (26). PAI-1 is synthesized by endothelial cells and human hepatocytes in culture and several other cell-types (85,86,87). It is also present in blood platelets in a 20-fold higher concentration than in plasma (88). The active form of PAI-1 reacts with u-PA and t-PA, resulting in complexes without plasminogen activator activity (89). PAI-1 is cleared from the circulation by the liver and its half life in plasma is about 20-40 minutes (28,90).

A deficiency of PAI-1 may lead to a haemorrhagic tendency, resulting in persistent bleeding after minor trauma (91). The role of PAI-1 levels in thrombo-embolic disease is not yet clear. It has been suggested that a high basal PAI-1 level blocks the normal local increase in t-PA activity in venous stasis, and that this may be associated with idiopathic deep venous thrombosis (27,92). Recently, it was suggested that increased PAI-1 levels could predispose to the development of acute myocardial infarction (31,93).

f) Plasminogen activator inhibitor-2

Plasminogen activator inhibitor-2, formerly called placenta-type plasminogen activator inhibitor, is a glycoprotein with a molecular mass of 48,000 and is most probably synthesized by macrophages, especially in human placenta (94). Recently PAI-2 was also found to be synthesized by lymphoma cells (95). PAI-2 inhibits u-PA more effectively than t-PA (94). PAI-2 only occurs in plasma during pregnancy. A concentration of PAI-2 of 100 μg/L has been found in the third trimester of pregnancy, which returns to nonpregnant values after delivery (96). The physiological role of PAI-2 during pregnancy has not yet been established.

g) Plasminogen activator inhibitor-3

Recently evidence has been obtained that plasminogen activator inhibitor-3 is identical with protein C inhibitor (97). PAI-3 is a single chain glycoprotein with a molecular mass of 50,000. It is synthesized by the liver and occurs in plasma at a concentration of 2-5 µg/L (97). It is involved in two antithrombotic mechanisms, i.e. the protein C system and the fibrinolytic system. It is the major inhibitor of activated protein C and is involved in inhibition of u-PA (97). Its physiological role is not clear, since no deficiencies of PAI-3 have been described so far.

2.3 ROLE OF THE LIVER IN THE REGULATION OF FIBRINOLYSIS

2.3.1. LIVER PHYSIOLOGY

The liver has several functions, such as the metabolism of amino acids and carbohydrates, synthesis and clearance of proteins, metabolism of drugs and hormones, and regulation of the lipid and cholesterol metabolism. In this paragraph we will focus on the role of the liver in the metabolism of the proteins of the haemostatic mechanism, especially of those of the fibrinolytic mechanism.

Coagulation: The liver is the source of many coagulation factors, including fibrinogen, factor II, V, VII, VIII, IX, X, XII, XIII and prekallikrein and high molecular weight kininogen (98,99). The liver is the sole source of some of these proteins. However, some proteins are also synthesized elsewhere, for instance factor VIII:vWF by endothelial cells and factor XI and XII by the reticulo-endothelial system. The coagulation factors II, VII, IX and X are vitamin K-dependent factors (100). Inhibitors of the coagulation system, such as antithrombin III, an inhibitor of factor IIa, Xa and XIa (99), as well as protein C, an inactivator of factor Va and VIIIa, and its co-factor protein S are also derived from the liver (98).
Additionally, the reticulo-endothelial system of the liver is involved in the clearance of activated coagulation factors and their degradation products from the circulation (101,102).

Fibrinolysis: In 1964, von Kaulla stressed the important role of the liver in the regulation of fibrinolysis. He demonstrated the inhibitory role of the liver on the fibrinolytic mechanism in a patient undergoing a homotransplantation of the liver (103). Current insights into the involvement of the liver in fibrinolysis are summarized in table 1 and 2. As can be seen, the liver is involved in the synthesis and clearance of most proteins of the fibrinolytic mechanism.

a) Synthesis function of the liver

The liver synthesizes plasminogen (104,105) and the fibrinolysis inhibitors α_2-antiplasmin (106,107), histidine-rich glycoprotein (63), α_2-macroglobulin (71) and C_1-inactivator (108) (table 1).

Table 1. Involvement of the liver in fibrinolysis: synthesis*

PROTEIN	SYNTHESIS		MAJOR REGULATION FACTORS	REFERENCE
	LIVER	OTHER CELLS		
Plasminogen	hepatocytes		pos acute phase estrogen anabolics	104,105
Urokinase-type plasminogen activator	hepatoma cells	epithelial cells fibroblasts pneumocytes endothelial cells kidney cells		33,34 114 35 35a
Tissue-type plasminogen activator		endothelial cells	venous stasis thrombin DDAVP, anoxia	20,23,24 25
Histidine-rich glycoprotein	hepatocytes	macrophages platelets [1]	sex steroids neg acute phase	63 115
α_2-Antiplasmin	hepatocytes	platelets [1]	pos acute phase	106,107
α_2-Macroglobulin	hepatocytes	monocytes fibroblasts, macrophages, lymphocytes	sex steroids	71,116
C_1-Inactivator	hepatocytes hep G2 cells	monocytes U93 lymphoma megacaryocytes	anabolics	108,78
Plasminogen activator inhibitor-1	hepatocytes hepatoma cells	endothelial cells platelets [1] other cell types	pos acute phase insulin anabolics	86,87 88 review 85
Plasminogen activator inhibitor-2		macrophages [2] leucocytes other cell types	sex steroids endotoxins	94 117
Plasminogen activator inhibitor-3	hepatoma cells			87

* This table summarizes the previous paragraphs on liver involvement in fibrinolysis. This table is, however, not complete. Other proteins with a (possible) regulatory role in fibrinolysis, such as protein C and S, tetranectin, F XII, F XIIIA, kallikrein and high molecular weight kininogen (HMWK) are also (in part) synthesized by the liver. Extensive description is outside the scope of this review.
[1] found to be present in platelets
[2] reported in placental macrophages

Additionally, it may account for a part of the synthesis of PAI-1 (87). Presuming that PAI-3 is identical with protein C inhibitor, the liver also synthesizes PAI-3 (98). We can conclude that the liver is the major site of synthesis of the presently known proteins of the fibrinolytic cascade. Only tissue-type plasminogen activator, plasminogen activator inhibitor 1 and 2 and pro-urokinase are solely or in part synthesized outside the liver.

b) Clearance function of the liver

The other function of the liver in fibrinolysis is the clearance of several fibrinolytic proteins such as the plasminogen activators urokinase (109) and t-PA (27,28,110,111), and the inhibitor PAI-1 (28,90). The evidence for the clearance function of the liver has been obtained from both clinical and experimental studies (table 2).

Additionally, the reticulo-endothelial cells of the liver can clear enzyme-inhibitor complexes, such as plasmin-α_2-antiplasmin complexes or complexes of α_2-macroglobulin with different proteases (112,113).

Table 2. Involvement of the liver in fibrinolysis: clearance

PROTEIN	PLASMA HALF LIFE	REFERENCE
Tissue-type plasminogen activator	2 - 4 min	27,28,110,111
Pro-urokinase, urokinase	15 min	109
Plasminogen activator inhibitor-1	20-40 min	28,90
Plasmin-α_2-antiplasmin complexes	12 hours	112
Protease-α_2-macroglobulin complexes	5 min	113

2.3.2 LIVER DISEASE

Liver diseases can be subdivided in acute and chronic disorders.

Chronic liver disease:

The most common chronic liver disease is cirrhosis of the liver. Cirrhosis of the liver is defined as a diffuse process characterized by fibrosis and a conversion of normal architecture into structurally abnormal nodules. In most of the patients, cirrhosis is caused by chronic alcohol abuse or viral hepatitis (postnecrotic cirrhosis). Other types of cirrhosis are primary biliary cirrhosis, pigment cirrhosis (hemochromatosis), cirrhosis associated with Wilson's disease or α_1-antitrypsin deficiency and cryptogenic cirrhosis.

Cirrhosis of the liver results in a reduced synthesis activity, caused by a decrease in the number as well as in the function of the hepatocytes. A reduced dietary intake of amino acids may contribute to deficient protein synthesis.

As a result of nodular regeneration and of the abnormal architecture of the liver, the vascular resistance in the sinusoidal bed of the liver increases. This causes a rise of the portal venous pressure (portal hypertension). Collateral vessels increase in size, which results in portal systemic shunting. Portal hypertension is usually associated with reduced hepatic clearance of substances including peptides which are rapidly cleared by the healthy liver.

Acute liver disease:

Fulminant or acute hepatic failure is often viral or toxic in origin and is defined as the occurence of encephalopathy secondary to severe liver dysfunction within 8 weeks of the onset of symptoms and in the absence of pre-existing liver disease. It can be due to viral hepatitis (A,B or non-A,non-B virus), drugs or chemical intoxication or circulatory changes (118). In acute severe liver disease, there is marked hepatocellular damage, which results in a decreased synthesis function of the liver and decreased clearance capacity, often without portal hypertension (119).

2.4 COAGULOPATHY IN CHRONIC LIVER DISEASE

2.4.1 COAGULATION

Since the liver is involved in the synthesis of many proteins of the haemostatic system it is evident that loss of liver function can result in decreased levels of coagulation factors (1,2,3). The coagulopathy in liver disease is usually multifactorial. In addition to the decreased synthesis there may be decreased clearance of activated coagulation factors and synthesis of abnormal clotting factors (e.g. dysfibrinogenemia) (120,121). In addition platelet number and platelet function may be decreased (122). Finally it has also been suggested that disseminated intravascular coagulation may contribute to the coagulopathy in liver disease (123). As several excellent reviews on the changes of the haemostatic mechanism in liver disease have been published, we will discuss mainly the changes in the fibrinolytic system (1,2,3,123).

2.4.2 FIBRINOLYSIS

Since the liver is involved in the regulation of the fibrinolytic cascade, liver disease can result in changes in fibrinolysis, often manifesting as enhanced fibrinolysis. Most studies have been performed in patients with chronic liver failure, as will be reviewed below. Fibrinolysis in other types of liver disease, such as acute hepatic failure and obstructive jaundice will be discussed separately.

2.4.2.1 Historical background

The first studies on fibrinolysis in liver disease were mostly performed by clot-lysis experiments on plasma of patients with various liver diseases. The assessed fibrinolysis (fibrinolytic activity) in vitro, however, reflects the fibrinolytic potential in plasma of these patients, but does not necessarily reflect the situation in vivo. The assessment of reaction products of fibrinolysis may indicate fibrinolysis in vivo. In this paragraph the early findings of studies on fibrinolysis are briefly reported. Goodpasture was the first to demonstrate that enhanced fibrinolysis

in vitro was common in liver cirrhosis (124). In 1914 he reported spontaneous lysis of plasma clots of four patients with liver cirrhosis. The clotted plasma was incubated at 37°C and lysed within sixteen hours. In contrast, clots of healthy volunteers remained unlysed for many days. Since then, several authors have reported enhanced fibrinolysis in liver cirrhosis (125-135). Rattnoff described clot lysis studies in patients with alcoholic cirrhosis in comparison with normal volunteers. He found rapid fibrinolysis in almost all the cirrhotics (125).

Kwaan et al studied the fibrinolytic mechanism in 30 patients with liver cirrhosis (126). Fibrinolytic activity was measured by a quantitative technique in which plasma was clotted by the addition of thrombin, and fibrin levels were measured before and after 6, 12 and 24 hours incubation at 37°C. The cirrhotics had an increased fibrinolytic activity compared to healthy volunteers. They found no relation between the severity of the disease and the intensity of fibrinolysis (126).

The increase of fibrinolysis was, however, not found by all investigators (136-138). In most of the studies some patients had accelerated fibrinolysis, whereas others had normal rates (138,139). Grossi et al found in 51 patients with liver cirrhosis an abnormal spontaneous proteolytic activity in 49% of the patients (131). In 1971 Van de Loo and Schmiessing reviewed the results of 20 studies on fibrinolysis in liver disease (139). They concluded that most studies showed enhanced fibrinolysis. Their own study of 80 patients with various liver diseases showed no correlation between the severity of disease and fibrinolytic activity (139).

The mechanism of enhanced fibrinolysis in liver diseases has been a subject of many studies. Van de Loo mentioned three mechanisms for increased fibrinolysis:

1. Increase of activators of the fibrinolytic system due to a decreased clearance or an increased synthesis of activators.
2. Reduced inhibition of fibrinolysis due to a decreased synthesis or increased consumption of inhibitors.
3. Local mechanisms giving rise to increased fibrinolysis within the liver, for instance a local increased level of plasminogen activator. This last mechanism is no longer considered to be of major importance.

ad 1) The decreased hepatic clearance of plasminogen activators in cirrhosis as the pathogenetic mechanism of hyperfibrinolysis was suggested firstly by Fletcher et al (140). They showed an abnormal fibrinolytic response in cirrhotic patients, who were injected with nicotinic acid, which is known to enhance the levels of plasminogen activator in plasma. This was ascribed to a defective clearance of plasminogen activator by the cirrhotic liver (140). These results were confirmed by Tytgat et al (136). Das et al showed that after a physiological stimulus such as exercise, the plasminogen activator response in some of the cirrhotic patients was excessive in terms of either the initial response and/or in the prolongation of the recovery period (141). Kwaan et al showed that infusion of adrenalin resulted in a higher and prolonged response (i.e. increased fibrinolytic activity) in patients with liver disease as compared with healthy individuals. This increased response may be due to a decreased clearance of plasminogen activators by the liver in cirrhotic patients, since adrenalin infusion increases the levels of plasminogen activator in blood (24,25).

Mowat et al and Ogston et al demonstrated increased levels of plasminogen activator activity in blood of patients with liver cirrhosis (134,138). In all the above mentioned studies only plasminogen activator activity was measured. As a consequence they do not reveal whether this increase in activity is due to an increased concentration of activators or to a decrease of activator inhibitors.

ad 2) Several authors suggested that low levels of inhibitors of fibrinolysis were the cause of hyperfibrinolysis in cirrhosis (103,142). O'Connell et al showed a decrease of inhibitors of fibrinolysis in serum of patients with alcoholic cirrhosis (142). They measured the inhibitors of plasminogen activators as well as of fibrinolysin (plasmin). This was confirmed in several other studies (126,128,136). Tytgat et al suggested that the decrease of inhibition of fibrinolysis might contribute to the excessive response of plasma fibrinolytic activity of cirrhotic patients (136).

ad 3) Denk et al demonstrated fibrinolytic activity in liver biopsies of cirrhotic patients (143). Their study revealed that this was due to local increase of plasminogen activator. It has been suggested by Astrup et al that new blood vessels with high

fibrinolytic activity may grow into the liver during tissue repair in cirrhosis (128). This proposed mechanism for the increase of fibrinolytic activity has been abandonned.

2.4.2.2 Recent advances

The increased interest in the fibrinolytic system in the late seventies and eighties has led to more studies and enwidened knowledge on fibrinolysis in liver disease. Several new factors have been purified and the role of these factors is becoming more clear (144). We have subdivided the studies performed in patients with liver cirrhosis in recent years in three groups: studies on the availability of plasminogen, on the plasminogen activators and on the inhibition of fibrinolysis in blood.

a) Plasminogen in liver disease

Liver disease is associated with a decrease in the levels of plasminogen in plasma (140,145). This decrease can be explained by the fact that plasminogen is synthesized by the liver. In patients with severe liver cirrhosis there is in addition an increased consumption of plasminogen (146,147). In theory this should result in a decreased fibrinolytic potential and these changes in plasminogen levels are apparently in contradiction to the growing evidence of enhanced fibrinolysis in liver disease.

b) Plasminogen activators in liver disease

As summarized above, several investigators have found increased levels of plasminogen activator activity in blood of patients with liver disease (134,138,140). With recently developed assays it is possible to assess whether this is due to increased concentrations of plasminogen activators or to decreased concentrations of their inhibitors. In addition, the types of plasminogen activator responsible for the activity can be discriminated.

Tissue-type plasminogen activator (t-PA): Juhan-Vague et al showed an increase of t-PA antigen in liver disease (148). Booth et al found that t-PA antigen was considerably increased in patients with alcoholic liver cirrhosis (149). All patients in their study had an increased fibrinolytic activity in vitro as judged by a fibrin plate assay (149). Boks et al failed to find increased fibrinolytic

activity in patients with non-alcoholic or alcoholic liver cirrhosis. Although a large variability in values was observed, the mean values tended to be lower than that of normal controls (150). However, the levels of t-PA antigen were significantly increased in alcoholic liver cirrhosis (150). Francis et al also found increased t-PA antigen levels in patients with liver disease of various types. In about 50 % of the patients with liver disease, irrespective of the presence of disseminated intravascular coagulation, t-PA activity was detectable (151). The reason for the discrepancy between antigen and activity levels of t-PA in liver disease could be the concomitant rise in plasminogen activator inhibitor in plasma (151). Hersh et al confirmed these results and suggested that plasminogen activator inhibitor played a critical role in fibrinolytic activity observed in patients with liver cirrhosis (152).

Urokinase-type plasminogen activator (u-PA): U-PA antigen levels were found to be normal in both alcoholic and non alcoholic liver cirrhosis by Kirchheimer et al (153). In contrast, Booth et al and Dooijewaard et al showed increased levels of u-PA antigen levels (149,36). The latter found increased levels of both pro-urokinase and urokinase-inhibitor complexes in patients with liver insufficiency. This suggests that the clearance of pro-urokinase by the diseased liver is decreased in severe situations, and/or that the synthesis is increased (36).

c) Inhibition of fibrinolysis in liver disease

α_2-Antiplasmin (α_2-AP): In 1978, Aoki and Yamanaka found that levels of the major plasmin inhibitor in blood, α_2-AP were decreased in chronic liver disease (55). They suggested that this decrease might contribute to enhanced fibrinolysis in these patients. This result was confirmed in several other studies, in which also the cause of the decrease in α_2-AP was studied (45,154,155,156). Marongiu et al found that a decrease of α_2-AP is associated with elevated levels of fibrinopeptide-A (FpA) (156). Since FpA is a marker for thrombin activity in vivo, they proposed that the decrease of α_2-AP is a secondary phenomenon, i.e. secondary to disseminated intravascular coagulation.

However, in a kinetic study on the metabolism of α_2-AP in patients with mild liver cirrhosis, Knot et al found that the α_2-AP decrease was not caused by enhanced consumption in the central compartment (blood), but by a decreased synthesis, perhaps in combination with a change in transcapillary flux ratio (45). It is concluded that the α_2-AP decrease in these patients is due to a decreased synthesis and in patients with severe cirrhosis consumption might contribute to the decrease.

Histidine-rich glycoprotein (HRG): The levels of HRG play a role in the availability of plasminogen. Previous studies on HRG in liver diseases have given contradictory results. In four studies a decrease of HRG was found in patients with liver diseases (62,68,149,157). Saito et al suggested that this decrease of HRG could contribute to enhanced fibrinolysis in patients with advanced liver cirrhosis (157). In contrast, Gram et al did not find a decrease of mean HRG level in a group of patients with various liver diseases (158). All studies showed wide variability in HRG levels in the patients, ranging from 21 % to 165 % of normal pooled plasma. Therefore it is still not clear whether changes in HRG levels indeed play a role in enhanced fibrinolysis in liver disease.

Plasminogen activator inhibitors (PAI): Only a few studies on PAI levels in patients with liver cirrhosis have been published. Boks et al reported that there was a wide range in levels of PAI activity in patients with alcoholic and non-alcoholic liver cirrhosis, and that the means of PAI levels in both patient groups were higher than normal (150). A correlation between the levels of PAI and the occurence of bleeding could not be demonstrated. Hersh et al studied 30 patients with liver cirrhosis and subdivided these patients into three groups with either markedly, moderate or no accelerated fibrinolysis, as judged by the diluted whole blood clot lysis time (DWBCLT) (151). They found a correlation between the levels of total t-PA inhibitory capacity of plasma and the DWBCLT. T-PA inhibition of plasma was significantly lower in the patients with markedly accelerated fibrinolysis compared to the patients with no enhancement of fibrinolysis. They suggested therefore that tissue plasminogen activator inhibitor plays a critical role in the

pathogenesis of accelerated fibrinolysis in liver cirrhosis (151). Recently Suzuki reported reduced levels of protein C inhibitor, in patients with liver disease (159).

Other inhibitors of fibrinolysis: The levels of other inhibitors of fibrinolysis, such as α_2-macroglobulin and C_1-inactivator are also changed in patients with liver diseases. α_2-Macroglobulin levels can be increased in patients with hepatic cirrhosis (76,154) and therefore seems to play no major role in enhanced fibrinolysis in liver cirrhosis. Booth et al reported normal or raised C_1-inactivator levels in patients with alcoholic liver cirrhosis (148).

It can be concluded that several changes in fibrinolysis variables occur in liver cirrhosis. The historical studies already suggested an increase in plasminogen activator activity and a decreased inhibition of fibrinolysis in patients with liver disease. Recent studies have revealed an increase in t-PA and u-PA antigen, resulting in some patients in an increased fibrinolytic activity in vitro. In addition reduced PAI activity may occur in some patients with cirrhosis. α_2-AP and HRG levels are frequently reduced in patients with liver cirrhosis. Most studies have focused on changes of only one of these fibrinolysis variables. Therefore it cannot be concluded what the relevance of changes of individual factors will be for overall fibrinolysis. Furthermore it should be noted that fibrinolytic activity is measured in vitro, and it remains unclear whether this represents the situation in vivo.

2.5 FIBRINOLYSIS IN OTHER TYPES OF LIVER INJURY

2.5.1 FIBRINOLYSIS IN ACUTE HEPATIC FAILURE

So far we have discussed the fibrinolytic system in chronic liver disease (cirrhosis). Several studies have been performed in patients with acute hepatic failure. These have shown extensive changes in the haemostatic mechanism, which have mainly been ascribed to disseminated intravascular coagulation (118).
The first study on fibrinolysis in patients with acute hepatic failure was performed by Ratnoff, who found no evidence for increased fibrinolytic activity (125). In 13 patients with acute hepatitis due to various causes the clot lysis time was always within normal limits (125). Clark et al also failed to find lysis on fibrin plates in 12 patients with fulminant hepatic failure (160). Hillebrand et al found decreased plasminogen activator activity with decreased plasminogen and increased fibrin(ogen) degradation products in plasma in the majority of patients with acute hepatic failure (161,162). They postulated formation of local fibrin deposits in the liver, with adsorption of plasminogen activator, plasminogen, and plasmin on the fibrin clot, followed by lysis of the clot (162). In a recent review Rock stated, that in acute hepatic failure, fibrinolysis is mild and not frequently identified (163). The fibrinolysis may be more related to an ongoing mild consumptive coagulopathy secondary to necrosis of hepatic tissue and local fibrin deposition within the liver. He suggested that due to the large blood flow through the liver these activated and clotted materials may be washed out of the liver into the systemic circulation, appearing as systemic coagulopathy.
Recent studies also reported a normal fibrinolytic activity in acute hepatic failure (150). However, an experimental study in mice injected with carbon tetrachloride revealed fibrinolysis on thrombo-elastography (164). So although no increased fibrinolytic activity has been reported in most studies, enhancement of fibrinolysis has been detected. This can be ascribed to the strong decrease in α_2-AP in acute hepatic failure (55,150,164). Tomiya et al found increased plasmin-α_2-antiplasmin complexes in the circulation in acute hepatic failure (165). The decrease of α_2-AP in acute hepatic failure is ascribed to a reduced synthesis and

additionally increased consumption.
It can be concluded that acute hepatic failure is associated with extensive changes in coagulation and fibrinolysis, due to decreased synthesis of clotting factors and disseminated intravascular coagulation, however with small or no changes in fibrinolytic activity in vitro.

2.5.2 FIBRINOLYSIS IN OBSTRUCTIVE JAUNDICE

Several studies of the fibrinolytic system have been performed in obstructive jaundice. Ratnoff failed to find an increase of fibrinolytic activity in these patients (125). Later studies by Jedrychowski et al and Wardle, showed a decrease of fibrinolytic activity (166,167). Recently, the cause of the decreased fibrinolytic activity was elucidated by Colluci et al. They demonstrated an increase of plasminogen activator inhibitor (PAI) activity in plasma of patients with obstructive jaundice compared to non jaundiced patients (168). Relief of the obstruction was accompanied by a decrease in PAI activity, parallel to the decrease in bilirubin levels. Rabbits undergoing bile duct ligation, showed an early and progressive increase in plasma PAI activity (168). The early rise in PAI activity however may in part be ascribed to the earlier reported, normal post-operative increase of PAI (169). It can be concluded that obstructive jaundice may be associated with increased PAI levels, resulting in a decreased rate of fibrinolysis (168).

2.5.3 FIBRINOLYSIS IN MALIGNANCY

Several studies have been reported in which the fibrinolytic system in malignancy was investigated. It has been postulated that plasminogen activator activity can be used by tumor-cells for local invasive growth and the formation of metastases. A few studies on fibrinolysis in patients with primary liver malignancy have been reported. Kwaan et al found a decrease of fibrinolysis due to an increase of inhibitors of plasminogen activator in primary liver carcinoma (170). Aoki et al found normal levels of α_2-antiplasmin in carcinoma of the liver (55).
Tumour cell lines of the liver (hepatoma, Hep-G_2) are frequently

used for studying the influence of hormones and other substances on the synthesis and secretion of fibrinolysis variables. This has been and still is of major importance for the increase in knowledge of the metabolism of fibrinolytic factors by the liver.

2.6 CLINICAL SIGNIFICANCE OF ENHANCED FIBRINOLYSIS IN LIVER DISEASE

The enhancement of fibrinolysis in liver cirrhosis may contribute to the bleeding complications in these patients. Bergström et al gave a detailed report of a patient with liver cirrhosis, in whom the fibrinolytic activity varied considerably in intensity over the course of the disease. Bleeding (haematemesis or melena) was related to periods of increased fibrinolytic activity (130). Purcell et al reported that spontaneous fibrinolytic activity was present in many patients in whom cirrhosis was accompanied by severe haemorrhage (135). Francis et al provided further evidence for the clinical significance of enhanced fibrinolysis in liver diseases (171). They showed that enhanced fibrinolysis, as measured by the whole blood clot lysis time, predisposed to soft tissue haemorrhage after trauma. They also found a trend towards increased intracranial bleeding in patients with enhanced fibrinolysis. Another study reported a relation between mucosal bleeding and a quotient of fibrinolytic activity and a clotting test (Normotest^{R}) in patients with acute and chronic liver failure (150). However, Brommer et al recently showed that in a subgroup of the patients of the above mentioned study (150) with chronic liver failure, no relation could be found between fibrinolytic activity and the occurrence of capillary-type or variceal bleeding (172).
The changes in fibrinolysis may result in complications during surgery in patients with liver disease. Kwaan et al suggested that an increase of fibrinolytic activity during splenectomy in a patient with hepatic cirrhosis, might be responsible for severe oozing during surgery (126). In patients undergoing a portacaval shunt operation, increased fibrinolytic activity may contribute to the haemorrhagic complications of the operation (173,174). In about 10% of the patients undergoing a portacaval shunt operation uncontrollable oozing, which is associated with hypofibrinogenemia and high plasma fibrinolytic activity, developed during surgery (175). In patients undergoing an orthotopic liver transplantation

severe bleeding is the major cause of peri-operative death (for a review see 176). The haemorrhagic diathesis during the procedure may be due to disseminated intravascular coagulation, hyperfibrinolysis or a combination of these two processes (176). Recent studies suggest that enhancement of fibrinolysis during the anhepatic and early post-anhepatic period, due to an extensive increase of tissue-type plasminogen activator, may be the major cause of sustained bleeding (177).

2.7 MANAGEMENT OF ENHANCED FIBRINOLYSIS IN LIVER DISEASE

As outlined above, bleeding is a major complication in patients with liver disease. This seems to be related both to mechanical factors, as in variceal bleeding, and to changes in the haemostatic mechanism (capillary-type bleeding). It is generally accepted that there is little indication for prophylactic treatment of the coagulopathy in most patients with liver disease, unless they bleed, or have to undergo surgery or biopsy. The enhancement of fibrinolysis in patients with liver cirrhosis cannot be regarded separately from the other changes of the haemostatic mechanism. In table 3, the various possible treatments of coagulopathy in patients with liver disease are summarized.
Since a reduction in the vitamin K dependent coagulation factors is common in both acute and chronic liver disease and vitamin K absorption may be decreased in liver disease, vitamin K should be administered to all these patients before surgery or biopsy or if they have features of vitamin K deficiency.

Table 3: Therapeutic possibilities in the management of coagulopathy in liver disease

Vitamin K
Fresh frozen plasma
Prothrombin complex
Cryoprecipitate
Platelets concentrates
Heparin
Epsilon amino caproic acid / Tranexamic acid

In patients who bleed extensively, replacement therapy with fresh frozen plasma or concentrates is indicated. A major problem is the volume of fluid and the quantity of the proteins which has to be administered. Prothrombin complex, however, carries the risk of triggering disseminated intravascular coagulation by the presence of activated coagulation factors in the concentrate (178).
Cryoprecipitate, containing factor VIII and fibrinogen can also be given to correct coagulation factor deficiencies. If a decrease of platelets is of major concern, depending on platelet count and bleeding time, platelets concentrates can be given. One of the major risks of giving plasma or concentrates is the transmission of viral hepatitis or AIDS.
Heparin is potentially dangerous, since it can worsen the bleeding, and should therefore be reserved for patients with clear evidence of disseminated intravascular coagulation.
If fibrinolysis is enhanced, treatment with synthetic fibrinolysis inhibitors may be a solution. The agents most widely used are epsilon amino caproic acid (EACA) and tranexamic acid. They both inhibit the binding of plasminogen to the fibrin surface, which results in a slower fibrinolysis (179). Early studies have shown a beneficial effect of EACA on excessive fibrinolysis in liver patients (180,181). However, the treatment with EACA has to be very carefully controlled, since the development of microthrombi during EACA treatment of patients with liver disease has been reported (182,183). Microthrombi occurred especially when hyperfibrinolysis was secondary to disseminated intravascular coagulation. Therefore it has been suggested that in cirrhotic patients EACA should only be used in combination with heparin (184).
EACA treatment has been reported to be of benefit in patients undergoing liver surgery and liver transplantation, which is associated with increased fibrinolytic activity (175,185). However there have been no adequately controlled trials of synthetic fibrinolysis inhibitors in bleeding due to enhanced fibrinolysis in patients with liver cirrhosis.
It can be concluded that therapy for haemostatic defects in liver disease is necessary in bleeding patients or patients who have to undergo surgery or biopsy. The mainstay of therapy is infusion of fresh frozen plasma. Enhanced fibrinolysis may be treated with synthetic fibrinolysis inhibitors, but these drugs have to be used

with caution.

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CHAPTER 3

MILD HAEMOSTATIC PROBLEMS ASSOCIATED WITH CONGENITAL HETEROZYGOUS α_2-ANTIPLASMIN DEFICIENCY

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ABSTRACT

A Dutch family, of which 13 members are heterozygotes, deficient for α_2-antiplasmin (α_2-AP) is reported. Clinical studies showed that 2 heterozygotes had a mild bleeding tendency, which presented as bleeding episodes after tooth extraction and after surgery and, in one patient, also as excessive menstruation. Laboratory investigations revealed an α_2-AP activity of 62 % (51-71) (median and range) and an antigen level of 60 % (60-66). The plasminogen binding as well as the fibrin binding properties of α_2-AP were normal. Plasminogen concentrations were significantly higher in the heterozygotes compared to the other family members. However, free plasminogen not bound to histidine-rich glycoprotein was not significantly different between these two groups. We propose that in this family the deficiency of α_2-AP is due to a decreased synthesis of a normal α_2-AP molecule. This present study brings the frequency of heterozygous α_2-AP deficient patients with a bleeding tendency to 13 out of 59 heterozygotes reported in the literature.

INTRODUCTION

α_2-Antiplasmin (α_2-AP), the fast acting plasma inhibitor of plasmin (1,2) is synthesized in the liver (3,4) and has a plasma concentration of 1 μM (5,6). It's major function in vivo is the regulation of fibrinolysis by forming an irreversible 1:1 stoichiometric complex with plasmin (7,8). Two other properties of α_2-AP are the formation of a reversible complex with plasminogen (2,9) and crosslinking to fibrin mediated by factor XIIIA (10). The binding of α_2-AP to plasminogen reduces the binding of plasminogen to fibrin, thereby reducing fibrinolysis (11). Crosslinking of α_2-AP to fibrin additionally reduces the clot lysability (12).

In plasma, α_2-AP circulates as a plasminogen binding (PB) form and a non-plasminogen binding (NPB) form with a PB:NPB ratio of about 2.4 (8,14). The PB form of α_2-AP is synthesized in the liver (14) and has all three functions mentioned above, whereas the NPB form is formed in the circulation by conversion of the PB form (14) and the only function remaining is a slow inactivation of plasmin (8,15,16).

The first case of a patient with congenital deficiency of α_2-AP with a haemorrhagic diathesis was reported by Koie et al. and Aoki et al.(17,18). Since then congenital deficiencies have been reported in eight families (19-26,38,39). Homozygotes have a severe haemophilia-like bleeding tendency (17-22,38,39). In some reports of heterozygote cases, a mild bleeding tendency was described (20,21,25,26), whilst other reports indicated no bleeding tendency (18,22,23).

We report a Dutch family with 13 heterozygous α_2-AP deficient members of which only two have a mild bleeding tendency.

MATERIALS AND METHODS.

Venous blood was collected in plastic tubes containing trisodium-citrate 0.11 mol/l (9:1) and placed immediately on melting ice. Plasma was prepared by centrifugation at 2,000 g for 30 min. at 4°C and stored in small aliquots at -70°C. Venous blood was also collected in 0.045 ml 15% sol 6.75 mg EDTA (5 ml). Serum used for determining the fibrin binding of α_2-AP was prepared from blood

collected into tubes without anticoagulant. These were left standing at 37°C for 4 hours, after which the supernatant serum was obtained by centrifugation (room temperature, 10 min., 2,000 g). Reference pooled plasma was obtained from 40 healthy volunteers.
Routine coagulation tests were performed using standard techniques. Antithrombin III activity was measured according to Abildgaard et al. (27) using Coatest(R) antithrombin III, KabiVitrum, Amsterdam; α_2-AP activity was assayed according to Friberger et al. (28) using Coatest(R) antiplasmin (KabiVitrum). Plasminogen was measured according to Friberger et al. (28) using Kabikinase(R) for activation of plasminogen and S-2251 as a substrate, both of KabiVitrum. The three foregoing assays were modified for automated determination (PA 800, Vitatron, Dieren, The Netherlands).
α_2-AP antigen was assayed by Rocket immunoelectrophoresis according to Laurell (29) using an 0.5 % anti-α_2-AP antiserum (a gift from Dr.I. Clemmensen). Histidine-rich glycoprotein (HRG) was assayed by Rocket immunoelectrophoresis according to Laurell (29) using 0.5 % home made rabbit anti-human HRG antiserum (K4559). Calculation of free plasminogen was based on the equilibrium [Plasminogen-HRG] $\rightleftarrows$ [Plasminogen] + [HRG] with a K_d of 1.0 μmol/l and results expressed in percentage of total plasminogen (30). A qualitative assay for Factor XIII was performed by addition of 5 mol/l urea to clotted plasma.
Fibrin and fibrinogen degradation products (Total Degradation Products) were measured using an ELISA technique (Organon Teknika, Boxtel, The Netherlands) (31). Fibrinogen was measured according to Clauss (32).
Protein C antigen was assayed using an ELISA Protein C technique from Boehringer Mannheim diagnostica, West Germany (33).
Tissue-type plasminogen activator (t-PA) and t-PA inhibition of plasma were measured by spectrophotometric assays according to Verheijen et al (34,35). To determine the euglobulin clot lysis time (ECLT), standard euglobulin fractions of plasma were prepared at pH 5.9 with a plasma dilution of 1:10 (36). Precipitates were redissolved in Tris/Tween buffer (0.1 mol/l Tris/HCL ; 0.1 % Tween 80 (v/v) pH 7.5) and 0.2 ml of the euglobulin fractions were clotted by addition of 0.1 ml of calcium-thrombin solution ($CaCl_2$ 25 mmol/l and thrombin 10 NIH U/l). The lysis time of the clot was recorded. The disappearance of air bubbles was regarded as the

endpoint.
The diluted whole blood clot lysis time (DBCLT) was determined according to Chohan (37).
The ratio PB:NPB forms of α_2-AP was determined by modified crossed immunoelectrophoresis (mCIE) according to Kluft (13).
The PB:NPB ratio was calculated as the ratio of the surfaces of the two immunoprecipitation peaks. The surfaces were determined as products of height and width at a point which bisects the height.
Binding of α_2-AP to fibrin during clot formation in whole blood was calculated from the amount of α_2-AP in serum and citrated plasma (corrected for the haematocrite (Ht) and the citrate dilution) and expressed as the percentage of α_2-AP bound to fibrin according to the formula:

$$\% \text{ bound to fibrin} = \frac{\frac{(1 - Ht)}{(0.9 - Ht)} \times (\alpha_2\text{-AP act.plasma}) - (\alpha_2\text{-AP act.serum})}{\frac{(1 - Ht)}{(0.9 - Ht)} \times (\alpha_2\text{-AP act. plasma})}$$

RESULTS

Case history

The proposita, a 36 year old Caucasian woman, was referred to the department of gynaecology because of severe and prolonged menstrual blood loss for four years. This loss could not be controlled by hormonal therapy and caused an iron deficiency anaemia. Before this period, menstrual blood loss had been completely normal. Echographic examination showed a submucous uterine myoma of 4 cm diameter, which did not seem to explain the excessive blood loss. A bleeding tendency was suspected and she was referred to the department of haematology for further examination.
Her history did not reveal any sign of spontaneous bleeding, neither as a child nor as an adult. She underwent three tooth extractions without a bleeding episode. She had never been pregnant or undergone surgery or experienced trauma. On physical examination, she looked pale but otherwise no abnormality was found. Laboratory tests revealed a mild iron deficiency anaemia, a normal white blood cell count, normal renal and liver functions and

no paraproteinemia.

Laboratory investigation of haemostasis (Table 1) revealed a partial deficiency of α_2-AP. The activity and antigen levels were 62 % and 60 % respectively, which are below the reference intervals (85 - 120 %) (40 healthy volunteers; mean ± 2SD).

Assuming that the combination of α_2-AP deficiency and the submucus myoma might be the explanation for the excessive blood loss, she was treated with the fibrinolytic inhibitor tranexamic acid, 4 gr daily orally during the menstrual period for two cycles without any success. It was then decided to perform a hysterectomy. As she had no history of a bleeding tendency she was given routine antithrombotic prophylaxis with subcutaneous heparin 5,000 U twice daily. Postoperatively, the haemoglobin was followed twice daily and was stable. Fibrinolytic parameters during the postoperative period showed no significant changes (Fig.1).

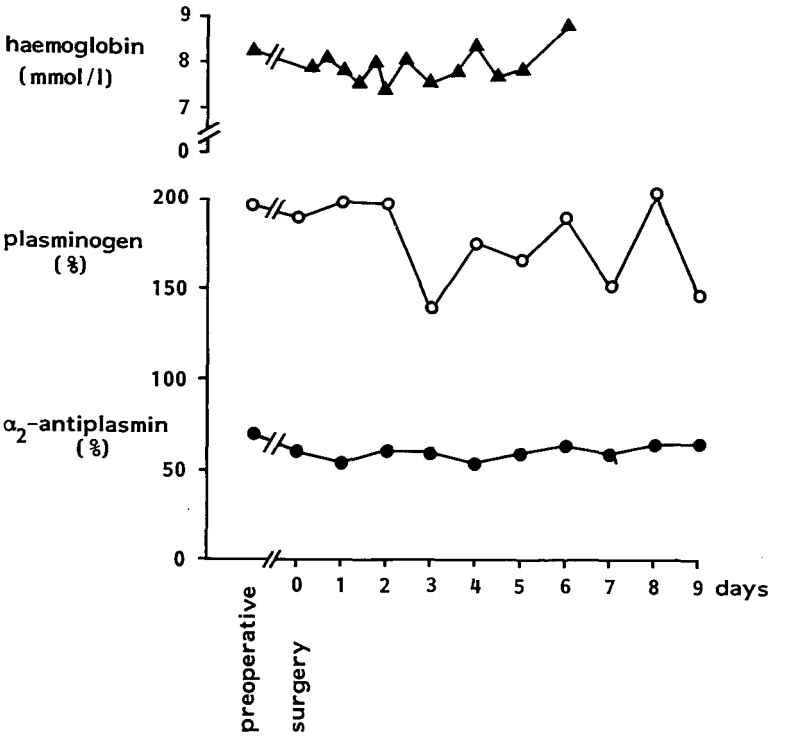

Figure 1: Haemoglobin concentration, α_2-antiplasmin activity and plasminogen before and after the hysterectomy of the proposita.

Table 1: Laboratory results: Some fibrinolysis parameters in the proposita, heterozygotes deficient for α_2-antiplasmin, and the other family members.

		Proposita	Heterozygous familymembers median (range)	n	Normal familymembers median (range)	n	Reference intervals
				13		12	
α_2-APactivity	%	62	62 (51-71)	13	111 (102-116)	12	85-120
α_2-APantigen	%	59.5	60 (60-66)	3	110/122	2	85-120
Ratio PB:NPB		2.1	2.1 (2.1-2.2)	3	2.1/2.3	2	2.1-2.8
α_2-AP binding to fibrin[1]	%	46	34 (27-46)	4	34 (30-47)	6	25-47
Plasminogen	%	208	116 (89-208)	10	99 (89-107)	7	85-120
Fibrinogen	g/l	3.2	2.8 (1.9-3.8)	12	2.5 (1.8-3.4)	12	1.8-3.6
TDP[2]	µg/ml	0	0.0 (0.0-0.5)	10	0.0 (0.0-0.6)	10	< 0.5
t-PA	mIU/ml	78	69 (0-327)	11	63 (0-240)	10	0-250
t-PA inhibition	IU/ml	0	5.3 (0-17.0)	10	6.0 (0-24)	8	0-9
DBCLT	min	175	177 (175-190)	3	150/155	2	> 90
ECLT	min	120	≥180 (n=4) 85/120/125/130	8	≥180 (n=6) 140	7	> 60
Factor XIII[3]		P	P	11	P	12	P
Protein C ag	U/ml	1.30	1.25 (0.81-1.53)	10	1.12 (1.06-1.33)	5	0.7-1.4
HRG[4]	%	94	95.2 (32-122)	10	97.0 (64-120)	10	53-145

[1]% of the initial plasma value; [2]TDP= Total (fibrin and fibrinogen) degradation products; [3]P=present; [4]HRG=Histidine-rich glycoprotein.

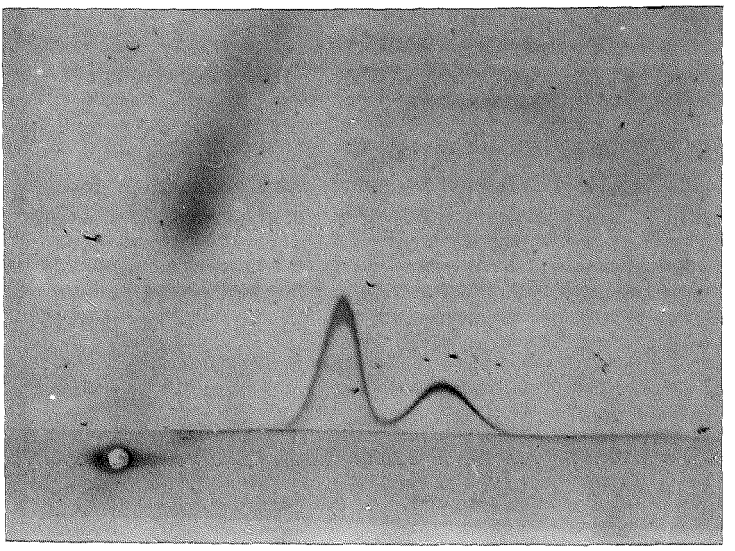

Figure 2: Modified crossed immunoelectrophoresis of plasma of the proposita. The first peak shows the plasminogen binding (PB) form and the second peak shows the non-plasminogen binding (NPB) form of α_2-antiplasmin.

Haemostasis studies

Fibrinolysis and some other parameters of the proposita and the other family members are shown in Table 1. Biological and immunological levels of α_2-AP of the proposita were both decreased with an activity / antigen ratio of 1.02.

Modified crossed immunoelectrophoresis showed a normal ratio of PB:NPB form of α_2-AP of 2.1 (normal range 2.1 - 2.8 (14)) (fig.2). The binding of α_2-AP to fibrin was 46 % of the initial plasma value which is in the normal range (25 - 47 ($x \pm 2$ SD;N=22)). The absolute amount of α_2-AP bound to fibrin however was half that of normal (23%).

Plasminogen concentration of the proposita was 208 % (normal range 85-120) and histidine-rich glycoprotein (HRG) was 94 %. Free plasminogen not bound to HRG was calculated to be 133 % (Fig. 3). Total fibrin and fibrinogen degradation products were not detectable (<0.5 µg/ml) with a sensitive ELISA technique.

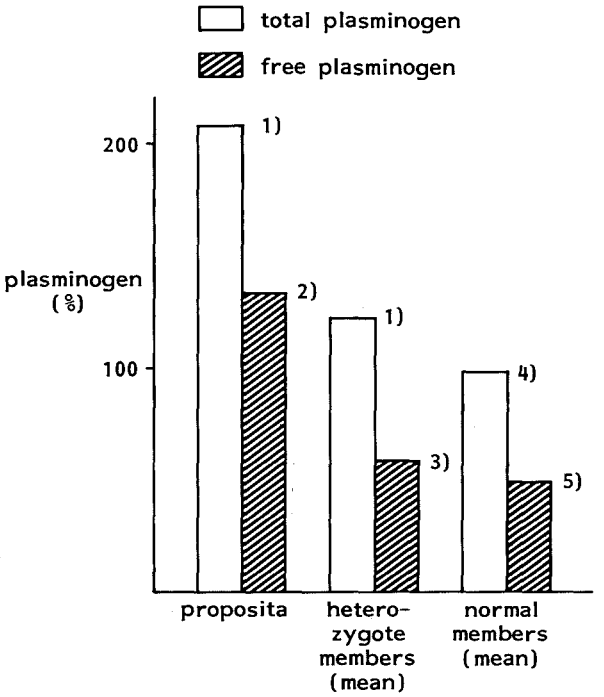

Figure 3: Total and free plasminogen of the proposita and the other family members. 1) is significantly different from 4) p <0.05 ; 2) is significantly different from 5) p <0.05 ; 3) is not significantly different from 5).

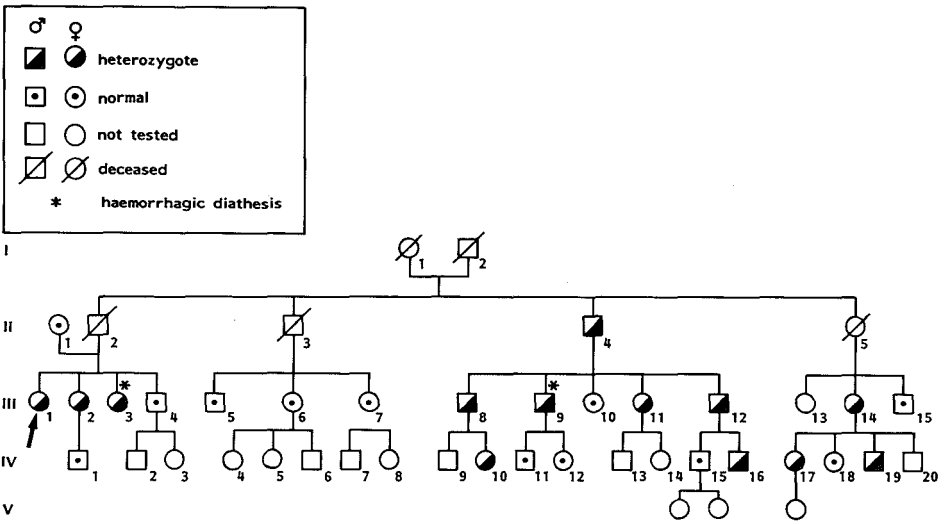

Figure 4: Pedigree of the family with a heterozygous α_2-antiplasmin deficiency. The arrow indicates the proposita.

Family Study

On studying the family, 12 more heterozygotes were diagnosed with α_2-AP activities ranging from 51-72 %, which are all below the normal range (85-120 %). The pattern of inheritance of the heterozygous α_2-AP deficiency was autosomal dominant (Fig. 4). A history of bleeding was established in 3 of the 12 heterozygotes by taking the patients' history and, in some cases, by consulting hospital records (Table 2). This was done before the α_2-AP activity was determined.

Member III-3 had excessive menstrual blood loss and had experienced a bleeding episode after surgical correction of the nasal septum. Member III-9 had had a severe bleeding episode for 1 day after tooth extraction (7 molars at one time) for which he consulted a general practioner. Another member (IV-16) had had severe bleeding episodes after a complicated tibia and fibula fracture. Six days after surgical treatment a large haematoma developed in the left leg while the patient was under prophylactic anticoagulant therapy (Thrombotest < 3 %). Several subsequent orthopaedic operations did not result in further bleeding episodes. Thus, in the proposita and in patient IV-16 causes other than the

α_2-AP deficiency alone were found that may explain the bleeding problems, in contrast to patients III-3 and III-9.

Other α_2-AP deficient family members and members with normal α_2-AP levels underwent several surgical interventions without a bleeding episode (Table 2). The results of the fibrinolysis study of the other heterozygotes are similar to the results of the proposita (Table 1). Median α_2-AP activity and antigen were 62 and 60 % respectively. Plasminogen concentration was significantly higher in the heterozygotes compared with the normal family members, 116 (89-208) % (median and range) and 99 (89-107) % respectively (Wilcoxon-test; $p<0.05$). HRG and the calculated free plasminogen levels were not significantly different between both groups (Fig. 3). The fibrinolytic activity, as measured by the ECLT and DBCLT, was normal in both groups. Platelet counts and functions, as well as the coagulation parameters, were normal in all family members.

No difference in fibrinolytic parameters was found between the bleeding and the non-bleeding heterozygotes.

Table 2: Clinical information of the heterozygous α_2-antiplasmin deficient family members. The numbers refer to the family pedigree in Fig 3. III-1 is the proposita.

Family-member	Clinical information	Tooth extractions	Menstruation
II-4	Deep venous thrombosis[1]	2-3x	-
III-1(prop)	Hysterectomy	3x	prolonged excessive
III-2	1 x Partus		
	Adenotonsillectomy, lipectomy	2x	normal
III-3	Nasal septum reconstruction B.E.[2]	5x	
	Haematomas, nose bleeding	2x B.E.	prolonged excessive
III-8	-	1x	-
III-9	-	3x B.E.	-
III-11			
III-12	Meniscectomy	2x	-
III-14	4 x Partus 1x B.E.		
	1 x Severe haematoma after trauma		
	Adenotonsillectomy		
	Cholecystectomy	3x	normal
IV-10	-	-	-
IV-16	Severe bleeding after complicated fibula and tibia fracture	-	-
IV-17	1 x Partus B.E.	-	normal
IV-19	-	1x	-

[1]Clinical diagnosis not confirmed by phlebography; [2]B.E.= bleeding episode afterwars.

DISCUSSION

Nine families with α_2-AP deficiency have been described. In eight of the described families the deficiency seems to be due to a decreased synthesis of a normal α_2-AP molecule (17-26) and in one the deficiency appears to be due to synthesis of an abnormal molecule (38,39).

The laboratory studies of our heterozygotes showed both a decreased α_2-AP activity and antigen. Plasminogen binding of α_2-AP, determined by the ratio of PB:NPB forms, was normal. Although the absolute amount of α_2-AP bound to fibrin was half of normal, the percentage α_2-AP bound was normal. Neither fibrin nor fibrinogen degradation products were detectable, indicating that the fibrinolytic system was not activated. We propose that in this family the deficiency is caused by a decreased synthesis of a normal α_2-AP molecule. Comparing the results of fibrinolysis and coagulation parameters between heterozygotes and normal members, we found a significantly higher plasminogen level in the heterozygotes. This is in accordance with the findings of Kluft et al. (20), who proposed that this could contribute to a haemorrhagic diathesis. Free plasminogen, not bound to HRG, was however not significantly different between both groups. There was no difference in the fibrinolytic activity, measured by the ECLT and the DBCLT, between both groups.

No difference was found in any of the measured haemostatic parameters between the bleeding and the non-bleeding heterozygotes.

Clinical studies in patients with α_2-AP deficiency show that all 7 homozygotes from 5 families described so far suffer from a severe haemophilia-like bleeding tendency (17-22,24). The clinical presentation of heterozygous α_2-AP deficient patients described to date is variable. In 6 families, forty-six heterozygotes with an α_2-AP activity ranging from 40-71% have been reported (18,20-23,25,26). Eleven of these heterozygotes from 3 families showed a mild bleeding tendency (20,21,25,26).

In our study, only 2 of the 13 heterozygotes showed a mild bleeding tendency, which can be attributed to α_2-AP deficiency with some certainty. The bleeding problems in these 2 patients (III-3 and III-9), compared with the bleeding symptoms described in some earlier reports (24,25), are not serious.

The proposita of our study did not have a bleeding tendency, as is demonstrated by her history, by unsuccesful treatment with tranexamic acid and by the uncomplicated per-and postoperative course of the hysterectomy using prophylactic anticoagulant therapy. The bleeding complications in the other patient (IV-16) may be explained by the severity of the trauma and the treatment with oral anticoagulant, but a role for the α_2-AP deficiency cannot be ruled out.

Thus in this family, only a minority of the heterozygotes have some mild bleeding problems. The results of this and foregoing studies suggest that an α_2-AP activity of 50-60 % is a threshold for normal haemostasis.

Through this study the frequency of α_2-AP deficient heterozygotes with a bleeding tendency is brought to 13 out of 59 (22%).

ACKNOWLEDGEMENT:

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CHAPTER 4

SEVERE THROMBOTIC TENDENCY ASSOCIATED WITH A TYPE I PLASMINOGEN DEFICIENCY

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ABSTRACT

We report a 45 year-old female patient with recurrent spontaneous deep vein thrombosis associated with an isolated hypoplasminogenemia (plasminogen activity and antigen level of 42 % and 37 %, respectively). The plasminogen molecule was normal as demonstrated by a normal activation by tissue-type plasminogen activator, electrophoretic mobilty on crossed immunoelectrophoresis, molecular weight and binding to lysine-Sepharose. All other hemostatic parameters predisposing to recurrent thrombosis were normal. A stimulation test with desmopressin acetate (DDAVP) showed a normal plasma rise of both tissue-type plasminogen activator and Factor VIIIR:WF.

This isolated plasminogen deficiency is apparently due to a decreased synthesis of a normal plasminogen molecule and is associated with a severe thrombotic tendency.

INTRODUCTION

A deficiency of plasminogen resulting from a dysfunctional molecule (dysplasminogenemia) is associated with a high risk for recurrent thrombosis (1). Congenital plasminogen deficiency resulting from decreased synthesis of a normal molecule (type I deficiency; hypoplasminogenemia) associated with thrombosis has been described only occasionally (2-4). One of the reported patients had a high level of plasminogen activator inhibitor additionally, which could have contributed to the thrombotic disease (4).

We report a patient suffering from recurrent episodes of objectively diagnosed recurrent deep vein thrombosis (DVT) and hypoplasminogenemia, without additional abnormalities in the hemostatic mechanism.

CASE HISTORY

A 45-year-old woman secretary was referred to our outpatient department because of recent complaints of pain, swelling and redness in the right calf. Her previous medical history revealed recurrent episodes of DVT in both legs (three times right, one time left) since the age of 38 years, despite long-term oral anticoagulant treatment. Her history was without predisposing factors for DVT. According to the patient, several members of her family had a history of DVT or pulmonary embolism.

DVT was suspected and later confirmed by impedance plethysmography (IPG) and venography. The venography of both legs showed severe abnormalities: no visualization of the entire deep vein system of the right leg, extreme formation of collateral veins in both legs, retroperitoneal collateral veins in the right iliacal region, and an obstruction of the vena saphena magna of the left leg. These severe abnormalities in the venous system are suggestive of recurrent thrombosis in both legs.

On account of the history of recurrent thrombosis in spite of anticoagulant therapy (thrombotest 7 % on referral, therapeutic range 5-10%) and a family history of thrombosis, extensive laboratory investigations for all known risk factors were performed. These studies revealed a partial deficiency of plasmino-

gen with an activity and antigen level of 42 % and 37 %, respectively. She was treated with heparin twice daily, 7500 U s.c., for 10 days and oral anticoagulant (OAC) treatment (Fenprocoumon) was continued. Although she was treated with OAC, she experienced recurrent episodes of spontaneous DVT in both legs since.

As soon as she developed complaints suspect for DVT, an IPG was made, and, if positive, she was treated with heparin, which she administers herself at home. Because of the venous insufficiency, she has developed a severe postthrombotic syndrome in both legs.

The brother of the patient (aged 43 years) was also partly deficient for plasminogen (activity 38 %), but has never experienced a thrombotic event to date. Of the family members with a history of DVT, unfortunately none were available for further investigations.

MATERIALS AND METHODS

Plasma

Venous blood, obtained by venapuncture in the antecubital vein, was collected into 0.11 mol/l (9:1) trisodium citrate and placed immediately on melting ice. Plasma was prepared by centrifugation at 2,000 g for 30 min. at 4°C and stored in small aliquots at -70°C. Reference pooled plasma was obtained from 40 healthy volunteers.

Hemostasis studies

Routine coagulation tests were performed according to standard procedures. All known risk factors for DVT were determined. Antithrombin III and protein C activity were measured using chromogenic assays (5,6). Protein C antigen was assayed by an ELISA protein C technique (7). Protein S antigen was determined using an immunoradiometric assay (performed by Dr. Engesser, Gaubius Institute) (8). Tissue-type plasminogen activator (t-PA) and t-PA inhibition of plasma were measured by spectrophotometric assays (9,10). A tissue thromboplastin inhibition test was performed to assess the presence of a circulating lupus anticoagulant (11). A stimulation test was performed by infusion of DDAVP (Desmopressin, Minrin(R), Pharmachemie, Haarlem, The Netherlands) intravenously at a dose of

0.4 μg/kg b.w. over a period of 20 minutes. Blood samples were taken before and every 20 min for up to 2 hrs after start of the infusion. Ristocetin cofactor activity (F VIIIR:WF) was determined by measuring the initial rate of agglutination of formalin-fixed platelets in the presence of 1 mg/ml ristocetin in the aggregometer (12). Factor VIII antigen was determined by an ELISA technique using antisera obtained from Dakopatts (Copenhagen, Denmark). Factor VIII activity was assayed in a one stage clotting assay on a coagulometer, using congenitally deficient plasma as a substrate.

Plasminogen determinations and purification

Plasminogen activity was measured using a chromogenic assay (13). Plasminogen antigen was measured by radial immunodiffusion technique (14) using goat anti-plasminogen antiserum from Nordic Immunology (Tilburg, The Netherlands). Crossed immuno-electrophoresis of the patient's plasma was carried out using the anti-plasminogen antiserum (1 %) from Nordic. Histidine-rich glycoprotein (HRG) was measured immunochemically using a home-made (Gaubius Institute) 0.5 % anti-HRG antiserum (15). Calculation of free plasminogen was based on the equilibrium [plasminogen-HRG] $\rightleftarrows$ [plasminogen] + [HRG] with a K_d of 1.0 μmol/l (16).
Plasminogen of the patient was purified from plasma by affinity chromatography on lysine-Sepharose (17). Sodium dodecyl sulphate (7%)-polyacrylamide gel electrophoresis (SDS-PAGE) (18) was used to determine the molecular weight of the patients' plasminogen. To study the functional activities of the patients' plasminogen, it was activated by tissue-type plasminogen activator with and without addition of fibrin fragment (FCB-2) (19). A stimulation factor, calculated as the quotient of the acceleration constants with and without addition of FCB-2, is a marker for the stimulatory effect of the fragment (normal value 40-60) (19).

RESULTS

Hemostasis studies

All known risk factors for DVT were normal. Antithrombin III activity was 103 % compared to normal pooled plasma. Protein C (activity 34 % and antigen 41 %) and protein S antigen (44 %) were

decreased from OAC treatment. The ratio protein C and factor X (5 %) was above 1.0. T-PA was 0.01 IU/ml (normal 0-0.25), and t-PA inhibition of plasma was 4.61 IU/ml (normal 0-9). α_2-Antiplasmin activity and antigen were 123 % and 106 %, respectively. α2-Macroglobulin antigen was 127 % compared to normal pooled plasma. Fibrinogen concentration was 4.0 g/liter (normal 1.2-4.8), and, because of a thrombin time of 10 sec (normal 9-13), dysfibrinogenemia could be excluded. Factor VIII activity was 118 %. A tissue thromboplastin inhibition test revealed no evidence of circulating lupus anticoagulant.

Plasminogen assays

Plasminogen activity and antigen levels were both decreased (42 % and 37 % respectively). On crossed immunoelectrophoresis, normal mobility of the patients' plasminogen was seen. Plasminogen activation with t-PA with and without addition of fibrin fragment FCB-2 showed a normal functioning plasminogen molecule. The calculated stimulation factor of FCB-2, known to enhance plasminogen activation, was 55.4 (normal 40-60). During the purification procedure, plasminogen of the patient had a normal binding to lysine-Sepharose. SDS-PAGE showed a normal molecular weight of approximately 90.000 D. The level of histidine-rich glycoprotein (HRG), which inhibits fibrinolysis by binding to plasminogen, thereby reducing the amount of "free" plasminogen, was 94 %. The calculated "free" plasminogen level was 16 %, which is strongly decreased, compared to reference pooled plasma.

DDAVP test

Infusion of DDAVP, to determine the capacity of vascular endothelium to release t-PA and Factor VIIIR:WF showed a normal rise in both t-PA and Factor VIIIR:WF to a maximum of 1.41 IU/ml after 25 min and of 320 % (compared to normal pooled plasma) after 45 min, respectively (Fig. 1) (20).
Factor VIII activity and antigen levels showed a maximum increase to 239 % and 261 % respectively (Fig. 1).

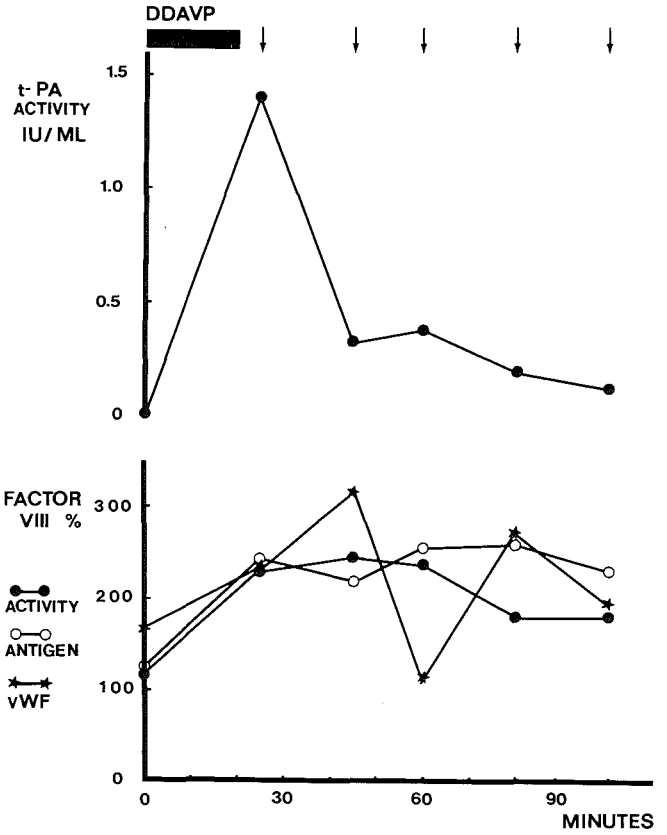

Figure 1: Laboratory results of factor VIII and tissue-type plasminogen activator after infusion of DDAVP (0.4 µg/kg b.w.) in the patient. Arrows indicate the times of blood sampling.

DISCUSSION

Several studies have shown that dysplasminogenemia may be associated with a thrombotic tendency (1). Only a few reports, however, have been published about hypoplasminogenemia associated with thrombosis (2-4). In one of the cases, hypoplasminogenemia was accompanied by an other abnormality in the hemostatic mechanism, i.e. high levels of plasminogen activator inhibitor (4). In this report we describe a patient with severe thrombotic disease, in spite of continuous OAC therapy, with a decreased activity and antigen level of plasminogen. Other risk factors for DVT were normal. The low levels of protein C, a vitamin-K dependent "inhibi-

tor" of coagulation, and its cofactor protein S were due to the OAC treatment. On account of a protein C/ factor X ratio of >1.0, a protein C deficiency can be ruled out. Normal levels of α_2-antiplasmin indicate that the fibrinolytic system was not activated. A DDAVP test, performed to determine the capacity of the vascular endothelium, showed a normal potential rise in t-PA and Factor VIIIR:WF.

Activation of the patients' plasminogen by t-PA with and without addition of FCB-2, showed a normal reactive molecule. Plasminogen had a normal affinity to lysine-Sepharose in the purification procedure, a normal molecular weight and a normal electrophoretic mobility on crossed immunoelectrophoresis. Based on these experiments, we propose that the plasminogen molecule is functionally normal and therefore suggest that the patient suffers from a type I plasminogen deficiency, due to a decreased synthesis of a normal molecule.

The clinical outcome in the proposita was severe. In spite of OAC treatment, she still experienced episodes of DVT. We have no explanation why the OAC prophylaxis has failed in this patient and, on the other hand, was of some benefit for another patient with hypoplasminogenemia (3).

Venography showed extreme abnormalities in the venous system in both legs, leading to severe venous insufficiency and postthrombotic syndrome. To release the patient from hospitalization, she is now given home treatment with heparin for each recurrent episode of DVT.

A familial deficiency is likely in this case, on account of the decreased plasminogen levels of the brother of the patient. He was not available for further study, and neither were the family members with, according to the patient, a history of DVT and pulmonary embolism.

This study demonstrates that an isolated plasminogen deficiency, apparently resulting from a decreased synthesis of a normal plasminogen molecule, may be associated with severe thrombotic tendency.

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CHAPTER 5

PLASMIN INHIBITORS IN THE PREVENTION OF SYSTEMIC EFFECTS DURING THROMBOLYTIC THERAPY BY THE PLASMINOGEN-BINDING FORM OF α_2-ANTIPLASMIN

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ABSTRACT

The aim of our study was to delineate the role of plasmin inhibitors, especially the two molecular forms of α_2-antiplasmin (α_2-AP), i.e. the plasminogen binding (PB) and the non plasminogen binding (NPB) forms, in control and prevention of systemic effects during thrombolytic therapy. We therefore studied both the consumption of plasmin inhibitors and the degree of fibrinogen breakdown in patients with acute myocardial infarction treated with tissue-type plasminogen activator (t-PA) or streptokinase (SK). At a low degree of plasminogen activation, for instance in patients treated with t-PA, PB-α_2-AP is consumed first. At a higher degree of plasminogen activation, PB-α_2-AP becomes exhausted (<20%), and other plasmin inhibitors, i.e. NPB-α_2-AP and α_2-macroglobulin, are consumed. After extensive plasminogen activation, for instance in patients treated with SK, PB-α_2-AP consumption is complete, and NPB-α_2-AP and α_2-macroglobulin are consumed about 30-50% of the pretreatment level. No significant C_1-inactivator consumption occurred, even at extreme degrees of plasminogen activation. Fibrinogen breakdown, taken as marker for systemic effects, correlated strongly with consumption of PB-α_2-AP. Fibrinogen breakdown did occur, but only when the amount of PB-α_2-AP was decreased to less than 20%. The other plasmin inhibitors were not able to prevent fibrinogen breakdown. These results were confirmed by in vitro studies. We conclude that PB-α_2-AP is the most important inhibitor of plasmin in the circulation and that, in order to obtain systemic effects, the amount of PB-α_2-AP, which is a threshold in circulation, has to be overcome. This indicates that PB-α_2-AP may be a new marker for systemic effects during thrombolytic therapy.

INTRODUCTION

Thrombolytic therapy with plasminogen activators is becoming a generally accepted treatment for patients suffering from acute myocardial infarction (AMI). Other diseases involving a pathological thrombus, such as venous thrombosis (1,2), thrombotic stroke (3,4), pulmonary embolism (5,6) and several other conditions (7), are also increasingly subject to thrombolytic treatment.

Several thrombolytic agents are studied and are in use. They comprise streptokinase (SK), acylated plasminogen streptokinase activator complex (APSAC), tissue-type plasminogen activator (t-PA) and (pro)-urokinase (u-PA, pro-u-PA).

One main difference among these frequently used thrombolytic agents is the degree of fibrin specificity and the degree of systemic effects at effective dosages. Roughly two categories of lytic agents can be distinguished with SK, APSAC and u-PA on the one hand with more extensive systemic effects, and t-PA and pro-u-PA on the other hand with a smaller degree of systemic lytic effects (8).

The systemic effects concern amongst other things, digestion of fibrinogen, resulting in appearance of (anticoagulant) split products (9), and of the coagulation factors V and VIII (10,11). This degradation of factors is due to the proteolytic action of plasmin, formed out of plasminogen in large amounts by the thrombolytic agents (12). More recently other effects of such excessive plasmin formation have also been described. It has been shown that plasminogen activation at the platelet surface leads to degradation of glycoprotein Ib and IIb/IIIa complex, which inhibits platelet adhesion and aggregation (13). In addition, platelet mass may dissolve, due to the proteolytic effect of plasmin on thrombospondin (14).

The significance of systemic effects in thrombolytic therapy has not yet been satisfactorily clarified. It has been suggested that the systemic effects are a disadvantage and may contribute to bleeding, the main side effect of thrombolytic treatment (15-19). However, data are controversial in this respect, since data which show the contrary have also been published (20,21).

Alternatively, it has been suggested that the systemic effects may be beneficial, for instance in obtaining recanalization (22-25). It has even been proposed that the systemic effects and recanalization

have to correlate, because of the key role of plasmin (26). In addition, the proteolytic damage of plasmin improves rheology of blood (27,28) and results in impairment of the coagulation system, thereby reducing the risk of rethrombosis (25). It can be concluded that further study on the occurrence and clinical significance of systemic effects is required.

Since plasmin is the key enzyme in fibrinolysis, and since it is responsible for the systemic effects, plasmin inhibitors may have a regulatory role in both processes. Several inhibitors of plasmin circulate in blood: α_2-antiplasmin, α_2-macroglobulin, C_1-inactivator and α_1-antitrypsin. The two functional forms of α_2-antiplasmin, the main inhibitor of plasmin, the plasminogen binding (PB-α_2-AP) and the non plasminogen binding (NPB-α_2-AP) form, are present in circulation in a concentration of 0.7 μM and 0.3 μM respectively (29). In vitro experiments in purified systems have shown that these two forms have a different mechanism of plasmin inhibition (30). PB-α_2-AP, which is the most active form, inhibits plasmin by rapid formation of an irreversible 1:1 stoichiometric complex (31). The function of NPB-α_2-AP is still unclear. In vitro studies have shown that it is less active than PB-α_2-AP and that complex formation with plasmin proceeds more slowly (30). Recent developments in the assay of α_2-AP have made it possible to determine the two molecular forms separately. This separate assessment of PB- and NPB-α_2-AP may give information on the consumption of the two forms of α_2-AP during thrombolytic therapy.

The aim of our study was to delineate the role of plasmin inhibitors in control and prevention of the systemic effects, for which fibrinogen breakdown was chosen as marker. We investigated, both in vivo and in vitro, one representative agent from each of the two groups of thrombolytic agents with different systemic effects (e.g. t-PA and SK).

METHODS

Patients

We have studied the relationship between the consumption of plasmin inhibitors and the extent of fibrinogenolysis in 35 patients suffering from acute myocardial infarction and receiving

thrombolytic treatment. These patients were participating in clinical studies in the United Kingdom and The Netherlands, and gave their informed consent (32,33). The selection criteria of both studies are described in detail elsewhere (32,33). Nine patients were treated with streptokinase (SK) (Kabikinase^R, KabiVitrum, Stockholm, Sweden) at a dose of 0.2 to 3.0 x 10^6 IU infused intravenously over 60 min. Twenty patients were treated with 100 mg recombinant tissue-type plasminogen activator (t-PA) (BW-t-PA, Wellcome, Beckenham, United Kingdom) infused i.v. over 90 min. In addition, six patients, who received either 20 mg (n=4) or 50 mg (n=2) t-PA were studied.

Plasma

Blood samples of the patients with AMI were taken just before the start of infusion and at the end of infusion (t=60 min. for SK or 90 min. for t-PA). Venous blood was collected in trisodiumcitrate (9:1 v/v) and placed immediately on melting ice. Plasma was prepared by centrifugation at 2000g for 30 min at 4 °C and frozen in small aliquots at - 70°C. It was thawed at 37 °C immediately before use. To exclude plasminogen activation in vitro, inhibitory polyclonal t-PA antibodies (250 μg) were added immediately after obtaining plasma of the patients treated with t-PA (34).
Pooled normal plasma was obtained from 20 healthy volunteers (male:female ratio 1.0).
Plasma artificially depleted in PB-α_2-AP was obtained by affinity chromatography of pooled plasma depleted in plasminogen (by chromatography on lysine-Sepharose (35)) on Kringle I-III-Sepharose. Fractions of 4 ml were collected and the fractions with the highest A_{280} were pooled and, after dialysis, concentrated to the original plasma volume. Before incubation experiments, plasminogen was added to the plasma to a final concentration equal to that of pooled normal plasma.

Assays

The concentrations of the PB and NPB forms of α_2-AP were determined by modified crossed immunoelectrophoresis (CIE). This technique has been described earlier in detail by Kluft and Los (36) and is described here only briefly. In this modified method plasminogen was added to the first dimension gel, resulting in reduction of

electrophoretic mobility of PB-α_2-AP. NPB-α_2-AP retains its normal mobility and, as is shown in figure 1, the two forms can be determined separately. Before second dimension electrophoresis, an intermediate gel containing 2% antiserum against Lys-plasminogen (a gift of Dr. D.W. Traas from the Gaubius Institute) was poured to capture and precipitate plasmin-α_2-antiplasmin (PAP-) complexes. The gel for the second dimension contained 1.5% rabbit anti human α_2-AP antiserum (Nordic Immunology, Tilburg, The Netherlands). Trasylol 1000 kIU/ml (Bayer, Leverkusen, FRG) was added to the agarose to inhibit proteolytic activities. The concentrations of the PB and the NPB forms of α_2-AP were determined by measuring the immunoprecipitation peaks with a computerized program using a Hipad digitalizer (Geveke Electronics, The Netherlands). For standardization purposes, modified CIE of plasma before and after treatment of one patient were run under identical conditions on one glass plate. By measuring the surface of the immunoprecipitation peaks before (pretreatment level is 100 %) and after thrombolytic treatment, it is possible to determine the consumption of both forms of α_2-AP. As can be observed in figure 1, a vague peak (arrow) is seen on modified CIE, that most likely represents degradation products of α_2-AP, which do not disturb the determination of the two forms.

α_2-AP activity was measured with a modified immediate plasmin inhibition test (IPIT) (37). α_2-Macroglobulin antigen levels were assayed according to Laurell using a 2% anti-α_2-macroglobulin-antiserum from Nordic Immunology, Tilburg, The Netherlands (38). C_1-Inactivator was measured by single radioimmunodiffusion technique (Mancini) using an anti C_1-inactivator antiserum from Behringwerke, Marburg, FRG (39). Plasminogen was measured according to Friberger et al. (40) using Kabikinase^R for activation of plasminogen and S-2251 as a substrate, both of KabiVitrum Haematology, Amsterdam, The Netherlands. Fibrinogen was measured according to Clauss (41). Total (fibrin and fibrinogen) degradation products were measured using an ELISA technique (Organon Teknika, Boxtel, The Netherlands) (42).

In Vitro Studies

In vitro studies were performed by addition of different dosages of Steptokinase (Kabikinase^R) or single chain melanoma t-PA (sc-t-PA)

(43) to pooled plasma and incubation for 90 min at 37 °C. The dosages of SK and sc-t-PA added to pooled plasma varied from 5-1000 IU/ml and 60-9000 IU/ml respectively.

Statistical evaluation.

For statistical analysis, the Wilcoxon rank sum test and the Spearman rank sum test were used. A p-value of <0.05 was considered to be significant. Data are given as median and range, unless otherwise stated.

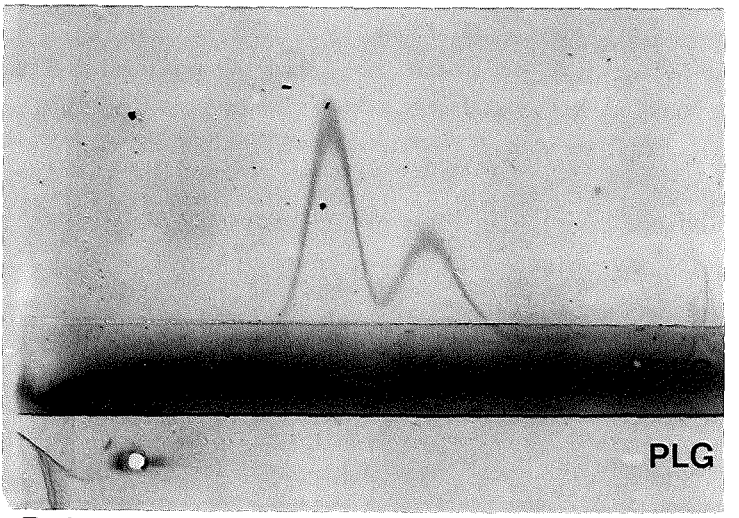
RESULTS

α_2-Antiplasmin consumption during streptokinase treatment of patients

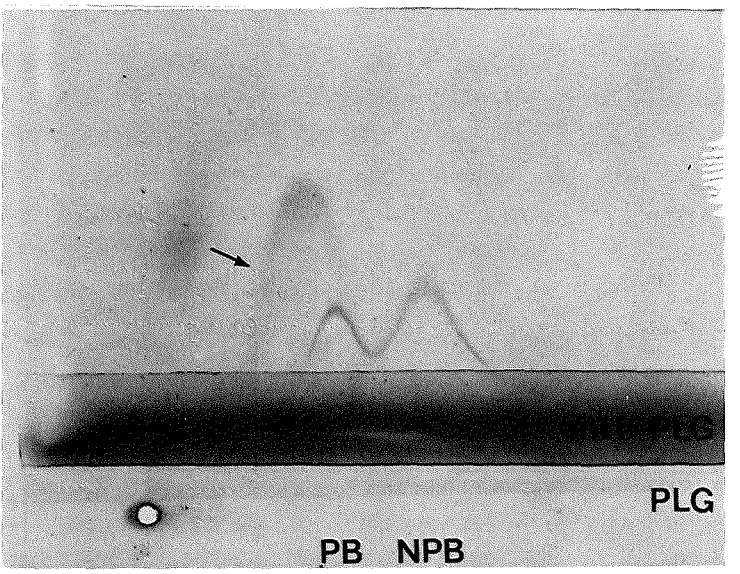
Nine AMI patients treated with a wide range of streptokinase dosages were studied. Treatment comprised 0.2 x 10^6 IU (n=1), 0.75 x 10^6 IU (n=4), 1.5 x 10^6 (n=3) and 3.0 x 10^6 IU (n=1) over 60 min. A strong decline in plasminogen was found in all individual patients (from 94% (74-111) to 6% (2-49) (figure 2). The rapid plasmin inhibitory capacity (α_2-AP activity level) decreased from 102% (83-129) to 11% (4-28). The consumption of PB-α_2-AP was complete in all patients. NPB-α_2-AP decreased to 57% (36-72) of the pretreatment level. The concentration of α_2-macroglobulin decreased from 83% (60-145) to 46% (33-85), whereas C_1-inactivator did not decrease (from 97% (89-103) to 95% (86-111).

In all patients treated with SK, practically all circulating fibrinogen was degraded, independent of the pretreatment levels of fibrinogen (mean pretreatment level 85% (54-104) (not shown).

Therefore, despite the wide range of SK-dosages, the patients treated with SK show a homogeneous response in consumption of PB-α_2-AP (complete), in degradation of fibrinogen (complete) and in consumption of C_1-inactivator (nihil). NPB-α_2-AP and α_2-macroglobulin are only partially consumed.



Before t-PA treatment



After t-PA treatment

Figure 1: Modified crossed immunoelectrophoresis of plasma of a patient with AMI, before and after treatment with 100 mg t-PA. The consumption of both forms of α_2-antiplasmin (α_2-AP) is measured by calculating the difference between the surface of the immunoprecipitation peaks before and after treatment. PB = plasminogen binding form of α_2-AP, NPB = non-plasminogen binding form of α_2-AP. PLG= plasminogen, anti-PLG= gel containing antibodies against plasminogen. The arrow indicates a vague peak, probably representing degradation products of α_2-AP.

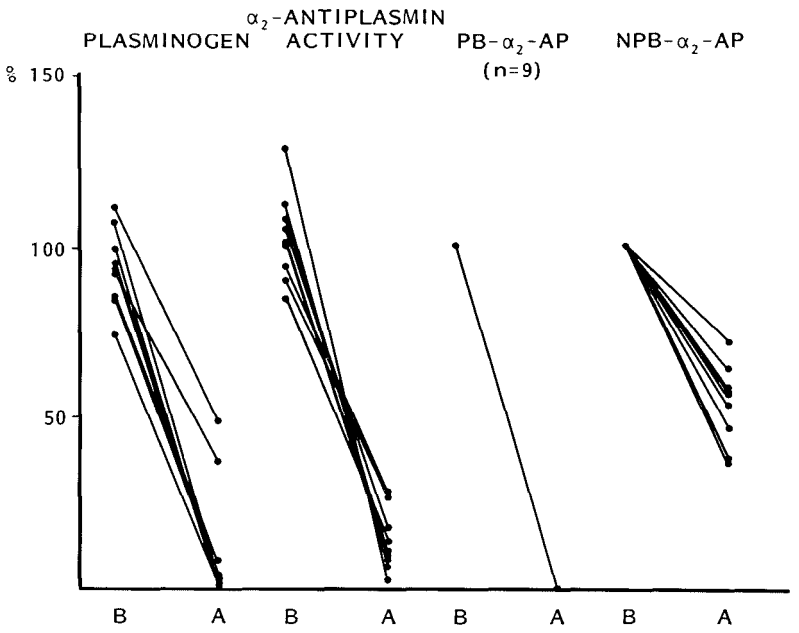


Figure 2: The levels of plasminogen, α_2-antiplasmin (α_2-AP) activity and the plasminogen binding (PB) and the non-PB forms of α_2-AP of all individual patients with AMI before (b) and after (a) streptokinase treatment. Results are expressed in % of normal pooled plasma. The values of both forms of α_2-AP are expressed as % of pretreatment level.

α_2-Antiplasmin consumption during tissue-type plasminogen activator treatment of patients

Twenty AMI patients, treated with 100 mg t-PA (in 90 min), showed less consumption of plasminogen and plasmin inhibitors than the patients treated with SK. The plasminogen concentration decreased from 104% (70-134) before treatment to 75% (59-97) after treatment (figure 3). α_2-AP activity decreased from 97% (76-129) to 33% (7-84) after treatment. The consumption of α_2-AP mainly concerned the PB-form of α_2-AP. This predominant consumption of PB-α_2-AP is illustrated in figure 1. The shown figure is representative for all twenty patients: in only one patient was total consumption of PB-α_2-AP observed. In the 20 patients the residual PB-α_2-AP level was

15% (0-39) of the pretreatment level. NPB-α_2-AP decreased only slightly to 88% (60-100) of the pretreatment level. α_2-Macroglobulin, as well as C_1-inactivator levels, did not show a significant decrease in the t-PA treated patients. The pretreatment levels for α_2-macroglobulin and C_1-inactivator were 89% (60-119) and 95% (54-162) respectively, and the post-treatment levels were 78% (52-105) and 88% (46-115).

Fibrinogen levels in the patients after treatment with t-PA varied strongly from an increase of fibrinogen in only one patient of 31% to a maximum breakdown of 79%. The median fibrinogen breakdown was 26% of the pretreatment plasma level.

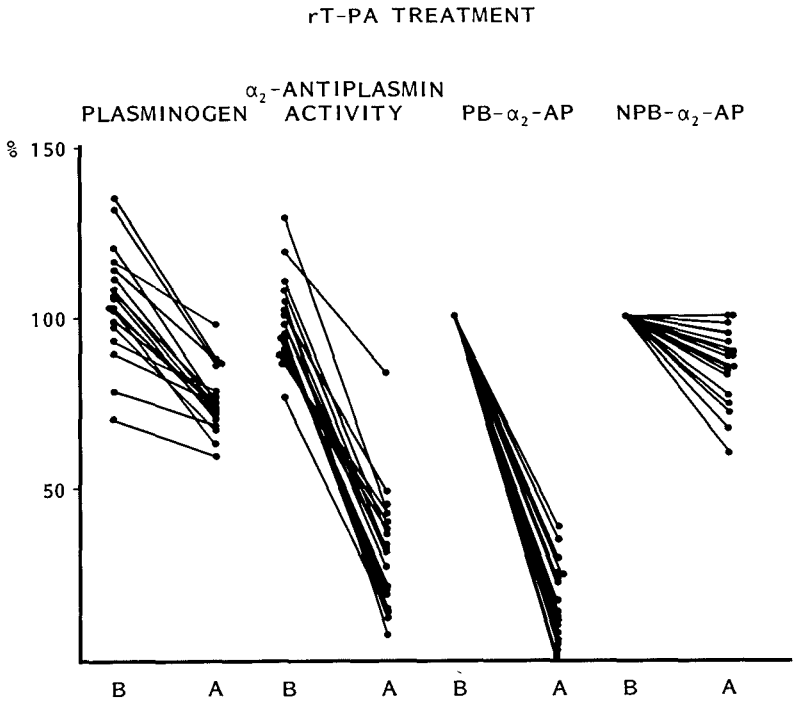


Figure 3: The levels of plasminogen, α_2-antiplasmin (α_2-AP) activity, and the plasminogen binding (PB) and the non-PB forms of α_2-AP of all individual patients before and after treatment with 100 mg rt-PA. Results are expressed in % of normal pooled plasma. The values of the two α_2-AP forms are given as % of pretreatment levels.

Thus, despite the fixed dosage of t-PA studied, the systemic effects as derived from fibrinogen breakdown are strongly variable. Further, the consumption of PB-α_2-AP and plasminogen varied considerably between patients.

Variability in fibrinogen degradation in t-PA treated patients

In investigating the reason for the variability in fibrinogen degradation in the t-PA treated patients, we explored correlations with endogenous fibrinolytic parameters of the patients. A correlation was found between the extent of fibrinogen breakdown and both the pretreatment concentrations of fibrinogen ($r=0.59;p<0.01$) and of plasminogen ($r=0.55;p<0.02$). No significant correlation was found between the extent of fibrinogen breakdown and the pretreatment levels of α_2-AP ($r=0.38$), α_2-macroglobulin ($r=-0.05$) and C_1-inactivator ($r=-0.18$). The consumption of plasminogen, as a percentage of the pretreatment value, ($r=0.58;p<0.01$) and α_2-AP ($r=0.55;p<0.01$) also correlated with the extent of fibrinogen breakdown, but the correlation with the lowest p-value was found between the consumption of the plasminogen binding form of α_2-AP and the amount of fibrinogenolysis ($r=0.68;p<0.002$).

In order to investigate further the apparent relationship between PB-α_2-AP consumption and fibrinogen breakdown, we measured the consumption of PB-α_2-AP in patients with AMI who were treated with low dosages of t-PA. The consumptions of PB-α_2-AP and fibrinogen are also given in figure 4. The less extensive activation of the fibrinolytic system in these patients was reflected by a lower consumption of PB-α_2-AP and fibrinogen. As mentioned before, in the SK-treated patients, all PB-α_2-AP was consumed, along with a total breakdown of fibrinogen, as determined by the Clauss method. The correlation of PB-α_2-AP consumption and fibrinogen consumption in all patients with AMI who were treated with plasminogen activators (both SK and t-PA) was highly significant ($r=0.88;p<0.001;n=35$).

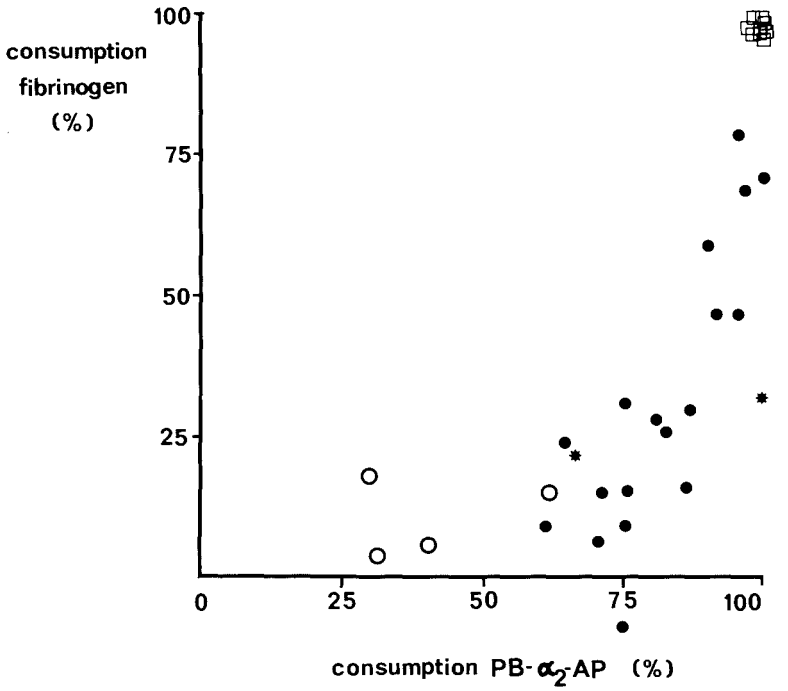


Figure 4: The relative breakdown of fibrinogen plotted against the consumption of the plasminogen binding form of α_2-antiplasmin (α_2-AP) of all patients with AMI, receiving thrombolytic treatment. □= various dosages streptokinase ● = 100 mg rt-PA; ✱ = 50 mg rt-PA; O = 20 mg rt-PA. A strong correlation between consumption of PB-α_2-AP and the percentage breakdown of fibrinogen was found ($r=0.88$; $p<0.001$).

IN VITRO STUDIES

Streptokinase

In order to investigate whether the findings in the patients receiving thrombolytic therapy could be confirmed in vitro, we incubated pooled normal plasma with SK and t-PA.

Incubation of pooled normal plasma with SK resulted in a strong plasminogen activation and a decrease of both α_2-AP forms. At low dosages, PB-α_2-AP is preferentially consumed; NPB-α_2-AP is consumed only at higher dosages SK (figure 5). Maximum consumption of NPB-α_2-AP stabilizes at about 50%, even after addition of very high dosages of SK, which coincides with a depletion of plasminogen. In

these in vitro experiments, no fibrinogen breakdown occurred until PB-α_2-AP was reduced to less than about 20-25 % of the initial level.

Addition of even very low dosages (10 IU/ml) of streptokinase to plasma artificially depleted in PB-α_2-AP resulted in breakdown of all fibrinogen.

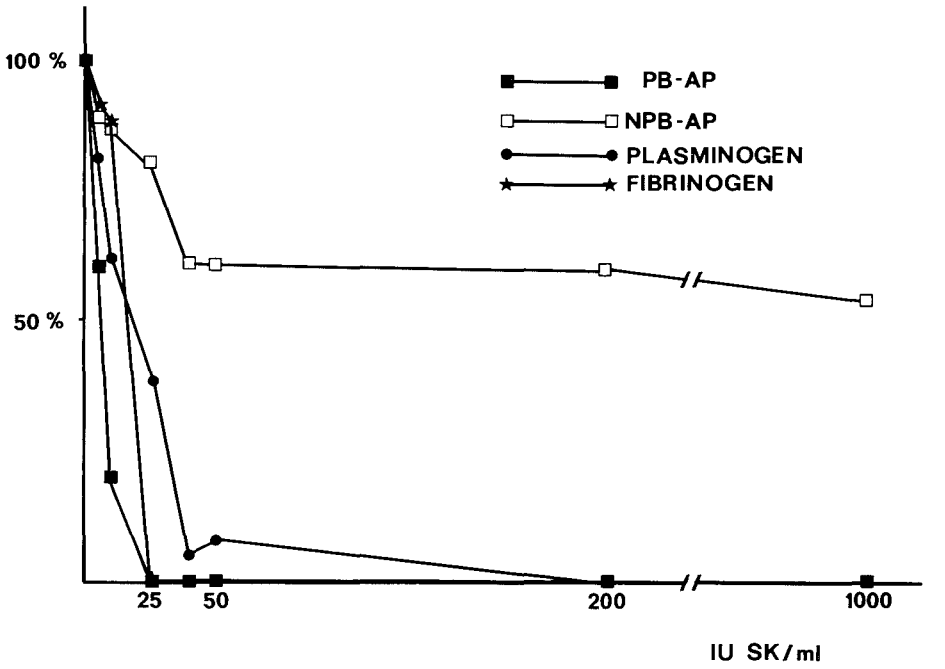


Figure 5: Results of the in vitro experiments with streptokinase. Experiments were performed by addition of various dosages of streptokinase to normal pooled plasma, which was incubated for 90 minutes at 37°C.

Tissue-type plasminogen activator

Incubation of plasma with single chain t-PA showed the same pattern as with SK. However, extremely high dosages of t-PA were required to achieve consumption of NPB-α_2-AP. Further, these incubation experiments showed that fibrinogen breakdown occurred after PB-α_2-AP reduction to 25% or less (figure 6). Accordingly, incubation of PB-α_2-AP depleted plasma with low dosages of t-PA (600 mIU/ml) resulted in consumption of all fibrinogen.

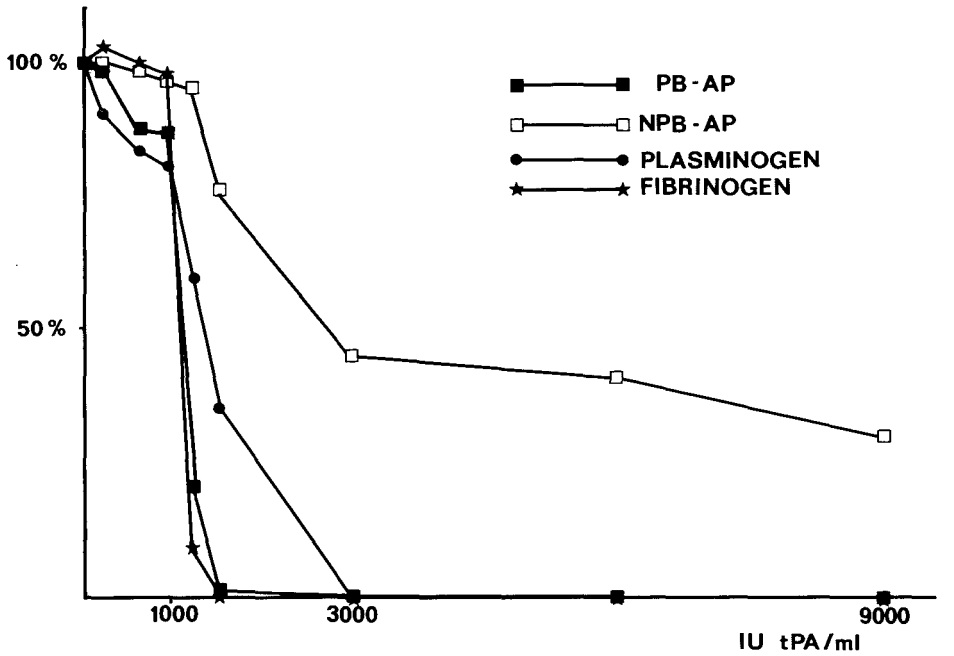


Figure 6: Results of the in vitro experiments with tissue plasminogen activator. Experiments were performed by addition of various dosages of single-chain t-PA to normal pooled plasma, which was incubated for 90 minutes at 37°C.

DISCUSSION

All thrombolytic agents in use show systemic effects at clinically effective dosages. However, the degree of systemic effects is strongly variable. This may be ascribed to differences in fibrin specificity of the various agents, but differences in endogenous (fibrinolytic) factors among patients may also contribute to the variability in systemic effects.

The aim of our study was to delineate the role of endogenous factors, in particular physiological inhibitors of plasmin, in controlling systemic effects during thrombolytic therapy. We therefore investigated both the degree of consumption of various plasmin inhibitors and the degree of fibrinogen breakdown, by activating plasminogen in plasma in vivo and in vitro. This was carried out firstly in patients with acute myocardial infarction, treated with various dosages of different thrombolytic agents, and, secondly, by in vitro experiments, in which plasma was incubated with various amounts of thrombolytic agents.

For the quantification of α_2-AP consumption, we studied for the first time the two molecular forms of α_2-AP separately, by using modified CIE (36). Most of the activity assays for α_2-AP used nowadays, are not specific for one of the two forms, but measure both forms. Additionally, these methods are frequently disturbed by other plasmin inhibitors, for instance α_2-macroglobulin. In an immunological assay, as in CIE, the presence of plasmin-α_2-AP (PAP) complexes in plasma may disturb in vitro studies, but may also disturb in vivo studies due to the rather long half life of about 12 hours of the complexes in circulation (44). By using an intermediate gel containing anti-plasminogen antibodies to capture and precipitate these complexes, it was possible to study the consumption of both forms of α_2-AP. α_2-Macroglobulin levels were determined only in vivo by immunoelectrophoresis (39), which was not disturbed by plasmin-α_2-macroglobulin complexes, because of the rapid clearance of the latter from the circulation (45).

Fibrinogen consumption was recorded by the Clauss clotting rate assay. This assay can be disturbed by high levels of fibrin(ogen) degradation products, which occurred in the patients treated with streptokinase (total (fibrin and fibrinogen) degradation products (TDP) were 124 μg/ml, range 6-270). However, in the patients

treated with t-PA, total amount of split products were low, 3.1 μg/ml (0.5-19.2), and outside the range that affects the Clauss method. This was confirmed for one of the patients' plasma sample with the highest levels of TDP. At the dilution used in the Clauss assay, the dose response curve of this plasma was parallel to the standard curve (data not shown). The low levels of fibrin(ogen) degradation products in the plasma samples of the patients treated with t-PA may be due to the addition of polyclonal antibodies against t-PA immediately after obtaining plasma, to prevent fibrinogen breakdown in vitro. The results are therefore representative for the actual situation in vivo.

The studies in patients treated with t-PA and SK and the incubation experiments in vitro showed similar results. Activation of plasminogen results firstly in consumption of PB-α_2-AP only, without any role for NPB-α_2-AP and α_2-macroglobulin. This was demonstrated in some patients treated with 100 mg t-PA and patients treated with lower dosages t-PA. This finding is in accordance with previous studies, in which α_2-macroglobulin is found to play only a minor role in plasmin inhibition, as long as α_2-AP is present in circulation (46). Higher degrees of plasminogen activation in the t-PA treated patients resulted in the onset of exhaustion of PB-α_2-AP (<20%), and consumption of NPB-α_2-AP and α_2-macroglobulin. After extensive plasminogen activation, as in patients treated with SK, the consumption of PB-α_2-AP was complete and consumption of both NPB-α_2-AP and α_2-macroglobulin was partial. The consumption of NPB-α_2-AP amounted to about 50 % (0.15μM) and of α_2-macroglobulin to about 30% (1.0μM). This indicates that both inhibitors are about equally effective, but that, because of its higher concentration, α_2-macroglobulin binds more plasmin. The consumption of both inhibitors is only partial because of an excess of the inhibitors over the amount of plasminogen available for activation (figure 7). Accordingly, we found in vitro an increased consumption of NPB-α_2-AP, if the plasminogen consumption was increased by addition of exogenous plasminogen to the test plasma (data not shown). It should be noted that it is demonstrated for the first time that NPB-α_2-AP inhibits plasmin in vivo. There was no C_1-inactivator consumption in our study at any degree of plasminogen activation, and C_1-inactivator seemed to play no important role in plasmin inhibition in vivo.

The pattern of fibrinogen degradation during plasminogen activation was similar both in vivo and in vitro. Both studies revealed that fibrinogen consumption was strongly correlated with the consumption of PB-α_2-AP. Fibrinogen breakdown occurred only when more than about 80 % of the PB-α_2-AP was consumed. The fact that other plasmin inhibitors in plasma were still present in a concentration of 50-90% of the pretreatment level, even at the highest degree of plasminogen activation, indicates the minor importance of these inhibitors in preventing systemic effects. It also indicates that only PB-α_2-AP plays an important role in control and prevention of systemic effects of plasmin in circulation and that the amount of PB-α_2-AP seems to be a threshold which has to be overcome to obtain systemic effects. PB-α_2-AP may therefore be used as a new marker for systemic effects during thrombolytic therapy. Although the assay (mCIE) used in this study is useful, it is a very complicated one. At present we are developing a new biological immunoassay specifically for the determination of PB-α_2-AP.

In figure 7 the concentrations of the various inhibitors of plasmin and plasminogen in plasma are shown. It shows that plasminogen (1.5 μM) is present in more than twofold concentration compared to PB-α_2-AP (0.7 μM). Since systemic effects do occur when more than 80 percent of PB-α_2-AP is consumed, about 35 % of the total plasminogen pool in plasma has to be activated to obtain systemic effects. It would be of interest to measure the rate of change in concentration of PB-α_2-AP in relation to fibrinogen breakdown during the course of infusion.

Apart from a clear relationship between the consumption of PB-α_2-AP and fibrinogen degradation, other endogenous factors may also contribute to the variability in systemic effects. We observed for instance, that the pretreatment levels of α_2-AP showed a large variability. This wide range may, because of the threshold function of PB-α_2-AP, contribute to the variability in systemic effects. However, the pretreatment values of α_2-AP did not show a significant correlation with fibrinogen consumption. The variability may also be dependent upon the individual levels of the thrombolytic agents in the circulation (21) and the degree of plasminogen activation achieved in the patient. However, we did not study the individual levels of the thrombolytic agents.

We found a significant correlation between the amount of activated

plasminogen and fibrinogen consumption (0.58;p<0.01), similar as has been previously reported (11). A significant correlation was also found between plasminogen activation and pretreatment levels of plasminogen (r=0.76;p<0.001). This is in accordance with the kinetics of plasminogen activation, which is directly related to plasminogen levels in the circulation. In accordance with the aforementioned correlations, a correlation between pretreatment levels of plasminogen and the consumption of fibrinogen was also detected.

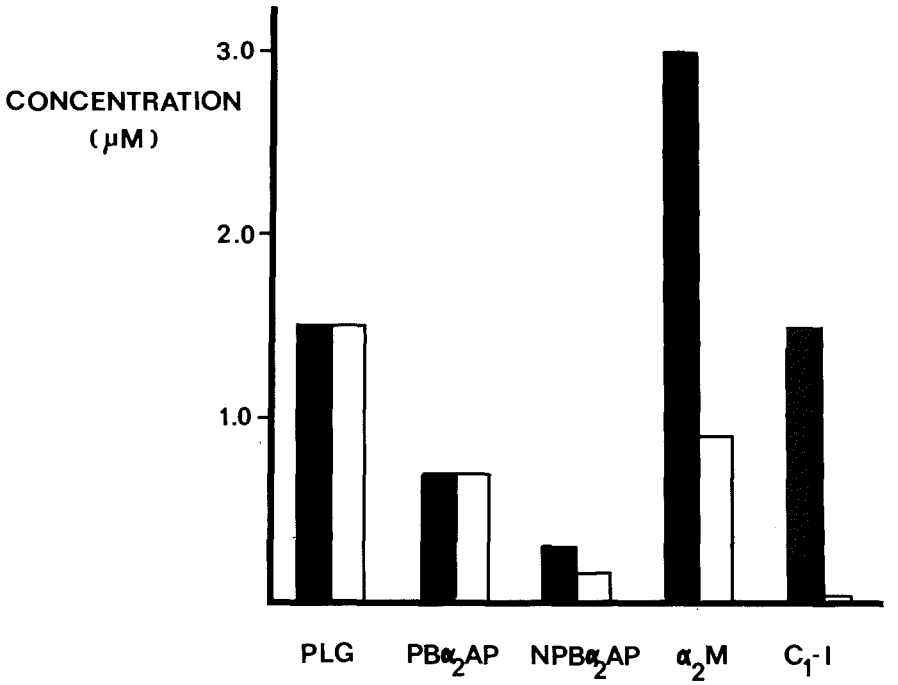


Figure 7: Average plasma concentration (in μM) of plasminogen and plasmin inhibitors (black bars). The open bars indicate the maximum consumption of plasminogen and the plasmin inhibitors after excessive plasmin formation, as was the case in the patients treated with streptokinase. In plasma, plasminogen is present in a twofold concentration compared to the plasminogen-binding form of α_2-antiplasmin (α_2-AP). C_1-I=C_1-inactivator; α_2-M=α_2-macroglobulin; NPB-α_2-AP=non-plasminogen binding form of α_2-AP; PB-α_2-AP=plasminogen binding form of α_2-AP.

Our study provides insight in the variability in systemic effects during thrombolytic therapy and shows the important role of PB-α_2-AP in the control and prevention of systemic effects. The clinical significance of the systemic effects is unknown, since it is doubtful whether these effects are beneficial or disadvantageous in obtaining recanalization, the pathogenesis of bleeding and the ocurrence of rethrombosis. It is therefore unclear whether or not the systemic effects should be prevented. It can be concluded that, in order to obtain systemic effects, all PB-α_2-AP, the most important inhibitor of plasmin in the circulation, has to be overcome. PB-α_2-AP may therefore be used as a new marker for systemic effects in patients undergoing thrombolytic therapy.

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CHAPTER 6

APPLICATION OF A MONOCLONAL ANTIBODY AGAINST THE COOH-TERMINAL SITE OF α_2-ANTIPLASMIN TO STUDY ITS MOLECULAR FORMS

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ABSTRACT

We have studied the different molecular forms of α_2-antiplasmin (α_2-AP), the major inhibitor of plasmin, in plasma and in other body fluids of healthy individuals and in patients with various diseases. We used a modification of the crossed immuno-electrophoresis technique (mCIE), by addition of monoclonal antibodies (MAb) against the COOH-terminal part of α_2-antiplasmin to the gel of the first dimension electrophoresis. The addition of MAb resulted in a mobility reduction of the plasminogen binding (PB) form of α_2-AP, whereas the non-plasminogen binding form (NPB-α_2-AP) retained its normal mobility. A two-peak pattern is obtained and the relation between the molecular forms of α_2-AP in plasma, serum and other body fluids can be studied.

We studied the conversion of PB-α_2-AP into NPB-α_2-AP, using this mCIE technique and found evidence that the in vivo and in vitro conversion have different mechanisms.

INTRODUCTION

α_2-Antiplasmin (α_2-AP), the main inhibitor of plasmin (1,2,3), circulates in plasma in two different molecular forms: A plasminogen binding (PB) form and a non-plasminogen binding (NPB) form (4). The concentrations of PB-α_2-AP and NPB-α_2-AP in the circulation are 0.67 μM and 0.30 μM respectively (5). PB-α_2-AP is the most active form. It rapidly forms a stable 1:1 complex with plasmin (6), and is able to bind reversibly to plasminogen (7) and crosslink to fibrin. The latter is mediated by factor XIIIA (8). NPB-α_2-AP is the less active form, with a slow rate of complex formation with plasmin and no plasminogen or fibrin binding properties (9). PB-α_2-AP is synthesised by the liver (10) and converted to NPB-α_2-AP in the circulation (11). The conversion of PB-α_2-AP into NPB-α_2-AP has been demonstrated both in vitro and in vivo and is temperature dependent (11). PB-α_2-AP has a halflife of about 8 days at 37°C (11). The mechanism of the conversion of PB-α_2-AP into NPB-α_2-AP is still unknown.

Until recently the two molecular forms of α_2-AP could only be studied using a modification of the crossed immuno-electrophoresis (CIE) technique according to Weeke (12). In this method plasminogen is added to the first dimension gel (13). The binding of PB-α_2-AP with plasminogen results in a reduced mobility of PB-α_2-AP compared to NPB-α_2-AP, giving two immunoprecipitation peaks. In this study we have used modified crossed immunoelectrophoresis (mCIE), and added monoclonal antibodies against the COOH-terminal part of α_2-AP, which harbours the plasminogen binding site (14), in the first dimension gel. The aim of the study was to obtain more insight in the behaviour of the molecular forms of α_2-AP. Therefore we studied the molecular forms of α_2-AP in plasma and serum of healthy individuals and patients with various diseases, and in other body fluids. We also followed the conversion of PB-α_2-AP into NPB-α_2-AP in vitro and in vivo and found evidence that the mechanisms of the in vivo conversion is different from the in vitro conversion.

MATERIALS AND METHODS

Materials

Agarose for electrophoresis (Electran) was obtained from BDH Chemicals, Poole, England. Trasylol was purchased from Bayer Ag (Leverkusen, FRG). Glu-plasminogen was prepared from human Cohn fraction III by affinity chromatography on lysine-Sepharose (15). Monoclonal antibodies against α_2-AP were obtained from Serotherapeutisch Institut (Vienna, Austria) (16). Polyclonal rabbit anti-human α_2-AP antiserum was obtained from Dakopatts (ITK, Uithoorn, The Netherlands) (I), Stago (Paris, France) (II), Behringwerke (Marburg, FRG) (III), Nordic Immunology (Tilburg, The Netherlands) (IV) and American Diagnostica Inc (New York, USA) (V). Nonadsorbed antiserum raised in rabbits against Lys-plasminogen was a gift of Dr. D.W. Traas of the Gaubius Institute. Purified PB-α_2-AP was obtained from Biopool (Umea, Sweden).
Plasma of patients with various disorders, was obtained according to standard procedures (17,18). Reference normal plasma was obtained by pooling plasma, obtained from 20 healthy volunteers. Plasminogen-free plasma was prepared by affinity chromatography of normal pooled plasma on lysine-Sepharose (15). This plasminogen-free plasma was then used to prepare plasma depleted in PB-α_2-AP, by affinity chromatography on kringle I-III Sepharose. To the plasma depleted in both PB-α_2-AP and plasminogen, plasminogen was added to a concentration of 100% compared to pooled normal plasma. Plasma depleted in NPB-α_2-AP was obtained by addition of purified PB-α_2-AP (in a concentration of 0.7 μM) to plasma of a patient with a homozygous type I α_2-AP deficiency (19).

Standard procedure of the assay:

The gel for the first dimension was 1% agarose in 0.03 mol/l sodium diethylbarbiturate/HCl, pH 8.6, containing 20 μg (50 nM) monoclonal antibody, which was added to the gel just before pouring the gel. Into a punched well 5 μl plasma was applied just before electrophoresis. First dimension electrophoresis was run for 4 hrs at 100 V at 12°C. The gel for the second dimension contained polyclonal anti-human α_2-AP antiserum. The second dimension was run overnight at 90 V at 12°C. Trasylol (1000 KIU/ml) was added to the agarose to inhibit proteolytic activities. The peak surfaces were

determined by a computerized program, using a Hipad digitalizer (Geveke, Amsterdam, The Netherlands). The PB:NPB ratio was determined by dividing the surface of the immunoprecipitation peak of PB-α_2-AP by the surface of the NPB-α_2-AP peak. Plasmin-α_2-AP (PAP-) complexes could be captured and precipitated, using an intermediate gel, containing 2% of a non-adsorbed anti-plasminogen antiserum, which was poured just before the second dimension electrophoresis.

RESULTS

MCIE to study the molecular forms of α_2-antiplasmin.

In normal crossed immunoelectrophoresis of plasma, α_2-AP has α_2 electrophoretic mobility (not shown). When modified crossed immunoelectrophoresis (mCIE) of pooled normal plasma against α_2-AP was carried out with plasminogen in the first dimension gel, two immunoprecipitation peaks were obtained (Figure 1a). These two peaks represent the plasminogen binding form, the retarded peak, and the non-plasminogen binding form of α_2-AP, the non-retarded peak. If monoclonal antibodies (MAb) against the COOH-terminal part of α_2-AP, harbouring the plasminogen binding site, were added to the first dimension gel, the same pattern was obtained (figure 1b). Plasma samples containing PB-α_2-AP or NPB-α_2-AP only, gave single immunoprecipitation peaks. The mobility of PB-α_2-AP was strongly reduced compared to the mobility of α_2-AP in CIE (figure 1c), while no reduction of mobility was found of the NPB-α_2-AP immunoprecipitation peak (figure 1d).

We studied the PB:NPB ratios of plasma and serum in 19 healthy volunteers (using antiserum I). The PB:NPB ratio of plasma was 2.95 ± 0.56 and of serum 2.28 ± 0.45.

The PB:NPB ratio is strongly dependent upon the polyclonal antibodies used in the second dimension gel. In table 1 the PB:NPB ratios of normal pooled plasma measured with various antibodies are given.

The separation of the two molecular forms of α_2-AP was accomplished by a reduction of the electrophoretic mobility of PB-α_2-AP by binding to MAb. This mobility reduction was dependent upon the

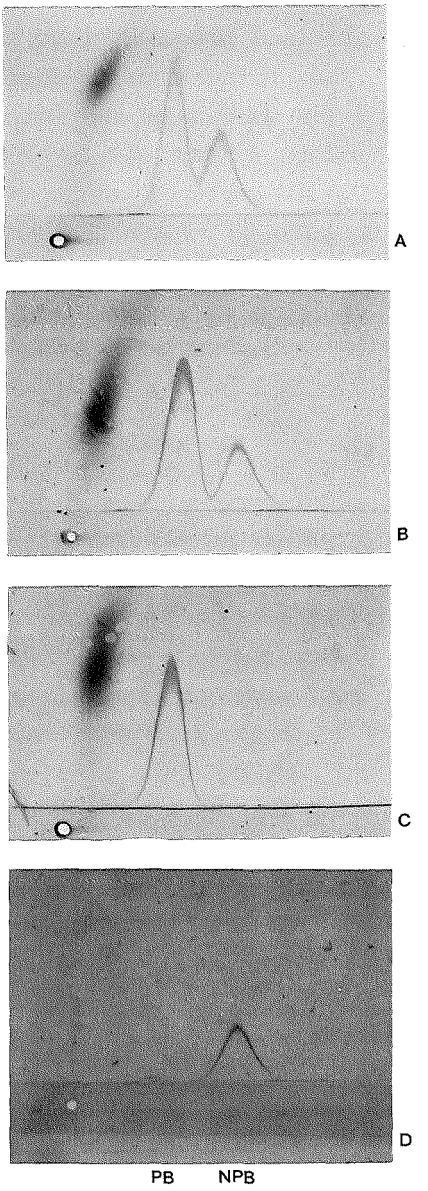

Figure 1: a) Modified crossed immunoelectrophoresis (mCIE) of pooled plasma with plasminogen added to the first dimension gel. This results in two immuno-precipitation peaks: the plasminogen binding (PB) and the non-plasminogen binding (NPB) forms of α_2-AP respectively. b) MCIE of pooled plasma with monoclonal antibodies (MAb) added to the first dimension gel. c) MCIE with MAb of plasma containing PB-α_2-AP only. d) MCIE with MAb in the first dimension gel of plasma depleted in PB-α_2-AP. Polyclonal antibodies against α_2-antiplasmin (α_2-AP) are added to the second dimension gel in all mCIEs. The mCIEs were not run under identical conditions.

concentration of MAb in the first dimension gel (Figure 2). By increasing the MAb concentration in the gel, a maximum mobility reduction for PB-α_2-AP of 35% was obtained. The half maximum mobility change was at a MAb concentration of 12.5 nM, which represents an apparent dissociation constant of the PB-α_2-AP-MAb complex. For routine use of the test 20 μg (50 nM) of the MAb solution was added to the first dimension gel.

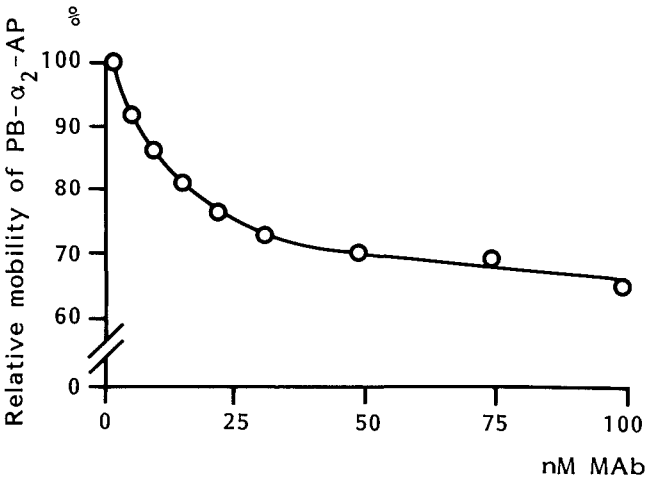

Figure 2: The relative mobility of PB-α_2-AP in mCIE with various concentrations of MAb in the first dimension gel, compared to CIE without addition of MAb. The maximum mobility reduction is 35 %.

The variation in the concentration of MAb in the first dimension gel did not have any effect on the ratio between the two molecular forms of α_2-antiplasmin (figure 3). However, the PB:NPB ratio was strongly dependent upon the concentration of plasminogen in the first dimension gel. An increase of the plasminogen concentration

resulted in decreasing ratios (fig 3). This was found to be dependent upon the antiserum used in the second dimension. Use of antiserum II, instead of antiserum I, resulted in more stable ratios ranging from 1.42 to 1.22 with plasminogen concentrations varying from 0.4μM to 1.2μM (data not shown).

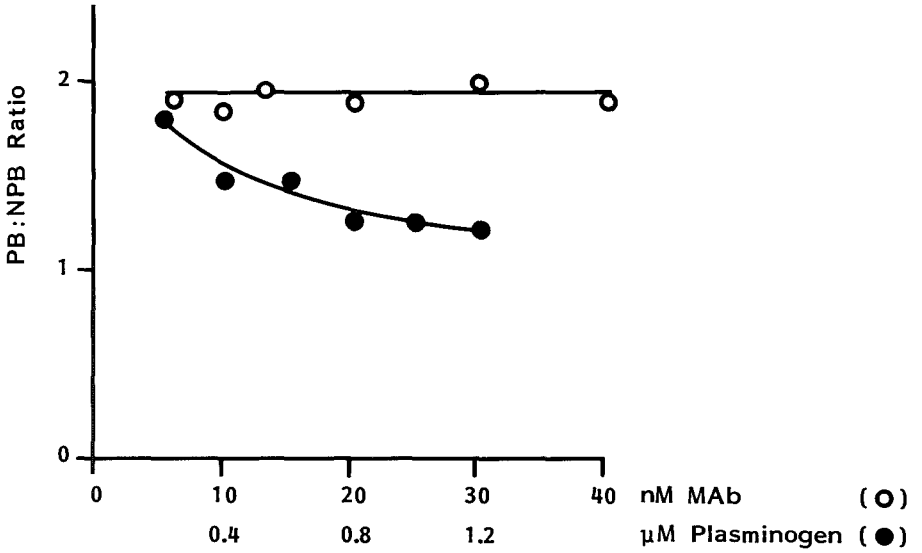

Figure 3: PB:NPB ratio of pooled normal plasma determined by mCIE with MAb or plasminogen in the first dimension gel. In both mCIEs, antiserum I was used in the second dimension. It shows that the PB:NPB ratios are independent of the MAb concentration used. The ratio is however dependent upon the plasminogen concentration used in the first dimension gel.

Table 1: Modified crossed immunoelectrophoresis of normal pooled plasma with MAb added to the first dimension gel and various polyclonal anti α_2-AP antisera added to the second dimension gel.

Antiserum (second dimension)	PB:NPB ratio	number of measurements
I	2.12	6
II	1.67	4
III	2.11	3
IV	1.96	5
V	1.33	2

PB:NPB α_2-AP in disease.

We studied the behaviour of the two molecular forms of α_2-antiplasmin in two disease states, known to be associated with changes in the fibrinolytic system: liver cirrhosis and chronic renal failure. The PB:NPB ratio increased in patients with mild and severe liver cirrhosis, compared to a control group of healthy individuals (table 2). In patients with chronic renal failure, the ratio PB:NPB also increased (table 2).

Table 2: PB:NPB ratio of plasma of patients with mild and severe liver cirrhosis and chronic renal failure. MAb were used in the first dimension gel.

Patient group	number	PB:NPB ratio (median;range)	antiserum (second dimension)
liver cirrhosis			
mild	12	2.8; 2.1-3.7	IV
severe	12	4.0; 1.7-15.4	IV
control group	12	2.2; 1.8-2.7	IV
chronic renal failure			
patients	23	2.6; 1.9-3.7	III
control group	6	2.2; 1.8-2.7	III

PB:NPB α_2-AP in other body fluids.

It is known that α_2-antiplasmin is present in various body fluids, including synovial fluid and ascites (20,21). We studied the two molecular forms in these body fluids with the above mentioned method. In figure 4a and 4b pictures of a mCIE of ascites of a patient with severe cryptogenic cirrhosis and of synovial fluid of a patient with active rheumatoid arthritis are given. By using an intermediate gel containing anti-plasminogen antibodies, plasmin-α_2-AP (PAP) complexes were captured and precipitated, as is shown in figure 4c. These pictures indicate that α_2-AP is present in synovia mainly in complex with plasmin or as NPB-α_2-AP, and not as PB-α_2-AP.

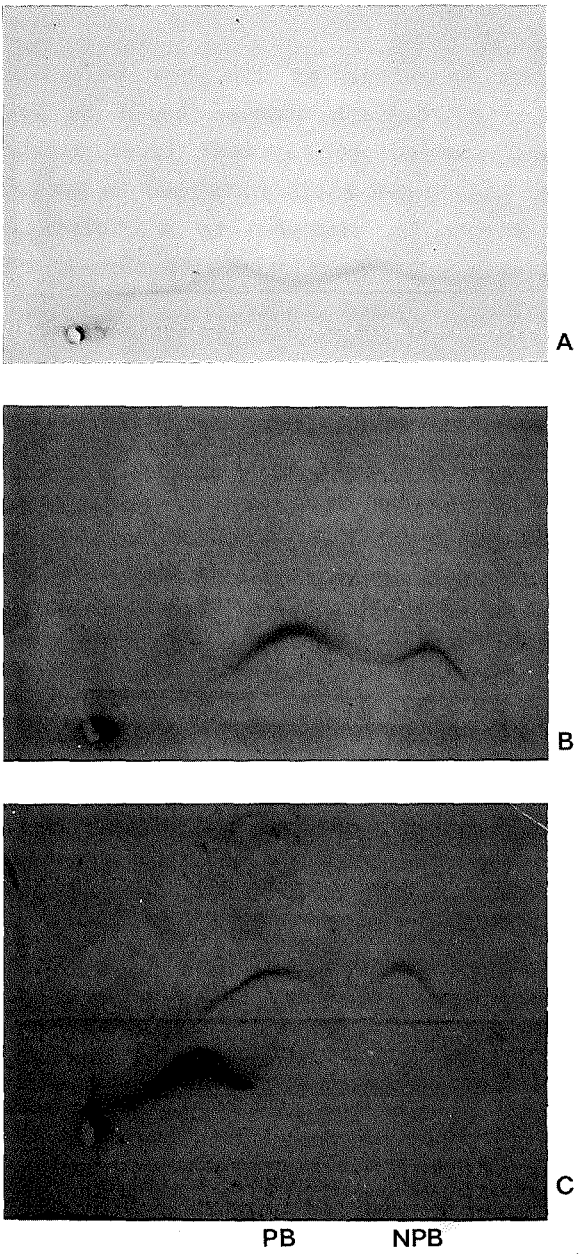

Figure 4: a) MCIE of ascites of a patient with severe cryptogenic cirrhosis. b) MCIE of synovial fluid of a patient with rheumatoid arthritis. c) MCIE of synovial fluid of a patient with rheumatoid arthritis, using an intermediate gel containing anti-plasminogen antibodies. Plasmin-α_2-antiplasmin complexes are precipitated in the intermediate gel.

Investigations on the conversion of PB-α_2-AP into NPB-α_2-AP

Up till now it was assumed that the conversion of PB-α_2-AP into NPB-α_2-AP was the same in vitro and in vivo (10).

In figures 5a and 5b mCIEs with plasminogen in the first dimension gel are given of plasma before and after 10 days incubation at 37°C. It can be seen that PB-α_2-AP, by losing its plasminogen binding property, is converted into NPB-α_2-AP.

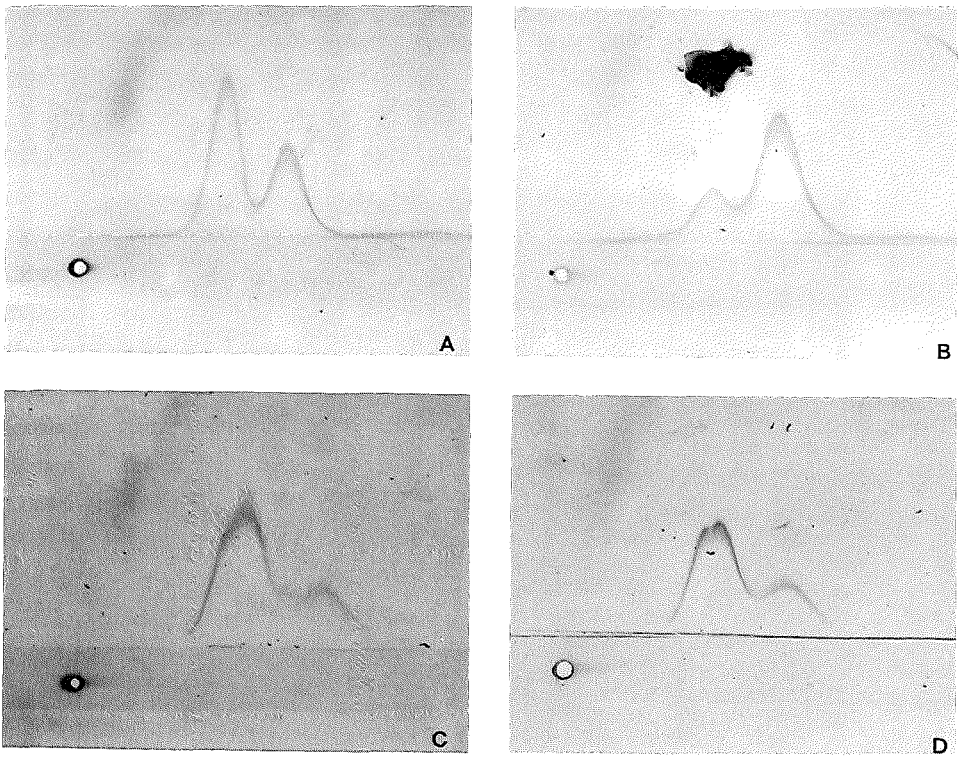

Figure 5: a and b) MCIEs of normal plasma with plasminogen in the first dimension gel before and after 10 days incubation at 37°C. This resulted in the conversion of PB-α_2-AP into NPB-α_2-AP. c and d) mCIEs of normal plasma with MAb in the first dimension gel before and after 10 days incubation at 37°C. The conversion of PB-α_2-AP could not be detected, indicating that the NPB-α_2-AP obtained by in vitro conversion retains its MAb binding property.

In figures 5 c and d MCIEs with MAb in the first dimension gel are given of plasma before and after 10 days incubation at 37°C. The pattern of the two immunoprecipitation peaks is not changed upon incubation in vitro. This indicates that the plasminogen binding property of PB-α_2-AP is lost upon incubation, while the MAb binding site is still functional.

By pouring a first dimension gel, containing plasminogen and MAb, three peaks could be demonstrated in the incubated plasma samples. They represent PB-α_2-AP, NPB-α_2-AP binding to MAb and NPB-α_2-AP neither binding to plasminogen nor to MAb (figure 6).

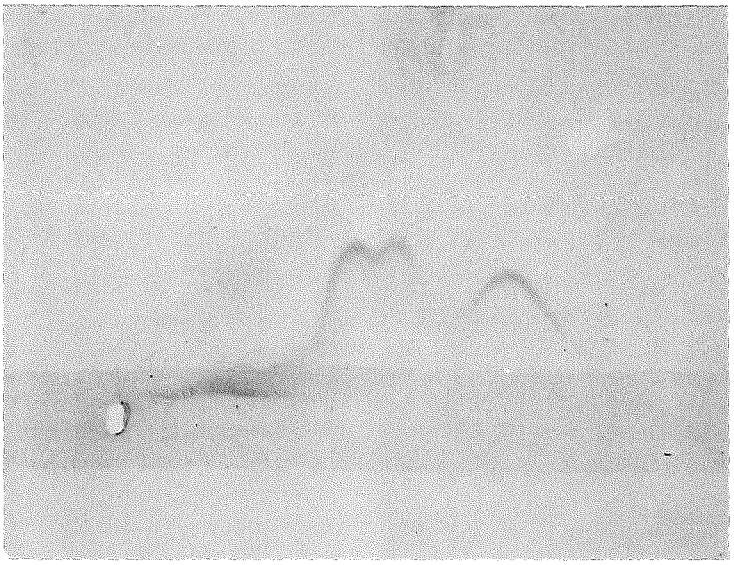

Figure 6: MCIE with MAb and plasminogen in the first dimension gel of plasma incubated for several days at 37°C. The addition of both MAb and plasminogen resulted in the appearance of a third peak. The peaks represent PB-α_2-AP, NPB-α_2-AP$^+$ (with MAb binding property) and NPB-α_2-AP$^-$ (without MAb binding property) respectively.

DISCUSSION

Earlier studies on α_2-AP revealed the existence of two molecular forms. The plasminogen binding (PB) form is the most active by a very fast complex formation with plasmin. The non-plasminogen binding (NPB) form is the less active. PB-α_2-AP is synthesized by the liver and NPB-α_2-AP is formed in the circulation by conversion of PB-α_2-AP (11).

Previously we were able to study the two molecular forms of α_2-AP with modified crossed immunoelectrophoresis using plasminogen in the first dimension gel (13). The addition of plasminogen in the first dimension gel resulted in binding of PB-α_2-AP to plasminogen, and thus a reduced electrophoretic mobility of PB-α_2-AP. The two-peak pattern obtained with mCIE showed the PB-α_2-AP and the NPB-α_2-AP peak respectively (figure 1a). This method has been used to assess the concentrations of the two forms in healthy individuals (11,13), to study the complex formation of α_2-AP with plasminogen (13,22) and to study the conversion of PB-α_2-AP into NPB-α_2-AP (11). MCIE was also used to study the complex formation of histidine-rich glycoprotein and plasminogen (22).

In the method presented here, we used a commercially available monoclonal antibody (MAb) directed against the COOH-terminal part of α_2-antiplasmin in the first dimension gel. The COOH-terminal site of α_2-antiplasmin harbours the plasminogen binding site. The binding of the COOH-terminal site of PB-α_2-AP to MAb resulted in a decreased electrophoretic mobility of PB-α_2-AP. MCIE with MAb in the first dimension gel could therefore be used to study the relation between the molecular forms of α_2-AP. We first studied the ratio between PB-α_2-AP and NPB-α_2-AP in pooled normal plasma. The PB:NPB ratio was dependent upon the polyclonal anti-α_2-AP antiserum used in the second dimension. This is in accordance with the findings of Kluft et al, who demonstrated differences in specificity of various anti-α_2-AP antisera for the two molecular forms of α_2-AP (5).

We studied the PB:NPB ratio in plasma and serum of healthy volunteers and found lower PB:NPB ratios in serum compared to plasma. This can be explained by the fact that PB-α_2-AP, in contrast to NPB-α_2-AP, has the ability to crosslink to fibrin during clot formation. The amount of PB-α_2-AP in serum is therefore

reduced compared to plasma, resulting in a decreased PB:NPB ratio in serum.

In figure 3 it was shown that mCIE with plasminogen revealed different PB:NPB ratios dependent upon the plasminogen concentration used. The variability in the PB:NPB ratio was found to be dependent upon the antibodies used in the second dimension gel. Plasminogen bound to α_2-AP probably interferes with the assay by changing the height of the PB-α_2-AP peak. This indicates that it is of importance to use constant and appropriate concentrations of plasminogen in the first dimension gel. Additionally the use of different antibodies in the second dimension gel can disturb the assay. Therefore studies using different antisera cannot be compared with each other.

We studied the PB:NPB ratio of α_2-AP in two different disease states: chronic renal failure and chronic liver disease (cirrhosis). Chronic renal failure is known to be associated with a decreased fibrinolysis, for which the cause is still unclear (23). We found a higher PB:NPB ratio compared to a control group of healthy volunteers (table 2), which was accompanied by high α_2-AP activity (17).

We also studied the PB:NPB ratios in patients with cirrhosis of the liver. This was also associated with an increase of the ratio, although the activity levels of α_2-AP decreased, due to a decreased concentration of α_2-AP (18). The increase of the PB:NPB ratio may be a non-specific reaction, caused by an acute phase reaction. This is in contradiction with earlier findings, in which patients with liver cirrhosis were thought to be in a steady state, associated with normal PB:NPB ratios (10).

MCIE can also be used to study the PB:NPB ratio in other body fluids, such as ascites and synovial fluid. In figure 4b, a mCIE is given of synovial fluid of a patient with active rheumatoid arthritis, in which both forms of α_2-AP are present in an other concentration compared to plasma. In figure 4c the PAP-complexes are precipitated, by using an intermediate gel containing anti-plasminogen antibodies. It can be concluded from these pictures that α_2-AP is present in synovial fluid mainly in complex with plasmin (PAP-complexes) or as NPB-α_2-AP. In patients with rheumatic disease, fibrin deposition is one of the major pathogenetic mechanisms of cartilage destruction (24). The fibrinolytic system

may therefore be of importance in rheumatic disease and the concentration of the major plasmin inhibitor α_2-AP in synovial fluid may influence the extent of fibrin depositions.

The conversion of PB-α_2-AP into NPB-α_2-AP

Earlier studies have shown that the conversion of PB-α_2-AP in circulation (in vivo) generates NPB-α_2-AP, which has lost the rapid plasmin inactivating, plasminogen-binding and fibrin cross-linking properties (11). The conversion of the active PB form into the less active NPB form is associated by a decrease of the α_2-antiplasmin activity (11). The in vitro studies on the conversion of PB-α_2-AP into NPB-α_2-AP showed that, using mCIE with plasminogen in the first dimension gel, the conversion could be followed visually (figure 5a and 5b). However with MAb in the first dimension gel, no conversion could be visualized (figure 5 c and 5d). Thus, during in vitro conversion the formed NPB-α_2-AP retains its MAb binding site. As was shown in figures 1 a and b, MCIEs with MAb and plasminogen in the first dimension gel of fresh obtained plasma show a similar pattern of immunoprecipitation peaks. This indicates that NPB-α_2-AP in vivo has no MAb binding property, in contrast to NPB-α_2-AP obtained by in vitro incubation. Therefore we can conclude that the in vivo conversion of PB-α_2-AP is another process than the in vitro conversion.

After incubation of plasma for several days, we could, by addition of plasminogen and MAb into the first dimension gel, detect three immunoprecipitation peaks. These represented PB-α_2-AP, NPB-α_2-AP, which has a MAb binding site and NPB-α_2-AP, without a MAb or plasminogen binding site, respectively (figure 6). We propose to call these two NPB-α_2-AP forms NPB-α_2-AP$^+$ (+MAb binding) and NPB-α_2-AP$^-$ (-MAb binding). We tried to demonstrate the NPB-α_2-AP$^+$ form in plasma in vivo, by using MAb and plasminogen in the first dimension gel. If NPB-α_2-AP$^+$ is present in vivo, one would expect to find a third peak, between the PB-α_2-AP peak and the NPB-α_2-AP$^-$ peak. However we could not demonstrate this in plasma and serum of healthy individuals or patients nor in synovial fluid (data not shown).

We hypothesized on the mechanism of the conversion and arrived at two possible explanations: conversion of PB-α_2-AP is accomplished by (a) two consecutive steps of conversion, of which the last one

is only found in vivo, or (b) two different conversion steps in vivo and in vitro.

a) in vivo PB-α_2-AP is converted into NPB-α_2-AP$^+$, thereby losing its rapid plasmin inactivating and plasminogen binding properties, but retaining its MAb binding property, followed by conversion into another NPB-α_2-AP$^-$, which in addition has lost its MAb binding property.

b) the conversion mechanism in vivo and in vitro are totally different processes. This would be in accordance with our findings that NPB-α_2-AP$^+$ could not be demonstrated in vivo.

The conversion can be accomplished by a molecular rearrangement or by splicing of a peptide of the COOH-terminal site.

An earlier study on NPB-α_2-AP showed that a peptide was split of the COOH-terminal site of the molecule. NPB-α_2-AP in this study was obtained by purification of NPB-α_2-AP out of plasma which was incubated for 20 days at 37°C (14). This NPB-α_2-AP, formerly thought to be the same as NPB-α_2-AP in the circulation, is however NPB-α_2-AP$^+$. The conversion in vitro therefore seems to be accomplished by splicing of a peptide. More studies on the two molecular forms are necessary to reveal the mechanism and the clinical relevance of conversion of PB-α_2-AP into NPB-α_2-AP in vivo.

The method presented here is a new, useful and easy assay. It opens new possibilities to study the different molecular forms of α_2-antiplasmin and hence to obtain more insight into the behaviour of the molecular forms of α_2-AP in health and disease.

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CHAPTER 7

HISTIDINE-RICH GLYCOPROTEIN IS ELEVATED IN MILD LIVER CIRRHOSIS AND DECREASED IN MODERATE AND SEVERE LIVER CIRRHOSIS

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ABSTRACT

Histidine-rich glycoprotein (HRG) reduces by complex formation with plasminogen the amount of "free" plasminogen in circulation and is therefore considered an inhibitor of fibrinolysis. We studied the levels of both HRG and plasminogen in patients with different degrees of liver cirrhosis to assess the role of HRG in enhanced fibrinolysis in these patients. In mild (Child A) cirrhosis, HRG levels unexpectedly were significantly increased. The total plasminogen level and the calculated amount of "free" plasminogen were decreased. In moderate (Child B) cirrhosis, both HRG levels and total plasminogen levels were reduced, resulting in a normal amount of "free" plasminogen in circulation. In severe (Child C) cirrhosis, HRG level, total plasminogen level, and "free" plasminogen level were all decreased. Because the HRG level is increased in Child A liver cirrhosis, we suggest that other mechanisms, other than simply a decreased synthetic capacity of the liver, contribute to the changes in HRG levels in patients with liver disease. Because of the reduction of "free" plasminogen levels in severe liver cirrhosis, we propose that the decrease in HRG levels in liver cirrhosis plays no role in enhanced fibrinolysis in these patients. The increase in the HRG level in liver cirrhosis Child A may be of importance in future studies on familial thrombosis associated with elevated levels of HRG, where mild liver dysfunction should at least be excluded.

INTRODUCTION

Histidine-rich glycoprotein (HRG) is a glycoprotein with a molecular mass of 80,000 D that circulates in plasma in a concentration of approximately 1.8 μmol/l (1) and is thought to be synthesized in the liver (2). By interacting reversibly with the lysine binding sites of plasminogen, HRG reduces by complex formation the amount of "free" plasminogen in circulation. Plasminogen bound to HRG is not available for binding to fibrin and subsequent activation to plasmin at the fibrin surface, and therefore, HRG is considered to be an inhibitor of fibrinolysis (3).

The clinical role of HRG has not yet been established. An increase in HRG is reported to be associated with a prothrombotic state (4). Because of the fibrinolysis inhibiting property of HRG, a decrease in HRG levels theoretically can result in enhanced fibrinolysis and possibly contribute to a bleeding tendency. HRG levels are reduced in several disease states, including sepsis (1), disseminated intravascular coagulation (5), systemic lupus erythemathosus (5); these levels are also reduced in women who take oral contraceptives (6,7), and during pregnancy (8) and are reduced as a result of a negative acute phase reaction, for instance, after acute myocardial infarction (9).

The first studies on HRG in patients with liver insufficiency revealed that HRG levels were decreased (1,5,10). Saito et al (10), who observed only patients with severe liver failure, suggested that this acquired HRG deficiency could contribute to enhanced fibrinolysis in patients with liver diseases. A more recent study of Gram et al. (11) in 28 patients with various liver diseases revealed that mean HRG was not reduced; however, in this study no distinction was made according to the severity of the disease.

In our study we determined both HRG and plasminogen levels in patients with various degrees of liver cirrhosis to clarify the contradictory results of previous studies and to assess the putative role of HRG in enhanced fibrinolysis in liver cirrhosis.

METHODS

Patients

A total of 40 patients with liver cirrhosis participated in the study. Before patients participated in the study, informed consent was obtained, and research was carried out according to the principles of the Declaration of Helsinki. The individuals in the study were consecutive outpatients with biopsy-proven liver cirrhosis who were in a stable condition and who did not receive blood products for at least two weeks before blood sampling. The patients were divided into three groups according to the modification of Pugh et al. of the Child classification (12). This classification subdivides patients with liver diseases in three classes (A,B,C) by using bilirubin and albumin levels, the grade of encephalopathy, the presence of ascites and a test for the extrinsic coagulation pathway, for which we used the Normotest and Thrombotest (Nycomed, Oslo) (12). Nineteen patients had mild cirrhosis (Child A), nine had moderate cirrhosis (Child B), and twelve had advanced cirrhosis (Child C). The causes of cirrhosis included alcohol abuse (in 18 patients), viral hepatitis (in 11 patients), and autoimmune hepatitis (in 8 patients). In two other patients, the causes were unknown, in one patient, the cause was α_1-antitrypsin deficiency (Table I).

Plasma

Blood, obtained by venapuncture, was collected in trisodium citrate (1:9) and placed immediately on melting ice. Plasma was prepared by centrifugation at 2000g for 30 min. at 4°C. The samples were stored at -70°C and thawed immediately before use. For reference values, we collected blood samples from 15 healthy volunteers (male/female ratio 1.1; age range 25 to 45 years). One of the female volunteers had a HRG level of 161 %, which is above the normal range in our laboratory of 65 to 135 % (n=150) (13). In retrospect, this woman was found to have received treatment for thyriod illness and was excluded from the study. A laboratory reference value of 100% was obtained from pooled normal plasma.

Assays

HRG was measured immunochemically by Rocket immunoelectrophoresis

according to Laurell done with a homemade 0.5 % rabbit anti-human anti-HRG antiserum (14). Plasminogen was measured using streptokinase for activation of plasminogen and HD-Nva-CHA-Lys-pNA as a substrate, both from Behringwerke (Marburg, West Germany) (15). Calculation of "free" plasminogen was based on the equilibrium [Plasminogen-HRG] $\rightleftarrows$ [Plasminogen] + [HRG] with a K_d of 1.0 µmol/l and results were expressed as percentage of total plasminogen (16). HRG and plasminogen levels were expressed in percentage of pooled normal plasma, in which the concentration of HRG, plasminogen and "free" plasminogen were 1.8µmol/l, 1.5 µmol/l and 0.74 µmol/l, respectively. The antithrombin III level was determined by using substrate HD-CHA-But-Arg-pNA obtained from Behringwerke (17). Albumin levels were determined by using the Bromocresol green principle on a chemistry analyzer (SMAC; Technicon Instruments Corp., Tarrytown, N.Y.). The albumin levels were taken as a marker for the synthesis capacity of the liver.

Statistical evaluation

For statistical evaluation, the rank sum (Wilcoxon) and Spearman correlation tests were used, and a p value of <0.05 was considered to be significant. All values were expressed as mean ± SD.

Table 1: Causes of liver cirrhosis of all individuals in the study.

Causes of cirrhosis	all patients n=40	severity groups Child A n=19	Child B n=9	Child C n=12
alcohol abuse	18	8	5	5
viral hepatitis	11	6	2	3
autoimmune hepatitis	8	4	2	2
other causes	3	1	0	2
albumin g/l *	34 ± 7	40 ± 4	31 ± 2	26 ± 3

The patients are subdivided using the Child classification, modified according to Pugh et al (12). *The albumin levels in the various groups are given as mean ± SD. The albumin level of the reference group was 43 ± 2 g/l.

RESULTS

The reference group

The results of the patients with cirrhosis were compared with those from a reference group consisting of 14 healthy volunteers, who were sampled in the same period (albumin levels 43 ± 2 g/l;mean ± SD). The levels of HRG, total plasminogen and "free" plasminogen of all individuals are given in figure 1. In the reference group, the mean HRG level was 105 ± 25%. The mean of total plasminogen was 91 ± 15% and that of "free" plasminogen 44 ± 12%.

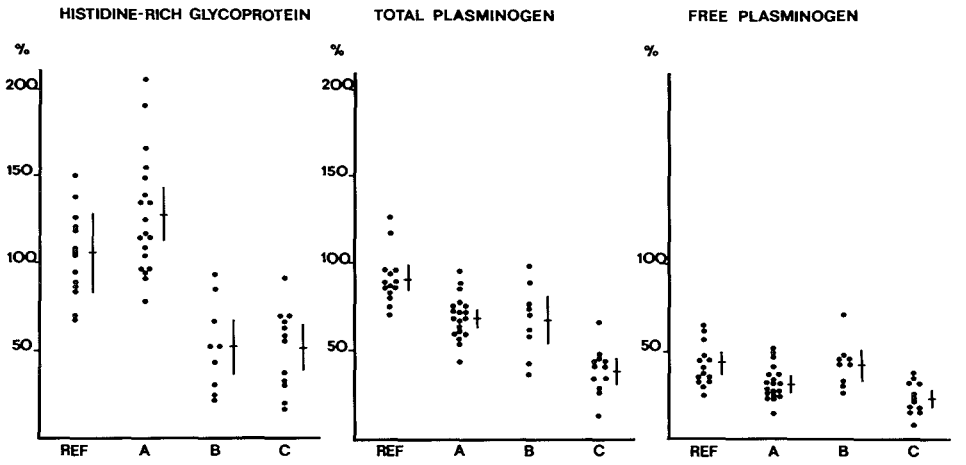

Figure 1. Histidine-rich glycoprotein, total plasminogen and "free" plasminogen in the individual patients with liver cirrhosis, subdivided in Child A, B and C liver cirrhosis compared to a reference group of 14 healthy volunteers (REF). The horizontal lines indicate the mean level, the vertical lines indicate the 95% confidence limits (mean ± 2 SEM). Data are presented in percentage of pooled normal plasma in which the HRG, plasminogen and "free" plasminogen concentrations are 1.8μM, 1.5μM and 0.74μM respectively.

Child A cirrhosis

In patients with Child A liver cirrhosis, higher HRG levels were found in comparison with those of the reference group ($p<0.05$). The mean level of HRG was 127 ± 34%. Plasminogen levels were decreased in these patients, to a mean of 69 ± 12% ($p<0.001$). The mean calculated "free" plasminogen level was 32 ± 10%, which is reduced in comparison with that of the reference group ($p<0.01$) (Figure 1).

Child B cirrhosis

The nine patients with Child B liver cirrhosis had reduced levels of both HRG and plasminogen. HRG levels were decreased to a mean of 52 ± 26% ($p<0.005$). Plasminogen levels were decreased to a mean level of 68 ± 20% ($p<0.001$). The "free" plasminogen levels in these patients were normal (43 ± 14%) when compared with the reference group (Figure 1).

Child C cirrhosis

In Child C liver cirrhosis, levels of HRG and total plasminogen were decreased. The mean HRG level was 51 ± 23 % ($p<0.001$) and mean level of total plasminogen was 39 ± 13% ($p<0.001$). In spite of the strong reduction in HRG levels in patients with severe liver cirrhosis, the calculated levels of "free" plasminogen were also decreased. The mean level of "free" plasminogen, expressed as the percentage of total plasminogen, was 24 ± 9% ($p<0.01$) (Figure 1).

Total patient group

We also calculated the mean levels of the fibrinolytic parameters in all patients with liver cirrhosis. The mean HRG level of all patients was 87 ± 48%, which was not significantly different from the levels in the reference group. Total plasminogen levels were reduced in all patients with cirrhosis, to a mean of 60 ± 20% ($p<0.001$), and the calculated "free" plasminogen level was decreased to a mean of 32 ± 13% ($p<0.005$).

HRG and liver synthesis capacity

A reduced synthesis capacity of the liver in the patients with cirrhosis was illustrated by decreased levels of albumin (Table I). In figure 2, the albumin levels of all individuals are plotted against HRG levels. A significant correlation was found

($r=0.66; p<0.001$).

We compared the mean plasma levels of HRG of the Child A, B and C cirrhosis groups with plasminogen and antithrombin III, two hemostasis proteins, known to be synthesized by the liver (Figure 3) (18). Although the levels of antithrombin III, plasminogen and HRG decreased to about the same extent in moderate and severe cirrhosis, a clear discrepancy between the levels of these parameters compared with HRG was observed in Child A liver cirrhosis.

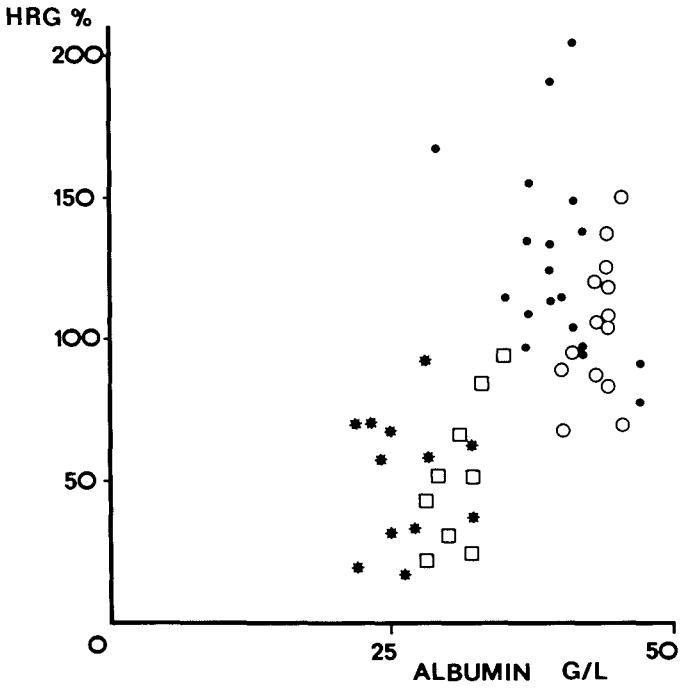

Figure 2. Individual plasma levels of histidine-rich glycoprotein (HRG) of all patients suffering from liver cirrhosis and healthy volunteers plotted against the levels of albumin, a marker for the synthesis capacity of the liver. HRG levels are expressed as percentage of pooled normal plasma in which the HRG concentration is 1.8μM. The correlation coefficient for the total group was 0.66 ($p<0.001$). O = reference group; ● = Child A cirrhosis;□ = Child B cirrhosis; ✱ = Child C cirrhosis.

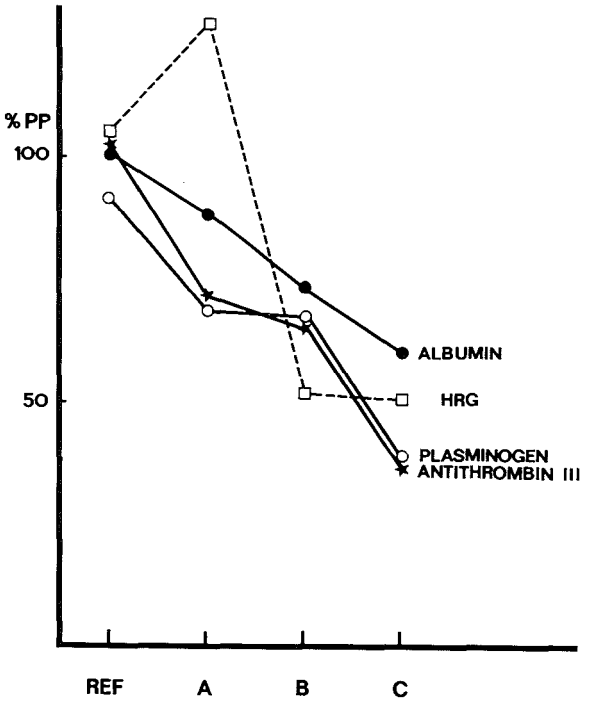

Figure 3. Mean levels of histidine-rich glycoprotein, total plasminogen, antithrombin III and albumin of the patients with Child A, B and C liver cirrhosis. The values of albumin are given as percentage of the mean level of the reference group, which is set at 100 %. (REF = reference group). HRG, plasminogen and "free" plasminogen levels are given in percentage of pooled normal plasma, in which the concentrations are 1.8μM, 1.5μM and 0.74μM respectively.

DISCUSSION

In patients with severe liver failure, enhanced fibrinolytic activity is frequently seen and is considered an additional cause for the bleeding problems observed in these patients (19,20). Several mechanisms for this enhanced fibrinolysis have been suggested, such as reduced clearance of plasminogen activators and reduced inhibition of fibrinolysis (21,22,23). Histidine-rich glycoprotein (HRG), a protein that acts as an inhibitor of fibrinolysis, could also play a role in the enhanced fibrinolysis in patients with cirrhosis, because an increase of "free" plasminogen, resulting from a reduced level of HRG, would suggest an increased fibrinolytic potential by the increased availability of plasminogen.

Previous studies on HRG in patients with liver disease have given contradictory results. Most studies have revealed a reduced level of HRG in patients with liver cirrhosis (1,5,10). Saito et al. (10) proposed that the decrease in HRG could contribute to enhanced fibrinolysis in patients with advanced liver cirrhosis. Although they found a parallel decrease of HRG with other proteins synthesized by the liver, a wide range in plasma HRG levels was seen in these patients. In contrast, Gram et al. (11) reported that in patients with various kinds of liver disease, the mean HRG level was not reduced. They found HRG levels ranging from 35 to 165 % compared with normal pooled plasma.

In our study we made a distinction in the severity of liver disease and found an increase in the HRG levels in patients with mild liver cirrhosis (Child A) and a strong decrease in HRG levels in patients with moderate and severe liver cirrhosis (Child B and C). As is shown in figure 2, the HRG levels are significantly correlated with albumin levels in the total patient group ($r=0.66; p<0.001$). A correlation was also found in the Child B subgroup, but no correlation was found in the Child C group, which is possibly a result of the small number of patients in this group (24). Because HRG levels are not decreased in Child A cirrhosis, we suggest that the synthesis capacity of the liver may not be the only mechanism that contributes to the changes in plasma levels of HRG in liver cirrhosis Child A. The increase in the HRG level in Child A cirrhosis cannot be the result of an acute phase reaction, because

HRG is known as a negative acute phase reactant (9). We have no explanation for this increase; however theoretically a hormonal influence on the remaining functioning hepatocytes may be responsible. Platelet involvement is not likely, because platelets contain only about 0.15 % of the total plasma HRG level (25). To elucidate the mechanism of the changes in HRG levels in cases of mild and severe liver cirrhosis, metabolism studies are required.

We suggest that the contradiction of earlier reports about HRG in liver diseases can be ascribed to a lack of differentiation of patient groups. The large range of HRG levels in earlier studies (1,5,10,11) can be explained by assuming that patients with different levels of severity of disease were studied. In our study we also would have found a normal mean level of HRG if no distinction had been made among the severity of cirrhosis in our patient group.

In our study, the total plasminogen concentration was significantly decreased in all patients with liver cirrhosis. The calculated "free" plasminogen levels were normal in Child B cirrhosis and decreased in Child A and C cirrhosis. This decrease of "free" plasminogen results in less plasminogen available for binding to fibrin and activation to plasmin at the fibrin surface. We therefore propose that a decrease in HRG level does not play a role in the enhanced fibrinolysis in severe liver failure.

In addition to binding to plasminogen, HRG can also bind to heparin (26) and thrombospondin (27). HRG, immobilized by these interactions and possibly by other interactions, can principally form a local focus of plasmin activity, because plasmin can be bound or remain bound to HRG and is then protected from inactivation by α_2-antiplasmin (28,29). How reductions in plasma levels of HRG influence such local events of proteolysis is difficult to assess. At least the binding of HRG to thrombospondin will not be affected significantly by changes in plasma levels of HRG, because the reported dissociation constant is very low (12.5 nmol/l) (30).

Finally, it should be noted that liver cirrhosis Child A is the second disease state, next to thrombophilia, in which elevated HRG levels are found. This may be of importance in future studies on familial thrombosis associated with elevated levels of HRG, where mild liver dysfunction should at least be excluded.

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CHAPTER 8

A SHIFT IN BALANCE BETWEEN PROFIBRINOLYTIC AND ANTIFIBRINOLYTIC FACTORS CAUSES ENHANCED FIBRINOLYSIS IN LIVER CIRRHOSIS: ROLE OF TISSUE-TYPE PLASMINOGEN ACTIVATOR, PLASMINOGEN ACTIVATOR INHIBITOR AND α_2-ANTIPLASMIN

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ABSTRACT

In patients with liver diseases, the liver-dependent metabolism of various fibrinolytic proteins may be altered. We tried to assess the effects of these changes by considering the balance between profibrinolytic and antifibrinolytic factors at two levels of the fibrinolytic cascade: firstly, at the tissue-type plasminogen activator (t-PA) and plasminogen activator inhibitor-1 (PAI-1) level and, secondly, at the plasminogen and α_2-antiplasmin (α_2-AP) level. Two groups of 12 patients with mild and severe liver cirrhosis, and, as reference 12 healthy volunteers, were studied.
Antigen levels of both t-PA and it's inhibitor (PAI-1) were increased in mild as well as severe cirrhosis. Some patients had increased t-PA activity levels, however, median t-PA activity and PAI-1 activity levels were normal in mild and severe liver cirrhosis. The specific activity of t-PA and PAI (activity/antigen quotient) was reduced in either mild cirrhosis or severe cirrhosis. This indicates that more enzyme-inhibitor complexes circulate in blood of patients with liver cirrhosis than in normal individuals. The balance between t-PA and PAI-1 changed in favour of t-PA, as calculated by increased t-PA:PAI-1 ratios in the patients.
Plasminogen and α_2-antiplasmin antigen and activity levels were decreased in patients with mild and severe cirrhosis. The absolute amount of α_2-AP bound to fibrin was also decreased in all patients. The decreased crosslinking of α_2-AP to fibrin, may result in fibrin clots, more susceptible to lysis. In addition in vitro experiments were performed, which showed that an equal decrease in the levels of plasminogen and α_2-AP resulted in enhanced fibrinolysis, indicating the important role of α_2-AP in the control of fibrinolysis.
We conclude that various changes in the balance between pro- and anti-fibrinolytic factors occur in liver cirrhosis. We suggest that increased t-PA activity in a subgroup of patients and decreased levels of α_2-AP in all patients are of major importance in the enhancement of fibrinolysis in patients with liver cirrhosis.

INTRODUCTION

Patients with liver cirrhosis may have disturbances in the coagulation mechanism, which may contribute to their bleeding problems (1,2). These changes are mainly due to a reduced synthesis of coagulation factors and a reduced clearance of activated clotting factors, which in turn may lead to disseminated intravascular coagulation, with consumption of coagulation factors. Inhibitors of coagulation, such as antithrombin III (3), protein C (4) and protein S (5) are also reduced in liver disease, altering the balance between procoagulant and anticoagulant factors (1).
In addition to the coagulation abnormalities, increased fibrinolysis may also be present. As early as 1914, Goodpasture found that blood clots of cirrhotic patients lysed more rapidly than blood clots of normal controls (6). The occurence of enhanced fibrinolysis in liver disease was confirmed in several other reports (7,8). Francis et al.(9) found in patients with severe liver disease that enhanced fibrinolysis predisposed to soft tissue hemorrhage after trauma. They also found a trend towards increased intracranial bleeding, which indicates the clinical significance of enhanced fibrinolysis. Boks et al. (10) failed to find a relation between fibrinolytic activity and variceal bleeding, but did find a relation between a quotient of fibrinolytic activity/ Normotest and mucosal bleeding, thereby substantiating the importance of fibrinolytic activity in liver diseases. Although several mechanisms have been proposed, such as reduced inhibition of fibrinolysis and reduced clearance of plasminogen activators, the cause of enhanced fibrinolysis in liver disease is still not clear (11,12).
In recent years, new fibrinolytic components and interactions have been discovered, resulting in increased complexity of the fibrinolytic system, and the liver seems to be involved in the synthesis and clearance of most proteins. Therefore it remains difficult to establish the exact cause of enhanced fibrinolysis in liver disease. Since impairment of liver function may result in several changes in the fibrinolytic system, it is not sufficient to study one single component of the fibrinolytic system; these components have to be studied in coherence.
We therefore investigated the balance between several pro-and anti-fibrinolytic factors of the fibrinolytic cascade. Firstly, the

balance between the plasminogen activator, t-PA (tissue-type plasminogen activator) and its inhibitor, PAI-1, (plasminogen activator inhibitor-1) was studied (13). Both t-PA and PAI-1 are synthesized and released mainly by endothelial cells and are cleared by the liver (14,15). An increase in "free" t-PA in circulation may result in enhanced fibrinolysis and a bleeding tendency (16), whereas an increase in PAI-1 levels is associated with an increased risk of thromboembolic disease (17).
Secondly, we studied the balance between plasminogen and α_2-antiplasmin (α_2-AP). Plasminogen is the pro-enzyme of plasmin, the key enzyme of the fibrinolytic cascade. A decreased level of plasminogen in circulation has been reported to be associated with deep venous thrombosis (18,19). α_2-AP is the most important inhibitor of plasmin and is synthesized by the liver as a very active plasminogen binding (PB) form, which is converted in circulation to a less active non-plasminogen binding (NPB) form (20). The PB form of α_2-AP has, next to its plasmin inactivating and plasminogen binding properties, also the ability to crosslink to fibrin, mediated by coagulation factor XIIIA (21). All these three properties of α_2-AP make the fibrin clot more resistant to lysis and so a reduced level of α_2-AP may result in enhanced fibrinolysis. Accordingly an inherited deficiency of α_2-AP is known to be associated with a haemorrhagic diathesis (22,23,24).
The approach of studying fibrinolytic proteins together may reveal the relevance of the changes in fibrinolytic components in patients with liver disease and might therefore result in a better understanding of the mechanism of enhanced fibrinolysis in these patients.

PATIENTS AND METHODS

Patients

After obtaining informed consent, in conformance with the Declaration of Helsinki, 24 patients with liver cirrhosis participated in the study. These patients were outpatients in a stable condition with biopsy proven liver cirrhosis of different etiology: alcohol abuse (n=10); viral hepatitis (n=5); autoimmune hepatitis (n=6); unknown (n=3). The patients were classified

according to Pugh's modification of the Child classification (25). Clinical information was obtained from hospital records. Twelve patients suffering from mild cirrhosis (Child A) and 12 patients suffering from severe cirrhosis (Child C), were studied. Twelve healthy volunteers served as control group.

Plasma

Venous blood, obtained by venapuncture between 10.00 A.M. and 12.00 noon, was collected in plastic tubes containing trisodiumcitrate 0.11 mol/l (9:1) and placed immediately on melting ice. Plasma was prepared by centrifugation at 2000 g for 30 min. at 4°C and stored at -70°C until use. A laboratory reference value of 100 % was obtained from pooled normal plasma.

Methods

Plasminogen was measured using streptokinase for activation of plasminogen and HD-Nva-CHA-Lys-pNA as a substrate, both obtained from Behringwerke (Marburg,FRG) (26). Tissue-type plasminogen activator (t-PA) activity and t-PA inhibition (PAI-1) of plasma were measured by spectrophotometric assays according to Verheijen et al. (27,28). T-PA antigen levels were determined using an enzyme linked immunosorbent assay (Biopool, Umea, Sweden) (29). Plasminogen activator inhibitor-1 antigen was measured using a TintElize TM PAI-1 assay from Biopool (Umea, Sweden). Specific activity of t-PA and PAI-1 was calculated as the ratio activity:antigen of t-PA and PAI-1 respectively. To determine the euglobulin clot lysis time (ECLT), standard euglobulin fractions of plasma were prepared at pH 5.9 with a plasma dilution of 1:10 (30). Precipitates were redissolved in Tris/Tween buffer (0.1mol/l Tris/HCl; 0.1% Tween 80 (v/v) pH 7.5) and 0.2 ml of the euglobulin fractions were clotted by addition of 0.1ml of calcium-thrombin solution ($CaCl_2$ 25 mmol/l and thrombin 10 NIH/l). The lysis time of the clot was recorded. The disappearance of air bubbles was regarded as the endpoint.

α_2-AP activity was measured with Behrichrom^R α_2-antiplasmin, using HD-Nva-CHA-Lys-pNA as a substrate (Behringwerke, Marburg, FRG) (31). α_2-AP antigen was measured by Rocket immunoelectrophoresis according to Laurell, using an 1.5 % anti-α_2-AP antiserum (Nordic Immunology, Tilburg, The Netherlands) (32). The ratio of the two

functional forms of α_2-AP (i.e. the plasminogen binding (PB) and the non plasminogen binding (NPB) forms) was determined by modified crossed immunoelectrophoresis (CIE), according to Kluft and Los (33). In this method we added monoclonal antibodies, directed against the C-terminal part, the plasminogen binding site, of α_2-AP (34) in the first dimension gel, and polyclonal antiserum (Nordic Immunology) against α_2-AP in the second dimension. The two obtained immunoprecipitation peaks represent the PB and the NPB forms of α_2-AP respectively. The PB:NPB ratio was calculated as the ratio of the surfaces of the immunoprecipitation peaks, which were determined with a computerized program using a Hipad digitalizer (Geveke Electronics, The Netherlands). Binding of α_2-AP to fibrin was studied by clotting 180 μl plasma with 120 μl of a mixture of thrombin (4.17 NIH/ml) and $CaCl_2$ (10.4 μM) in 0.11 M NaCl, which was left standing at 37°C for 1 hour. In the serum supernatant and in a plasma sample incubated with 120 μl 0.15 M NaCl, α_2-AP activity was assayed as described above. The difference represented the amount of α_2-AP bound to fibrin, and was expressed relative to the patients own plasma level (relative value) and in absolute amounts (absolute value). Factor XIIIA was determined according to Laurell using an anti Factor XIIIA antiserum from Behringwerke (32). α_2-Macroglobulin was assayed according to Laurell, using a 2% rabbit anti-human α_2-macroglobulin antiserum (Nordic) (32). Fibrin degradation products were measured in plasma using an enzyme immunoassay (Organon Teknika, Boxtel, The Netherlands) (35). Albumin levels were determined using the BCG (Bromocrysolgreen) principle on a Technikon/SMAC analyser.

In vitro studies

Clot lysis experiments were performed to determine the effect of decreasing levels of plasminogen and α_2-AP on the rate of fibrinolysis. Plasma artificially depleted in plasminogen and PB-α_2-AP was obtained by affinity chromatography on Lysine Sepharose (35), followed by affinity chromatography on Kringle I-III Sepharose. To this plasma different amounts of purified Glu-plasminogen and PB-α_2-AP were added to concentrations of 12.5 %, 25 %, 50 %, 100 % and 150 % of both plasminogen and α_2-AP. Plasminogen was prepared from human Cohn III fraction (36) and purified PB-α_2-AP was obtained from Biopool, Umea, Sweden. Ninety μl of these

artificially made plasma samples was clotted by addition of a mixture of 0.3 NIH thrombin and 4.5 IU single chain melanoma t-PA (37) in 7.5 μl of a 0.2 M $CaCl_2$ solution and 37.5 μl phosphate buffered saline. These plasma samples were incubated at 37°C and fibrin degradation products were measured after 10 minutes and every 5 min thereafter.

Statistical evaluation

Statistical analysis was performed using the Wilcoxon rank sum test and the Spearman rank correlation test. A p value of <0.05 was considered to be significant. Unless otherwise stated, the values are expressed as median and range.

RESULTS

T-PA and PAI-1 levels in liver cirrhosis

In both mild and severe liver cirrhosis, t-PA antigen levels, which include free t-PA and t-PA bound to inhibitors, were increased compared to the control group (table 1). A wide variability of t-PA activity levels was found in mild and severe cirrhosis, resulting in a median normal t-PA activity level in both groups. The specific activity, calculated as the quotient t-PA activity: t-PA antigen, was reduced in mild cirrhosis ($p<0.05$) and a similar trend was seen in severe cirrhosis ($p=0.06$;n.s.).

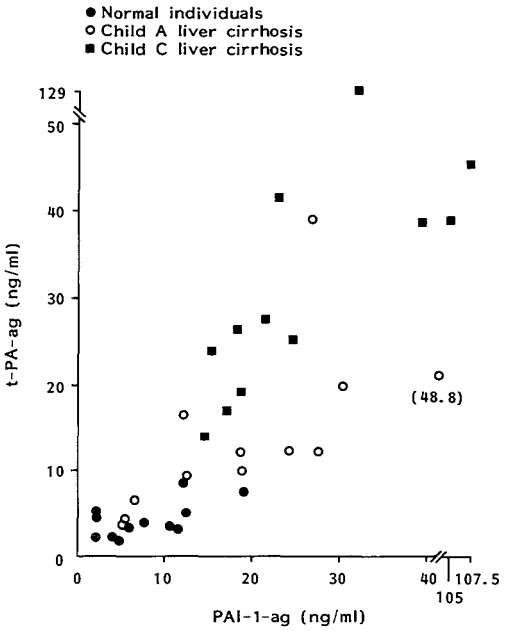
PAI-1 antigen levels were increased in both the patient groups with mild and severe liver cirrhosis (table 1). PAI-1 activity levels showed a wide variability in the patients with cirrhosis, but the median values of both groups were not significantly elevated (table 1). PAI-1 specific activity was reduced in severe liver cirrhosis (table 1).

Balance between t-PA and PAI-1

In figure 1, it can be seen that the balance between t-PA antigen and PAI-1 antigen changes in patients with liver cirrhosis.

Figure 1a shows that both t-PA antigen and PAI-1 antigen levels increase in patients with liver cirrhosis compared to the normal individuals, and the increase is dependent upon the severity of the disease. In figure 1b, we calculated the ratio between t-PA antigen

and PAI-1 antigen in mol/mol, showing a trend towards increased levels of t-PA compared to PAI-1. The t-PA antigen and PAI-1 antigen levels in the total group of patients with liver cirrhosis correlated significantly (r=0.71; p<0.001).



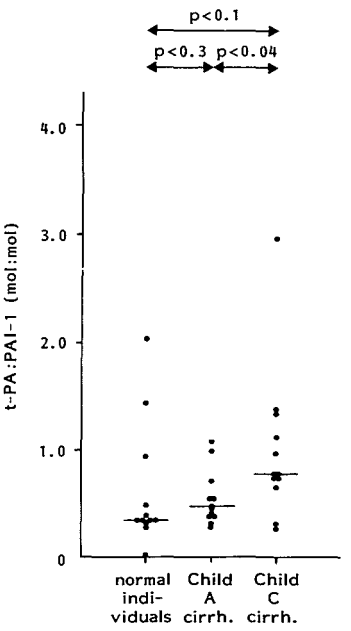


Figure 1: a) Antigen levels of tissue-type plasminogen activator (t-PA) and its inhibitor (PAI-1) in healthy individuals and patients with mild (Child A) and severe (Child C) liver cirrhosis. The correlation coefficients of the normal individuals, mild and severe cirrhosis are 0.48 ($p<0.05$), 0.83 ($p<0.001$) and 0.83 ($p<0.001$) respectively. b) The ratio's between t-PA and PAI-1 antigen levels (t-PA:PAI-1) of all healthy individuals (N) and the patients with mild (Child A) and severe (Child C) liver cirrhosis, expressed in mol/mol. The horizontal lines indicate the median. PAI-1= plasminogen activator inhibitor-1; t-PA= tissue-type plasminogen activator.

In figure 2 the individual levels of t-PA activity and PAI activity of healthy volunteers and patients with severe cirrhosis are given. As can be observed, t-PA activity levels correlated negatively with PAI activity levels in both controls ($r=-0.75;p<0.01$) and patients ($r=-0.68;p<0.02$). In patients suffering from severe cirrhosis, low levels of PAI activity (< 2 IU/ml), were associated with extreme high levels of t-PA activity. In the other patients (PAI-1 activity levels > 2 IU/ml), t-PA activity levels were not increased. When a curve is plotted through the individual levels of tPA and PAI of the control and the group of patients with severe cirrhosis, a shift in the balance of these parameters can be observed (figure 2).

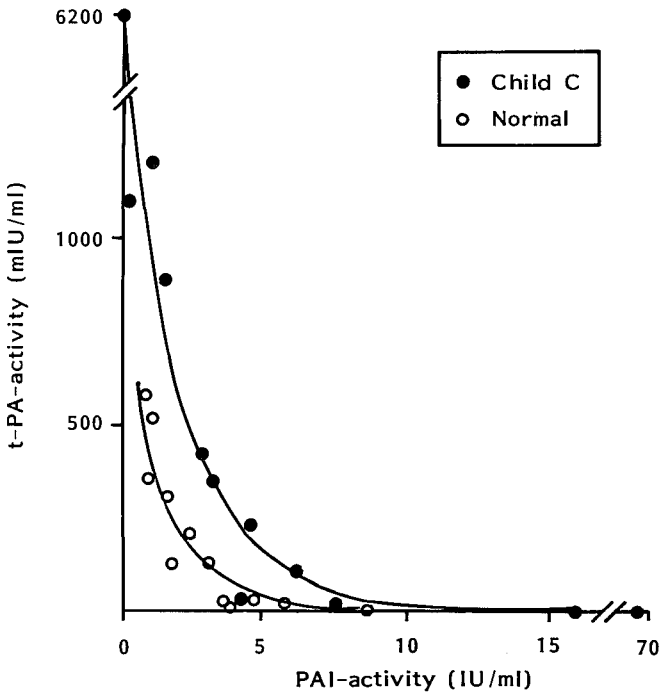


Figure 2: Levels of tissue-type plasminogen activator activity (t-PA) and plasminogen activator inhibition of plasma (PAI) of individual patients with severe liver disease and the normal subjects.

The net result of these changes in t-PA and PAI-1 on overall fibrinolytic activity was measured by the euglobulin clot lysis time. The ECLT was > 180 min in all healthy individuals. In mild cirrhosis, the ECLT was > 180 min in seven patients and between 107 and 172 min for the other five patients. In severe cirrhosis, the ECLT was > 180 min in 6 patients, and between 18 and 130 min in the other 6 patients. The four patients with the highest t-PA activity had all a shortened ECLT (18, 27, 51 and 73 min).

Table 1: Results of tissue-type plasminogen activator (t-PA) antigen and activity and plasminogen activator inhibition (activity and antigen levels) of plasma in patients with Child A and Child C liver cirrhosis compared to a reference group.

		Control n=12	Child A n=12	Child C n=12
t-PA antigen ng/ml	mean	4.2	13.6[a]	36.8[b]
	median	3.8	11.7	27.1
	range	2.0 - 8.0	3.1 - 38.1	13.5 - 129
t-PA activity mIU/ml	mean	196	221[c]	884[c]
	median	127	28	295
	range	1 - 590	0 - 1770	1 - 6220
t-PA specific activity mIU/ml	mean	73	29[d]	22[c]
	median	41	2	12
	range	0 - 295	0 - 183	0 - 84
PAI antigen ng/ml	mean	8.2	19.5[e]	35.8[a]
	median	7.0	18.5	21.6
	range	2.3 - 18.5	5.0 - 48.8	13.3 - 108
PAI activity IU/ml	mean	2.8	5.5[c]	9.8[c]
	median	2.5	4.6	4.2
	range	0.9 - 7.8	0 - 22.0	0 - 67.8
PAI specific activity IU/ml	mean	0.35	0.22[c]	0.19[d]
	median	0.31	0.24	0.18
	range	0.16 - 0.96	0 - 0.45	0 - 0.65

The specific activity of both t-PA and PAI was calculated as the activity/antigen ratio and expressed in mIU/ml and IU/ml respectively;
[a] $p<0.002$, [b] $p<0.0001$, [c] not significant, [d] $p<0.05$, [e] $p<0.01$, all versus controls.

Balance between plasminogen and α_2-antiplasmin

The amount of plasminogen was decreased in both mild (66;42-95 %:$p<0.005$) and severe (41;13-67 %;$p<0.0001$) liver cirrhosis, compared to the control group (88;71-126 %). In mild and severe liver cirrhosis both α_2-AP activity and antigen were decreased (figure 3).

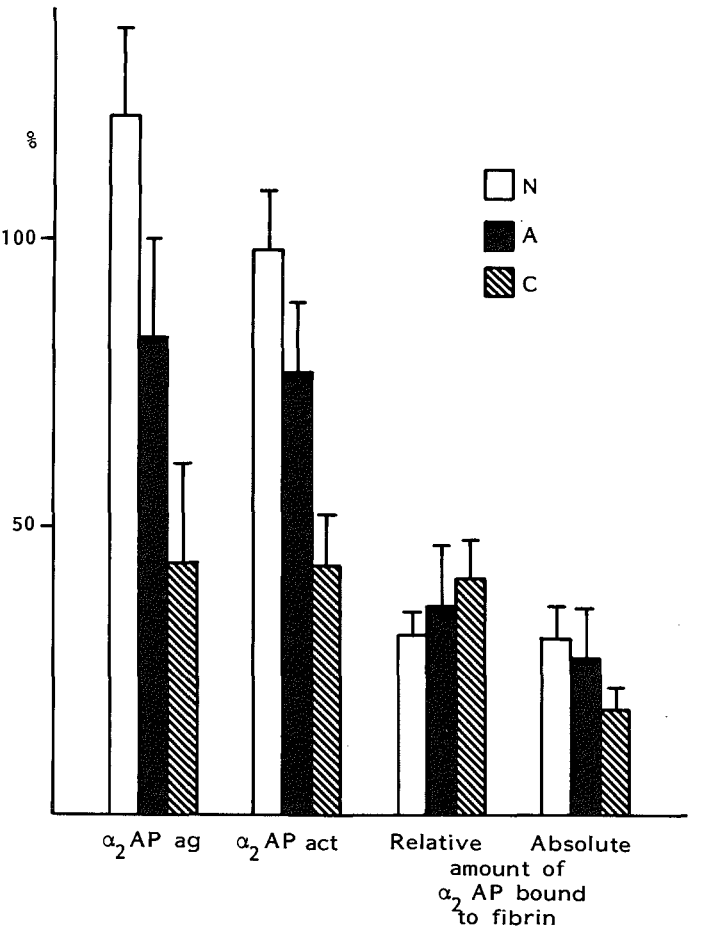


Figure 3: α_2-Antiplasmin (α_2-AP) in liver cirrhosis. α_2-AP activity and antigen levels and the relative and absolute amount of α_2-AP bound to fibrin in normal subjects (control; n=12), Child A liver cirrhosis (n=12) and Child C liver cirrhosis (n=12). The bars indicate mean values, the lines indicate the SD.

The ratio between the two molecular forms (plasminogen binding: non plasminogen binding form) was measured by mCIE and an example is given in figure 4. The PB:NPB ratio was increased in mild cirrhosis (2.8;2.1-3.7 :$p<0.001$) and in severe liver cirrhosis (4.0;1.7-15.4: $p<0.005$) (control 2.2;1.8-2.7).
The relative amount of α_2-AP bound to fibrin is normal in mild, but increased in severe cirrhosis (figure 3). Coagulation factor XIIIA, which mediates the binding of α_2-AP to fibrin, is decreased in mild (76;52-119%;$p<0.025$) and severe (76;41-116%;$p<0.005$) liver cirrhosis, compared to the control group (94;81-122%) (data not shown). Although the relative amount of α_2-AP bound to fibrin is increased (at least in severe cirrhosis), the absolute amount is decreased, due to the strong reduction of total α_2-AP.
α_2-Macroglobulin, also capable of inhibiting plasmin, was increased in mild liver cirrhosis (132;98-257%;$p<0.0005$) and normal in severe liver cirrhosis (105;82-178%) (control 89;59-127%) (data not shown).

<u>In vitro studies</u>
The influence of changes in plasminogen and α_2-AP levels on the rate of fibrinolysis was investigated by clot lysis experiments. Plasma samples containing various, but equal, amounts of plasminogen and PB-α_2-AP, were clotted and the lysis of the clots was recorded by measuring fibrin degradation products (FDP) in the fluid phase of the clot. The results are given in figure 5, in which lysis of the clots is given as the percentage of the maximum FDP levels after complete lysis of the plasma clot. As can be observed, although the levels of plasminogen and α_2-AP are constantly in balance with each other, the rate of fibrinolysis is increased at reduced levels of both proteins.

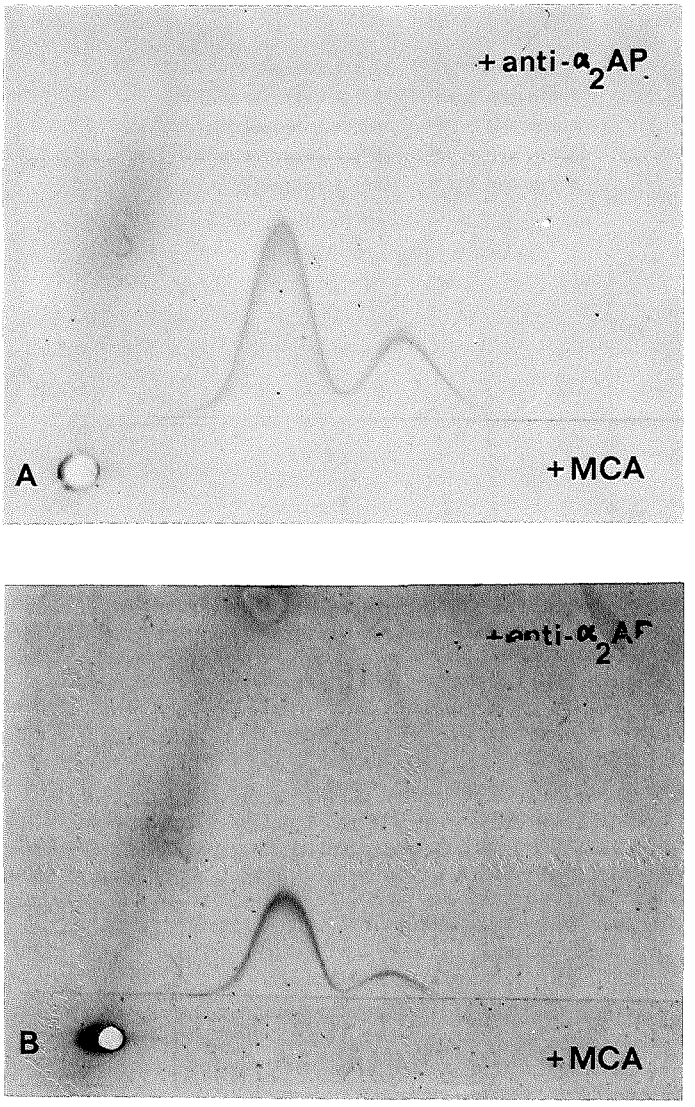


Figure 4: The two molecular forms of α_2-antiplasmin: Modified crossed immunoelectrophoresis to assay the plasminogen binding (PB) and the non-PB forms of α_2-antiplasmin (α_2-AP) in plasma of a healthy volunteer, with a PB:NPB ration of 2.3 (A) and of a patient with severe liver cirrhosis, with a PB:NPB ratio of 4.0 (B). Monoclonal antibodies (MCA) were added to the first dimension gel. In the second dimension gel polyclonal antibodies against α_2-AP were added. The ratio PB:NPB forms of α_2-AP was calculated as the ratio of the surfaces of the immunoprecipitation peaks.

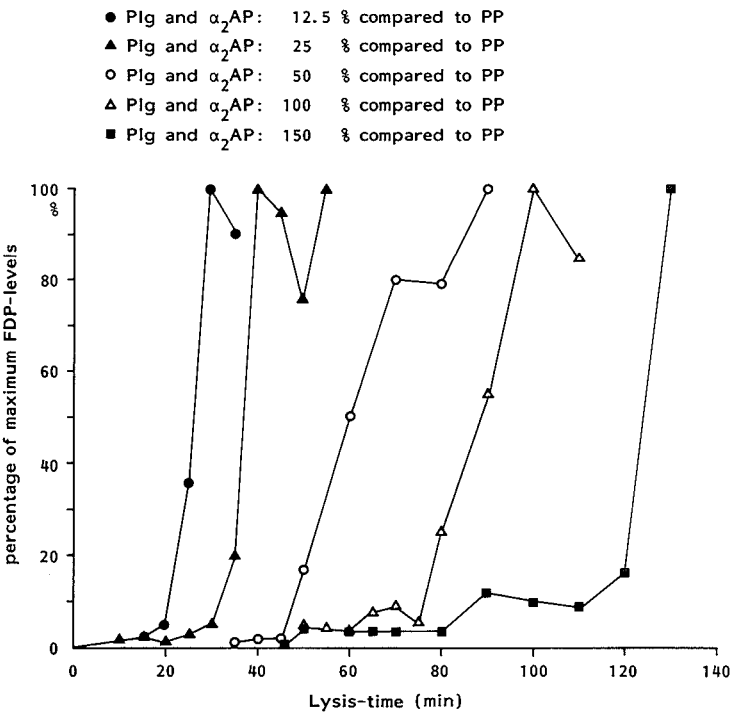


Figure 5: Results of the in vitro plasma clot lysis experiments. Plasma samples containing various, but equal concentrations of PB-α_2-AP and plasminogen were clotted and incubated at 37°C. The lysis of the clots was recorded by measuring fibrin degradation products (FDP) in the fluid phase of the clot. The FDP levels, expressed as percentage of the maximum FDP level (obtained after complete lysis of the clot), are given at the vertical axis. FDP levels were measured every five minutes up till 140 min incubation.

DISCUSSION

In this study we investigated fibrinolysis in patients with different degrees of liver cirrhosis, in order to try to contribute to the understanding of enhanced fibrinolysis in severe liver failure. Several mechanisms have been proposed to be responsible for increased fibrinolysis. An inappropriate inhibition of fibrinolysis was suggested by Arnman et al (38) and Aoki et al. (39), who attributed it to a decrease of the fast acting inhibitor

of plasmin, α_2-antiplasmin, in circulation. Saito et al. (40) proposed that reduced levels of histidine-rich glycoprotein (HRG) could play a role in enhanced fibrinolysis in severe liver disease. They suggested that the decrease of HRG could result in increased levels of "free" plasminogen available for binding to fibrin and activation to plasmin, thereby contributing to enhanced fibrinolysis. However in a recent study (41), we found reduced levels of both HRG and total plasminogen in patients with severe liver cirrhosis, resulting in decreased levels of "free" plasminogen, and we therefore concluded that HRG plays no role in enhanced fibrinolysis in liver disease. Booth et al. (11) reported that increased levels of plasminogen activators were responsible for enhanced fibrinolysis in alcoholic liver cirrhosis. This increase was proposed to be due to a reduced clearance of plasminogen activators in patients with liver cirrhosis. A recent report of Hersch et al. (42) showed that the inhibitor of plasminogen activators played a critical role in enhanced fibrinolysis in liver cirrhosis. However all these studies focussed mainly on one of the components of the fibrinolytic system only. In our study, we considered various components together, notably the pro- and antifibrinolytic factors at two levels of the fibrinolytic cascade. In the present study we found several changes in the t-PA- PAI-1 balance in patients with liver cirrhosis. In the patients both t-PA antigen and PAI-1 antigen were increased. The antigen levels of both proteins were increased concomitantly with the severity of disease. This has two possible explanations: a decreased clearance of both proteins by the diseased liver or an increased synthesis. The latter would fit with a parallel regulation as suggested from the findings of homologous regulation elements in the t-PA and PAI-1 gene (43). This is supported by earlier reported correlations between blood levels of t-PA antigen and PAI-1 activity or antigen in both healthy individuals and several patient groups (44-46). In our study however, the balance between t-PA and PAI-1 antigen changes slightly in favour of t-PA. This change in balance is expressed in figure 1b, showing the ratios of t-PA antigen:PAI-1 antigen, which are increased in most patients. The mechanism of this change is not elucidated by our study. It may be caused by a selective increase in t-PA synthesis or in a selective change in the clearance by the diseased liver of t-PA versus PAI-1.

The used antigen assays for both t-PA and PAI-1 are recording the respective molecules equally effective, both free in blood or in complex with each other (t-PA-PAI-1 complex). This excludes the possibility that a change in the composition towards more t-PA-PAI-1 complexes is the origin of the above discussed difference. This specificity of both antigen assays is important, since we provided evidence for a change in the composition of the circulating amounts of t-PA and PAI-1. As is indicated in table 1, the specific activity of both t-PA and PAI-1 was decreased in the patients, indicating larger amounts of enzyme-inhibitor complexes in the patients compared to the healthy individuals. It is of particular interest how this changed situation reflects itself in the activity levels of both the enzyme and the inhibitor. As indicated in figure 2, the balance between active t-PA and PAI is shifted, but the median values of both are not significantly different in the patient groups with mild and severe cirrhosis compared to the controls (table 1). The consequence of our evaluation of the t-PA-PAI-1 balance is that patients should be evaluated as individuals rather than as groups. In individual patients the change in balance can express itself in an elevated t-PA activity (figure 2) and a concomitant shortening of the euglobulin clot lysis time. Only these patients may be at risk for a bleeding tendency due to increased fibrinolysis. In figure 1b it is evident that in most patients the ratio between t-PA antigen and PAI-1 antigen is increased. Since the patients with liver cirrhosis have higher levels of both t-PA and PAI-1 than the normal individuals, only a small increase in the ratio may result in an absolute high free t-PA level. Only these patients where PAI-1 control of t-PA is weak, may be at risk for bleeding. In future studies it has to be evaluated whether this situation is longitudially stable or not and whether the patients with an inappropriate PAI-1 control may be at risk for bleeding.

The second level at which we studied the balance between pro-and antifibrinolytic factors was at the plasminogen and α_2-AP level.

The inhibition of plasmin in liver cirrhosis was decreased. In mild and severe cirrhosis, the activity and antigen levels of the most important inhibitor of plasmin, α_2-AP were decreased. The ratio of the active plasminogen binding form and the less active non-plasminogen binding form of α_2-AP is slightly increased in mild

cirrhosis and strongly increased in severe cirrhosis. This might be due to a decreased conversion of the PB into the NPB form, which results in a higher PB:NPB ratio. The mechanism of the conversion of PB-α_2-AP to NPB-α_2-AP is still unknown, but our findings may indicate that the liver, or a factor derived from the liver is involved in this proces. The relative amount of α_2-AP bound to fibrin, is increased in liver cirrhosis. This might be attributed to the relative increase of PB-α_2-AP compared to NPB-α_2-AP, reflected by the higher PB:NPB ratio, since only the PB form of α_2-AP binds to fibrin (21). Therefore relatively more α_2-AP is bound to fibrin, compared to normal controls. However, due to the low levels of total α_2-AP, the absolute amount of α_2-AP bound to fibrin is strongly decreased. Since alterations in α_2-macroglobulin were only mild, α_2-macroglobulin plays no major role in changes in the fibrinolytic system in patients with liver cirrhosis.

The reduced inhibition of plasmin, both in circulation, due to a decrease in total α_2-AP, and at the fibrin surface, due to a reduced crosslinking of α_2-AP to fibrin, may make fibrin more susceptible to lysis and may lead to enhanced fibrinolysis. At the other hand, plasminogen levels were decreased in the patients with liver cirrhosis, which may result in a decreased fibrinolytic potential in these patients.

To evaluate the relative importance of decreased plasminogen levels and decreased α_2-AP levels, we performed in vitro plasma clot lysis studies. Since the levels of both plasminogen and α_2-AP are decreased about equally in patients with liver cirrhosis, we mimiced this situation by decreasing both factors in plasma artifically. In these experiments an equal decrease of plasminogen and PB-α_2-AP resulted in increased rate of fibrinolysis. Therefore we suggest that the net effect of decreased levels of both plasminogen and α_2-AP is an enhancement of fibrinolysis, due to a dominating role of α_2-AP.

In conclusion our study showed that several changes occur at the two studied levels of the fibrinolytic mechanism. The changes include increased t-PA and PAI-1 antigen levels, abnormal correlation between t-PA and PAI blood levels, reduced plasminogen and α_2-AP levels, a changed balance between PB-α_2-AP and NPB-α_2-AP and a reduced binding of α_2-AP to fibrin. Although it remains difficult to assess the relative importance of these changes on

overall fibrinolysis, we suggest that the increased t-PA activity in a subgroup of patients and reduced levels of α_2-AP in all patients are of major importance in the enhancement of fibrinolysis in patients with liver cirrhosis.

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CHAPTER 9

GENERAL DISCUSSION

GENERAL DISCUSSION

Congenital and acquired disorders of fibrinolysis

Congenital and acquired disorders of the fibrinolytic system can have major clinical effects: either bleeding or thrombo-embolic complications.

A haemorrhagic diathesis due to congenital disorders of fibrinolysis has been ascribed to an excess of plasminogen activator (1), a decrease of the functional activity of plasminogen activator inhibitor (2) or α_2-antiplasmin (α_2-AP) deficiency (3,4). A severe, haemophilia-like bleeding tendency occurs in patients with homozygous α_2-AP deficiency. So far only a few patients with homozygous α_2-AP deficiency (type I and II) have been described (5-9). In heterozygous α_2-antiplasmin deficiency the clinical expression is variable. Our large family study (chapter 3) revealed a mild bleeding tendency in only 2 out of 13 heterozygous family members (10). In some earlier reported family studies, none of the heterozygotes had a bleeding tendency (5,8). On the other hand some heterozygotes of other families were admitted to hospital, because of a life-long bleeding tendency (11,12). A total of 59 patients with heterozygous α_2-AP deficiency have been reported so far: 13 patients (22%) had a (mild) haemorrhagic diathesis (3,4). We conclude that heterozygous α_2-antiplasmin deficiency may result in a haemorrhagic diathesis, which can be variable in severity.

Congenital and acquired disorders of fibrinolysis may be associated with thrombo-embolic complications. Recently an inappropriate tissue-type plasminogen activator (t-PA) response to venous stasis was found in patients with deep venous thrombosis (13,14). Decreased levels of t-PA in patients recovering from a first myocardial infarction were reported to be associated with recurrence of myocardial infarction (15). Increased levels of plasminogen activator inhibitor (PAI) have been reported to predispose to the development of acute myocardial infarction (16). A familial increase of histidine-rich glycoprotein has been found in families with thrombophilia (17,18).

It has also been suggested that plasminogen deficiency is associated with thrombosis (19,20,21,22). We reported a study of a patient with a severe thrombotic tendency, in whom only a decreased plasminogen activity and antigen was found (chapter 4). This report

gives additional information on the association between plasminogen deficiency and thrombosis (23). However, a causal relation between plasminogen deficiency and thrombosis is not proven. The identification of genetic risk factors for hereditary thrombophilia is mainly based on studies of the hereditary nature of the defect and a significant association between the defect and the presence of thrombotic disease, as outlined by Bertina (24). Although several reports on plasminogen deficiency associated with thrombosis have been published, there is still a lack of adequate family studies (25). Although we described a dramatic case history on plasminogen deficiency associated with thrombosis (23), family studies are required to definitely identify plasminogen deficiency as a risk factor for the development of thrombosis.

An acquired disorder of fibrinolysis is obtained as a side effect during thrombolytic therapy with plasminogen activators in patients with acute myocardial infarction. The aim of the activation of the fibrinolytic system is the dissolution of the pathological thrombus. However the extensive activation of circulating plasminogen into plasmin may result in breakdown of fibrinogen and coagulation factors V and VIII in the systemic circulation, giving a systemic lytic state (26). The clinical relevance of these systemic effects is still not clear. It has been suggested that the systemic effects are of benefit for obtaining recanalization and preventing reocclusion, but they increase the risk of bleeding. In a study on the consumption of plasmin inhibitors during thrombolytic therapy, we found that the plasminogen binding form of α_2-AP (PB-α_2-AP) plays a critical role in the prevention of the systemic effects (chapter 5). As long as 20-25 % of PB-α_2-AP is present in the circulation systemic effects do not occur. Other plasmin inhibitors, including the non-plasminogen binding form of α_2-AP (NPB-α_2-AP) are not able to prevent systemic effects. We conclude that PB-α_2-AP seems to be the most important plasmin inhibitor in the circulation, and may be used as a marker for the occurrence of systemic effects during thrombolytic therapy.

It is therefore of interest to study the molecular forms of α_2-AP separately. Using a newly developed assay, the different molecular forms of α_2-AP in plasma and other body fluids were studied (chapter 6). This revealed that the conversion of the active plasminogen binding form into the less active non-plasminogen

binding form of α_2-AP are different processes in vivo and in vitro, which is in contradiction with earlier concepts (27). Future studies will have to reveal the clinical relevance of the conversion of PB-α_2-AP into NPB-α_2-AP.

Fibrinolysis in liver disease

Acquired disorders of fibrinolysis frequently occur in patients with liver disease (28). Reviewing the literature on fibrinolysis in liver disease, we concluded that, although many studies have been performed, the mechanism of enhanced fibrinolysis in liver disease is still unclear. Early studies have suggested two mechanisms of enhanced fibrinolysis in liver disease: increased plasminogen activator activity and reduced inhibition of fibrinolysis.

Recent developments and the discovery of new fibrinolytic proteins provide new opportunities to further characterize the cause of enhanced fibrinolysis.

So far, studies have mainly focussed on single components of the fibrinolytic cascade. In our studies we investigated several fibrinolytic proteins in a theoretical coherence: the balance between pro- and antifibrinolytic factors.

This approach was used to study the levels of histidine-rich glycoprotein (HRG) in relation to the levels of plasminogen in patients with liver cirrhosis (chapter 7). We found increased levels of HRG in patients with mild liver cirrhosis and decreased levels in moderate and severe cirrhosis. Previous studies on HRG in several groups of patients with liver disease revealed decreased or normal levels (29-32). The decrease of HRG in severe cirrhosis was previously suggested to result in enhancement of fibrinolysis (29). We found that due to a concommitant decrease of plasminogen in severe liver cirrhosis, "free" plasminogen levels are decreased. It is therefore unlikely that the decreased levels of HRG contribute to the enhanced fibrinolysis in severe liver cirrhosis (33).

A study of the balance between tissue-type plasminogen activator (t-PA) and its inhibitor (PAI-1) revealed that antigen levels of both proteins were increased in liver cirrhosis. The antigen levels increased gradually with the severity of the disease. The increase of both proteins can be due to a decreased clearance by the liver

or an increased synthesis (release) by endothelial cells. The median activity levels of both t-PA and PAI were not increased in mild or severe cirrhosis. However, the balance between t-PA activity and PAI activity changed in the patients with liver cirrhosis compared to a control group. This resulted in high activity levels of t-PA in only a small number of patients. In most patients t-PA and PAI increase concomitantly, resulting in normal t-PA activity levels (34,35,36).

A study on the α_2-antiplasmin and plasminogen balance showed decreased levels of both proteins, which may be due to a decreased synthesis, but may also be due to increased consumption, especially in severe liver cirrhosis (37). As has been outlined in chapter 3 and 4 of this thesis, deficiencies of both proteins have different effects on the rate of fibrinolysis and, accordingly, a different clinical outcome. Therefore we studied in vitro the net effect of a decrease of both α_2-AP and plasminogen on the rate of fibrinolysis. This revealed that an equal decrease in both proteins resulted in an increased rate of fibrinolysis. With regard to the above mentioned studies in patients with liver cirrhosis, we conclude that:

1. HRG levels are not important in the mechanism of enhanced fibrinolysis in liver cirrhosis.
2. Increased t-PA activity, due to extremely high levels of t-PA or an inappropriate inhibition by PAI, may result in enhanced fibrinolysis in some patients with liver cirrhosis.
3. A decrease of α_2-AP, which we found in all patients with liver cirrhosis, plays a role in enhanced fibrinolysis in liver cirrhosis.
4. Since the changes in fibrinolysis, especially in the t-PA and PAI-1 balance, are strongly individually variable and not, for example, dependent upon the severity of the disease, we propose that the patients should be evaluated as individuals rather than as groups.
5. Some of these patients may be at risk for bleeding, especially during surgery or biopsy. This seems most likely in patients with high t-PA activity levels associated with decreased α_2-AP levels, or patients with extreme low α_2-AP levels. Those patients should be treated pre-operatively or in case of bleeding. The first choice is substitution with

fresh frozen plasma. In case of primary fibrinolysis, without evidence of disseminated intravascular coagulation, treatment with fibrinolysis inhibitors, such as epsilon-amino caproic acid or tranexamic acid can be considered.

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This thesis comprises studies on congenital and acquired disorders of fibrinolysis, with emphasis on the changes in fibrinolysis variables in patients with liver cirrhosis.

In chapter 1 evidence for the relevance of the fibrinolytic system for normal haemostasis is briefly summarized. Examples of congenital disorders of fibrinolysis show that fibrinolytic disorders can have two different clinical outcomes: an increased fibrinolysis may lead to a bleeding tendency, whereas a decreased fibrinolysis can result in thrombo-embolic complications. In addition the objectives of the study are stated in this chapter.

In chapter 2 the physiology of fibrinolysis is reviewed, in particular the role of the liver in the regulation of fibrinolysis. The last part of the chapter is a review of the literature on fibrinolysis in liver disease.

In chapter 3 a family with congenital heterozygous α_2-antiplasmin deficiency is reported and the clinical outcome of an α_2-antiplasmin deficiency is described. Thirteen family members were heterozygotes, deficient for α_2-antiplasmin. Two of these patients had a mild haemorrhagic diathesis, presenting as bleeding episodes after tooth extraction and after surgery and, in one patient as excessive menstruation. Both α_2-antiplasmin activity and antigen levels in the heterozygotes were about 60 % of normal. The plasminogen and fibrin binding properties of the α_2-antiplasmin molecule were normal. We concluded that the deficiency in this family is caused by a decreased synthesis of a normal α_2-antiplasmin molecule.

In chapter 4 a case history of a patient with recurrent deep venous thrombosis is presented. Laboratory investigations on risk factors for thrombosis revealed no abnormality, except for a plasminogen activity and antigen level of about 40 % of that of normal pooled plasma. The plasminogen molecule was apparently normal as demonstrated by a normal activation by tissue-type plasminogen activator, electrophoretic mobility on crossed immunoelectrophoresis, molecular weight and binding to lysine-Sepharose. We proposed that the deficiency in this patient is due to a decreased synthesis of a normal functioning plasminogen molecule, and that

this is associated with an increased tendency to venous thrombosis.

In chapter 5 a study on the consumption of plasmin inhibitors during thrombolytic therapy in patients with acute myocardial infarction is described. The aim of the study was to delineate the role of the two molecular forms of α_2-antiplasmin in the prevention of systemic effects during thrombolytic therapy. These systemic effects are due to the proteolytic degradation of clotting factors, including fibrinogen, Factor V and VIII, by plasmin in the circulation. This study revealed that no systemic effects occured as long as more than 20% of the plasminogen binding form of α_2-antiplasmin was present in the circulation. It was also demonstrated that the non-plasminogen binding form of α_2-antiplasmin played no role in the control of systemic effects. In addition we found that the other studied plasmin inhibitors could not prevent fibrinogenolysis. We concluded that the plasminogen binding form of α_2-antiplasmin has to be consumed before systemic effects can occur. The plasminogen binding form of α_2-antiplasmin may therefore be used as a marker for systemic effects during thrombolytic therapy.

In chapter 6 we described a study of the different molecular forms of α_2-antiplasmin, using a newly developed method. In this investigation we found indications that the conversion of the plasminogen binding form into the non-plasminogen binding form of α_2-antiplasmin in vivo is different from the conversion in vitro.

In chapter 7 and 8 the results of studies on the balance between pro- and antifibrinolytic factors in liver cirrhosis are described. In chapter 7 a study is presented on the role of histidine-rich glycoprotein (HRG), an inhibitor of fibrinolysis, in enhanced fibrinolysis in patients with liver cirrhosis. The study showed that HRG is increased in patients with mild liver cirrhosis, compared to a control group. HRG is decreased in patients with moderate and severe liver cirrhosis. This decrease is accompanied by a decrease in the levels of total plasminogen in blood. The net effect is a decrease of the amount of "free" plasminogen in patients with severe liver cirrhosis. We therefore conclude that the decrease of HRG in blood of patients with liver cirrhosis is not of importance in the pathogenesis of enhanced fibrinolysis in these patients.

In chapter 8 we described studies on the balance between pro- and antifibrinolytic factors in patients with liver cirrhosis at two levels of the fibrinolytic mechanism: tissue-type plasminogen activator (t-PA) and plasminogen activator inhibitor (PAI-1), and plasminogen and α_2-antiplasmin. Although both t-PA and PAI-1 antigen are increased in patients with liver cirrrhosis, the balance between these two proteins is altered. Median activity levels of t-PA and PAI are not increased in patients with mild or severe cirrhosis, as compared to a control group. In a few patients the shift in balance between t-PA and PAI-1 results in increased fibrinolytic activity. In all patients with liver cirrhosis the levels of both plasminogen and α_2-antiplasmin are decreased. We mimicked this situation in vitro in clot lysis experiments, and found that an equal decrease in plasminogen and α_2-antiplasmin results in an enhancement of fibrinolysis. This study shows that several changes occur in the fibrinolytic mechanism in patients with liver cirrhosis. Since these changes, especially in the t-PA and PAI-1 balance, are individually variable and not, for example, dependent upon the severity of the disease, patients should be evaluated as individuals rather than as groups. It is concluded that an increased t-PA activity in some patients and decreased levels of α_2-antiplasmin in all patients play a major role in the enhancement of fibrinolysis in patients with liver cirrhosis.

Dit proefschrift omvat een aantal studies naar aangeboren èn verworven afwijkingen van de fibrinolyse. De nadruk wordt daarbij gelegd op de veranderingen in fibrinolyseparameters bij patienten met levercirrose.

In hoofdstuk 1 wordt het belang van de fibrinolyse voor het normaal functioneren van het haemostasemechanisme kort uitgelegd. Afwijkingen in de fibrinolyse kunnen zich op twee manieren uiten: een te snelle fibrinolyse kan leiden tot een bloedingsneiging, terwijl een te langzame fibrinolyse kan leiden tot thrombo-embolische complicaties. Tevens worden de doelstellingen van de studie besproken.

In hoofdstuk 2 wordt de fysiologie van de fibrinolyse kort beschreven, met nadruk op de rol van de lever in de regulatie van de fibrinolyse. Het laatste deel van dit hoofdstuk geeft een overzicht van de literatuur over fibrinolyse bij patienten met leverziekten.

In hoofdstuk 3 wordt een familie met een heterozygote α_2-antiplasmine deficiëntie beschreven. Aan de hand van dit familieonderzoek worden de klinische gevolgen van de deficiëntie getoond. In deze familie waren 13 leden heterozygoot deficiënt voor α_2-antiplasmine. Twee hadden een milde haemorrhagische diathese, die zich uitte in langdurige bloedingen na kiesextracties en operatieve ingrepen en bij één patiente in een overvloedige menstruatie. Zowel de α_2-antiplasmine activiteit als concentratie was ongeveer 60 % van normaal. De plasminogeen- en fibrinebindende functies van het α_2-antiplasmine molecuul waren normaal. Wij concluderen dat de deficiëntie bij deze familieleden wordt veroorzaakt door een verminderde synthese van een normaal functionerend α_2-antiplasmine molecuul.

In hoofstuk 4 wordt de ziektegeschiedenis beschreven van een patiente met een ernstige thromboseneiging. Het orienterend haemostaseonderzoek naar risicofactoren voor thrombose leverde geen bijzonderheden op, behoudens een plasminogeen activiteit en concentratie van ongeveer 40 % van normaal. Het plasminogeen molecuul leek een normale functie te hebben, zoals bleek uit een normale activatie door weefsel-plasminogeenactivator, een normale

mobiliteit bij immuno-electrophorese, een normaal molecuulgewicht en een normale binding aan lysine-Sepharose. Wij concluderen dat de deficiëntie bij deze patiente wordt veroorzaakt door een verminderde synthese van een normaal functionerend plasminogeen molecuul en dat dit geassocieerd is met een verhoogde neiging tot diepe veneuze thrombose.

In hoofdstuk 5 werd een onderzoek beschreven naar het verbruik van plasmineremmers tijdens thrombolytische therapie bij patienten met een acuut myocardinfarct. Het doel van de studie was te onderzoeken het belang van de verschillende moleculaire vormen van α_2-antiplasmine bij het voorkómen van systemische effecten, i.c. fibrinogenolyse, gedurende thrombolytische therapie. Uit deze studie komt naar voren dat zolang meer dan 20 % van de plasminogeen-bindende vorm van α_2-antiplasmine in het bloed aanwezig is, geen fibrinogenolyse optreedt. Tevens blijkt uit deze studie dat geen van de andere bestudeerde plasmineremmers, waaronder de niet-plasminogeenbndende vorm van α_2-antiplasmine in staat is fibrinogenolyse te voorkómen. Wij suggereren dat de plasminogeenbindende vorm van α_2-antiplasmine moet worden verbruikt, voordat fibrinogenolyse kan optreden en dat de plasminogeenbindende vorm van α_2-antiplasmine gebruikt zou kunnen worden als nieuwe maat voor het optreden van systemische effecten gedurende thrombolytische therapie.

In hoofdstuk 6 wordt een studie beschreven naar de verschillende moleculaire vormen van α_2-antiplasmine, die bestudeerd zijn met een nieuw ontwikkelde methode.

In deze studie worden aanwijzingen gevonden dat de in vivo omzetting van de plasminogeenbindende vorm naar de niet-plasminogeenbindende vorm van α_2-antiplasmine een ander proces is dan de omzetting in vitro.

In hoofdstuk 7 en 8 worden de resultaten beschreven van studies naar de balans tussen pro-en antifibrinolytische factoren bij patienten met verschillende ernst van levercirrose. In hoofdstuk 7 wordt het onderzoek beschreven naar de rol van histidine-rijk glycoproteine (HRG), een remmer van de fibrinolyse, in het optreden van een versnelde fibrinolyse bij patienten met levercirrose. Het onderzoek toonde aan dat HRG is verhoogd bij patienten met een milde levercirrose in vergelijking met een controle groep. Daarentegen is HRG verlaagd bij patienten met matig ernstige en

ernstige levercirrose. Deze verlaging gaat samen met een verlaging van de totale plasminogeen concentraties bij deze patienten. Het netto effect is een verlaging van de hoeveelheid "vrij" plasminogeen bij patienten met ernstige levercirrose. Wij concluderen hieruit dat veranderingen in HRG in bloed van patienten met levercirrose niet van belang zijn in de pathogenese van versnelde fibrinolyse bij deze patienten.

In hoofdstuk 8 wordt onderzoek beschreven naar de balans van de fibrinolyse op het niveau van weefsel-plasminogeenactivator (t-PA) en plasminogeenactivator-remmer (PAI), alsmede op het niveau van plasminogeen en α_2-antiplasmine bij patienten met levercirrose.

Bij patienten met levercirrose blijkt, ondanks het feit dat de concentratie van zowel t-PA als PAI-1 toeneemt, de balans tussen deze beide te veranderen. De mediaan van de t-PA en PAI activiteit is niet verhoogd bij patienten met levercirrose. Bij sommige patienten leidt de verandering in de t-PA-PAI-1 balans tot verhoogde fibrinolytische activiteit. Bij alle patienten met levercirrose is zowel de hoeveelheid plasminogeen als α_2-antiplasmine in bloed verlaagd. Met behulp van stolsel-lysis experimenten in vitro toonden wij aan dat een evenredige daling van plasminogeen en α_2-antiplasmine in plasma, zoals wordt gevonden bij patienten met levercirrose, tot gevolg heeft dat de lysistijd van het stolsel wordt verkort. Deze studie toont aan dat diverse veranderingen optreden in de fibrinolyse bij patienten met levercirrose. Omdat deze veranderingen, met name in de t-PA-PAI balans, individueel erg variabel zijn, en bijvoorbeeld niet afhankelijk zijn van de ernst van de ziekte, vinden wij dat patienten moeten worden bestudeerd als individu en niet als groep. Wij concluderen dat de verhoogde t-PA activiteit in sommige patienten en de verlaagde concentraties van α_2-antiplasmine in alle patienten de belangrijkste rol spelen in het mechanisme van een versnelde fibrinolyse bij patienten met levercirrose.

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De auteur van dit proefschrift werd op 17 juli 1963 te 's Gravenhage geboren. Hij volgde zijn middelbare schoolopleiding aan de scholengemeenschap "Het Nieuwe Lyceum" te Bilthoven en behaalde in 1981 het eindexamen Gymnasium β.

In dat zelfde jaar werd een aanvang gemaakt met de studie Geneeskunde aan de Erasmus Universiteit te Rotterdam. Gedurende 3 jaar was hij als student-assistent werkzaam op de afdeling Anatomie I (destijds hoofd: Prof. Dr. P. Krediet). In het kader van het keuzepracticum, onderdeel van het kandidaats-examen, deed hij onder leiding van Dr. J. Stibbe onderzoek op de afdeling Hematologie van het Academisch Ziekenhuis Rotterdam "Dijkzigt" (Hoofd: Prof. Dr. J. Abels) naar haemostase afwijkingen tijdens open-hart chirurgie en streptokinase behandeling van het acute myocardinfarct. Tijdens de doctoraalfase van zijn studie was hij als student-assistent werkzaam op de afdeling Inwendige Geneeskunde II (Hoofd: Prof. J.H.P. Wilson). Hij werkte onder leiding van Dr. E.A.R. Knot mee aan klinische onderzoeken op het gebied van de fibrinolyse. Daar werd tevens de aanzet gegeven tot het in dit proefschrift vastgelegde onderzoek. In oktober 1986 behaalde hij het doctoraal-examen Geneeskunde. Het onderzoek werd daarna voortgezet op de afdeling Inwendige Geneeskunde II en tevens op het Gaubius Instituut TNO te Leiden (Hoofd: Prof. Dr. P. Brakman), waar hij twee jaar onder leiding van Dr. C. Kluft als wetenschappelijk medewerker werkzaam was.

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